VASCULAR INFLAMMATION IN AGING AND NEURODEGENERATION

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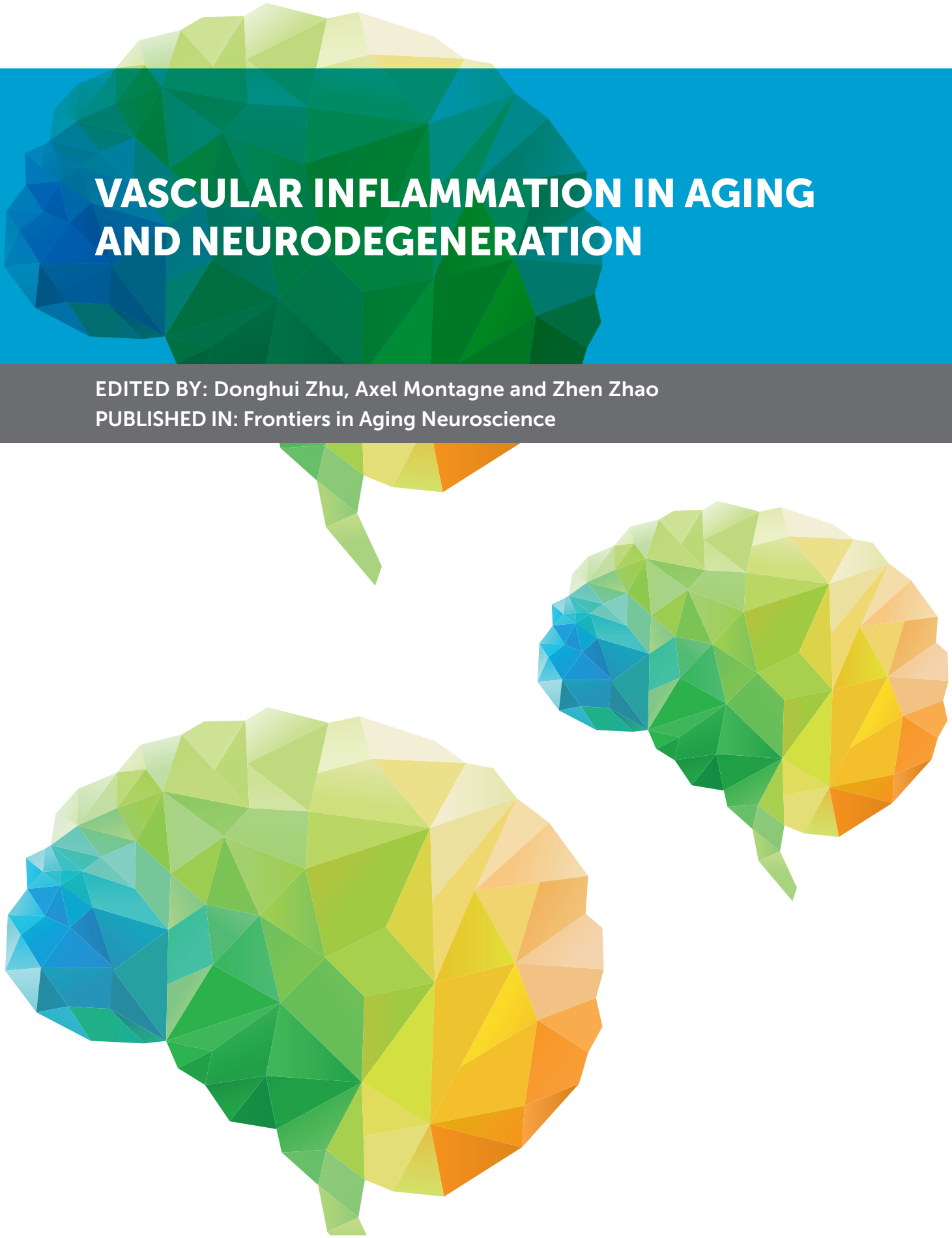

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VASCULAR INFLAMMATION IN AGING AND NEURODEGENERATION

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ORIGINAL RESEARCH
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Shifted Dynamics of Glucose Metabolism in the Hippocampus During Aging

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Aging is a process that adversely affects brain functions such as cognition. Brain activity is highly energy consuming, with glucose serving as the main energy source under normal circumstances. Whether the dynamics of glucose metabolism change with aging is not well understood. This study sought to investigate the activity-dependent changes in glucose metabolism of the mouse hippocampus during aging. In brief, after 1 h of contextual exploration in an enriched environmental condition or 1 h in a familiar home cage condition, metabolites were measured from the hippocampus of both young adult and aged mice with metabolomic profiling. Compared to the home cage context, the enriched contextual exploration condition resulted in changes in the concentration of 11 glucose metabolism-related metabolites in the young adult hippocampus. In contrast, glucose metabolism-related metabolite changes were more apparent in the aged group altered by contextual exploration when compared to those in the home cage condition. Importantly, in the aged groups, several key metabolites involved in glycolysis, the TCA cycle, and ketone body metabolism accumulated, suggesting the less efficient metabolization of glucose-based energy resources. Altogether, the analyses revealed that in the aged mice altered by enriched contextual exploration, the glucose resource seems to be unable to provide enough energy for hippocampal function.

Keywords: aging, glucose metabolism, hippocampus, energy, enriched contextual exploration

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HIGHLIGHTS

- Glycolysis becomes less efficient in the aged hippocampus altered by contextual exploration.
- TCA cycle is altered in the aged hippocampus by contextual exploration.
- Ketone body metabolism is elevated in the aged hippocampus altered by contextual exploration.

INTRODUCTION

Aging is a degenerative process that affects almost all biological organisms (Mattson and Arumugam, 2018). During the aging process, there is a buildup of biological waste products and metabolites that likely contribute to the development of degenerative diseases. For instance, aging has been associated with diseases such as atherosclerosis with the accumulation of oxidized lipid

particles in the subintimal layer of the arterial wall (Tyrrell and Goldstein, 2021), and age-related macular degeneration with the accumulation of cholesterol-rich drusen deposits in the central area of the retina (Al-Zamil and Yassin, 2017). Importantly, age-related degeneration has a particularly prominent effect on the brain and can negatively impact important functions such as cognition and sleep (Mattis and Sehgal, 2016; Mander et al., 2017; Schaum et al., 2020). Although many molecular mechanisms underlying brain degeneration have been discovered, the mechanisms involved in possible changes in energy-related metabolism remain largely unknown.

Since neurons require high energy consumption to function normally, notably to drive a sodium gradient across the cell membrane via the Na+/K+-ATPase, the cell's energy production capability is vital for the execution of brain physiological functions (Dienel, 2019). Although previous research demonstrates that energy metabolism is altered in the aged brain compared to the young adult brain, a more specific and quantitative description of energy metabolic pathways in the brain (Hoyer, 1990; Camandola and Mattson, 2017; Drulis-Fajdasz et al., 2018; Manza et al., 2020), especially in animals completing behavioral tasks, is not well-understood. Glucose is the major energy resource responsible for supporting brain activity under non-starvation conditions (Mergenthaler et al., 2013; Dienel, 2019). Previous non-invasive imaging techniques provided direct and indirect evidence of glucose level changes in the brain during neural activity (Choi et al., 2001; Raichle and Mintun, 2006; Magistretti and Allaman, 2015). It is also important to note that several other studies showed that activity led to increases in blood flow and glucose, but only slightly increases oxygen consumption. This elucidates the hypothesis that both oxidative and non-oxidative glucose metabolism might be involved in providing energy for neural activity (Belanger et al., 2011; Figley and Stroman, 2011). However, detailed metabolic mechanisms underlying these changes are lacking.

To better understand the possible changes in metabolites during hippocampal activity, we used a metabolomics method to analyze the dynamics of energy-related pathways in the mouse brain. In this present study, we performed a global metabolomics analysis to investigate the differences in the hippocampal energy metabolism of both young adult (6-week-old) and aged (78-week-old) mice under resting and hippocampal-engaged contextual exploration conditions. Metabolites in the glucose metabolic pathway, tricarboxylic acid (TCA) cycle, and ketone body metabolism were measured in both age categories as well as both conditions. The activity-dependent dynamics were compared between the two conditions. Strikingly, we found substantially more changes in concentrations of metabolites in the aged group altered by contextual exploration compared to that of the young adult group. Interestingly, in the aged group, the glycolytic efficiency decreased while the ketone body metabolism increased. While changes in the overall energy production remain unknown, a clear shift in the energy production pathway was observed.

MATERIALS AND METHODS

Experimental Animals

Mice used for the metabolomics were either 6-week (young adult) or 78-week (aged) C57BL/6 male mice (The Jackson Laboratory). Animals were housed under a 12-h light/dark cycle cage and given *ad libitum* access to food and water. All experimental procedures were carried out in accordance with guidelines from the National Institutes of Health and approved by the Stony Brook University Animal Care and Use Committee.

Enriched Contextual Exploration

Young adult and aged mice cohorts that were set as a contextual exploration group were given 1 week to become familiar with the exploration task. We arranged a cohort of 12 male mice that were 6-weeks-old and another cohort of 12 male mice that were 78-weeks-old. Six of the mice of each age were housed in a cage with 4–6 novel objects (wood blocks and plastic balls, 100–200 cm^3), defined as an enriched environment group (EE), to increase context exploration as we have previously described (Shen et al., 2019; Wang et al., 2020). All mice were tested in a plastic cage (24.5 × 47 cm) with wood bedding. The other six mice of each age were placed in a cage with only bedding, defined as a home cage group (HC or resting group). One hour after the mice were left in these two conditions, we sacrificed the mice and promptly collected the entire hippocampi for metabolomic analysis. All experiments were performed during the night cycle of mice and animals were sacrificed in a random order.

Metabolomic Profiling

Animals were anesthetized with urethane (i.p. 1.5g/kg) and transcardially perfused with ice-cold phosphate-buffered saline (PBS) buffer. In each mouse, two hippocampi were separated immediately from both hemispheres on ice and directly frozen by dry ice before being stored in a −80°C freezer until processing. For sample extraction, several recovery standards were used before the first step in the extraction process to allow confirmation of extraction efficiency (MicroLab STAR system, Hamilton Company). To remove protein, small molecules bound to protein were dissociated or trapped in the precipitated protein matrix, and chemically diverse metabolites were recovered, with samples precipitated with methanol for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. Samples were then placed briefly on a TurboVap (Zymark) to remove the organic solvent content and then frozen and dried with nitrogen. Instrument variability was determined by calculating the median relative standard deviation for the standards added to each sample before injection into the mass spectrometers. Overall process variability was determined by calculating the median relative standard deviation for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled matrix samples. Metabolomic profiling analysis utilized multiple platforms, including ultra-high performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) methods and hydrophilic interaction chromatography

(HILIC)/UPLC-MS/MS, which was performed by Metabolon, Inc. Aliquots of the sample were analyzed using a Waters Acquity UPLC (Waters Corp.) and LTQ mass spectrometer (MS) (Thermo Fisher Scientific, Inc.), which consisted of an electrospray ionization source and a linear ion-trap mass analyzer. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion; scanning varied slightly between methods but covered 70–1,000 mass-to-charge ratio.

A global metabolite class within carbohydrates and energy categories was investigated. Compounds were identified by comparison to library entries for purified standards, including retention time/index, mass-to-charge ratio, and chromatographic data (including MS/MS spectral data) using Metabolon's hardware and software. Peaks were quantified using the area under the curve. Comprehensive metabolomic data analysis was performed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca). Principal component analysis (PCA) was performed using MATLAB and applied for a preliminary evaluation of data quality between groups. Hierarchical cluster analysis (based on *t*-tests) was performed to create a heatmap of differentially expressed metabolites using MetaboAnalyst 5.0.

Statistical Analysis

The original values for each metabolite are normalized in terms of raw area counts. All metabolites are rescaled to set the median equal to 1 and the missing values are imputed with the minimum. The data was analyzed with Student's *t*-tests (unpaired, two-tailed). A value of $p < 0.05$ was considered statistically significant. All data are presented as mean ± standard error of the mean (SEM).

RESULTS

Metabolomics of the Aged Hippocampus Altered by an Enriched Contextual Exploration

Endogenous neurogenesis, neural circuit plasticity, and cognitive function in the hippocampus all decline during aging (Mattson and Arumugam, 2018). Recent studies, including the work from our group, have shown that the hippocampus, a key brain area for episodic memory, exhibits dramatic changes in concentration of metabolites related to energy consumption altered by a behavior task (McNay et al., 2000; Wang et al., 2020). While previous studies have shown the declining functionality of the aging hippocampus, the causes underlying this decay remain largely unknown. The impaired energy supply may contribute to the dysfunction of the aged hippocampus given that neuronal coding is an energy-intensive activity. We, therefore, aim to analyze the dynamics of energy production-related metabolites in the aged hippocampus in comparison to those of the young adult hippocampus altered by enriched contextual exploration, which serves as a stimulus to activate the hippocampus.

To analyze the dynamics of these metabolites in the hippocampus across aging, we first profiled metabolites of the hippocampus after hippocampus-engaged behaviors as outlined in **Figure 1A**. At both ages, out of the 593 analyzed metabolites, we sorted out 54 metabolites closely linked with carbohydrate metabolism and energy production. Using PCA analysis of six biological replicates of each group, we found a clear separation in the young adult and aged group under EE but not the HC condition (**Figure 1B**). Of the 6-weeks old mice groups, 11 metabolites had a significant difference ($p < 0.05$, unpaired *t*-test) between EE and HC conditions. In contrast to the changes in metabolite levels in young adult mice, the changes were more pronounced in aged mice after EE (**Figure 1C**). As shown in the hierarchical clustering heatmap (**Figure 1D**), we discovered activity-dependent changes in metabolites in the aged hippocampus that are involved in multiple pathways including glycolysis, pyruvate metabolism, galactose metabolism, TCA cycle, and oxidative phosphorylation.

Taken together, our metabolomic analysis of the young adult and aged hippocampus revealed substantial changes in levels of energy production-related metabolites in the aged mice when compared to those in young adult mice after enriched contextual exploration. This analysis suggests a possible energy metabolism shift in the aged hippocampus altered by a hippocampus-engaged exploratory behavior.

Decreased Glycolytic Dynamics in the Aged Hippocampus Altered by an Enriched Contextual Exploration

Substantial changes in metabolites in the activated hippocampus of both young adult and aged groups altered by enriched contextual exploration motivated us to explore the metabolism of glucose, an essential carbohydrate metabolite. Importantly, the mammalian brain heavily depends on glucose as its main source of energy, although it can adapt to utilize ketone bodies during periods of starvation (Hasselbalch et al., 1994; Mergenthaler et al., 2013). It has been generally considered that the brain glucose metabolism starts with glycolysis in the astrocytes (Belanger et al., 2011). The end product of glycolysis, pyruvate, is then catalyzed into lactate, which is then transported into neurons to enter the TCA cycle for further metabolization. TCA cycle metabolites are then used to generate ATP via the electron transport chain in the mitochondria.

Therefore, we first analyzed the metabolites involved in glycolysis as summarized in **Figure 2A**. The concentrations of glucose and glycolytic metabolites between HC and EE were compared. We found that in the young adult hippocampus there was a slight increase of glucose and metabolites involved in glycolysis, including glucose-6-phosphate, dihydroxyacetone phosphate (DHAP), 3-phosphoglycerate, and phosphoenolpyruvate altered by enriched contextual exploration (**Figures 2B–G**). A significant increase of pyruvate was observed in these mice under EE. In contrast, in the aged hippocampus, a substantial increase in hippocampal glucose concentration was observed altered by contextual

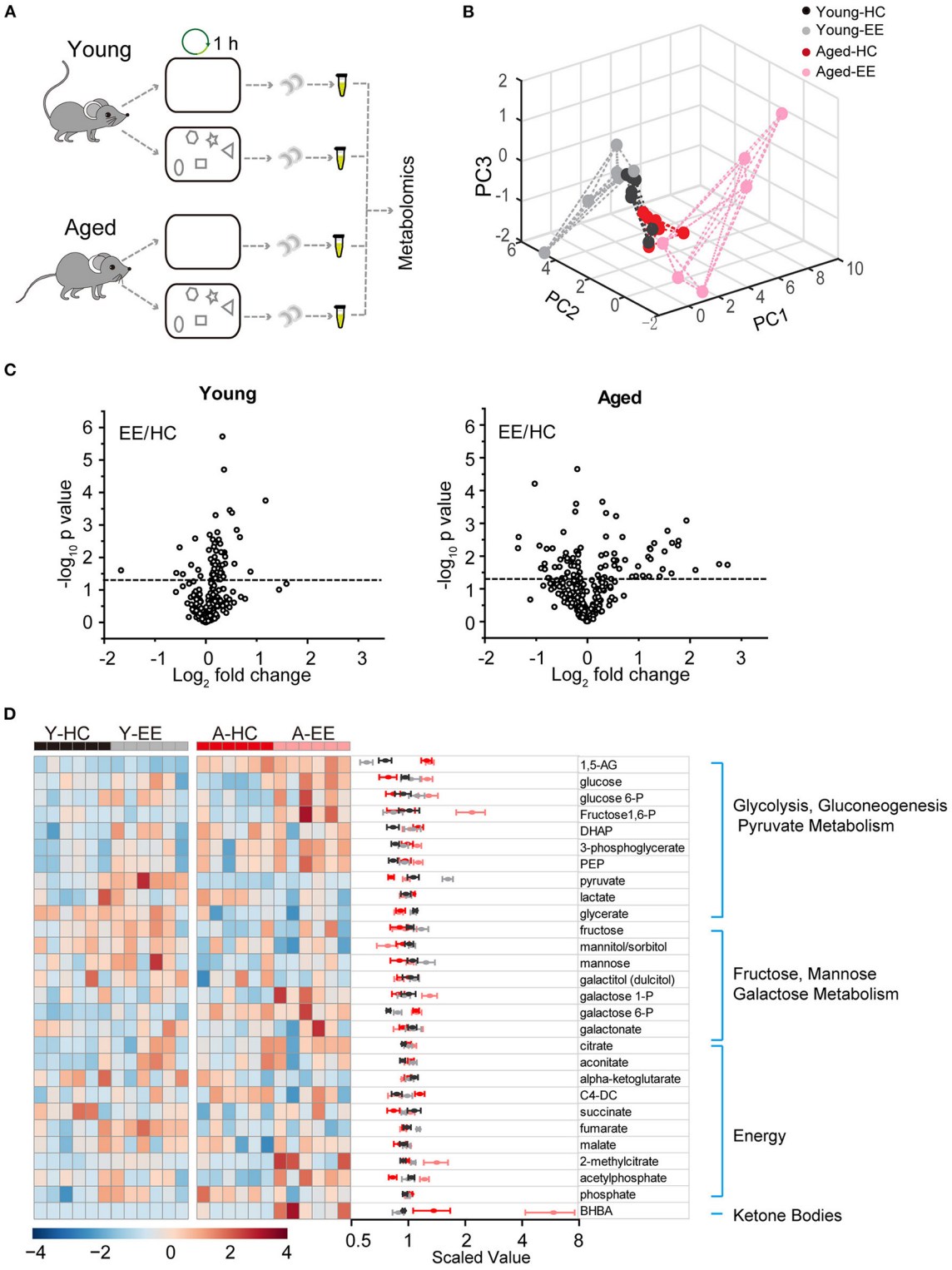

FIGURE 1 | Metabolic analysis of the hippocampus altered by content exploration in aged mice. **(A)** Experimental design schematic for the metabolic analysis of aged hippocampus. **(B)** PCA analysis of six repeats of young adult and aged group underlying home cage (HC) and enriched environment (EE) conditions. **(C)** Volcano plot of the fold-change and *p*-value for all 54 metabolites between HC and EE conditions. The horizontal dashed line represents the significance cutoff specified in the analysis ($p < 0.05$). HC, $n = 6$; EE, $n = 6$; unpaired *t*-test. **(D)** Hierarchical clustering heatmap and averaged value of metabolites between HC and EE conditions.

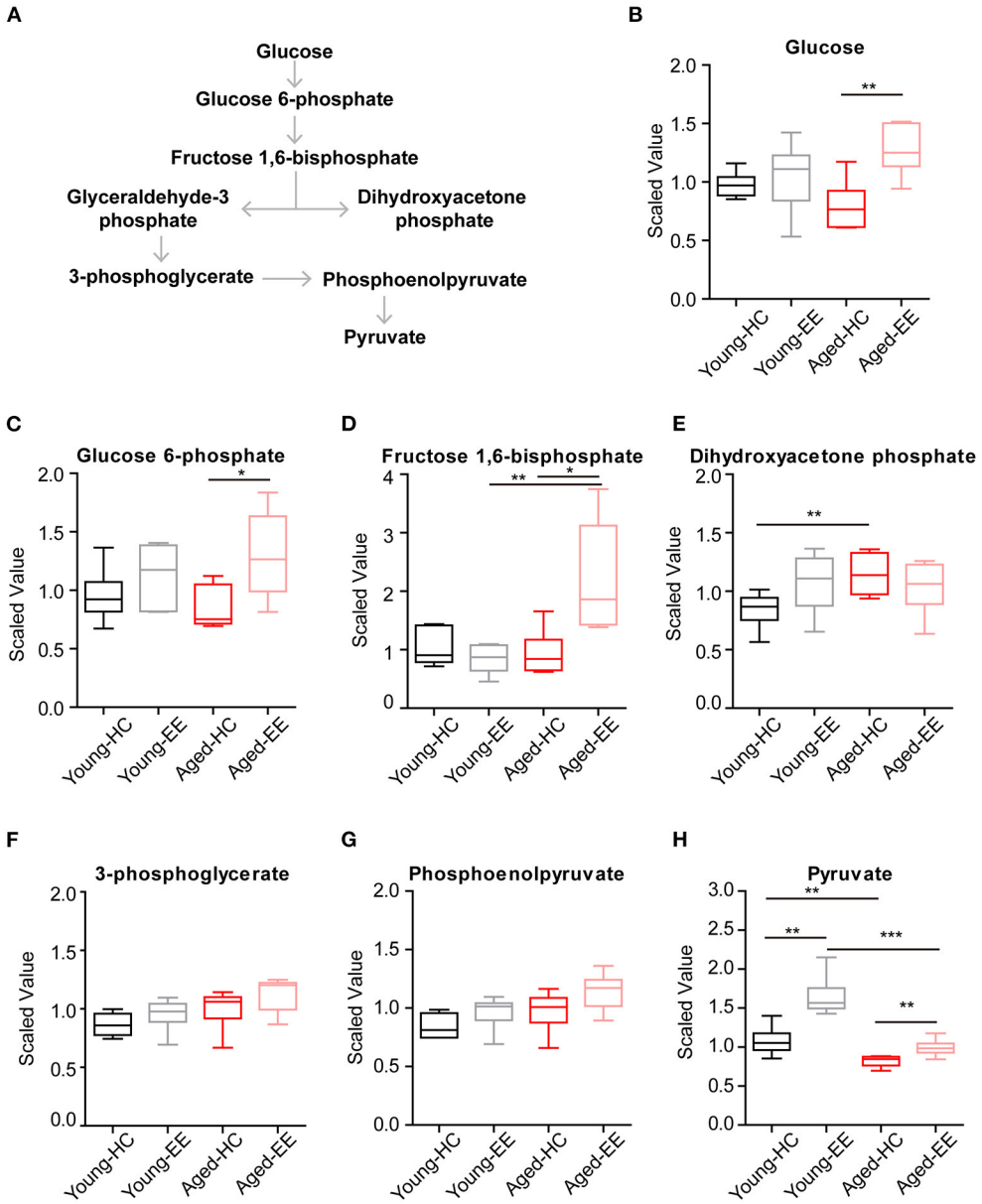

FIGURE 2 | Concentration of metabolites related with glycolysis of aged hippocampus. **(A)** Schematic drawing of the general glycolytic metabolism pathway. **(B–H)** Box plots of the levels of glucose **(B)**, glucose-6-phosphate **(C)**, fructose 1, 6-bisphosphate **(D)**, dihydroxyacetone phosphate (DHAP) **(E)**, 3-phosphoglycerate **(F)**, phosphoenolpyruvate **(G)**, and pyruvate **(H)** in young adult and aged mice under HC and EE condition. HC, $n = 6$; EE, $n = 6$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; unpaired *t*-test.

exploration (**Figure 2H**). In turn, levels of glucose 6-phosphate and fructose 1,6-bisphosphate had increased substantially (**Figures 2C,D**). Interestingly, we discovered that levels of DHAP and 3-phosphoglycerate, which are downstream of glucose 6-phosphate and fructose 1,6-bisphosphate, surprisingly remained unchanged (**Figures 2E,F**). Furthermore, levels of phosphoenolpyruvate also remained unchanged during EE conditions (**Figure 2G**). We should point out that an increase in pyruvate (**Figure 2H**), the end product of glycolysis, was discovered. This suggests a compensation mechanism from other energy sources to supplement the shortage in pyruvate production during glycolysis.

Altogether, the analyses revealed that glycolysis, the first step of glucose metabolism for energy production, changed during EE conditions and ended with elevated pyruvate levels in young adult mice. However, although the directional dynamics remained similar in the early steps of glycolysis in the aged mice, there was a significant break from the trend in the cleavage from fructose 1,6-bisphosphate to DHAP, suggesting a possible defect in the cellular mechanisms of the aged animals.

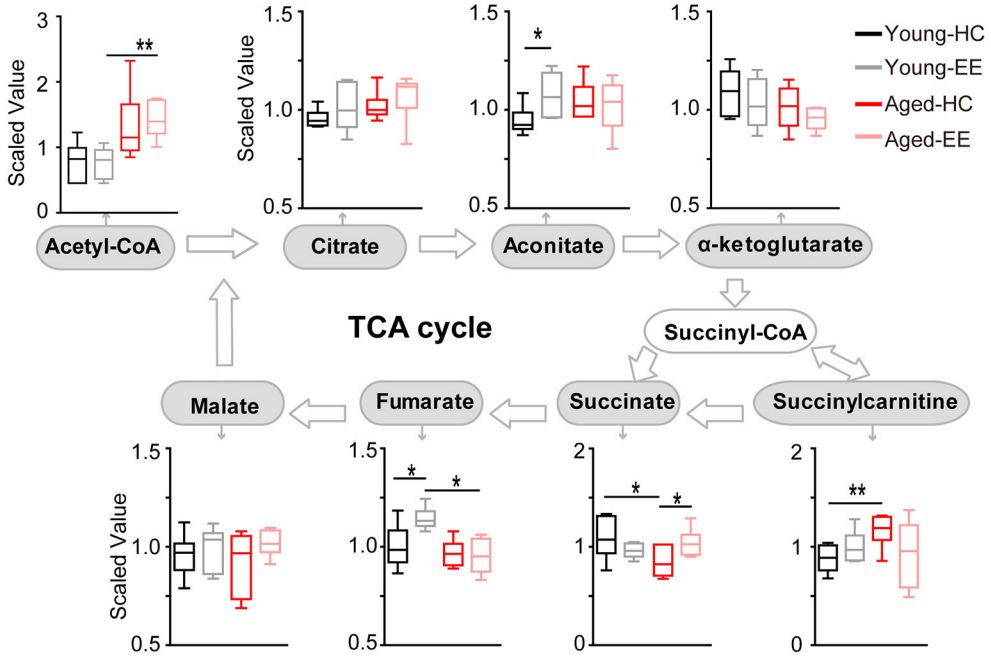

FIGURE 3 | Concentration of metabolites related to the TCA cycle of the aged hippocampus. Box plots of metabolites involved in the TCA cycle show the concentration change between young adult and aged hippocampus under HC and EE condition. TCA cycle key metabolites, including acetyl-CoA, citrate, aconitate, alpha-ketoglutarate, succinate, fumarate, and malate were plot according to the metabolic pathway. $^{*}p < 0.05$, $^{**}p < 0.01$; unpaired *t*-test.

Disrupted Cellular Respiration Pathway in the Aged Hippocampus in Comparison to That of the Young Adult Altered by an Enriched Contextual Exploration

To further analyze the changes in the metabolic pathway between young adult and aged mice altered by enriched contextual exploration, we analyzed the pathway following the production of pyruvate, the end product of glycolysis. Pyruvate serves as an essential substrate for ATP production via the TCA cycle. In the presence of oxygen, the mitochondrion facilitates aerobic respiration via the TCA cycle. Acetyl-CoA is first produced from pyruvate, which then enters the TCA cycle and is then oxidized to CO_2 while reducing NAD to NADH and FAD to $FADH_2$, as illustrated in **Figure 3**. NADH and $FADH_2$ are then used by the electron transport chain to generate ATP as part of oxidative phosphorylation. In the TCA cycle, the metabolites of citrate, aconitate, alpha-ketoglutarate, succinate, succinylcarnitine, fumarate, and malate were detected and analyzed in the hippocampus of the four experimental conditions.

In the young adult hippocampus, a slight increase in metabolite levels involved in the TCA cycle was observed, including aconitate and fumarate altered by enriched contextual exploration (**Figure 3**). In contrast, in the aged hippocampus, a visible increase in succinate was found altered by contextual exploration. Succinyl-CoA was preferentially diverted into succinylcarnitine as opposed to succinate in the aged home cage condition animals as compared that of the young adult. This suggests that the TCA cycle does not run as efficiently in aged animals in comparison to young adult animals. Succinylcarnitine, a metabolic "dead end," accumulates in aged control hippocampi, suggesting less energy production via the TCA in aged animals. Importantly, this diverted effect seemed to be "rescued" in aged animals by placing them under EE conditions, leading to increased succinate and decreased succinylcarnitine.

Altogether, the analyses revealed that certain metabolite levels in the TCA cycle significantly increased in young adult mice altered by contextual exploration. However, these significant increases were not paralleled in the aged mice placed under the same conditions. In the aged hippocampus, the TCA cycle may be diverting metabolites to succinylcarnitine, making it less efficient at running aerobic metabolism, suggesting possible additional mechanisms to compensate the short of TCA-based energy production.

Elevated Ketone Metabolism in the Aged Hippocampus in Comparison to That of the Young Adult Altered by an Enriched Contextual Exploration

In the aged hippocampus, both glycolysis and the TCA cycle seemingly become less efficient altered by contextual exploration and are unable to supply the energy demands of the brain. The brain might supplement its energy requirements by utilizing ketone bodies when glucose levels are insufficient. Therefore, we set out to measure ketone concentrations in the hippocampus across aging and hippocampal stimulation.

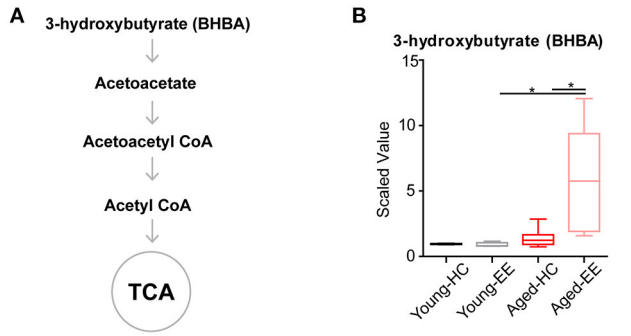

FIGURE 4 | Concentration of metabolites related to acetyl CoA of aged hippocampus. **(A)** Schematic drawing of metabolic pathway including ketone body metabolism linking with acetyl CoA. **(B)** Box plot of the levels of 3-hydroxybutyrate (BHBA) in young adult and aged mice. HC, $n = 6$; EE, $n = 6$; $^*p < 0.05$; unpaired t-test.

Ketone bodies include acetoacetate and beta-hydroxybutyrate (BHBA). Acetoacetate can be transformed into acetyl-CoA to enter the TCA cycle in the absence of sufficient ATP production from glycolysis and the TCA cycle (**Figure 4A**). In our metabolomics assay, we successfully detected BHBA (**Figure 1**). In the young adult brain, there were few changes observed in the concentration of BHBA altered by contextual exploration. However, a substantial increase in BHBA concentration was observed in the aged brain altered by contextual exploration (**Figure 4B**). The oxidation of BHBA eventually leads to the production of acetyl-CoA, which requires the transfer of CoA from succinyl-CoA. This may result in a higher concentration of succinate (**Figure 3**). This is a possible cause of the increase in succinate observed in the TCA cycle.

Altogether, the analyses revealed that the level of the ketone body BHBA remains comparable in the aged brain as compared to the young adult brain during HC. Additionally, there was no change in the level of BHBA in the young adult brain between HC and EE. However, the level of BHBA in the aged hippocampus is substantially elevated during EE.

DISCUSSION

How does energy metabolism change in the aged brain, especially during cognitive activity? In this study, using a metabolome analysis method, we measured the changes in the levels of various metabolites under enriched contextual exploration conditions in both the young adult and aged hippocampus. The entire metabolite profiling displayed substantial changes in concentrations of metabolites involved in energy production pathways. We first explored changes in glucose metabolism by analyzing the levels of metabolites involved in the glycolytic process. The levels of certain metabolites experienced significant changes and we discovered an overall decrease in efficiency of pyruvate production in the aged brain. Furthermore, we analyzed levels of metabolites in the TCA cycle, finding an increase in succinate in the aged brain. This suggests a secondary pathway, namely from ketone bodies, that bolstered the concentration of succinate. Together, our results suggest impairments in specific enzymes of metabolic pathways that lead to the overall decrease in energy metabolism in the aged brain at baseline and after contextual exploration.

During hippocampus-engaged behaviors, it has been shown that glucose levels decrease substantially in the extracellular compartment (McNay et al., 2000, 2001), and glucose utilization is increased to meet the elevated neural activities (De Bundel et al., 2009; Belanger et al., 2011; Figley and Stroman, 2011), suggesting elevated glucose-related metabolism. Thus, to better investigate this, we analyzed the levels of various metabolites in the glucose-related metabolism to gauge the changes in these levels after hippocampus-engaged behaviors. Using metabolomic analyses, we found that EE led to changes in the concentration of a variety of energy production-related metabolites in the young adult hippocampus. Interestingly, in the aged hippocampus, we observed an apparent diversion of pyruvate and acetyl-CoA toward ketone metabolism in the aged brain, especially under conditions of enriched environmental exploration, as compared to the young adult brain. Previous studies indicated that physical activity regulates cerebral blood flow which affects oxygen level and brain metabolism (Querido and Sheel, 2007; Overgaard et al., 2012). Although we conducted experiments during the dark cycle when mice were more active, aged mice tend to exercise less vigorously and exhibit more variable exploratory behavior. Aging related changes in behavior and blood flow may involve in shifted energy metabolism in the brain.

During glycolysis, although there was a substantial increase in pyruvate concentration during the EE condition in the aged hippocampus, the absolute levels of pyruvate between the young adult and aged hippocampus show a lower metabolism efficiency in the aged hippocampus (**Figure 2H**). While there were no significant differences in the levels of glucose between the young adult and aged hippocampus under both HC and EE conditions, there were major differences in the levels of pyruvate under both conditions. In the aged hippocampus, the glucose concentration was substantially increased under EE, possibly reflecting increased blood glucose delivery to the hippocampus, but impairment in the ability to utilize this glucose for aerobic metabolism, which is consistent with previous observations in humans (Goyal et al., 2017).

Astrocytes take up glucose from the bloodstream and fuel neurons with lactate, which serve as a crucial way to provide energy in the brain. A lot of delicate experiments showing how lactate is generated and utilized in neuron-astrocyte coupling and serve as fuel for mitochondria to provide neuron energy (Diaz-Garcia et al., 2017; Descalzi et al., 2019). Previous studies have shown that the aged hippocampus increases glycogen metabolism enzyme concentrations and shifts in these enzymes' localization from astrocytes to neurons (Drulis-Fajdasz et al., 2018). However, given the decrease of pyruvate in the aged cohort despite a substantial increase in glucose levels, we hypothesize a decrease in efficiency of glucose metabolism in the aged brain. Future work should aim to specifically measure glucose consumption in different brain areas across ages, for example, by measuring glucose transporter kinetics and glucose labeling to trace the accumulation of metabolic by-products.

In the TCA cycle of the young adult brain, there was an increase in the levels of aconitate and fumarate during the EE condition. In the TCA cycle of the aged brain, there was a substantial increase in succinate concentration during EE conditions (**Figure 3**). The question remains regarding the cause of this buildup of succinate. One possibility is that succinate dehydrogenase is less efficient in the aged brain as compared to the young adult brain. Alternatively, we hypothesize that this increased succinate may result as a byproduct of BHBA metabolism. In our further analysis, we found that BHBA levels had a sharp, significant increase from the aged HC to the aged EE condition (**Figure 4B**). Since the breakdown of BHBA to acetyl-CoA involves changing succinyl-CoA to succinate, this may serve as a possible mechanism to explain the significant increase in succinate in the aged cohort.

Neurodegeneration is the progressive decline of functionality due, in part, to alterations in cellular metabolism and respiration (Procaccini et al., 2016). The functionality of the brain fundamentally relies on energy that is metabolized from various sources. This current study adds to a growing body of literature using a metabolomics approach to understand the energy supply dynamics. This work provides insights into metabolic derangements associated with aging and degeneration which may lead to the development of therapeutic strategies to help mitigate such degeneration.

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 animal study was reviewed and approved by Stony Brook University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

IG and XW carried out the experiment and data analysis. IG, GK, and XW wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

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Association of Aortic Stiffness and Cognitive Decline: A Systematic Review and Meta-Analysis

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Background: Increased aortic stiffness has been found to be associated with cognitive function decline, but the evidence is still under debate. It is of great significance to elucidate the evidence in this debate to help make primary prevention decisions to slow cognitive decline in our routine clinical practice.

Methods: Electronic databases of PubMed, EMBASE, and Cochrane Library were systematically searched to identify peer-reviewed articles published in English from January 1, 1986, to March 16, 2020, that reported the association between aortic stiffness and cognitive function. Studies that reported the association between aortic pulse wave velocity (PWV) and cognitive function, cognitive impairment, and dementia were included in the analysis.

Results: Thirty-nine studies were included in the qualitative analysis, and 29 studies were included in the quantitative analysis. The aortic PWV was inversely associated with memory and processing speed in the cross-sectional analysis. In the longitudinal analysis, the high category of aortic PWV was 44% increased risk of cognitive impairment (OR 1.44; 95% CI 1.24–1.85) compared with low PWV, and the risk of cognitive impairment increased 3.9% (OR 1.039; 95% CI 1.005–1.073) per 1 m/s increase in aortic PWV. Besides, meta-regression analysis showed that age significantly increased the association between high aortic PWV and cognitive impairment risk.

Conclusion: Aortic stiffness measured by aortic PWV was inversely associated with memory and processing speed and could be an independent predictor for cognitive impairment, especially for older individuals.

Keywords: aortic stiffness, pulse wave velocity, cognitive impairment, vascular dementia, aging

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INTRODUCTION

With the population aging, an increasing number of older adults suffer from cognitive impairment and dementia, which substantially reduce the quality of life in the elderly and bring a substantial medical burden to their family and the whole society (Langa and Levine, 2014). It is of great significance to recognize the risk factors to prevent cognitive impairment and dementia (Livingston et al., 2017).

In recent years, through the growing investigations and the more in-depth understanding of aortic stiffness, it was found that aortic stiffness is not only related to increased risk of cardiovascular

diseases and related mortality (Vlachopoulos et al., 2010) but also involved in the aging changes of brain and cognitive function (Vlachopoulos et al., 2010; Zeki Al Hazzouri et al., 2013; Yukutake et al., 2015; Iulita et al., 2018; Rouch et al., 2018). With advancing age, the aortic vessel wall's elastic fibers are gradually reduced and replaced by collagen fibers or deposition of calcification, which impairs elastic aorta's elasticity and causes aortic stiffness (Thorin-Trescases and Thorin, 2016). The stiffening and loss of recoil in the aorta would transmit excessive and damaging pulsatile load to the peripheral arteries of body organs. Theoretically, the brain is more susceptible to pulsatile damage due to its low-resistance and high-flow characteristics (Thorin-Trescases and Thorin, 2016; Iulita et al., 2018). Aortic stiffness was reported to be closely associated with cerebral structural changes, primarily the cerebral small vessel disease and brain atrophy (Mitchell et al., 2011; Webb et al., 2012; van Sloten et al., 2015, 2016). There have been studies that focus on the relationship between aortic stiffness and cognitive function. However, their results were inconsistent (Poels et al., 2007; Singer et al., 2013; van Sloten et al., 2015).

Among various pulse wave velocity (PWV) measurements for aortic stiffness, carotid-femoral PWV (cfPWV) that measure the PWV along the aortic and aortoiliac pathways is the recommended gold-standard non-invasive technique to assess aortic stiffness because of its reliability and feasibility, which is highly related with magnetic resonance imaging (MRI) directly measuring PWV (Laurent et al., 2006; Boutouyrie et al., 2014). While brachial-ankle PWV (baPWV) or femorotibial PWV (ftPWV), the commonly used PWV index measured outside the main aortic track, reflects mainly the stiffness of the small arteries rather than pure aortic stiffness, its predicted value in cardiovascular disease is still controversial (Boutouyrie et al., 2014; Iulita et al., 2018). Thus, considering the validation in clinic practice, we performed a systematic review and meta-analysis about the association between aortic stiffness measured using the validated aortic PWV and cognitive function, risk of cognitive impairment, or dementia to help clarify the association between aortic stiffness and cognitive function in the aging process.

METHOD

This systematic review and meta-analysis was reported, adhering to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement and Meta-analysis of Observational Studies in Epidemiology (MOOSE) checklist (Stroup et al., 2000; Liberati et al., 2009).

Search Strategy and Data Source

We searched for articles published from January 1, 1986, to March 16, 2020, through electronic databases, including PubMed, Cochrane Library, and EMBASE using "aortic stiffness" and "cognitive impairment" as major themes (precise search terms are provided in the **Supplementary Material**). The search was restricted to articles published in the English language. Also, we reviewed the reference lists of all relevant articles for potentially eligible studies.

Selection Strategy and Criteria

Two investigators (QL and CHC) independently screened all relevant studies and determined eligibility based on the title, abstract, and full texts. Studies were included if they matched the following inclusion criteria: (1) human studies and full-length publications in peer-reviewed journals; (2) cross-sectional or longitudinal designed studies; (3) in order to comprehensively explore the association between aortic stiffness and cognition in adults, we included the studies with participants aged 18 years or older, regardless of sample size and types of population (including general population or targeting on a particular population with a risk factor or disease); (4) reporting an association between aortic stiffness and cognitive function; (5) evaluating aortic stiffness exclusively using validated PWV measurement along aorta; (6) cognitive function were assessed with validated scales, and mild cognitive impairment (MCI) or dementia was diagnosed based on clinic diagnostic standards or guidelines. Studies were excluded if they met the exclusion criteria: (1) case–control study or placebo-controlled clinical trial (involving a specific intervention); (2) the article did not report an association between aortic stiffness and cognitive impairment; and (3) the aortic stiffness was assessed using PWV measured outside the aortic track, at the upper (baPWV) or lower limb (ftPWV).

Data Extraction

Two investigators (QL and CHC) independently extracted data from each eligible study. Any disagreements were resolved by consensus or consultation with a third investigator (JHF). The following information was extracted from each eligible study: authors, published year, design (follow-up years for longitudinal studies), country, study population, sample size, male (%), mean age, mean or median aortic PWV value (m/s), cognitive tests, adjusted covariates, and main results. The outcomes for meta-analysis were various domains of cognitive function, cognitive impairment, and dementia. In cognitive function domains, we focused on attention, executive function, global cognitive function, memory, processing speed, and visuospatial ability. For those studies that published more than one article from the same cohort, (1) if they had the same study design and cognitive outcomes, we included only the one with the results either that could be included in meta-analysis or with the largest sample size; (2) if they reported on different cognitive outcomes, we included different cognitive domains' data in each of these articles separately in the analysis. Required metrics not reported in the article were requested from the corresponding authors by email.

Quality Assessment

Two investigators (QL and JHF) independently assessed the quality of included studies using the modified version of Newcastle Ottawa Scale (NOS) (Wells et al., 2014) (see the **Supplementary Material**). The NOS includes items on participant selection, the validity of measurements, and whether adjusting associations by systolic and/or mean blood pressure (MBP), age and education, and assessment of outcomes. For cross-sectional studies, the maximum score was 5 points, and scores <3 points were considered as high risk of bias. For

longitudinal studies, the maximum score was 8 points, and scores <4 points were considered as high risk of bias.

Statistical Analysis

All analyses were performed using Comprehensive Meta-Analysis software version 3 (CMA 3.0, Biostat Inc., Englewood, NJ, USA). For cross-sectional studies, Pearson's r correlation coefficients were pooled as the effect size to show the association between aortic PWV and cognitive function (attention, executive function, global cognitive function, memory, processing speed, and visuospatial ability). Multiple scales for one cognitive domain in each study were collapsed into a single effect size (Borenstein and Wiley, 2009). Negative associations indicated that greater stiffness (aortic PWV) was associated with worse cognitive function. Additionally, the r correlation coefficients and 95% CI between aortic PWV and Mini-Mental State Examination (MMSE) scores were synthesized. For longitudinal studies, the odds ratios (ORs) were pooled as effect size to show the association between aortic PWV (the highest stiffness group vs. the lowest group) and risk of cognitive impairment and dementia. Since most studies reported ORs of continuous aortic PWV metric, we also pooled the adjusted ORs per absolute aortic PWV (1 m/s) to explore between constant aortic PWV values and the risks of cognitive impairment or dementia. A random-effects model was used to pool these effect estimates when significant heterogeneity existed among studies.

Among the included studies, a few studies recruited participants specifically with chronic kidney disease (including end-stage renal disease), hypertension, and complain of memory loss. Thus, we divided these studies into three categories according to their participants' condition: chronic kidney disease, hypertension, and complaint of memory loss. Sensitivity analyzes were performed by excluding these three categories of studies one by one from the pooled estimates to show the influence of these specific conditions on the overall effect size and 95% CI. Moreover, we performed subgroup analysis when there were more than three papers in each of the above three categories. Meta-regression analysis was conducted to evaluate the influence of mean age, percentage of male, MBP, and percentage of education of high school or less on the association between aortic stiffness and cognitive decline.

Q test and I-squared statistics were used to examine the heterogeneity across studies, with a $P \leq 0.10$ and $I^2 \geq 50\%$ indicating significant heterogeneity (Higgins et al., 2003; Borenstein and Wiley, 2009). Egger's test and funnel plot were used to evaluate publication bias, and $P < 0.05$ of Egger's test and/or funnel plot asymmetry was considered as the existence of publication bias (Borenstein and Wiley, 2009).

RESULTS

Qualitative Summary Characteristic of Included Studies

As shown in the diagram of the selection process (**Figure 1**), among 2,869 records, 65 articles were evaluated based on full text. Finally, 39 studies were summarized for detailed review, including 28 studies with 29,955 participants from 14 different countries in the cross-sectional analysis (Hanon et al., 2005; Scuteri et al., 2005; Poels et al., 2007; Elias et al., 2009; Kearney-Schwartz et al., 2009; Triantafyllidi et al., 2010; Watson et al., 2011; Singer et al., 2013; Nilsson et al., 2014, 2017; Zhong et al., 2014; Cooper et al., 2016; Geijselaers et al., 2016; Lim et al., 2016; Pase et al., 2016b; Riba-Llena et al., 2016; Tasmoc et al., 2016; Kim et al., 2017; Meyer et al., 2017; Suleman et al., 2017; Karasavvidou et al., 2018; Kennedy et al., 2018; Muela et al., 2018; Araghi et al., 2019; DuBose et al., 2019; Palta et al., 2019; Dixon et al., 2020; Zijlstra et al., 2020) and 16 studies with 23,448 participants from seven different countries in the longitudinal analysis (Poels et al., 2007; Scuteri et al., 2007, 2013; Waldstein et al., 2008; Watson et al., 2011; Zeki Al Hazzouri et al., 2013; Watfa et al., 2015; Hajjar et al., 2016; Pase et al., 2016a; Tsao et al., 2016; Kim et al., 2017; Nilsson et al., 2017; Cui et al., 2018; Rouch et al., 2018; Araghi et al., 2019; Menezes et al., 2019) (**Supplementary Tables 1**, **2**). Only the study of Zijlstra et al. measured aortic PWV using MRI and showed median PWV of 9.6 m/s (interquartile range: 7.8–13.0) (Zijlstra et al., 2020). The other studies used cfPWV and showed mean or median PWV ranging from 4.96 to 14.3 m/s. For the cross-sectional study, modified NOS scores ranged from 1 to 5 points, and 10 studies (35.7%) (Scuteri et al., 2005; Kearney-Schwartz et al., 2009; Tasmoc et al., 2016; Kim et al., 2017; Suleman et al., 2017; Karasavvidou et al., 2018; Muela et al., 2018; Araghi et al., 2019; Dixon et al., 2020; Zijlstra et al., 2020) had a low score of 1–2 points. For longitudinal studies, the score ranges from 3 to 8 points, with two studies (12.5%) (Kim et al., 2017; Araghi et al., 2019) having a low score of 3 points (**Supplementary Tables 1**, **2**).

In the cross-sectional studies, some reported a significant association between aortic PWV and cognitive function or cognitive decline (Hanon et al., 2005; Scuteri et al., 2005; Elias et al., 2009; Kearney-Schwartz et al., 2009; Watson et al., 2011; Nilsson et al., 2014; Zhong et al., 2014; Cooper et al., 2016; Lim et al., 2016; Pase et al., 2016b; Tasmoc et al., 2016; Meyer et al., 2017; Kennedy et al., 2018; Muela et al., 2018; Araghi et al., 2019; DuBose et al., 2019; Palta et al., 2019; Dixon et al., 2020; Zijlstra et al., 2020), while some did not support significant association between aortic PWV and cognitive function or dementia (Poels et al., 2007; Triantafyllidi et al., 2010; Singer et al., 2013; Geijselaers et al., 2016; Riba-Llena et al., 2016; Kim et al., 2017; Nilsson et al., 2017; Suleman et al., 2017) (**Supplementary Table 1**). Among them, studies that included exclusive participants with chronic kidney disease (Tasmoc et al., 2016; Kim et al., 2017; Karasavvidou et al., 2018; Zijlstra et al., 2020) or hypertension (Triantafyllidi et al., 2010; Riba-Llena et al., 2016; Muela et al., 2018) mainly did not support the association between aortic stiffness and cognitive decline. However, those studies with participants complaining of memory loss showed a significant association (Hanon et al., 2005; Scuteri et al., 2005; Kearney-Schwartz et al., 2009; Dixon et al., 2020) (**Supplementary Table 1**).

In longitudinal studies, 13 studies with 17,727 participants (followed up 1–15 years) suggested significant associations between aortic PWV and cognitive decline or dementia (Scuteri et al., 2007, 2013; Waldstein et al., 2008; Watson et al., 2011; Zeki Al Hazzouri et al., 2013; Watfa et al., 2015; Hajjar et al., 2016;

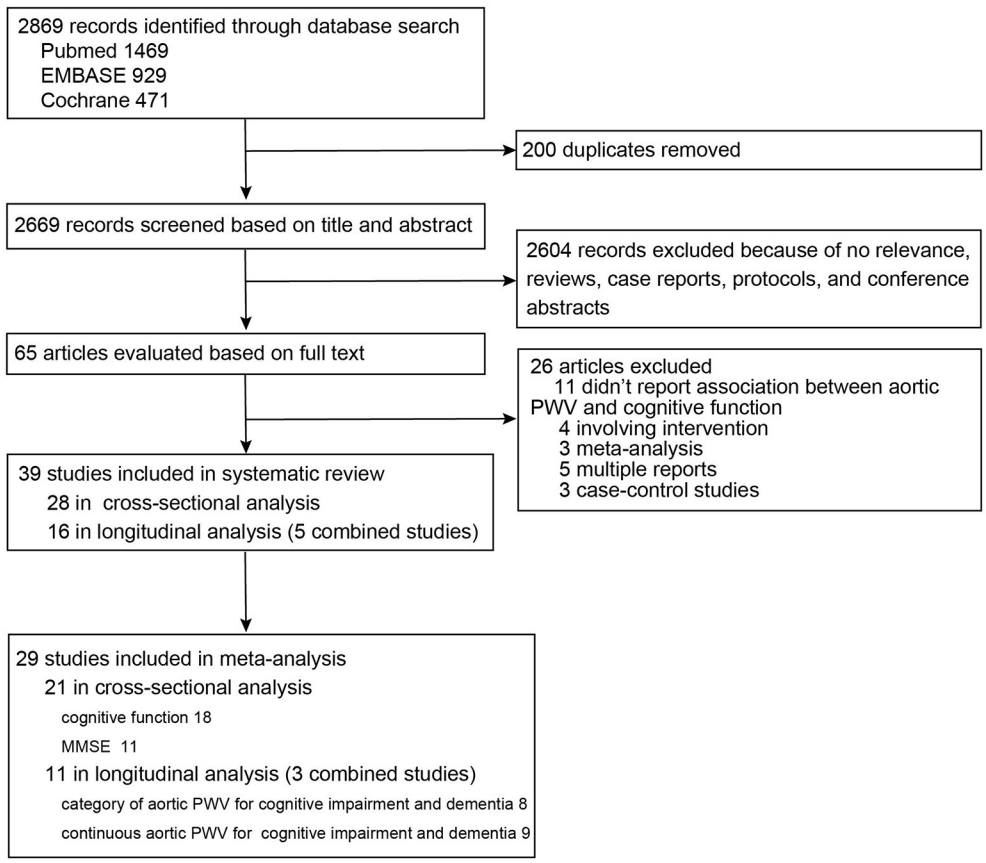

FIGURE 1 | Flow diagram of study selection for systematic review and meta-analysis.

Pase et al., 2016a; Tsao et al., 2016; Cui et al., 2018; Rouch et al., 2018; Araghi et al., 2019; Menezes et al., 2019), in which three studies included participants with complaints of memory loss (Scuteri et al., 2007, 2013; Rouch et al., 2018), whereas two studies with 5,721 participants (followed up about 4 years) (Poels et al., 2007; Nilsson et al., 2017) and one study with 135 hemodialysis participants (followed up 1 year) (Kim et al., 2017) did not find association between aortic PWV and cognitive impairment (**Supplementary Table 2**).

Aortic Pulse Wave Velocity and Cognitive Function

Eighteen studies with 15,489 participants were eligible for meta-analysis of association between aortic PWV and cognitive function (Hanon et al., 2005; Poels et al., 2007; Elias et al., 2009; Watson et al., 2011; Singer et al., 2013; Nilsson et al., 2014; Zhong et al., 2014; Cooper et al., 2016; Geijselaers et al., 2016; Lim et al., 2016; Pase et al., 2016b; Riba-Llena et al., 2016; Tasmoc et al., 2016; Kennedy et al., 2018; Muela et al., 2018; DuBose et al., 2019; Dixon et al., 2020; Zijlstra et al., 2020). Among them, there were 157 chronic kidney disease participants (Tasmoc et al., 2016; Zijlstra et al., 2020), 976 hypertension participants (Riba-Llena et al., 2016; Muela et al., 2018), and 364 participants complaining of memory loss (Hanon et al., 2005; Dixon et al., 2020). As shown in **Figure 2**, we detected a significant association between aortic PWV and attention ($r = -0.174$), global cognitive function ($r = -0.122$), memory ($r = -0.061$), and processing speed ($r = -0.119$) in all participants (**Figure 2**, **Table 1**). Nevertheless, significant heterogeneity existed among studies (**Supplementary Table 3**). After studies with participants of chronic kidney disease, of hypertension, and complaining loss of memory were excluded, the aortic PWV was still statistically associated with memory ($r = -0.022$) and processing speed ($r = -0.048$) (**Table 1**), without significant heterogeneity and publication bias (**Supplementary Table 3**).

A significant association between aortic PWV and MMSE scores was detected among 11 studies with 9,034 participants ($r = -0.11$, 95% CI -0.15 to -0.07) (Hanon et al., 2005; Scuteri et al., 2005; Poels et al., 2007; Triantafyllidi et al., 2010; Nilsson et al., 2014; Zhong et al., 2014; Lim et al., 2016; Tasmoc et al., 2016; Karasavvidou et al., 2018; Muela et al., 2018; Dixon et al., 2020), but there were significant heterogeneity and publication bias (**Supplementary Table 4**, **Supplementary Figure 5**). After studies with participants with specific disease or condition (Hanon et al., 2005; Scuteri et al., 2005; Triantafyllidi et al., 2010; Tasmoc et al., 2016; Karasavvidou et al., 2018; Muela et al., 2018; Dixon et al., 2020) were excluded, the significance disappeared, while in the subgroup

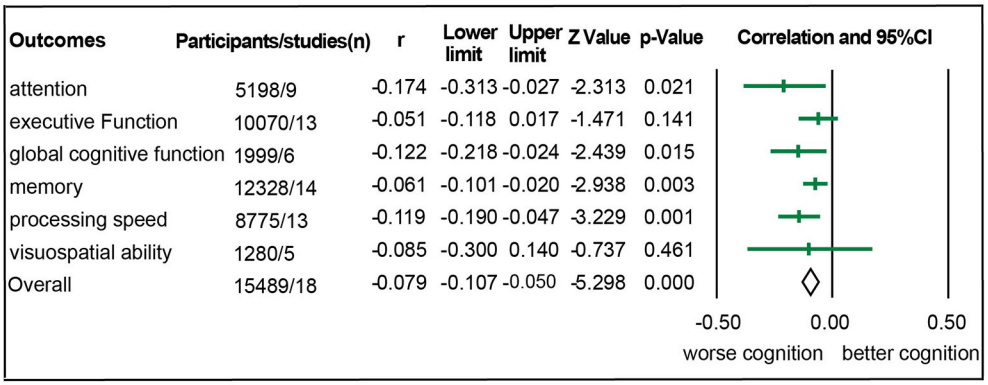

FIGURE 2 | Forest plot for association between aortic stiffness and domains of cognitive function.

of participants complaining of memory loss (Hanon et al., 2005; Scuteri et al., 2005; Dixon et al., 2020), the aortic PWV was significantly associated with MMSE score ($r = -0.27$), and there were no heterogeneity and significant publication bias (**Supplementary Table 4**). These results supported aortic stiffness associated with cognitive impairment. However, as a screening test for dementia, the MMSE scale might not be a validated and sensitive tool for detecting cognitive impairment in the general population (Waldstein et al., 2008; Pase et al., 2012).

Aortic Pulse Wave Velocity and Cognitive Impairment or Dementia

Six studies with 13,648 participants were included in synthesizing adjusted ORs of the highest vs. lowest category of aortic PWV to cognitive impairment (Scuteri et al., 2013; Zeki Al Hazzouri et al., 2013; Pase et al., 2016a; Cui et al., 2018; Araghi et al., 2019; Menezes et al., 2019), and three studies with 4,532 participants were synthesized for adjusted ORs for dementia (Pase et al., 2016a; Nilsson et al., 2017; Rouch et al., 2018). The pooled results showed that the highest category of aortic PWV independently increased risk of cognitive impairment (OR 1.44, 95% CI 1.124–1.845) and dementia (OR 2.1; 95% CI 1.159–3.804) than the lowest group of aortic PWV, though with moderate heterogeneity (**Figure 3**), but no significant publication bias (**Supplementary Figure 6**). After studies with participants complaining of memory loss (Scuteri et al., 2013; Rouch et al., 2018) were excluded, the significantly increased risk for cognitive impairment remained, but not for dementia (**Supplementary Figure 2**).

We also calculated the pooled ORs of continuous aortic PWV (m/s) to cognitive impairment or dementia. It showed that the cognitive impairment risk increased 3.9% (OR 1.039; 95% CI 1.005–1.073) per 1 m/s of aortic PWV increase from six studies (15,711 participants) reporting risk of cognitive impairment (Poels et al., 2007; Watson et al., 2011; Watfa et al., 2015; Pase et al., 2016a; Araghi et al., 2019; Menezes et al., 2019). There were moderate heterogeneity and no publication bias (**Figure 4**, **Supplementary Figure 6**). However, for the five studies with 7,655 participants reporting risk for dementia (Poels et al., 2007; Pase et al., 2016a; Nilsson et al., 2017; Cui et al., 2018; Rouch et al., 2018), we did not detect a significant association between continuous aortic PWV (m/s) and the risk of dementia (**Figure 4**, **Supplementary Figure 3**).

Meta-Regression Analysis

The meta-regression analysis showed that age, the proportion of males, and MBP significantly increased the association between aortic PWV and memory or processing speed in all participants (**Supplementary Table 5**). However, the significance disappeared after excluding studies investigating this association under specific diseases or conditions (**Supplementary Table 6**). Besides, we found that age significantly increased the risk of cognitive impairment of high vs. low aortic PWV in all participants (**Supplementary Table 5**, **Supplementary Figure 4**). The influence of age on this association remained after excluding participants with specific diseases or conditions (**Supplementary Tables 5**, **6**). However, neither age nor other listed variables had an impact of the association between continuous aortic PWV (m/s) and cognitive impairment risk (**Supplementary Tables 5**, **6**).

DISCUSSION

This comprehensive systematic review and meta-analysis showed that aortic stiffness measured with aortic PWV was inversely associated with the function of memory and processing speed, and aortic PWV was an independent predictor for cognitive impairment. Besides, age could increase the association between high aortic PWV and the risk of cognitive impairment.

The association between aortic stiffness and memory was thought to be mainly due to the microvascular injury in deep white matter (Mitchell et al., 2011; Kloppenborg et al., 2014; Cooper et al., 2016), and the statistical mediation analysis showed that cerebrovascular resistance (52% of indirect effect) and white matter hyperintensities (41% of indirect effect) accounted for major observed relation between cfPWV and memory (Cooper et al., 2016). Besides, the excessive pulsatile damage from aortic stiffness to the medial temporal lobe and hippocampus may

TABLE 1 | Sensitive analyses of association between aortic PWV and cognitive function.

Outcomes	Analysis 1			Analysis 2			Analysis 3			Analysis 4		
	Studies	Participants	*r* (95% CI)	Studies	Participants	*r* (95% CI)	Studies	Participants	*r* (95% CI)	Studies	Participants	*r* (95% CI)
Attention	9	5,198	−0.174 (−0.313, −0.027)*	7	5,041	0.006 (−0.051, 0.063)	5	4,335	0.031 (−0.009, 0.071)	4	4,279	0.034 (−0.012, 0.079)
Executive Function	13	10,070	−0.051 (−0.118, 0.017)	11	9,913	−0.006 (−0.061, 0.050)	9	9,207	0.017 (−0.043, 0.076)	8	9,151	0.003 (−0.054, 0.059)
Global cognitive function	6	1,999	−0.122 (−0.218, −0.024)*	6	1,999	−0.122 (−0.218, −0.024)*	4	1,293	−0.113 (−0.255, 0.034)	3	1,127	−0.055 (−0.143, 0.033)
Memory	14	12,328	−0.061 (−0.101, −0.020)*	12	12,171	−0.034 (−0.063, −0.004)*	10	11,465	−0.022 (−0.041, −0.003)*	9	11,409	−0.022 (−0.042, −0.001)*
Processing speed	13	8,775	−0.119 (−0.190, −0.047)*	11	8,618	−0.056 (−0.094, −0.017)*	10	8,476	−0.048 (−0.081, −0.016)*	9	8,420	−0.048 (−0.082, −0.014)*
Visuospatial ability	5	1,280	−0.085 (−0.300, 0.140)	5	1,280	−0.085 (−0.300, 0.140)	4	1,138	−0.073 (−0.328, 0.192)	4	1,138	−0.073 (−0.328, 0.192)

Analysis 1 included all the eligible studies; Analysis 2 excluded studies with participant of chronic kidney disease based on analysis 1; Analysis 3 further excluded studies with participants of hypertension based on analysis 2; Analysis 4 further excluded studies with participants complaining loss of memory based on analysis 3 and just included studies with participants from general older adults.

*$P < 0.05$.

contribute to poor memory as well (Wåhlin et al., 2014; Lilamand et al., 2016). It was reported that the major brain structural changes caused by aortic stiffness was white matter lesion (Tarumi et al., 2015; van Sloten et al., 2015), which was thought to preferentially cause the decline of processing speed and executive function (Kloppenborg et al., 2014; Biesbroek et al., 2017). However, we just detected a significant correlation between aortic stiffness and processing speed but not executive function. The non-significant association between aortic stiffness and executive function in our analysis may due to the heterogeneity among studies or the non-linear association (Nilsson et al., 2014; Zhong et al., 2014; Dixon et al., 2020), which prevented the detection of significant linear association using the correlation coefficient as the effect size. Several pathophysiologic mechanisms might be involved in the diminishing effect of aortic stiffness on cognitive function. Besides the microvascular damage caused by excessive pulsatile load (Mitchell et al., 2011; van Sloten et al., 2015; Lilamand et al., 2016), the reduced cerebral perfusion due to aortic stiffness may aggravate the white matter lesion and brain atrophy (Tarumi et al., 2011). Moreover, the Aβ deposition was found to play an important role in the association between aortic stiffness and cognitive impairment (Hughes et al., 2013, 2018).

Besides increased risk for cognitive impairment, our results indicated that the high aortic stiffness increased the risk of dementia by 2-fold. But the significant association disappeared after excluding the study of Rouch et al. We speculated that this may due to the limited studies for synthesized analysis and short follow-up years. Although the study by Rouch et al. just followed up in a relatively short period of 4.5 years, including specific participants of MCI in their study would make it more sensitive to detect the independent association between aortic stiffness and risk of dementia (Rouch et al., 2018).

Additionally, the meta-regression analysis indicated that age increased the risk of cognitive impairment caused by high aortic PWV. This is consistent with studies that showed that the interaction of PWV and age (PWV × age) increased the magnitude of associations between PWV and cognitive performance (Elias et al., 2009; Pase et al., 2016b; Menezes et al., 2019). Since both aortic stiffness and cognitive impairment are age-related changes (Langa and Levine, 2014; Iulita et al., 2018), there should be vicious loop between age, aortic stiffening, and cognitive decline. Thus, it is important to make early interventions to prevent progression of aortic stiffness to delay cognitive impairment and dementia. A recently published meta-analysis using Cohen's d index as effect sizes also showed a negative relationship between arterial stiffness with executive function and memory, but they did not find age to modify the strength of this association (Alvarez-Bueno et al., 2020). This might because they included studies with PWV index involving the stiffness of the peripheral arteries (i.e., baPWV), which could diminish the direct effect of age on aortic stiffness. Besides, they did not analyze the association between PWV and risk for cognitive impairment, a cumulative result of cognitive function decline, in which the effect of age on this association should be more pronounced.

A few limitations should be noticed. First, the limited amount of studies included in the meta-analysis may prevent the full

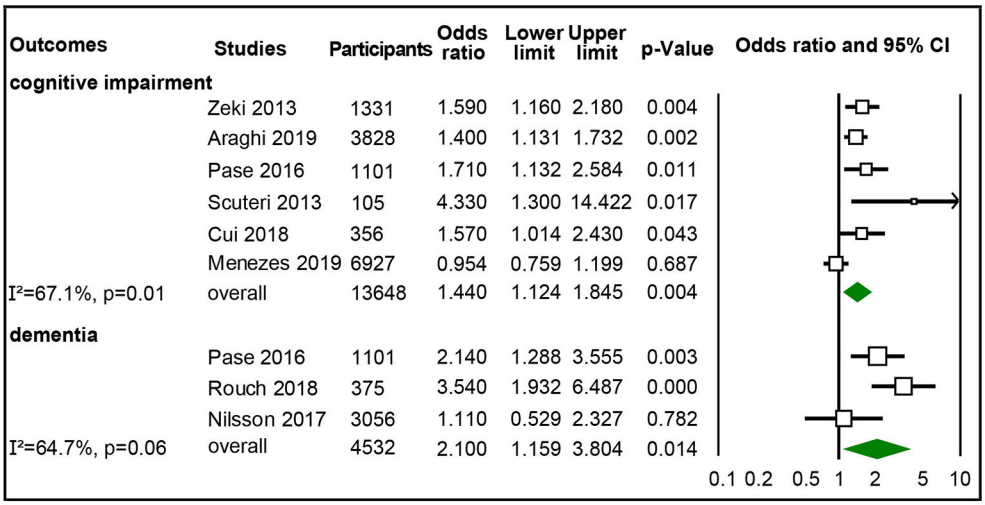

FIGURE 3 | Forest plot of association between categorical aortic pulse wave velocity (PWV) (high vs. low) and cognitive impairment and dementia.

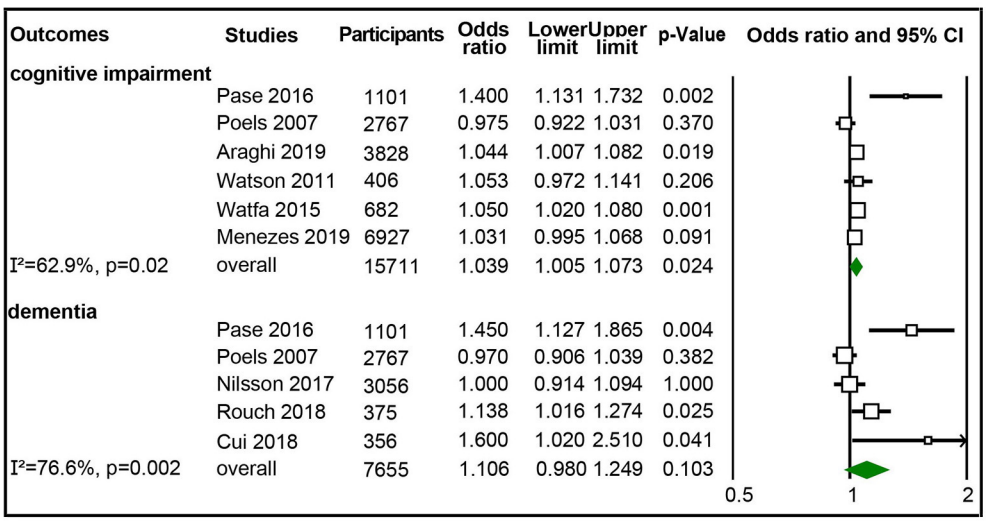

FIGURE 4 | Forest plot of association between continuous aortic pulse wave velocity (PWV) (m/s) and cognitive impairment and dementia.

interpretation of the results. But our results supported that the high aortic stiffness at least associated with memory and processing speed decline and increased cognitive impairment risk. Second, the variables for adjustment varied from study to study, which increased the heterogeneity of studies as well as the variation of true effect size. And it is possible that residual confounding remains in some studies, which may prevent detecting the statistical significance for some domains of function that would have had a significant association with aortic stiffness. But this should have less influence on the significance of association that we have already found in this meta-analysis. Finally, we did not pool the correlation of aortic PWV and domains of cognitive function in longitudinal studies due to the limited available data. Since there were studies that showed a faster decline in several domains of cognitive function with higher aortic stiffness (Hajjar et al., 2016; Menezes et al., 2019), it would be more convincing if we further confirmed this longitudinal association in our quantitative analysis.

CONCLUSION

In summary, this systematic review and meta-analysis suggested that aortic stiffness is inversely associated with cognitive function, an independent predictor for cognitive impairment, and a potential risk factor for dementia, especially in the elderly. This study supports the assessment of the aortic PWV in routine clinical practice for primary prevention to slow down early the progression of cognitive decline.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

QL and JF: study conception and design. NC and LH: supervision and administration. QL, JF, MM, and NC: writing-manuscript preparation and intellectual input. QL, JF, CC, NC, and LH: data interpretation. QL, JF, CC, and SD: data analysis. QL, JF, CC, SD, LG, JB, and YL: experiment or data collection. 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.2021.680205/full#supplementary-material

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Inflammatory Biomarkers Aid in Diagnosis of Dementia

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Dual pathology of Alzheimer's disease (AD) and vascular cognitive impairment and dementia (VCID) commonly are found together at autopsy, but mixed dementia (MX) is difficult to diagnose during life. Biological criteria to diagnose AD have been defined, but are not available for vascular disease. We used the biological criteria for AD and white matter injury based on MRI to diagnose MX. Then we measured multiple biomarkers in CSF and blood with multiplex biomarker kits for proteases, angiogenic factors, and cytokines to explore pathophysiology in each group. Finally, we used machine learning with the Random forest algorithm to select the biomarkers of maximal importance; that analysis identified three proteases, matrix metalloproteinase-10 (MMP-10), MMP-3 and MMP-1; three angiogenic factors, VEGF-C, Tie-2 and PLGF, and three cytokines interleukin-2 (IL-2), IL-6, IL-13. To confirm the clinical importance of the variables, we showed that they correlated with results of neuropsychological testing.

Keywords: Alzheimer's disease, vascular cognitive impairment and dementia, inflammation, diffusion tensor imaging, cerebrospinal fluid, white matter disease, machine learning

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HIGHLIGHTS

- Multimodal biomarkers facilitate biological classification of mixed dementia in cognitively impaired patients with Alzheimer's disease and vascular disease.
- Machine learning model aids in classification of this diverse group of patients by narrowing down a large number of biomarkers to those that are most important.
- Relevance of this approach is shown by correlation of those important biomarkers with neuropsychological test results.
- Proteases, angiogenic factors and cytokines in various patient groups suggest pathophysiology.

INTRODUCTION

The need to identify patients with dementia and to determine the cause of cognitive decline during life has greatly increased as a consequence of the increase in dementia due to the aging of the world's populations. Alzheimer's disease (AD) and vascular cognitive impairment and dementia (VCID) are the major causes of dementia (Snyder et al., 2015). While the need for earlier diagnosis to facilitate treatment is generally recognized, the overlapping of symptoms, beginning in midlife, has confounded attempts at early diagnosis, promoting a search for biomarkers to aid this process

(Jorgensen et al., 2020). While AD and VCID are the most common single forms of dementia, autopsy series show that mixed dementia (MX) due to dual pathologies is most common, making it important to be able to diagnose MX during life (Schneider et al., 2007; Toledo et al., 2013; Karanth et al., 2020).

Biomarkers facilitate the detection of multiple pathological processes that accumulate with aging; they provide a window on the earliest events at a time when separation of patients from effects of aging using clinical criteria alone is challenging (Sonnen et al., 2011). Biological criteria for diagnosing AD have been published in the National Institute of Aging-Alzheimer's Association (NIA-AA) research framework, which is based on the use of pathological proteins, amyloid-β (Aβ) and phosphorylated tau (pTau) in either the CSF or brain as shown by positron emission tomography (PET) along with evidence of neurodegeneration; the authors predicted that other pathological processes, such as vascular disease, could be added to the formula at a later time as new biomarkers are discovered (Jack et al., 2018). We adopted this approach to diagnose patients with MX involving dual pathology by combining white matter injury on MRI with the biological diagnosis of AD obtained from CSF. Then, to better understand the underlying pathophysiology in the expanded groups of AD, VCID, and MX, we used multiplex assays of biomarkers in CSF and blood (Craig-Schapiro et al., 2011; Pillai et al., 2019; Whelan et al., 2019; Elahi et al., 2020; Winder et al., 2020). Because of the large amount of information obtained from the multiplex assays, we used a machine-learning algorithm, Random Forests, to identify the variables of maximal importance for classifying patients into the three dementia groups. Finally, we demonstrated that the important variables had clinical relevance by correlating them with neuropsychological test results.

METHODS

Patients and Biomarkers

The study was approved by the University of New Mexico Human Research Review Committee. All patients gave informed consent to study procedures including a lumbar puncture. Patients were recruited from neurology clinics at the University of New Mexico Hospital and the Albuquerque Veterans Administration Hospital. Patients underwent neurological examinations, neuropsychological tests, a lumbar puncture to collect CSF, a venipuncture to collect blood plasma, and a MRI. All subjects were at least 50 years old. Controls for the imaging studies were recruited from community-based volunteers. Control CSF came from patients undergoing spinal anesthesia for orthopedic surgery. ApoE genotyping was not performed.

Cognitive Testing

Cognitive tests were administered by a trained research psychologist (JP) or trained research coordinators and scored according to standard procedures. Standardized (T) scores were calculated for each test. Averaged composite T-scores were calculated for separate cognitive domains: memory (Hopkins Verbal Learning Test-Delay, Rey Complex Figure Test-Long Delay), executive function [Digit Span Backwards, Trail Making Test B, Stroop, Controlled Oral Word Association (FAS)], attention (Digit Span Forward and Trial Making Test A), language [Boston Naming 60 item test, Controlled Oral Word Association (Animal)], and processing speed (Digit Symbol and Symbol Search, both based on WAIS-III). An overall cognitive composite score was derived as the mean of individual domain T-scores. Control participants for the MRI studies underwent the same neuropsychological test battery.

Blood and CSF Studies

Phosphorylated Tau and Aβ

A number of biomarkers were measured in CSF and blood plasma. CSF biomarkers were obtained by lumbar puncture performed in the morning after fasting by one of the authors (JCA). Blood draws were performed during the same patient visit. Samples were centrifuged, aliquoted, and stored at −80°C for later analysis.

Levels of CSF Tau protein phosphorylated at threonine position 181 (pTau) were measured using the Innotest Phospho-Tau (181P) ELISA (Fujirebio US; Malvern PA). Prior to analysis, all CSF underwent one freeze-thaw cycle. Assays were performed according to manufacturer protocols and were read with a Bio-Tek multimodal plate reader with absorbance at 450 nm. The output data were used to quantify the concentrations based on the supplied in-assay standard curve. We measured β-amyloid$_{1-42}$ (Aβ_{1-42}) and β-amyloid$_{1-40}$ (Aβ_{1-40}) to calculate the Aβ_{1-42}/Aβ_{1-40} ratio (V-PLEX Aβ Peptide Panel 1–6E10; MesoScale Discovery MSD, Rockville, Maryland). The output data were used to quantify the concentrations based on the 2-fold sample dilation and the supplied in-assay standard curve. All data were expressed as pg/mL, though the ratio is unitless.

Matrix Metalloproteinase, Angiogenesis, and Proinflammatory Assays

The biomarkers we selected were based on the MesoScale Discovery (MSD) multiplex assay kits. These have been adapted for use by the MarkVCID consortium. Matrix metalloproteinases (MMP-1, MMP-2, MMP-3, MMP-9, MMP-10) were measured with two ELISA kits (MSD; MMP 2-Plex and MMP 3-Plex). Angiogenic growth factors were measured by ELISA (MSD; Angiogenesis Panel 1). Similarly, multiple proinflammatory factors were measured with the Proinflammatory Panel 1 (MSD). For these assays, all CSF samples were run undiluted while all plasma samples were diluted 2-fold except for the MMP 3-Plex, in which case the plasma samples were diluted 10-fold. All data were expressed as pg/mL.

Fluid Sample Analyses

Assays were performed using established protocols on an MSD Quickplex SQ 120 plate reader, followed by analysis performed in the MSD Discovery Workbench 4.0 software that was used to quantify analyte concentrations and all data were expressed as pg/mL. Protein markers measured with MSD assays were subjected to intra-plate variability tests which calculated the coefficient of variation (CV), as determined by duplicate runs for each sample. Samples with a CV $\geq$ 15% were removed from further analysis. Another assessment involved two CSF

and two plasma pooled control samples run in duplicate on the same plate in all assays. These control samples were held to the same intra-plate CV (≥15%) and were also assessed for plate-to-plate variability.

MRI Studies

To obtain information on the integrity of the white matter, we used MRI scans that were performed on a Siemens 3T scanner. Initial scans were performed on a 12-channel radio frequency (RF) coil and later scans were acquired with a 32-channel RF coil. The imaging parameters with the two RF coils were closely matched. The 3D MPRAGE sequence had TR = 2530 ms, four echoes, and TI = 1200 ms with an acquisition time of 6.5 min. The 3D FLAIR sequence had a TR = 6000 ms, TE = 427 ms, and TI = 2000 ms. The diffusion data were collected with a FOV = 224, 2 mm isotropic resolution, and 72 slices for both RF coils. On the 12-channel coil, the diffusion protocol had a single-shell of b-value = 800 s/mm^2 with 30 volumes collected with different gradient directions and five volumes with b = 0. The acquisition time was 6.5 min. The experiments done on the 32-channel coil used a CMRR multi-band sequence, which enabled us to collect more gradient directions. On the 32-channel coil, we collected three shells with a maximum b-value = 3000 s/mm^2, 155 volumes with different gradient directions, and eight volumes with b = 0. The acquisition time was 12.5 min.

White matter hyperintensity (WMH) volume was calculated from FLAIR images based on JIM software (www.xinapse.com). The diffusion images were corrected for motion, distortion, and mean diffusivity (MD), and fractional anisotropy (FA) was calculated (www.fmrib.ox.ac.uk).

Statistical Methods

Patient data underwent transformation, outlier detection, selection, and missing value imputation. Fluid variables measuring concentration were transformed to the $\log_2$ scale to mitigate right skewness; the resulting roughly symmetric distributions satisfy statistical assumptions and afford straightforward visual comparisons. Univariate outliers were identified by visual inspection and replaced with a missing value code (to be imputed later) if it was likely due to measurement error by outlying from the majority of points by roughly greater than twice the range of the majority of points on the variable's original scale. This resulted in removing roughly one or two values from about half of the features, a total of 54 values over 55 features. Observations were filtered to include patients who did not have missing values for more than 30% of the features, retaining 86 observations for our three primary diagnosis groups and controls. Missing values were imputed using the method "Multivariate Imputation by Chained Equations" via the mice R package (van Buuren and Groothuis-Oudshoorn, 2011).

Patient classification based on fluid features used Random forests (RF), a supervised ensemble machine learning algorithm that is based on classification trees (Breiman, 2001) in which many classification trees (a "forest") are fit on bootstrapped samples of the original observations and randomly selected subsets of features. Each tree partitions the data based on a random subset of predictor variables in such a way as to obtain optimal separation between the diagnosis groups. RF provides a measure of variable importance (VIMP) for prediction accuracy, which is interpreted as the increase in prediction accuracy for decision trees within the forest with a given feature (variable) compared to decision trees without that feature; VIMP can be negative. RF also provides the marginal probability of group identity for values of each variable, and the bootstrap aggregating (bagging) technique keeps RF from overfitting. Furthermore, RF can perform multiclass prediction, automatically employs external cross-validation by predicting a patient diagnosis based on trees estimated without that patient, has minimal distributional model assumptions and is easy to implement. Variable selection improves classification and the reduced models based on classification accuracy are presented. RF was performed in R software using the package "randomForestSRC" function "rfsrc" with 10,000 trees (Ishwaran and Malley, 2014).

RESULTS

The three neurologists arrived at a consensus clinical diagnosis based on clinical history, neuropsychological tests and MRI FLAIR results. Initially, the results of the diffusion tensor MRI and some results of the CSF and blood studies were not available: AD CSF biomarkers were done initially, and the subsequent biomarkers in CSF and plasma were from the proteases, angiogenic factors and cytokines. Since VCID includes a number of forms of vascular disease, we focused on the small vessel form, subcortical ischemic vascular disease (SIVD), which can be detected by MRI and has a progressive course, making it more amenable to clinical trials (Pantoni, 2010). The diagnoses used were: (1) SIVD, indicating normal CSF AD proteins and abnormal white matter on FLAIR; (2) AD patients had abnormal CSF AD proteins and normal white matter; (3) MX patients had both AD proteins and white matter injury. We excluded large vessel infarcts and single strategic strokes without white matter injury. We also excluded several patients with abnormal FLAIR MRI without a cognitive deficit; they were considered white matter changes of aging.

Demographic and Cognitive Features

Eighty-six (86) subjects had complete data permitting a full analysis; the numbers in each category are shown in **Table 1**. Forty-five percent of the patients were female. The median patient age was 72 years; MX patients were 7 years older than either the SIVD or AD groups ($p = 0.010$) (**Table 1**). Controls performed significantly better across cognitive domains than all patient groups. Memory function in the AD group was lower than in SIVD and MX (30.0 vs. 44.0 and 36.0, $p < 0.001$). There were no significant between-group differences for other cognitive domains (T-executive, T-attention, T-language, and T-processing) and composite cognitive score (T-overall).

For the biomarkers, we performed several analyses. First, we compared the controls against the three patient groups combined using each of the CSF and plasma features; this showed that there were significant differences in the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio and pTau. In addition, CSF values for MMP-1, MMP-9, and MMP-10, VEGF-D, Flt-1, PlGF, IL-8, IL-10, and IL-13 were significantly

TABLE 1 | Features that are significant between diagnosis groups with Control reference values.

Features Demographics	SIVD (*N* = 17)	Mixed (*N* = 15)	AD (*N* = 19)	*p*	Control (*N* = 35)
Age at baseline	68.0 [60.0;74.0]	76.0 [73.0;79.5]	70.0 [66.0;74.0]	0.010	65.0 [62.0;68.5]
Sex				0.039	
Female	11 (64.7%)	3 (20.0%)	9 (47.4%)		24 (68.6%)
Male	6 (35.3%)	12 (80.0%)	10 (52.6%)		11 (31.4%)
Neuropsychological					
T-memory	44.0 [38.0;60.5]	36.0 [25.0;41.0]	30.0 [21.0;35.5]	0.000	**56.0 [46.5;61.5]
Alz disease proteins					
A Beta 42/40 ratio*	7.30 [3.45;9.30]	3.30 [2.85;4.10]	4.70 [4.00;6.15]	0.010	8.70 [6.80;10.40]
P-Tau	52.0 [44.0;56.0]	97.0 [64.0;121.0]	71.0 [53.0;100.5]	0.001	54.0 [42.0;64.0]
Protease					
MSD CSF MMP-10	52.8 [43.1;69.2]	98.9 [91.1;119.6]	81.6 [75.6;101.9]	0.001	46.5 [34.1;67.8]
MSD Plasma MMP-3*	14.4 [11.7;20.7]	23.7 [17.0;34.2]	20.8 [12.0;26.7]	0.054	15.1 [11.4;21.2]
Angiogenesis					
CSF VEGF-C	25.3 [14.2;40.6]	25.1 [18.6;34.8]	12.7 [5.0;15.2]	0.008	19.8 [13.4;28.3]
CSF Flt-1	67.9 [60.6;87.8]	110.8 [81.9;125.4]	82.4 [67.5;93.4]	0.051	68.9 [52.2;87.7]
CSF PlGF	28.5 [16.7;41.3]	33.4 [23.9;59.7]	21.4 [18.5;23.3]	0.018	16.0 [12.1;22.0]
Cytokine					
CSF IL-2*	7.94 [6.30;9.75]	4.81 [4.50;9.63]	4.50 [4.11;7.23]	0.075	4.50 [4.50;5.95]
Plasma IL-13	0.914 [0.395;1.995]	2.529 [1.391;7.923]	1.636 [0.606;4.795]	0.043	2.552 [1.070;4.842]

Numeric summaries are median and IQR bounds [Q1, Q3] (25th and 75th percentiles), or categorical frequencies and percentages. Three values were scaled for presentation in the table [, A Beta 42/40 ratio (value*100), MSD Plasma MMP-3 (value/1000), CSF IL-2 (value*100)]. Note that the Controls with neuropsychological measurements (**, N = 199) were distinct from those with fluid measurements analyzed in the manuscript and are included as an external reference. P-values reported from the Kruskal-Wallis test for continuous data and from the chi-square test with continuity correction for categorical data to compare between the three diagnosis groups.*

different from controls (**Figure 1**). In plasma, MMP-1, VEGF-A, VEGF-C, PlGF, bFGF, IL-8 and TNF-α were significantly different from controls (**Figure 1**). Comparing controls with each patient group revealed many differences in both CSF and plasma (**Figure 1**). Comparing between the three groups revealed a number of significant differences between the groups in both the CSF and plasma, which tended to be much more prominent in CSF (**Figure 1**).

Alzheimer's Biomarker Features

The $A\beta_{1-42}/A\beta_{1-40}$ ratio was lower in MX than in SIVD or AD ($p = 0.010$), while pTau was higher in the MX than in SIVD or AD ($p = 0.001$) (**Table 1**; **Supplementary Figure 2**). The $A\beta_{1-42}/A\beta_{1-40}$ ratio was negatively correlated with age but not with any of the cognitive features, while pTau was positively correlated with age, attention, executive function, and processing speed (**Figure 2**).

Protease Features

CSF MMP-10 was highest in MX and AD relative to SIVD ($p = 0.001$) (**Table 1**; **Supplementary Figure 3**). No other median differences between patient groups were observed, including CSF MMP-1,−2,−3, and−9, and Plasma MMP-1,−2,−9, and−10. CSF MMP-10 positively correlated with age and negatively with memory scores. There were no significant between-group differences in plasma MMPs except plasma MMP-3, which showed a trend toward significance ($p < 0.054$). Plasma MMP-2 negatively correlated with most of the cognitive measures (attention, executive function, language, and overall), and plasma MMP-3 positively correlated with age (**Figure 2**). CSF MMP-3 and MMP-10 correlated with plasma values for both proteases (**Figure 2**).

Angiogenesis Features

CSF Placental growth factor (PlGF) was elevated in MX relative to AD ($p = 0.018$) and CSF VEGF-C was lower in AD relative to SIVD and MX ($p = 0.008$) (**Table 1**; **Supplementary Figure 4**). No other median differences between patient groups were observed in CSF for the angiogenic features VEGF-A, VEGF-D, Tie-2, Flt-1, and bFGF. In addition, there were no significant between-group differences in plasma angiogenesis features (VEGF-A, VEGF-C, VEGF-D, Tie-2, Flt-1, PlGF, and bFGF). CSF PlGF was the only angiogenesis factor correlated (positively) with age. CSF VEGF-A was positively correlated with language, and CSF VEGF-C is positively correlated with memory (**Figure 2**). Plasma Tie-2 is positively correlated with language and memory and Plasma Flt-1 is negatively correlated with the overall cognitive features. CSF VEGF-D and PlGF correlated with plasma values (**Figure 2**).

Cytokine Features

None of the CSF cytokine features showed median differences (IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α). Plasma IL-13 was lower in SIVD relative to MX ($p = 0.043$) (**Table 1**; **Supplementary Figure 5**). No other median differences between patient groups were observed for plasma.

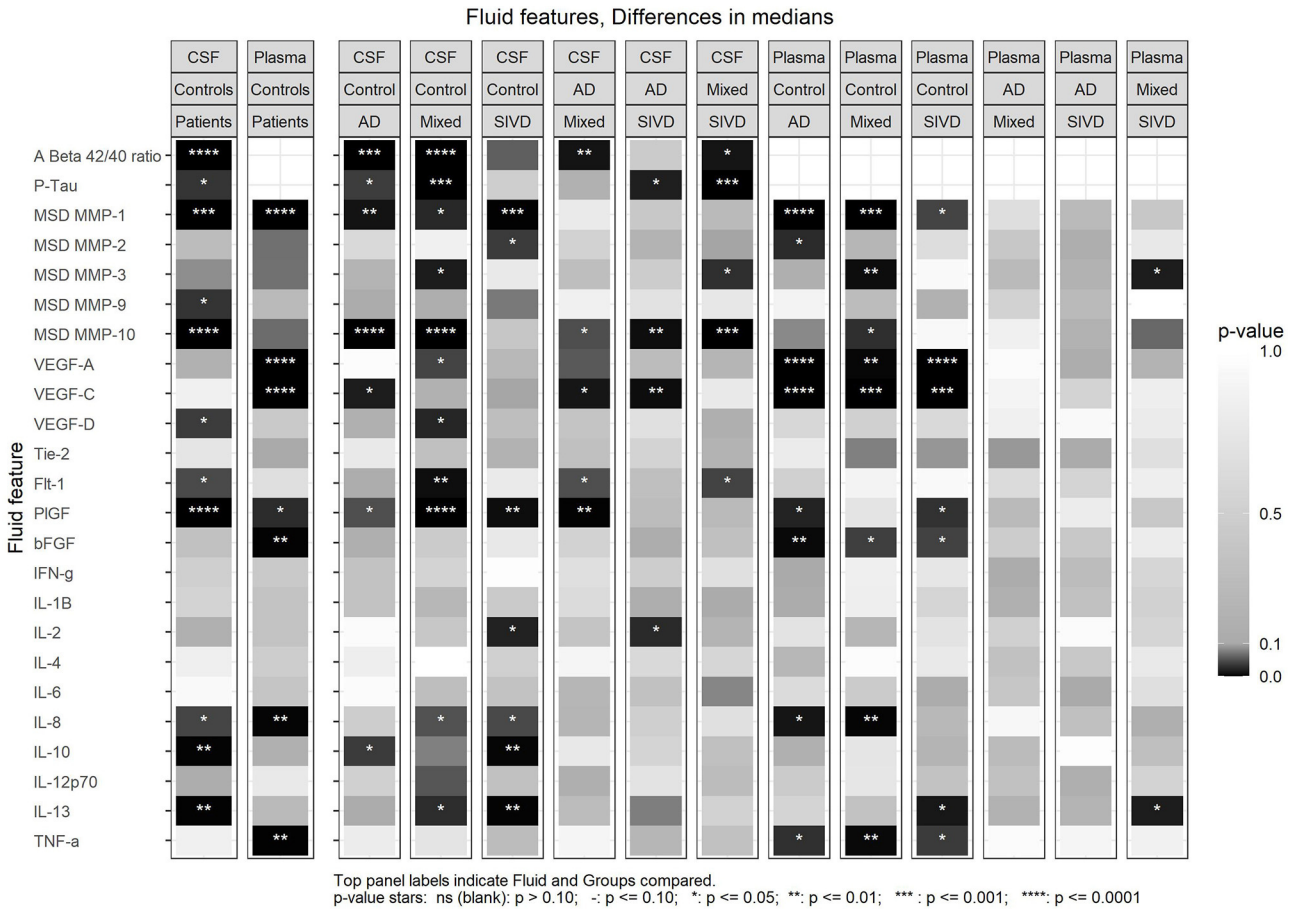

FIGURE 1 | Differences in medians for all CSF and Plasma features between patient and control groups indicated by *p*-value. The first set of two panels (left) compare the combined patients groups with the control group for CSF and Plasma. The next sets of panels compare the Control group with each patient group then AD group vs. MX and SIVD and MX vs. SIVD. Some of the data is included in **Table 1** and plots of all comparisons are in **Supplementary Figures 1–5**.

CSF IL-1β was positively correlated with executive function, language, and overall cognition, CSF IL-2 was positively correlated with memory, TNF-α was positively correlated with language, memory, and overall cognition, and CSF IL-13 was negatively correlated with attention, executive function, and processing speed (**Figure 2**). Plasma IL-1β, IL-2, IL-4, and IL-10 were positively correlated with language, plasma IL-4 alone was positively correlated with overall cognition, while plasma IL-6 was negatively correlated with memory. Plasma TNF-α was positively correlated with age.

Biomarker Stratification of Patients Into SIVD, MX, and AD

We performed supervised classification using Random Forests with subsets of features from CSF and plasma to classify diagnosis groups in three ways (SIVD vs. AD, SIVD vs. AD and MX, and SIVD vs. MX vs. AD). We considered three broad scenarios. First, we considered "All Factors" of CSF and plasma together, as well as CSF and plasma features separately. Second, we considered the separate "CSF Factors" of AD Proteins, Proteases, Angiogenesis, and Cytokines. Third, we considered the separate "Plasma Factors" of Proteases, Angiogenesis, and Cytokines. To improve classification accuracy, each model is first fit using the complete set of features and then we perform manual stepwise backward selection based on variable importance (VIMP) until all remaining variables have reliably positive VIMP values. The classification accuracy results for all scenarios are summarized in **Figure 3** with associated ROC curves for two-group models in **Figure 4**, then the variable importance values for the "All Factors" scenario are in **Table 2**.

In the "All Factors" scenario the All features (CSF and Plasma) and CSF alone features have similar accuracies of roughly 77%, 88%, and 67% for the three diagnosis groups, while Plasma alone features had much lower accuracies (56%, 73%, and 47%). Therefore, the Plasma features do not add additional classification benefits to the CSF features (**Figure 3**, left; **Table 2**, top row). Additionally, a sensitivity analysis was performed by excluding the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio and pTau from the modeling; accuracies were similar for both All features (75%, 84%, and 70%) and CSF features (75%, 84%, 67%). The ROC Curves indicate the optimal threshold (**Figure 4**, circle) and the

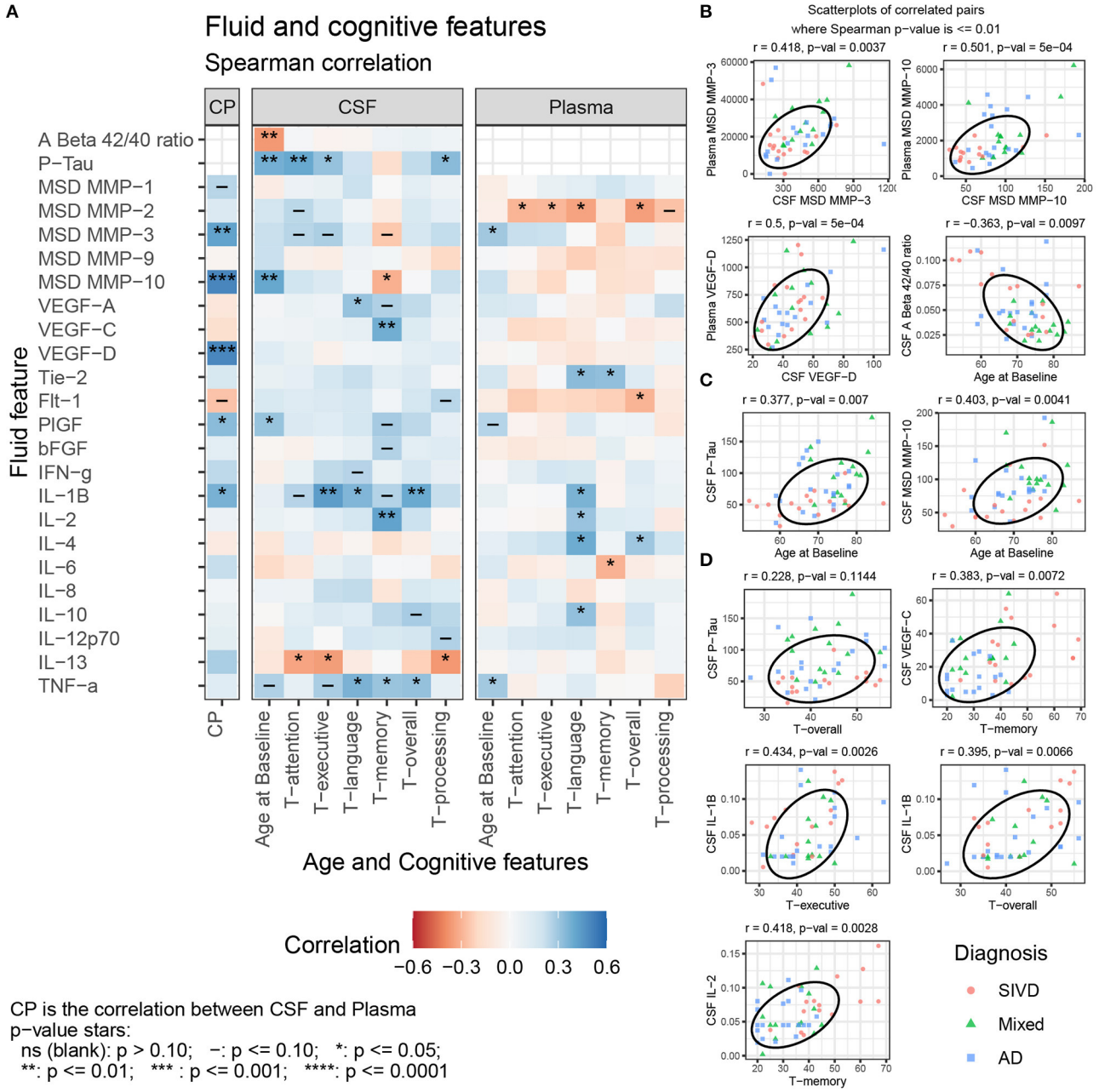

FIGURE 2 | (A) Spearman correlation of CSF and Plasma features with Age and Cognitive features for SIVD, Mixed, and AD patients. Selected scatterplots illustrate relationships at the $p \leq 0.01$ significance level: **(B)** same fluid features CSF vs. plasma, **(C)** fluid features with Age, and **(D)** fluid features with cognitive features.

area under the curve (AUC) as an indication of the quality of the classifier, with values closer to 1 being better. For the two two-group models, the All Factor and CSF Factor models have AUC values between 0.83 and 0.88, but the Plasma Factor model has AUC values between 0.70 and 0.74 (**Figure 4**). Additionally, the sensitivity analysis excluding the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio and pTau from the modeling were similar, between 0.84 and 0.86.

Variable importance (VIMP) values for the "All Factors" scenario for the three diagnosis group definitions are given in **Table 2**. The features contributing the most to accurate classification are similar for the All features and CSF alone features, with the most important being CSF MMP-10, pTau, and VEGF-C. Less important CSF features also include CSF PlGF, Tie-2, VEGF-D, IL-2, IL-13, and IL-1β. When CSF variables are in the model, demographic features of Age and Sex actually worsen classification accuracy (negative VIMP values). The most important Plasma-only features are Plasma Tie-2, MMP-1, and MMP-10. Less

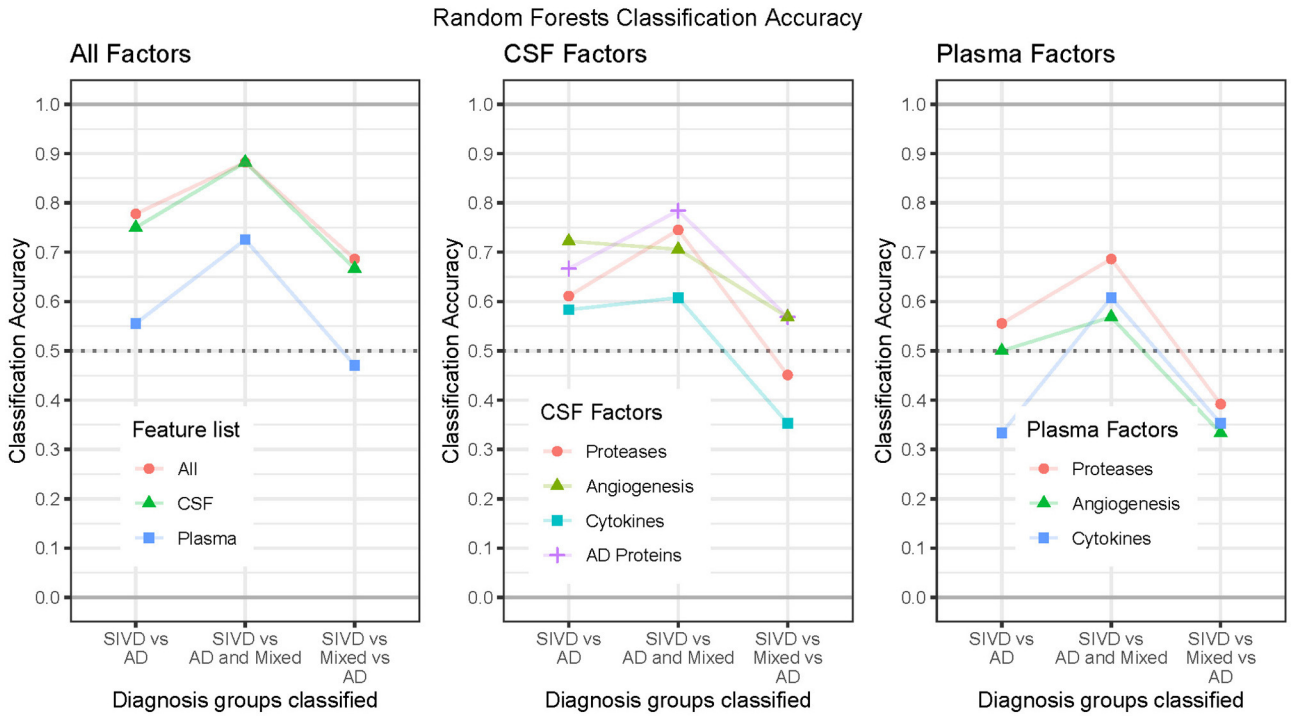

FIGURE 3 | Classification accuracy by feature list considered and diagnosis grouping.

important Plasma features include Age, MMP-3, IL-13, and IL-6.

In the "CSF Factors" scenario, separate models were considered for each set of features. The classification accuracy indicates that AD biomarkers and Angiogenesis factors are more predictive of diagnosis category than Proteases, with the Cytokines being the least predictive (**Figures 3**, **4**). In the "Plasma Factors" scenario, Proteases added some predictive ability, with Angiogenesis and Cytokines providing almost no predictive ability (**Figures 3**, **4**).

Features that did not improve classification (**Table 2**) because they contributed a classification of <0.3% for any diagnostic groups included Sex; Protease CSF MMP-1,−2, and−3, and Plasma MMP-9; Angiogenesis CSF VEGF-D, Flt-1, bFGF, and Plasma VEGF-A, VEGF-C, VEGF-D, Flt-1, and PlGF; and Cytokine CSF IFN-γ, IL-4, IL-6, IL-8, and IL-10, and Plasma IL-1β, IL-2, IL-4, IL-8, IL-12p70, and TNF-α.

DISCUSSION

Using the biological diagnosis to diagnose AD and MRI white matter injury to indicate vascular disease, we identified during life a group of cognitively impaired patients with dual pathology. Having separated patients into AD, VCID, and MX, we then used a large number of biomarkers determined in CSF and plasma with multiplex assay kits to determine the biomarkers of maximal importance. Finally, we used neuropsychological testing to validate the biomarkers identified. An important part of the study was the use of a statistical machine learning method to determine the relative importance of the biomarkers. In this manner, our study was a step in the realization of precision medicine for dementia studies.

We studied the variable importance of biomarkers in a diverse group of cognitively impaired patients classified into AD, SIVD, and MX. We included the MX group by expanding the AD biological research criteria to include a vascular factor to identify dual pathology patients (Jack et al., 2018). Commercially available multiplex assays identified proteases, angiogenic growth factors, and cytokines in CSF and plasma. A machine learning method, Random Forests, showed that the CSF variables of maximal importance, were MMP-1, MMP-3, MMP-10, VEGF-C, PlGF, IL-2, IL-6, and IL-13. By initially classifying patients into diagnostic groups, we were able to determine the levels of the biomarkers in each group, and showed that the highest values tended to be in the dual pathology patients. Our results show that the availability of multiplex assays to measure biomarkers in CSF and plasma during life provides data to compare with neuropathological studies, confirming the importance of multiple neuropathological processes in cognitively impaired patients (Toledo et al., 2013; Karanth et al., 2020).

The classes of biomarkers that we studied had inflammation and repair in common. We found that those with dual pathology had the highest values for the biomarkers, which is consistent with studies that show an acceleration of cognitive decline suspected to be due to the cumulative effects of the different pathological processes

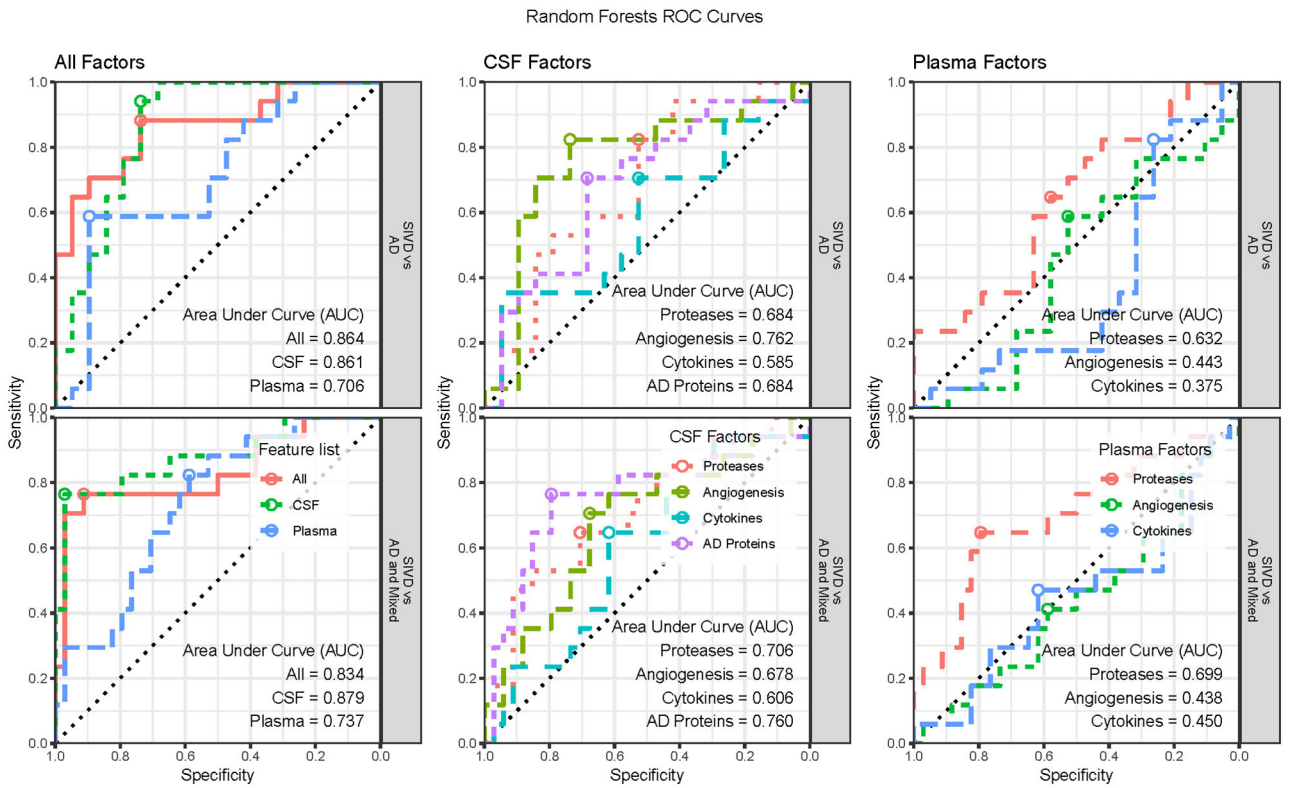

FIGURE 4 | Receiver Operator Characteristic (ROC) Curves for the two-group classification scenarios by feature list and groups classified. Three-group ROC Curves are not available. The optimal threshold is indicated with a circle and the area under the curve (AUC) statistic is provided for each set of features.

(Snowdon et al., 1997; Karanth et al., 2020). To obtain this data, we expanded the biological formula for AD to include a vascular factor, permitting the identification of patients with relatively pure AD and VCID as well as a group with dual pathology (Jack et al., 2018). Our results concur with other pathological and CSF studies that have identified proteolytic, angiogenic and inflammatory biomarkers as central features of the pathobiology of both AD and VCID (Tarkowski et al., 2002; Desai et al., 2009; Biron et al., 2011). Our results suggest that the MMPs and the angiogenic factors act together. The three MMPs that were most prominent, MMP-1, MMP-3, and MMP-10, are inducible enzymes with transcription factors, AP-1 and NF-kB, that would be important in inflammation; MMP-2, which was identified in plasma, but not CSF, is a constitutive enzyme that may have other roles (Candelario-Jalil et al., 2009).

Angiogenic factors have been identified in a number of studies in AD, but it is unclear whether they participate in injury or repair. It is possible to conceptualize a pathological scenario in which the growth of blood vessels begins with the proteolytic disruption of the extracellular matrix by one or more of the MMPs, which is analogous to vessel growth in tumors where the proteases remove pericytes and breakdown extracellular matrix proteins to prepare the vessels for sprouting under the control of angiogenic factors (Rundhaug, 2005). The angiogenic factors, VEGF, PlGF, and their receptors, Flt-1 and Tie-2, were identified: Flt-1 (elevated in CSF for MX compared to the other three groups) (**Supplementary Figure 4**), and Tie-2 (important in classification in plasma) (**Table 2**); they initiate vessel growth controlled by hypoxia-inducible factor-α under hypoxic conditions, which are present in both AD and VCID due to reduced cerebral blood flow as found in both conditions, but for different underlying mechanisms (Tomimoto, 2011; Iadecola, 2013).

Correlating biomarkers with neuropsychological testing was important in that it showed their clinical relevance. The relationship between fluid biomarkers and cognition is complex and, given modest correlations and small sample size, our data should be considered as hypothesis-generating rather than instructive. Positive correlations between cognitive performance and CSF levels of inflammatory cytokines pose a paradox if inflammation precedes injury to brain structure. Scatterplots in **Figure 2** suggest that elevated CSF cytokines (e.g., CSF IL-2) and VEGF-C may differentially affect cognition by patient group. For example, higher cognitive scores in SIVD with elevated inflammatory factors might indicate they play a reparative role in this group.

Our results reveal the role of the angiogenic factors. It is interesting that Flt-1 besides being the receptor for VEGF, is a signaling factor for microglia (Ryu et al., 2009). Similarly, the proteases probably have multiple roles; high levels of MMP-10

TABLE 2 | Variable Importance (VIMP) for important features used to classify each definition of diagnostic groups for the all factors classification scenario; cells are shaded darker for larger VIMP.

	All			CSF			Plasma		
Accuracy:	**77.8%**	**88.2%**	**68.6%**	**75.0%**	**88.2%**	**66.7%**	**55.6%**	**72.5%**	**47.1%**
Feature	**SIVD vs. AD**	**SIVD vs. AD and Mixed**	**SIVD vs. Mixed vs. AD**	**SIVD vs. AD**	**SIVD vs. AD and Mixed**	**SIVD vs. Mixed vs. AD**	**SIVD vs. AD**	**SIVD vs. AD and Mixed**	**SIVD vs. Mixed vs. AD**
Age at baseline			0.3%			−0.1%	2.8%	0.5%	2.6%
CSF A Beta 42/40 ratio		1.0%	1.2%		1.4%	1.4%			
CSF P-Tau	3.3%	2.9%	2.3%	4.0%	4.0%	2.8%			
CSF MSD MMP-9				0.4%					
CSF MSD MMP-10	3.4%	5.3%	4.6%	4.9%	6.8%	5.6%			
Plasma MSD MMP-1	1.4%	1.3%	0.8%				4.3%	3.0%	1.8%
Plasma MSD MMP-2	1.0%		0.5%					−0.4%	
Plasma MSD MMP-3		0.2%	0.1%					0.5%	0.7%
Plasma MSD MMP-10		0.2%					2.3%	1.2%	0.5%
CSF VEGF-A			0.3%			0.5%			
CSF VEGF-C	4.3%	0.7%	3.1%	3.4%	1.1%	3.8%			
CSF Tie-2	1.1%	0.4%	0.5%		0.6%	0.8%			
CSF PlGF	0.0%		1.8%	0.4%		1.8%			
Plasma Tie-2							5.2%	−0.3%	1.6%
Plasma bFGF									0.4%
CSF IL-1B	0.1%	0.0%	−0.1%	0.2%	0.6%	−0.1%			
CSF IL-2		0.7%	0.5%	1.6%	1.2%	0.7%			
CSF IL-12p70						0.4%			
CSF IL-13	1.6%		0.4%	0.7%		0.4%			
CSF TNF-a		0.1%			0.4%	−0.1%			
Plasma IFN-g			0.7%						
Plasma IL-6	0.6%	−0.1%	0.3%					0.0%	0.9%
Plasma IL-10									0.3%
Plasma IL-13		0.4%	0.3%					0.7%	1.5%

were found in CSF and plasma, and it correlated with pTau, suggesting importance in AD by a mechanism that remains to be determined. Others have reported MMP-10 elevations in patients with AD (Stomrud et al., 2010; Craig-Schapiro et al., 2011; Whelan et al., 2019). Several of the biomarkers showed a correlation between values in the CSF and plasma, suggesting that plasma may be able to be used instead of CSF, particularly with the ultra-sensitive assay platforms (Janelidze et al., 2016).

Random Forests, a machine learning method, selected several of the cytokines as variables of importance for distinguishing patient groups, including IL-2, IL-6, and IL-13. These may influence the inflammatory response: IL-2 amplifies T_{reg} cells that are linked to chemokines, CCL1 and CCL20, which suppress astrocytosis, contributing to repair (Ito et al., 2019); in animals with traumatic brain injury, IL-13 impacts microglia by converting M1/M2 microglia into anti-inflammatory M2 phenotype (Miao et al., 2020); IL-13 is found in resilient AD patients that have reduced glial activation, increased neuronal survival, and preserved cognition (Barroeta-Espar et al., 2019).

There are several caveats with our data. First, patients were from a single center and only a subset had complete CSF/plasma and MRI datasets, reducing the numbers available for statistical analysis. Second, biomarkers selected were those available from MesoScale Discovery and had been used by the MarkVCID consortium, which included our group; other biomarkers and platforms with different biomarkers could have been used. Furthermore, the study was cross-sectional rather than longitudinal, precluding inferences about the temporal dynamics of analyte levels. A major caveat is the small sample size, which was further hindered by forming an additional MX group. However, despite the small numbers, the results were statistically significant. A follow-up study on a larger population is necessary to further validate the results of this present study.

In conclusion, we expanded the biological definition of AD by adding vascular factors, allowing the identification of patients with dual pathology prior to autopsy. Using Random Forests, a machine learning method, we have determined the major proteases, angiogenic factors, and cytokines of importance in classification in a diverse group of dementia patients. Following an initial classification into diagnostic groups, we identified the proteases, MMP-1, MMP-3 and MMP-10, the angiogenic factors, VEGF-C, PlGF, Flt-1, Tie-2, and the cytokines, IL-2, IL-6, and IL-13. Our results suggest that the combined action of proteases and angiogenic growth factors may be important in dementia with

cytokines fueling the inflammatory processes. Further studies in larger numbers of patients will be needed to confirm these results.

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 New Mexico Human Research Review Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EE performed the statistical analysis and wrote a draft. JA and JK recruited the patients. AC performed the MRIs and analyzed the MRI data. JT and SH analyzed the CSF and blood. JP performed the neuropsychological testing. DS obtained the control CSF during surgery. GR obtained the funding, recruited patients, and contributed to the writing of the manuscript. 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.2021.717344/full#supplementary-material

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ORIGINAL RESEARCH
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Microglial TREM2 Mitigates Inflammatory Responses and Neuronal Apoptosis in Angiotensin II-Induced Hypertension in Middle-Aged Mice

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Growing evidence suggests that hypertension and aging are prominent risk factors for the development of late-onset Alzheimer's disease (LOAD) by inducement of neuroinflammation. Recent study showed that neuroinflammation *via* activated microglia induces reactive astrocytes, termed A1 astrocytes, that highly upregulate numerous classical complement cascade genes that are destructive to neurons in neurodegeneration diseases. Moreover, triggering receptor expressed on myeloid cells 2 (TREM2) is considered as one of the strongest single-allele genetic risk factors and plays important roles in neuroinflammation for LOAD. However, the mechanisms of microglia in the regulation of A1 astrocytic activation are still not clear. We introduced angiotensin II-induced hypertension in middle-aged mice and found that hypertension-upregulated TREM2 expression and A1 astrocytic activation were involved in neuroinflammation in the animal models used in this study. The *in vitro* results revealed that overexpression of microglial TREM2 not only mitigated microglial inflammatory response but also had salutary effects on reverse A1 astrocytic activation and neuronal toxicity.

Keywords: LOAD, hypertension, aging, TREM2, microglia, astrocyte, neuroinflammation

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INTRODUCTION

Alzheimer's disease is the most common cause of all types of dementia (Lane et al., 2018; Scheltens et al., 2021), and sporadic Alzheimer's disease (AD) usually occurs after the of 65, and is also named late-onset AD (LOAD) (Rabinovici, 2019). Growing evidence suggests that hypertension plays an important role in LOAD (Kruyer et al., 2015; Lüders and Schrader, 2015) by causing Aβ plaque deposits and cerebral amyloid angiopathy (CAA) (Tsukuda et al., 2009; Carnevale et al., 2012; Elias et al., 2012). Furthermore, clinical research investigators have confirmed that midlife hypertension is strongly correlated with late-life dementia in humans (Iadecola, 2014), and that effective treatment for hypertension in midlife can attenuate the risk of developing cognitive impairment in older age (Elias et al., 2012; Tzourio et al., 2014; Baranowski et al., 2018).

Although the mechanisms of hypertension causing dementia are still not fully understood, it has been found that neuroinflammation plays a pivotal role in the incidence and progression of AD (Iadecola, 2014; McMaster et al., 2015; Bolos et al., 2017). As inflammatory response cells in the brain, microglia play important roles in immune responses in the central nervous system and is involved in the pathogenesis of neurodegenerative and neuroinflammatory diseases. In response to inflammation, microglia can be activated and polarized into pro-inflammatory M1 phenotype or anti-inflammatory M2 phenotype. The M1 activation of microglia occurs in response to Aβ and other inflammatory stimuli, which have detrimental impacts on neurons, through the release of pro-inflammatory factors and various toxic substances (Sarlus and Heneka, 2017; Hansen et al., 2018).

A recent study has shown that M1 microglia activated astrocytes into A1 astrocytes, which highly upregulate numerous classical complement cascade genes that are destructive to neurons and oligodendrocytes in neurodegenerative diseases (Liddelow et al., 2017). Blocking M1 microglial-induced A1 astrocytic activation is considered to be an effective therapeutic strategy for AD (Baldwin and Eroglu, 2017; Clarke et al., 2018). Notably, TREM2 plays important roles on microglial functions such as phagocytosis, biosynthetic metabolism, and inflammatory response (Condello et al., 2018), and TREM2 deficiency exacerbates activated M1 microglial inflammatory cytokines release and neuronal apoptosis, but TREM2 overexpression markedly attenuated inflammation and neuronal death in AD model studies (Jiang et al., 2014; Jay et al., 2015, 2017). Furthermore, genetic studies unveil that rare coding variants in TREM2 in microglia increase the risk of developing LOAD by 3–4 folds (Jin et al., 2014; Song et al., 2017; Zhong et al., 2019). Although TERM2 is considered as one of the strongest single-allele genetic risk factors for LOAD (Jiang et al., 2016), its potential roles in the onset and progression of the disease still remain to be explored.

In this study, we introduced hypertension in middle-aged mice to investigate the mechanisms in the progression of LOAD. We found that hypertension upregulated TREM2 expression and A1 astrocytes in middle-aged mice for the first time. Based on the anti-neuroinflammatory role of TREM2 that has been proven in AD, in this study, we hypothesized whether TREM2 upregulation in microglia could be a potential strategy to impede M1 microglial-induced A1 astrocytes and neuronal toxicity. We then performed *in vitro* experiments and revealed that the overexpression of microglial TREM2 not only mitigated microglial inflammatory response, but also had salutary effects on reverse A1 astrocytic activation and neuronal toxicity.

MATERIALS AND METHODS

Materials

Chemicals

Lipopolysaccharides (LPS; L2880) and the anti-glial fibrillary acidic protein (anti-GFAP; G9269) antibody were purchased from Sigma (St. Louis, MO, United States). The complement C3 polyclonal antibody (sc-58926) was purchased from Santa Cruz Biotechnology (Dallas, TX, United States). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and penicillin-streptomycin, 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Life Technologies (Carlsbad, CA, United States). Anti-tubulin antibodies (2148S) were purchased from Cell Signaling Technology (Beverly, MA, United States). Goat anti-rabbit IgG H&L (Alexa Fluor 488), rabbit anti-rat IgG H&L (Alexa Fluor 555), TNF-α enzyme-linked immunosorbent assay (ELISA) kits, and IL-1α ELISA kits were purchased from Abcam (San Francisco, CA, United States). C1q ELISA Kit (mouse; HK211) was purchased from Hycult Biotech (Plymouth Meeting, PA, United States). The anti-aquaporin 4 (AQP4) (249-323) antibody (AQP-004) was purchased from Alomone Labs (Jerusalem, Israel). The TREM2 polyclonal antibody (PA5-87933) and lactate dehydrogenase (LDH) release were purchased from Thermo Fisher Scientific (Waltham, MA, United States). Cell Counting Kit-8 was purchased from Dojindo Molecular Technologies (Rockville, MD, United States).

Microglia BV2 Cell Line Culture, Treatment, and Transfection, and Conditioned Medium Preparation

In this study, BV2 cells were cultured in DMEM with 10% FBS and penicillin-streptomycin at 37°C with 5% CO2. Then, the BV2 cells were pretreated with serum-free DMEM containing LPS (0.1 ug/ml). After incubation for 24 h, the conditioned medium was collected and denoted as MCM. Meanwhile, the BV2 cell complete culture medium was denoted as MCM-control. The BV2 cells (4×10^5 cells per well) were seeded onto six-well plates overnight to reach 70% confluence for transfection. TREM2 (Myc-DDK-tagged) overexpression plasmid (MR202717) or pCMV6-Entry Mammalian Expression Vector (PS100001), which was purchased from OriGene Technologies, Inc. (Rockville, MD, United States), was transfected into cells for 4.5 h using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, United States) and then replaced with a complete medium to culture for another 48 h at 37°C, which was then collected for use in subsequent experiments. The C-Myc mouse monoclonal antibody (TA500002, OriGene Technologies, Inc., Rockville, MD, United States) was applied to confirm transduction efficiency. For combination treatments, LPS (0.1 ug/ml) was added to the BV2 cells after TREM2 (vector or overexpression) transfection for 24 h, and culturing was continued for another 24 h at 37°C. The above conditioned mediums containing LPS (0.1 ug/ml) and TREM2 (vector or overexpression) in BV2 cells were collected and were denoted as MCM + TREM2 (vector or overexpression).

Primary Astrocyte Cultures, Treatment, and Staining

Primary cortical astrocyte cultures were prepared using 1- to 3-day-old neonatal c57mouse brains as previously described (Schildge et al., 2013), which were obtained from Charles River Laboratories (Wilmington, MA, United States). Briefly, the mice were decapitated, the cerebral cortices were removed, and the meninges were carefully stripped off. Tissues were maintained in DMEM and nutrient mixture F12 (DMEM/F12) and dissociated into single cells in the DMEM/F12 medium supplemented with

10% FBS. The cultures were incubated at 37°C in a humidified 5% CO2, 95% air atmosphere. The cell culture medium was changed 24 h after plating and, subsequently, every 3 days, until confluence was reached, which usually occurs after 7–10 days. The flasks were shaken at 180 rpm for 30 min in an orbital shaker to remove microglia, and the supernatant containing microglia was discarded. Afterward, a 20-ml fresh astrocyte culture medium was added to the flakes, and the experiment was continued by shaking the flask at 240 rpm for 6 h to remove oligodendrocyte precursor cells. After that, cells were subjected to a passage to generate purified astrocyte cultures (secondary cultures), which constituted more than 95% of GFAP-positive cells. For the treatments, first, the astrocytes were treated with MCM to induce A1 astrocytic activation. After incubation for 24 h, the conditioned medium was collected and denoted as ACM. Second, the astrocytes were treated with MCM + TREM2 (vector or overexpression) for 24 h, and the above conditioned mediums containing MCM + TREM2 (vector or overexpression) in astrocytes were collected and denoted as ACM + TREM2 (vector or overexpression). For immunocytochemistry, the astrocytes were seeded at 0.8 × 106 on 1.5-mm^2 coverslips for 24 h. After treatment, the cells were fixed with 4% ice-cold paraformaldehyde for 20 min at 4°C and air dried. Then, blocking and permeabilization were performed with 1% BSA in PBS with.1% Triton for 30 min. After that, the astrocyte (A1) was stained with the C3 antibody (1:1,000) and GFAP antibodies (1:2,000) or the AQP4 antibody (1:500) for 24 h in a humidified chamber at 4°C. After three washes with.1% Triton in PBS, goat anti-rat IgG H&L (Alexa Fluor 555, 1:200) and goat anti-rabbit IgG H&L (Alexa Fluor 488, 1:200) was used for 1 hr. After DAPI staining for 10 min, the coverslips were transferred onto glass slides. Images were obtained using a digital microscope camera system (Nikon DS-Ri2, Nikon, Tokyo, Japan).

Cell Viability Assay

Murine neuronal-like (Neuro2a) cell line is widely used the neuronal cell line for investigation of neuronal survival, neuronal differentiation, and neuroprotection *in vitro* (Eom et al., 2015; Nicolas et al., 2015; Steiner et al., 2016). In this study, the viability of Neuro2A cells following incubation with MCM, ACM, TREM2 overexpression + MCM, and TREM2 overexpression + ACM was evaluated by lactate dehydrogenase (LDH) release and Cell Counting Kit-8 (CCK-8) assays. The amount of LDH released was expressed as a percentage of the value obtained in comparative wells where cells were 100% lysed by 1% Triton X-100. For the CCK-8 assays, data are presented as a percentage of the value obtained from cells incubated in a fresh medium only.

Animals

This study was approved by the Animal Research Committee of Sun Yat-sen University (Guangzhou, China; Committee Reference Number: SYSU-IACUC-2018-000093). All efforts were made to minimize the number and suffering of animals used in this study. Fourteen male C57BL/6J mice (30–35 g) obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Stock Number: SCXK2016-0006) were used. All the animals were used at 10 months of age (considered middle age in mouse), housed under a 12:12 h light-dark cycle (light from 07:00 to 19:00) with controlled temperature and humidity, and given food and water *ad libitum*.

Hypertension in Mouse Model Induced by Chronic Angiotensin Infusion

The hypertension mouse model was established as described previously (Gentile et al., 2009). Briefly, angiotensin II (AGT II,0.5 ng/kg/day in.9% NaCl, Sigma-Aldrich, St. Louis, MO, United States) was infused into a group of seven mice for 30 days with an osmotic mini pump (Durect, Cupertino, CA, United States) implanted subcutaneously. An additional group of seven mice was infused with saline vehicle alone as a control. Blood pressure was measured with the tail-cuff method on days 0, 7, 14, 21, and 30 during the 30-day infusion period.

Histology

The mice were perfused with 50 ml of ice-cold.9% saline followed by 50 ml of 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). Their brains were removed and incubated overnight in 4% paraformaldehyde and then dehydrated in 20–30% sucrose in PBS. Coronal brain slices (14-mm thick) from the right parietal cortex were sectioned with a frozen microtome (Leica Biosystems, Wetzlar, Germany) to produce consecutive frozen sections. For immunofluorescence staining, the sections were boiled in a citric acid buffer (pH 6) for 5 min in a microwave oven. After the sections were cooled, they were treated with.3% Triton X-100 and 10% goat serum for 1 h at room temperature. The sections were then incubated overnight at 4°C with a primary antibody (1:100 anti-ionized calcium binding adapter molecule 1, IBA1) antibody, catalog number 019-19741, Wako; 1:100 anti-glial fibrillary acidic protein (GFAP) antibody, catalog number AMAb91033, Sigma-Aldrich (St. Louis, MO, United States); 1:50 anti-amyloid antibody, β1-40, catalog number AB5074P, Millipore (Burlington, MA, United States); 1:50 anti-amyloid antibody, β1-42, catalog number AB5078P, Millipore (Burlington, MA, United States); 1:100 anti-c3 antibody, catalog number sc-58926, Santa Cruz Biotechnology (Dallas, TX, United States); or 1:200 anti-CD68 antibody, catalog number ab125212, Abcam (San Francisco, CA, United States), and then incubated for 1 h at room temperature with a secondary antibody (1:500 goat anti-rabbit IgG H&L, Alexa Fluor® 488), catalog number ab150077, Abcam (San Francisco, CA, United States); 1:500 goat anti-mouse IgG H&L (Alexa Fluor® 555), catalog number ab150118, Abcam (San Francisco, CA, United States); or 1:500 rabbit anti-goat IgG H&L (Alexa Fluor® 555), catalog number ab150146, Abcam (San Francisco, CA, United States) in PBS containing 10% blocking solution. The sections were mounted onto slides, stained with DAPI solution with antifade (Sigma-Aldrich, St. Louis, MO, United States), and covered with a coverslip. To evaluate the Aβ expression levels, the percentage of the Aβ40 or the Aβ42 plaque area was measured within the cortex and hippocampus. The percentage of GFAP-positive astrocytes with C3 localization among total GFAP-positive astrocytes was determined from the

images. A similar method was applied to analyze other positive markers of target cells.

TUNEL Assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling staining method is used to detect fragments of DNA in apoptotic cells in tissue samples (Kyrylkova et al., 2012). In this study, TUNEL staining was performed to detect DNA fragments during neuronal apoptosis. A TUNEL assay was performed according to the directions of the manufacturer using *In Situ* Cell Death Detection Kit (CAT: 11684817910, Roche, Indianapolis, IN, United States). In brief, the sections were washed three times for 10 min each. Then, the sections were incubated in.3% Triton-X and 0.1% sodium citrate and rinsed three times with PBS for 10 min each. The sections were incubated in.3% H2O2 in PBS for 30 min and then rinsed with PBS. The sections were then incubated with a 50-ml TUNEL reaction mixture in a humidified atmosphere for 60 min at 37°C in the dark. Then, the sections were rinsed three times with PBS. The sections were then observed under a digital microscope camera system (Nikon DS-Ri2, Nikon, Tokyo, Japan). A negative control was carried out by incubating the sections in 50 ml of a label solution without terminal transferase instead of the TUNEL reaction mixture. 4,6-Diamino-2-phenyl indole (DAPI, Sigma-Aldrich, St. Louis, MO, United States) was used for nuclear staining, and a primary antibody (anti-NeuN antibody [EPR12763], ab177487) was used for neuronal staining. To quantify the degree of apoptotic neuron death in the cortex and hippocampus, the percentage of cells labeled with both TUNEL-positive fluorescein and NeuN localized in the DAPI-stained nucleus out of the total number of NeuN-positive cells was determined from the images.

ELISA

The levels of C1q, TNF-α, and IL-1α were determined in the cell-culture medium obtained from the experiments. The culture medium was analyzed with a commercially ELISA kit according to the protocol of the manufacturer. Analysis of optical density or fluorescence was performed in a plate reader.

Western Blot Analysis

Cells were scraped and lysed in a RIPA lysis buffer on ice after treatment of 1 h at 4°C. Protein was extracted and quantified using a BCA assay kit (Thermo Scientific, Waltham, MA, United States) according to the instructions of the manufacturer. The cell lysates were solubilized in a sodium dodecyl sulfate (SDS) sample buffer (40 μg/lane) and separated by 10% SDS-polyacrylamide gel electrophoresis (110 V for 75 min). After electrophoresis, the protein was transferred to polyvinylidene difluoride (PVDF) membranes. Then, the membranes were blocked with 3% bovine serum albumin (BSA) and incubated with primary antibodies overnight at 4°C, followed by a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, and detected with the enhanced chemiluminescence plus detection system (Millipore, Billerica, MA, United States). The density of each band was quantified using the Quantity One image analysis software (Bio-Rad, Life Science, Hercules, CA, United States).

Data and Statistical Analyses

The ImageJ software (National Institutes of Health, Bethesda, MD, United States) was used to analyze the immunofluorescence results. To evaluate the immunofluorescence results, the number of target cells was counted using the ImageJ software. The significance of differences in data sets was analyzed by two-tailed Student's *t*-test or one-way analysis of variance tests. The data were expressed as the mean ± SD and analyzed using the GraphPad Prism 6 software (GraphPad, La Jolla, CA, United States). A p value < 0.05 was considered statistically significant.

RESULTS

Hypertension Exacerbates β-Amyloid Deposition and Causes Neuronal Apoptosis in Middle-Aged Mice

We first established hypertension, induced by angiotensin II, in the middle-aged mouse models. Systolic blood pressure (SBP) was measured on 0(98 ± 3.16 vs. 100 ± 5.51, $p = 0.47$), 7(99.5 ± 3.81 vs. 148.14 ± 8.45, $p < 0.01$), 14 (103.57 ± 3.69 vs. 149.42 ± 4.49, $p < 0.01$), 21(104.57 ± 5.01 vs. 147.42 ± 2.77, $p < 0.01$), and 30(103.28 ± 4.19 vs. 147.71 ± 5.96, $p < 0.01$) days during angiotensin II or saline (vector) osmotic mini pump administration. As shown in **Figure 1**, chronic infusion of angiotensin II with an osmotic mini pump evokes a significant increase in SBP, which reaches its peak at the seventh day of administration compared with the mice infused with saline. The hypertension effect was stable and continuous throughout the rest of the 30-day infusion period. Once hypertension was established, we assessed the effect of angiotensin II on Aβ deposition in the brain of the mice. As shown in **Figure 2**, the depositions of Aβ40 (3 ± 0.01% vs. 56% ± 0.04%, $p < 0.01$, cortex; 7 ± 0.02% vs. 39% ± 0.04, $p < 0.01$, hippocampus) and Aβ42 (6 ± 0.02% vs. 47 ± 0.04%, $p < 0.01$, cortex; 9 ± 0.03% vs. 52 ± 0.03%, $p < 0.01$, hippocampus) are significantly higher in the cortex and hippocampus of the mice in the hypertension group compared with the control group. Furthermore, neuronal apoptosis in the cortex and hippocampus was evaluated by TUNEL assay (**Figure 3**). The results revealed that hypertension markedly increased neuronal apoptosis in the cortex (13.71 ± 2.49% vs. 44.42 ± 3.86%, $p < 0.01$) and hippocampus (6.42 ± 2.50% vs. 27.57 ± 4.35%, $p < 0.01$, DG; 10.57 ± 1.9% vs. 24.28 ± 2.81%, $p < 0.01$, CA1; 6.57 ± 2.63% vs. 27.57 ± 2.63%, $p < 0.01$, CA3) of the mice in the angiotensin II infusion group compared with the mice in vehicle control.

Neuroinflammatory Network Under Hypertension in Middle-Aged Mice

M1 Microglia Activation

In this study, we used Iba1, a cytoplasmic protein marker of resting microglia (Bennett et al., 2016), and CD68, a lysosomal marker of microglial activation (Bennett et al., 2016), to characterize M1 microglia found in the cortex and hippocampus

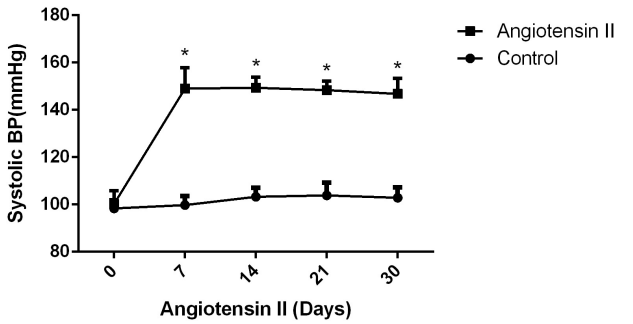

FIGURE 1 | Effect of angiotensin II on systolic blood pressure (SBP) in middle-aged mice. The systolic blood pressure of the middle-aged mice was measured 7, 14, 21, and 30 days after the infusion of saline or angiotensin II (n = 7 per group). Data are expressed as the means ± SD. * $p < 0.05$.

of mice in each group (**Figure 4A**). The microglia in the middle-aged mice with hypertension exhibited a more robust expression of CD68 (46.85 ± 8.23 vs. 266.28 ± 20.76, $p < 0.01$, cortex; 145.142 ± 15.61 vs. 389.71 ± 21.02, $p < 0.01$, CA1; 157.71 ± 11.51 vs. 324.57 ± 12.52, $p < 0.01$, DG; 160 ± 8.64 vs. 302.85 ± 27, $p < 0.01$, CA3), and Iba-1 (89.71 ± 9.75 vs. 301.71 ± 12.82, $p < 0.01$, cortex; 213.71 ± 19.43 vs. 452 ± 30.02, $p < 0.01$, CA1; 167.42 ± 13.15 vs. 370.28 ± 22.96, $p < 0.01$, DG; 171.42 ± 16.56 vs. 335.42 ± 12.09, $p < 0.01$, CA3) than control mice with vehicle, and the microglia in the mice with hypertension displayed a highly activated ameboid morphology with large bodies and few thick cellular processes, which indicates microglial activation.

Neurotoxic A1 Astrocytic Activation

According to previous research, A1 astrocytes highly upregulate numerous classic complement cascade genes that are destructive to neurons and oligodendrocytes. In particular, complement component 3 (C3) is one of the characteristic genes of A1 astrocytes (Liddelow et al., 2017). To investigate whether A1 astrocytes and C3 participated in neuro-inflammation in the animal models, we measured A1 astrocytes in hippocampal areas (DG, CA1, and CA3) by assessing the anti-GFAP and anti-C3 antibody staining through immunofluorescence. As shown in **Figure 5**, the reactive astrocytes overexpressing GFAP were found in hippocampal areas (DG, CA1, and CA3) of mice in each group. Furthermore, the A1 astrocytic marker C3 was obviously upregulated in the hippocampal areas of the middle-aged mice with hypertension compared with the very weak expression of C3 in the control group (31.14 ± 4.01% vs. 81.42 ± 3.33%, $p < 0.01$, DG; 17.71 ± 2.42% vs. 92 ± 4.84%, $p < 0.01$, CA1; 21 ± 2.3% vs. 56.71 ± 2.81%, $p < 0.01$, CA3).

Spatial Co-localization Between M1 Microglia and A1 Astrocytes

Because M1 microglial activation provokes neurotoxic astrocyte reactivity (Liddelow et al., 2017), we wondered whether there was a specific spatial co-localization promoting a microglia-astroglia interaction. As shown in **Figure 6**, although there are more activated microglia and reactive astrocytes in the hippocampal areas (DG, CA1, and CA3) of the mice in the hypertension group, their distance and the closeness of the connection between microglial cell branches and astrocytic end feet seem to be similar to those of the control group.

Microglial TREM2 Upregulation in Middle-Aged Mice With Hypertension

Furthermore, as revealed in **Figure 7**, the expression of TREM2 in microglia in the group of mice with hypertension is significantly higher compared with the control group (16 ± 5.65 vs. 280.57 ± 16.56, $p < 0.01$, cortex; 13.42 ± 4.57 vs. 348.57 ± 22.2, $p < 0.01$, hippocampus).

Microglial TREM2 Overexpression Reversed A1 Astrocytic Activation and Neuronal Toxicity *in vitro*

Based on the above histopathological findings, we proposed that the overexpression of microglial triggering receptor expressed on myeloid cells 2 (TREM2) might reverse M1 microglial-induced A1 astrocytic activation and neuronal toxicity. We first incubated the BV2 cells, a microglial cell line, in a serum-free medium containing LPS (0.1 ug/ml) for 24 h to imitate a neuroinflammation *in vitro* environment (Catorce and Gevorkian, 2016; Lopes, 2016). Because the LPS-activated M1 microglia conditioned medium can induce A1 astrocytic activation *via* the secretion of TNF-α, IL-1α, and C1Q *in vitro* (Liddelow et al., 2017), we then performed ELISA to detect the concentrations of TNF-α, IL-1α, and C1Q in the MCM. As shown in **Figures 8A–C**, the concentrations of TNF-α (6.701 ± 1.323 vs. 353.6 ± 11.84, $p < 0.05$), IL-1α (2.747 ± 0.3313 vs. 45.84 ± 0.7023, $p < 0.05$), and C1Q (2.175 ± 0.3398 vs. 4.206 ± 0.4517, $p < 0.05$) were significantly elevated in the MCM than the concentrations of above cytokines in the normal cell culture media controls. Further, we found that LPS inhibited the TREM2 expression of the BV2 cells in a time-dependent manner (**Supplementary Figure 1**). To test the hypothesis, we overexpressed TREM2 in the BV2 cells (**Figure 9**, $F = 11.32$, $p = 0.0092$), which were used for subsequent combination treatments. The results showed that the increase in the above cytokines are alleviated by the overexpression of TREM2 in the BV2 cells [**Figures 8A–C** ($F = 673.7$, $p < 0.001$), TNF-α; ($F = 812.8$, $p < 0.001$), IL-1α; ($F = 7.818$, $p = 0.0002$), C1Q], but there are no effects on TREM2 in the vector control group. These findings confirmed that TREM2-overexpressing microglia following LPS stimulation did decrease the key chemokines for A1 astrocytic activation.

As indicated in **Figure 10**, we confirmed that the A1 astrocytic marker C3 expression was significantly increased in MCM than that in control (1 vs. 2.363 ± 0.1056, $p < 0.05$). Afterward, a combination treatment with BV2 microglial TREM2 overexpression and MCM significantly reduced the A1 astrocytic marker C3 expression (2.363 ± 0.1056 vs. 1.107 ± 0.1854, $p < 0.05$), but no significant change in the C3 expression was noted after the TREM2 vector was combined with MCM treatment (**Figure 10**). Meanwhile, there was no significant change in C3 expression in astrocytes after treatment of LPS or TREM2 (vector or overexpression) transfection

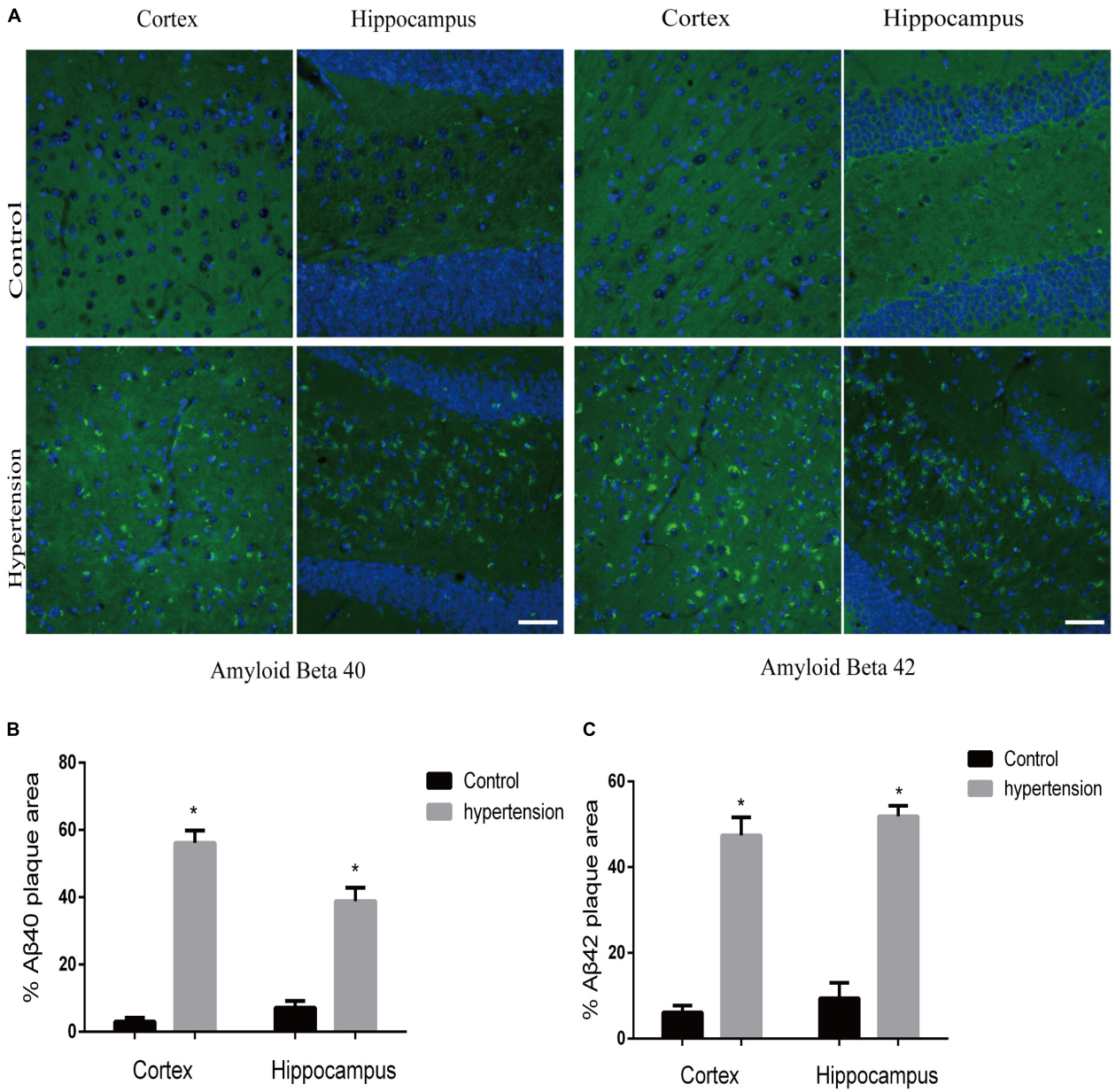

FIGURE 2 | Aβ40 and Aβ42 deposition in the brain. After 30 days of angiotensin II treatment in middle-aged mice, the brain slices were subjected to immunofluorescence staining with the anti-Aβ40 (green) or the anti-Aβ42 (green) antibody and DAPI (blue) to observe β-amyloid deposition in the cortex and hippocampus. **(A)** Representative images of Aβ40 or Aβ42 deposition in the control and hypertension groups (immunofluorescence, ×40, scale bar = 50 μm). **(B,C)** Histograms comparing the percentage of Aβ40 or Aβ42 plaque area in the cortex and hippocampus of the control and hypertension groups (n = 7 per group). Data are expressed as the means ± SD. * $p < 0.05$.

conditioned medium, which was served as the negative control (**Supplementary Figure 2**). Furthermore, immunofluorescence was used to further confirm the results of this study. As shown in **Figure 11A**, MCM significantly increased the A1 marker C3 expression, which was reversed by BV2 microglial TREM2 overexpression treated with MCM. Meanwhile, we also introduced water channel aquaporin 4 (AQP4), the most prevalent aquaporin channel specifically located at the astrocyte foot processes in the brain parenchyma (Saadoun and Papadopoulos, 2010), to investigate the A1 astrocytes. **Figure 11B** shows that the foot processes and AQP4 expression were reduced in the A1 astrocytes but rescued by the overexpression of BV2 microglial TREM2 treated with MCM.

Furthermore, we measured the viability of Neuro2A cells to confirm the findings. As shown in **Figures 12A,B**, the overexpression of TREM2 in the BV2 cells significantly protect the Neuro2A cells from the toxic effects of MCM and ACM, which are shown as increased cell viability (**Figure 12A**, $F = 65.14$,

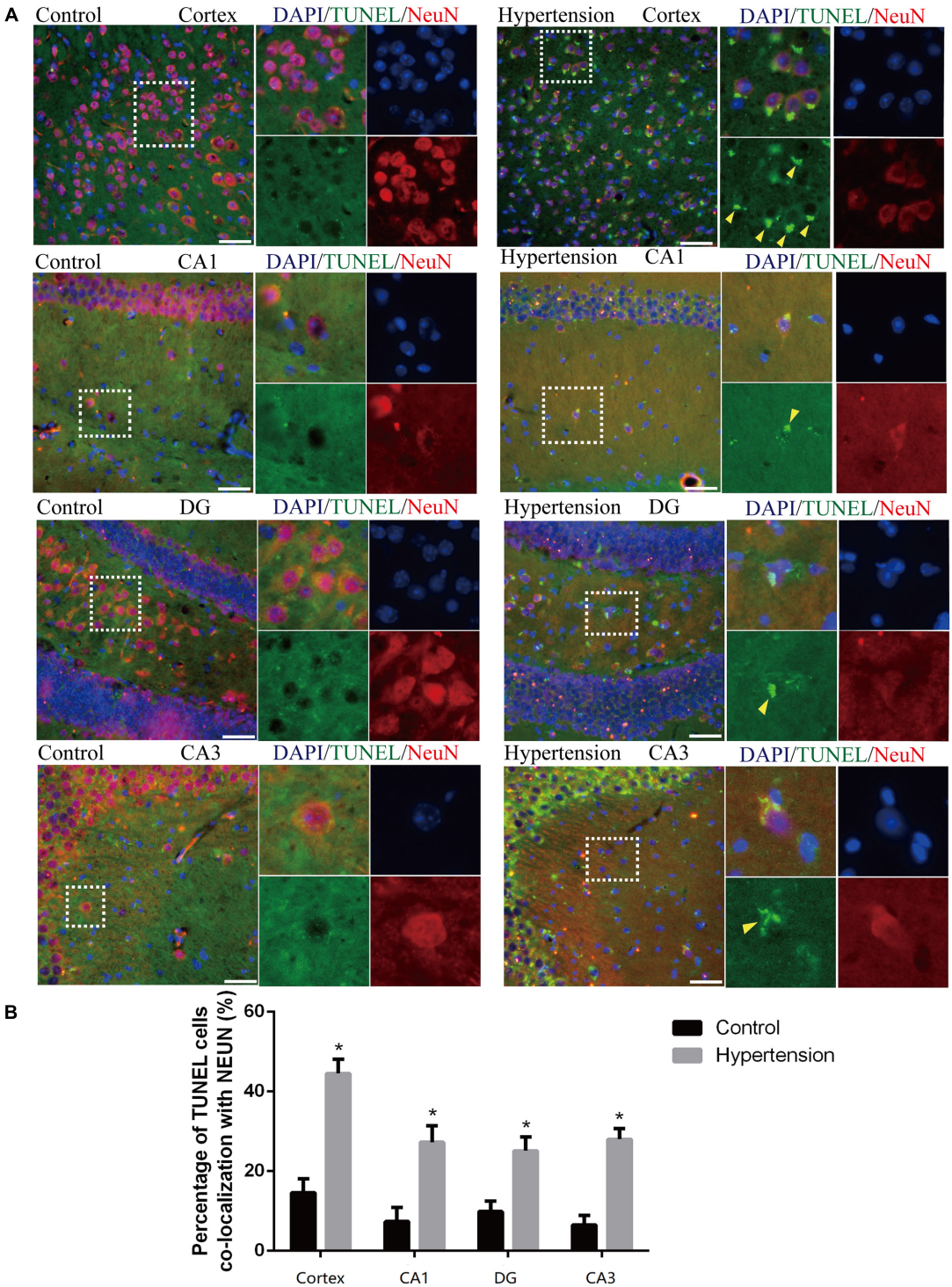

FIGURE 3 | Angiotensin II-induced hypertension promotes neuronal apoptosis in middle-aged mice. **(A)** Representative images of TUNEL-positive, fluorescein-labeled (green) neurons co-stained with the anti-NeuN antibody (red) in the cortex and hippocampus (CA1, DG, and CA3) of mice in the control and hypertension groups (immunofluorescence, ×20, scale bar = 50 μm). **(B)** Histograms comparing the level of positive TUNEL staining in neurons in the cortex and hippocampus (CA1, DG, and CA3) of mice in the control and hypertension groups. Data are expressed as the means ± SD. * $p < 0.05$. (Negative control image of TUNEL staining assay provided in **Supplementary Figure 3**).

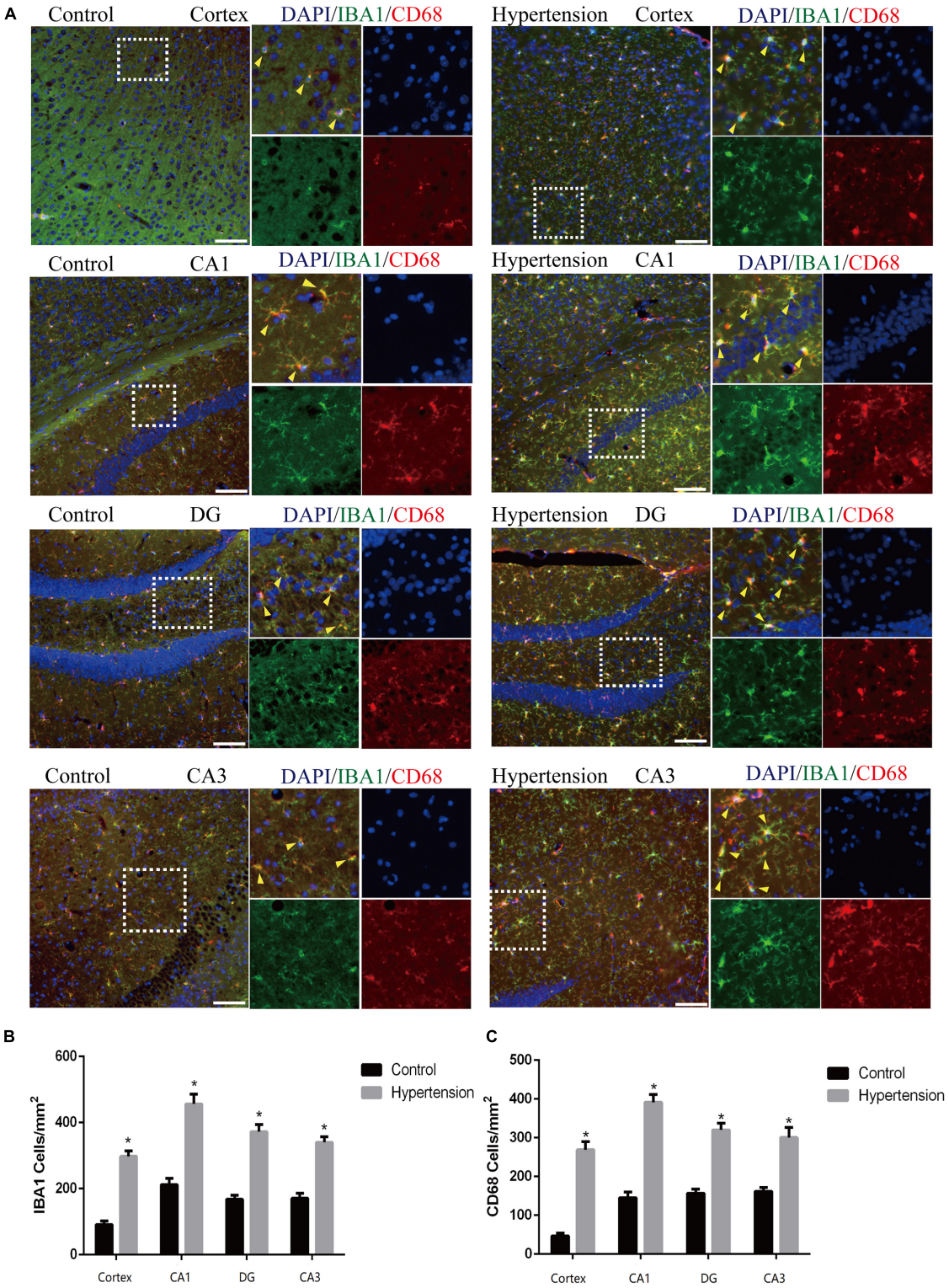

FIGURE 4 | Angiotensin II-induced hypertension upregulates the expression of microglial marker and promotes the activation of M1 microglial transformation in middle-aged mice. **(A)** Representative images of brain slices stained with the anti-IBA1 (green) and anti-CD68 antibodies (red) in the cortex and hippocampus (CA1, DG, and CA3) of mice in the control and hypertension groups (immunofluorescence, ×20, scale bar = 50 μm). **(B)** Histograms comparing the number of IBA1-positive cells in the cortex and hippocampus (CA1, DG, and CA3) of mice in the control and hypertension groups. **(C)** Histograms comparing the number of CD68-positive cells in the cortex and hippocampus (CA1, DG, and CA3) of mice in the control and hypertension groups. Data are expressed as the means ± SD. * $p < 0.05$.

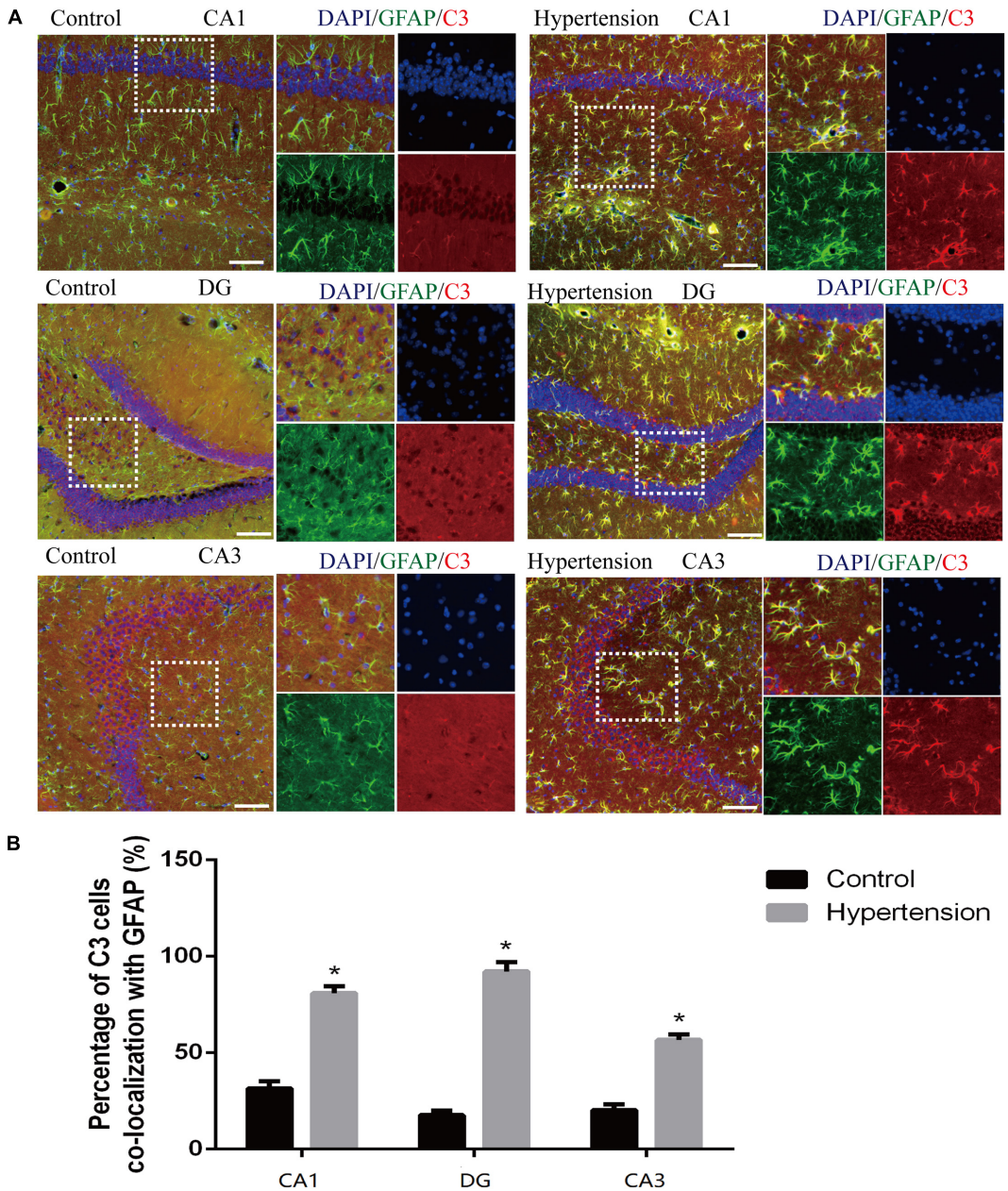

FIGURE 5 | Astrogliosis and notable reactive A1 astrocytes were found in the hippocampus of middle-aged mice after 30 days of angiotensin II treatment. **(A)** Representative images of immunofluorescence double staining with the anti-GFAP (green) and anti-C3 antibodies (red) in the hippocampus (CA1, DG, and CA3) of the mice in the control and hypertension groups (immunofluorescence, ×20, scale bar = 100 μm). **(B)** Histograms comparing C3 levels of astrocytes in the hippocampus (CA1, DG, and CA3) of the mice in the control and hypertension groups. Data are expressed as the means ± SD. * $p < 0.05$.

$p < 0.001$) and reduced LDH release (**Figure 12B**, $F = 129.2$, $p < 0.001$).

DISCUSSION

Alzheimer's disease consists of two categories, early-onset AD (EOAD, age < 65) and late-onset AD (LOAD, age ≥ 65). EOAD only comprises approximately 5% of AD cases (Mendez, 2017), shows a stronger tendency toward familial inheritance with more aggressive clinical course, and fewer overall comorbidities than LOAD (Smits et al., 2015; Gerritsen et al., 2016). In contrast, LOAD comprises approximately 95% of AD cases with not only genetic risk factors but also medical comorbidities, lifestyle, and other environmental factors contribute to its occurrence (Baumgart et al., 2015). Among the comorbidities, hypertension, a critical vascular risk factor, has been confirmed to cause age-related cognitive impairment and accelerate the development

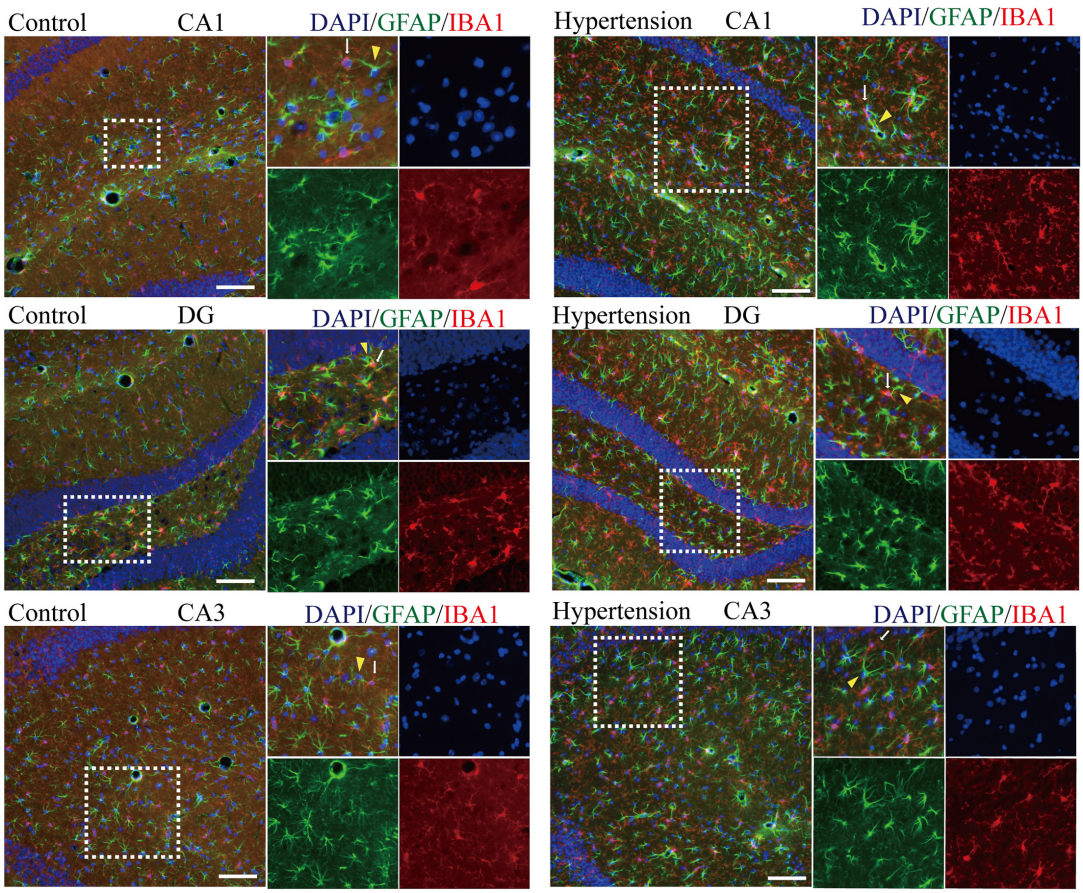

FIGURE 6 | Determination of spatial colocalization of microglia and astrocytes after 30 days of angiotensin II treatment in middle-aged mice. Representative images of immunofluorescence double-staining with anti-GFAP (green) and anti-IBA1 antibodies (red) in the hippocampus (CA1, DG, and CA3) of the mice in the control and hypertension groups (immunofluorescence, ×20, scale bar = 100 μm).

of LOAD by inducing cerebrovascular dysfunction in the early stage (Zlokovic, 2011; Baumgart et al., 2015; Csiszar et al., 2017). A variety of studies elucidated that hypertension causes cerebral microcirculation disorder (capillary rarefaction, blood-brain barrier disruption), neurovascular uncoupling (Csiszar et al., 2017; Hammer et al., 2017), cognitive impairment, Aβ deposition, and CAA (Gentile et al., 2009; Carnevale et al., 2012; Wenzel and Munzel, 2015; Cifuentes et al., 2017). However, the mechanisms of hypertension that cause LOAD are still not fully understood.

In this study, chronic infusion with angiotensin II *via* an osmotic mini pump was able to induce hypertension in middle-aged mice with the mechanism by which the renin-angiotensin system (RAS) regulates blood pressure in the human body (Yang and Xu, 2017), and the results were consistent with those of previous publications (Gentile et al., 2009; Chan et al., 2012; Lu et al., 2015). Hypertension significantly increased amyloid deposition and neuronal apoptosis in middle-aged mice compare with the control middle-aged mice. In addition, we observed that most Aβ40 was deposited in the brain parenchyma (**Figure 2A**), which is different from previous reports on CAA indicating that the location of Aβ40 deposition is outward of blood vessels (Watts et al., 2014). The difference in the location of Aβ40 deposition between CAA and hypertension may be due to different pathological changes. Although there are damages in vasculature in both cases, hypertension may cause more para-vascular damages and subsequently leads to more Aβ40 depositions in para-vascular tissue. Nevertheless, the accumulation of Aβ40 in the brain can lead to dementia at the end in both cases (Qiang et al., 2017).

In addition to Aβ deposition, we observed that hypertension also induces neuronal death by neuro-inflammation, which is in line with previous evidence that proved ANG II-dependent microglial activation induces neuroinflammation acts *via* cross-talk between central renin-angiotensin system type 1 receptors (AT1R) and toll-like receptor 4 (TLR4) (Biancardi et al., 2016; Liu et al., 2016).We further investigated the roles of neuroinflammation in the mouse models of this study. Consistent with previous studies (Heneka et al., 2010; Carnevale et al., 2012; Cai et al., 2014), activated M1 microglia were markedly upregulated in the cortex and hippocampus of the mice in the hypertension group (**Figure 4**). Importantly, in addition to more neuronal deaths, we found that the reactive astrocytic phenotype

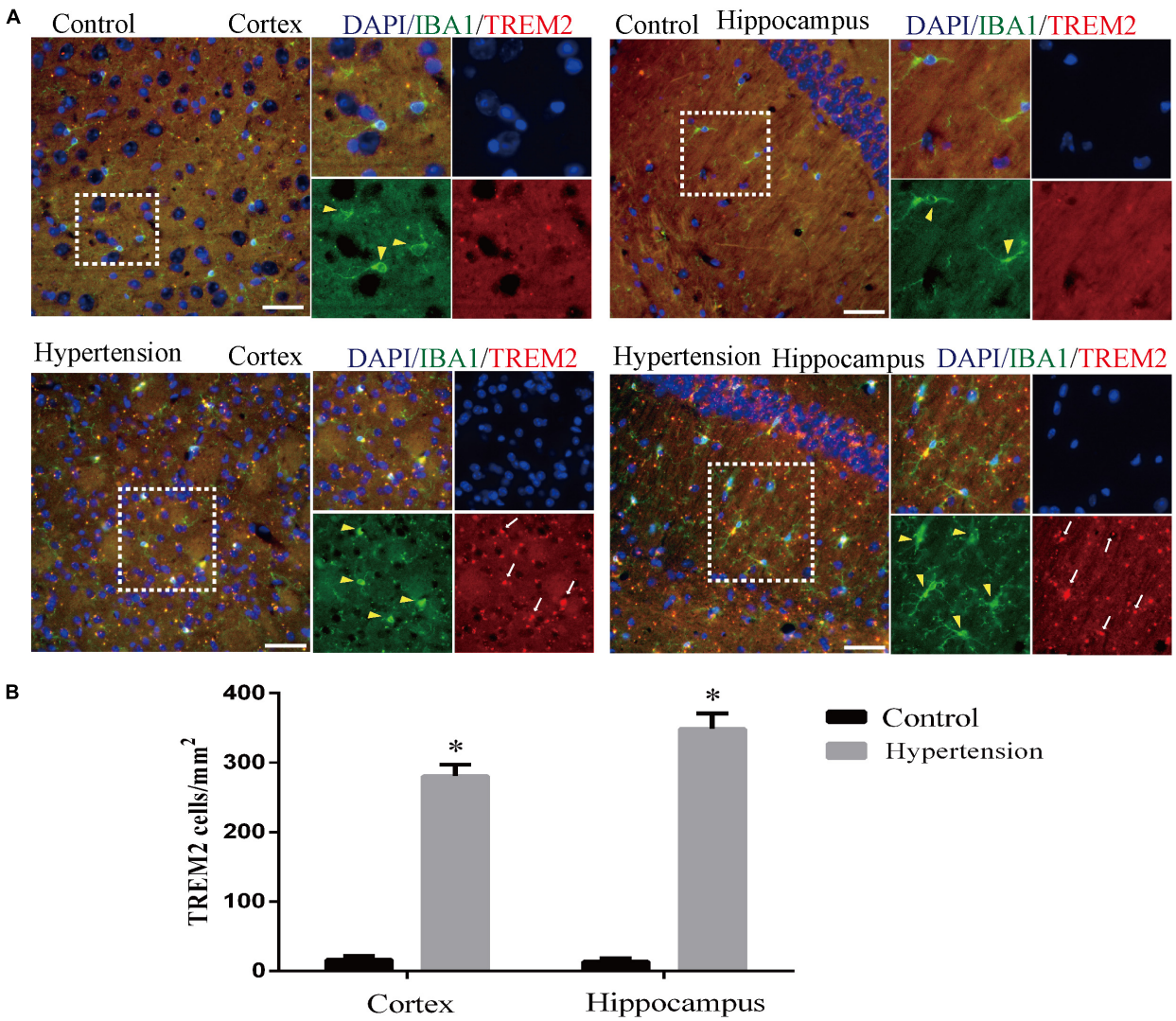

FIGURE 7 | Angiotensin II-induced hypertension increased the expression of triggering receptor expressed on myeloid cells 2 (TREM2) co-localization with microglia marker IBA1 in middle-aged mice. **(A)** Representative images of brain slices stained with the anti-IBA1 (green) and anti-TREM2 antibodies (red) in the cortex and hippocampus of the mice in the control and hypertension groups. **(B)** Histograms comparing the number of TREM2-positive cells in the cortex and hippocampus of mice in the control and hypertension groups (immunofluorescence, ×40, scale bar = 50 μm). Data are expressed as the means ± SD. *$p < 0.05$.

A1 astrocytes significantly increased in the hypertension group (**Figure 5**). Based on the above observation, we hypothesized that M1 microglia and A1 astrocytes may work collaboratively to induce neuronal death. However, further immunofluorescence did not demonstrate that the spatial associations and connections between M1 microglia and A1 astrocytes were closer or that their end feet engaged in more interactions. Then, we proposed that their communication may be through cytokine secretion.

In this study, we confirmed for the first time that microglial TREM2 was upregulated in the hypertension group. A similar regulatory pattern has been reported in an mouse model with AD, which is deemed to be an anti-neuroinflammatory response to M1 microglia (Wang et al., 2016; Yuan et al., 2016; Yeh et al., 2017). We then hypothesized that the overexpression of microglial TREM2 could not only mitigate microglial inflammatory response, but also had salutary effects on reverse A1 astrocytic activation and neuronal toxicity. To test the hypothesis, we incubated an immortalized mouse microglia-like BV2 cell line in an LPS-containing cell culture medium to mimic a neuroinflammatory environment (Lund et al., 2006). We found that the BV2 microglial cells mimicked primary microglia responses with high fidelity (Elkahloun et al., 2019) and were more tolerable for manipulating TREM2 overexpression. Because angiotensin II induces microglial activation through AT1R interacts with TLR4, and BV2 cells are devoid of AT1 receptor gene expression (Xu et al., 2015), BV2 cells are especially suitable for the present study to elucidate the mechanism focusing on TREM2-mediated microglial anti-inflammatory responses via LPS-ignited TLR4 pathway (Srinivasan et al., 2016; Zheng et al., 2016). In this study, we successfully induced A1

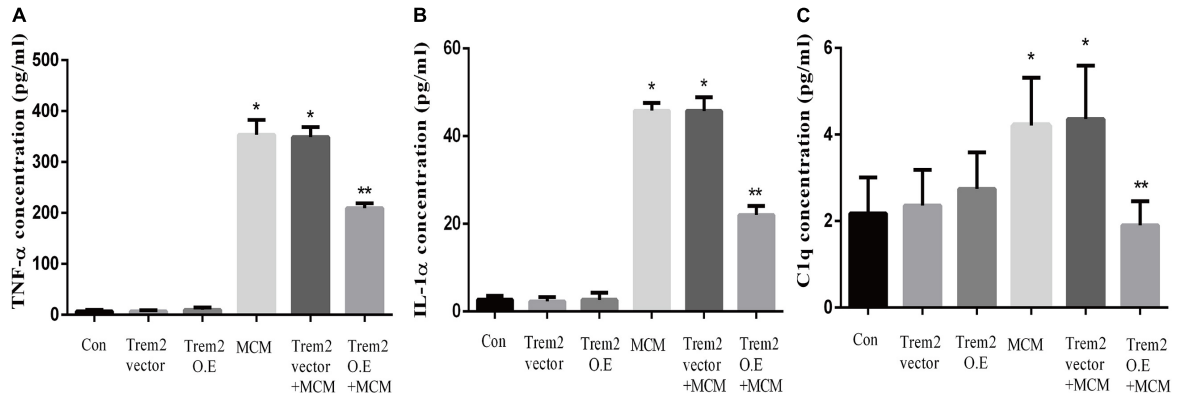

FIGURE 8 | Concentrations of IL-1α, TNF-α, and C1q were tested in MCM, Triggering receptor expressed on myeloid cells 2 (TREM2) (vector or overexpression) transfection-conditioned mediums, and TREM2 (vector or overexpression) + MCM. The concentration levels were measured using ELISA kits. **(A)** Concentration of TNF-α. **(B)** Concentration of IL-1α. **(C)** Concentration of C1q. Differences between the means were determined by *t* test. *$p < 0.05$ versus control at the match time point, ** $p < 0.05$ versus LPS (0.1 ug/m) at the match time point. The data are expressed as means ± SEM from three independent experiments. C1q: complement component 1q; IL-1α: interleukin 1α; TNF-α: tumor necrosis factor-α.

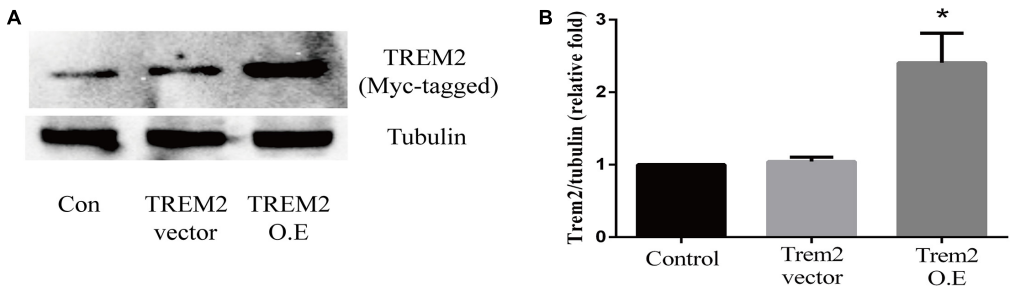

FIGURE 9 | Triggering receptor expressed on myeloid cells 2 (TREM2) protein level was quantitatively measured after TREM2 (vector or overexpression) plasmid transfection in BV2 cells. **(A)** Western blot analysis of TREM2 (Myc-tagged) protein expression. **(B)** Quantitative analysis of TREM2 (Myc-tagged) protein expression. *$p < 0.05$ vs. control of each protein. The data are expressed as means ± standard deviation (SD) from three independent experiments.

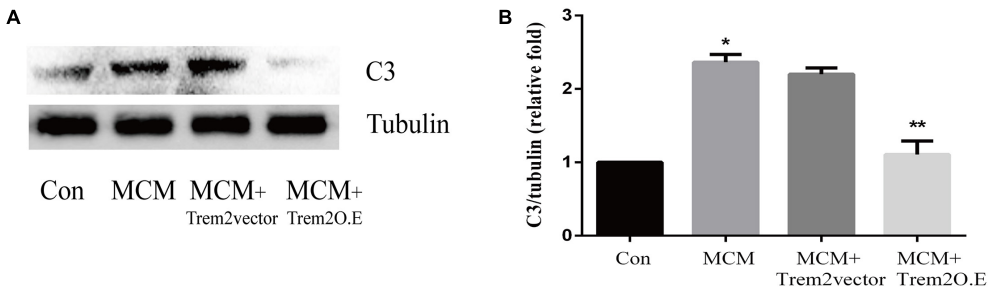

FIGURE 10 | A1 astrocytic marker C3 protein expressions were tested in MCM or triggering receptor expressed on myeloid cells 2 (TREM2) (vector or overexpression) + MCM after incubation for 24 h. **(A)** Western blot of C3 protein; tubulin was used as the internal loading control. **(B)** Quantitative analysis of the C3 protein expression. The data are expressed as means ± standard deviation (SD) from three independent experiments. *$p < 0.05$ versus control, ** $p < 0.05$ versus MCM. The data are expressed as means ± SD from three independent experiments.

astrocytic activation by LPS-induced M1 microglia (**Figure 10**), which was consistent with a previous study (Liddelow et al., 2017). Subsequently, the activation of both M1 microglia and A1 astrocytes was obviously alleviated by the overexpression of microglial TREM2 (**Figures 9**, **10**). Normally, astrocytes show a typical "star" cell shape and play an essential role in trophic support for neurons, metabolic regulation, and synaptic transmission (Liddelow and Barres, 2017; Morita et al., 2019). In this study, the A1 astrocytic marker C3 was highly expressed, and the "star" cell shape sharply diminished in A1 astrocytes (**Figure 11A**). In addition, AQP4, which is expressed in the end-feet of the astrocytes, plays a crucial in maintenance of the blood

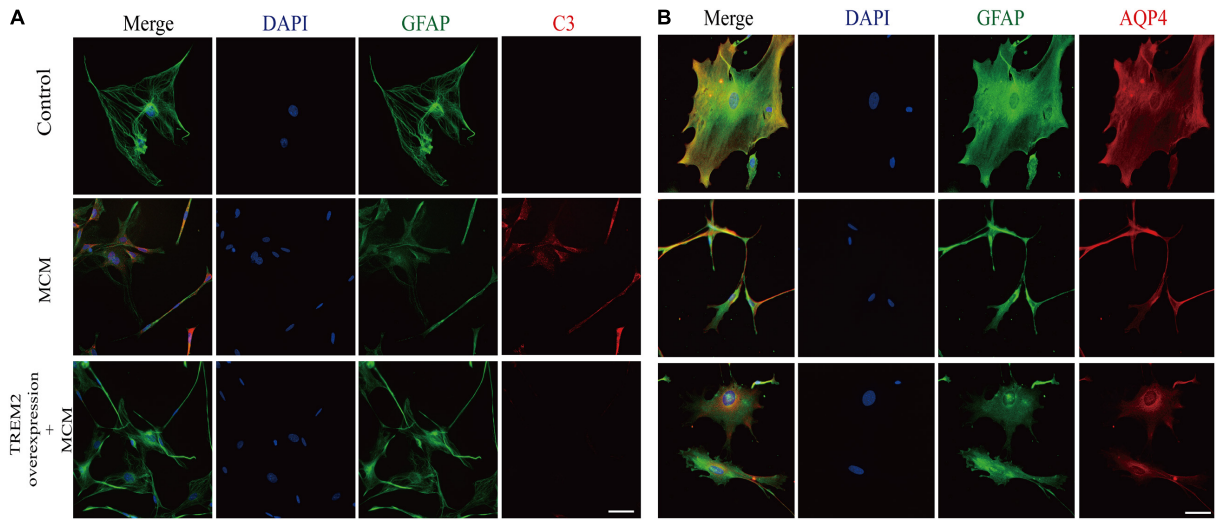

FIGURE 11 | After incubation with MCM or TREM2 overexpression + MCM for 24 h, the astrocytes were subjected to immunofluorescent staining with anti-GFAP (green), A1 anti-C3 (red; **A**), anti-AQP4 (red; **B**), and DAPI (blue) (immunofluorescence, ×40, scale bar = 10 μm).

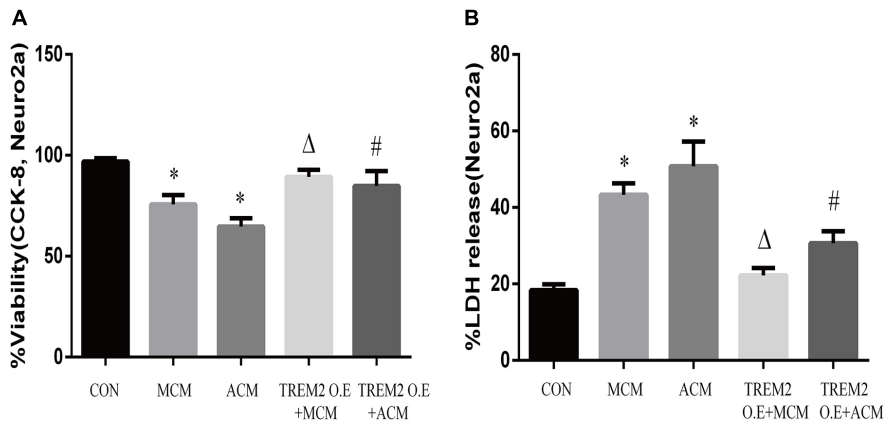

FIGURE 12 | Neuro2A cells were treated with MCM, ACM, triggering receptor expressed on myeloid cells 2 (TREM2) overexpression + MCM, or TREM2 overexpression + ACM, and then CCK-8 and lactate dehydrogenase (LDH) release assays were performed after 24 h. **(A)** CCK-8 assay indicated Neuro2A cells viability changes. **(B)** LDH release assay indicated Neuro2A cells toxicity changes. The data are expressed as means ± SEM from three independent experiments. $^{*}p < 0.05$ versus control, $^{\Delta}p < 0.05$ versus MCM. $^{\#}p < 0.05$ versus ACM.

brain barrier (BBB) (Garwood et al., 2017), and the results show that the foot processes and AQP4 expression were reduced in A1 astrocytes (**Figure 11B**). However, these phenomena of the AQP4 expression were rescued by the overexpression of microglial TREM2 treated with MCM (**Figure 11B**). To finally verify the findings, Neuro2A cell viability assays confirmed that the overexpression of TREM2 in BV2 cells could protect Neuro2A cells from the toxic effects of MCM and ACM (**Figures 12A,B**).

CONCLUSION

This present study showed that angiotensin II-induced hypertension significantly exacerbated M1 microglial activation, amyloid deposition, and neuronal apoptosis in middle-aged mice. Compared with previous studies that introduced hypertension in 4- to 12-week-old mice, which is considered young age in mouse, we believed that this middle-age hypertension mouse model shared the same principle of midlife hypertension for late life dementia in humans and is more suitable for investigating the relevant mechanisms in the progression of LOAD. Moreover, previous studies generally only have focused on activated microglial neuroinflammation-induced neuronal lesions, but new evidence has confirmed that activated microglia alone were insufficient to cause neuron death, and a more complicated network, such as A1 astrocytes, through complement components was involved in neuronal lesions and contributed to cognitive decline (Liddelow et al., 2017). Most intriguingly, we confirmed that neurotoxic A1 astrocytes were significantly upregulated in the middle-aged mice with

hypertension. The findings propose the concept that just as middle age creates "infertile soil, namely, a vulnerable brain microenvironment, hypertension acts as a "bad seed," a catalyst that quickly promotes amyloid deposition, neuroinflammation, and microcirculation disorder in the brain. Moreover, Aβ, the core pathogen of AD, acts on and stimulates M1 microglial activation, inducing a more serious neuroinflammation (Bagyinszky et al., 2017; VanItallie, 2017). This feedback creates a "vicious circle" between neuroinflammation and neuronal apoptosis, which eventually leads to late-life dementia. More importantly, microglial TREM2 upregulation was identified in middle-aged mice with hypertension. Contrastingly, it is worthy to point out that LPS inhibited microglial TREM2 expression in a time-dependent manner *in vitro* (**Supplementary Figure 1**), which was also consistent with previous studies (Gawish et al., 2015; Srinivasan et al., 2016). Then, we further overexpressed microglial TREM2 *in vitro* to confirm the hypothesis, and expanded the dimensions of evidence that TREM2 restrained the neuroinflammatory network *via* "cross-talk" between M1 microglia and A1 astrocytes to protect neurons. The above-mentioned findings implied that microglial TREM2 upregulation might be an anti-neuroinflammation response in middle-aged mice with hypertension. With progress in the "vicious circle," the anti-neuroinflammatory role of TREM2 will fade away in the late-life phase. Further study will be needed to validate the findings in more details of the current mouse model and address the following questions: what is the relationship between TREM2 and angiotensin II? Does TREM2 participate in the regulation of hypertension-induced cerebral microcirculation dysfunctions, or blood pressure? Is TREM2 an approach to regulate a neurovascular unit or a BBB function through restraining the neuroinflammatory network *via* "cross-talk" between M1 microglia and A1 astrocytes?

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study was approved by the Animal Research Committee of Sun Yat-sen University (Guangzhou, China; Committee Reference Number: SYSU-IACUC-2018-000093).

AUTHOR CONTRIBUTIONS

XX performed the experiments. All authors contributed to writing the manuscript and have approved the final version 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.2021.716917/full#supplementary-material

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ORIGINAL RESEARCH
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Glibenclamide Attenuates Neuroinflammation and Promotes Neurological Recovery After Intracerebral Hemorrhage in Aged Rats

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Intracerebral hemorrhage (ICH) is a common disease in the elderly population. Inflammation following ICH plays a detrimental role in secondary brain injury, which is associated with a poor prognosis of patients with ICH, and no efficient pharmacological preventions are available. Here, we investigated the effects of glibenclamide (GLC) on neuroinflammation in an autoblood-induced aged rat (18 months old) model of ICH. Rats were randomized into the sham, vehicle, and GLC groups. First, we investigated the expression level of sulfonylurea receptor 1 (Sur1) surrounding the hematoma after ICH. Then, neurological scores were calculated, and water maze tests, brain water content analysis, western blotting, and immunofluorescence assays were implemented to detect the neuroprotective effect of GLC. The expression of the Sur1-Trpm4 channel was significantly increased in the perihematomal tissue following ICH in aged rats. The GLC administration effectively reduced brain edema and improved neurofunction deficits following ICH. In addition, GLC increased the expression of brain-derived neurotrophic factors and decreased the expression of proinflammatory factors [tumor necrosis factor (TNF)-α,interleukin (IL)-1, and IL-6]. Moreover, GLC markedly reduced Ikappa-B (IκB) kinase (IKK) expression in microglia and nuclear factor (NF)-κB-P65 levels in perihematomal tissue. GLC ameliorated ICH-induced neuroinflammation and improved neurological outcomes in aged rats. In part, GLC may exert these effects by regulating the NF-κB signaling pathway through the Sur1-Trpm4 channel.

Keywords: intracerebral hemorrhage, glibenclamide, SUR1, neuroinflammation, aged rats

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INTRODUCTION

Intracerebral hemorrhage (ICH) is a subtype of stroke that leads to high rates of disability and death in humans (Xi et al., 2006). ICH is a common disease among the elderly population, and age is an essential factor that affects the prognosis of humans and animals after a stroke (Davis et al., 1995; Gong et al., 2004). Inflammatory responses play an important role in the pathogenesis after ICH (Aronowski and Hall, 2005; Hussain et al., 2009). Previous research has shown that nuclear factor

(NF)-κB plays a crucial role in secondary brain injury after ICH (Pozniak et al., 2014; Zhang et al., 2019). Therefore, it is necessary to research new therapies that target inflammatory responses to improve the prognosis of patients with clinical ICH.

Glibenclamide (GLC) is an oral hypoglycemic medicine that works by inhibiting sulfonylurea receptor 1 (Sur1) (Kurland et al., 2013). Sur1 forms two distinct ion channels: the Sur1-Trpm4 channel and Sur1-Kir6.2 channel (Chen et al., 2003). Many reports have shown that GLC protects against central nervous system (CNS) diseases, such as cerebral metastases, subarachnoid hemorrhage (SAH), traumatic brain injury, ischemic stroke, and status epilepticus (Simard et al., 2009; Thompson et al., 2013; Lin et al., 2017; Jha et al., 2020; Woo et al., 2020). For the first time, our previous research showed that the expression increased in the Sur1-Trpm4 channel in the perihematomal tissue following ICH in adult rats. GLC treatment improved neurological outcomes and protected the blood–brain barrier integrity following ICH, and these effects involved the expression of MMPs (Jiang et al., 2016). Previous research demonstrated that GLC treatment reduced tumor necrosis factor (TNF)-α, interleukin (IL)-6, and NF-κB following experimental cardiac arrest (Nakayama et al., 2018). However, no literature reported whether CLC participates in neuroinflammation following ICH in aged rats.

Thus, we hypothesize that GLC treatment alleviates secondary brain injury and improves neurofunction deficits after ICH in aged rats by suppressing neuroinflammation by inhibiting the Sur1-Trpm4 channel. An ICH model of an aged rat was used to verify this hypothesis.

MATERIALS AND METHODS

Ethics Statement

All the procedures in this study complied with the Guide for the Care and Use of Laboratory Animals. All the experiments were designed to minimize pain and animal numbers, and the study protocol was approved by the Animal Care and Use Committee at Army Medical University. The animals were housed under a 12-h light and 12-h dark cycle and were given free access to food and water.

Animals and Surgical Procedures

A total of 150 male Sprague-Dawley (SD) rats, weighing 450–550 g, were provided by the Army Medical University (Chongqing, China). Rats were randomly divided into three groups: the sham-operated group, ICH + vehicle group, and ICH + GLC group. To mimic the clinical condition of ICH, a model was established *via* injection of autogenous blood, as previously reported (Jiang et al., 2016). Briefly, the animals were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg). The body temperature of the animals was maintained at 37°C. The animals were positioned in a stereotaxic frame, a cranial burr hole (1 mm diameter) was drilled, and 100 μl autogenous arterial blood (obtained from the right femoral artery) was microinfused using a pump at a constant rate of 10 μl/min into the right caudate nucleus (coordinates: 3.5 mm lateral, 5.5 mm ventral, and 0.2 mm anterior to the bregma) through a 29-G needle. The sham-operated rats were only subjected to needle insertion. All rats survived the ICH induction and no mortality happened.

Glibenclamide Treatment

Glibenclamide (Tocris Bioscience, Ellisville, MO, United States) was administered as previously reported (Jiang et al., 2016). Briefly, dimethyl sulfoxide (DMSO) (50 mg/ml) was used to prepare stock solutions of GLC. The injection solution (200 ng/μl or 1 μg/ml) was made by dilution in 0.9% NaCl, and clarifying the solution using a few microliters of 0.1 N NaOH (final pH ∼ 8.5). At the end of the surgery, GLC was administered in a single loading dose (10 μg/kg intraperitoneal injection) plus continuous subcutaneous infusion (200 ng/h) *via* a mini-osmotic subcutaneous pump (Alzet, 2001, 1.0 μl/h; Alzet Corp., Cupertino, CA, United States). The vehicle group was treated with vehicle control solutions in the same manner.

Measurement of Brain Water Content

The brain water content was examined 72 h following ICH, as previously reported (Li et al., 2015). Briefly, the rats (n = 10/group) were euthanized and decapitated, the brains were quickly removed, and a 4-mm thick section of the coronal brain tissue surrounding the needle entry site was harvested. The brain tissue was divided into four parts: the contralateral cortex, contralateral basal ganglia, ipsilateral cortex, and ipsilateral basal ganglia. The cerebellum served as the internal control. The brain tissue weights were determined immediately after removal and after drying for more than 24 h at 100°C on an electronic analytical balance. The brain water content (%) was calculated using the following formula: (wet weight−dry weight)/wet weight × 100%.

Tissue Fixation and Immunofluorescence

Immunofluorescent labeling was conducted 24 or 72 h following ICH as previously described (Tang et al., 2015). About 18-μm thick brain tissue sections were prepared and stored at −20°C. The specimens were incubated with primary antibodies overnight at 4°C, and then with the appropriate secondary antibodies for 2 h at 37°C. Co-localization was examined by fluorescence microscopy (LSM780; Zeiss, Oberkochen, Germany).

Western Blot Analysis

Western blot analysis was conducted 24 h following ICH as previously described (Qi et al., 2019). The perihematomal brain tissues (4-mm-thick) were sampled. The relative densities of the bands were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, United States).

Real-Time PCR

PCR was performed to analyze Sur1 gene expression as previously reported (Jiang et al., 2016). Rats (n = 6/group) were sacrificed by decapitation 6, 12, and 24 h following ICH. The brain tissues were dissected (2 mm posterior and 2 mm anterior to the needle entry site) and immediately flash-frozen with liquid nitrogen. The primers for SUR1 were as

TABLE 1 | Primers used for real-time RT-PCR.

Gene	GenBank accession no.	Primer sequences
SUR1	NM_013039.2	F: CACAAGAAGCCCATCGACCT
		R: ATCGAAGGCCAAGCAGAGTC
GAPDH	NM_017008.4	F: TGAGGAGTCCCCATCCCAAC
		R: GATGGTATTCGAGAGAAGGGAGG
β-actin	NM_031144.3	F: GCAGGAGTACGATGAGTCCG
		R: ACGCAGCTCAGTAACAGTCC

F, forward primer; R, reverse primer.

follows: forward, 5′-CACAAGAAGCCCATCGACCT-3′; reverse, 5′-ATCGAAGGCCAAGCAGAGTC-3′ (**Table 1**). A positive standard curve was obtained using a serially diluted complementary DNA sample mixture. Gene expression was normalized by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression and quantified with standard samples. The data are expressed as a normalized messenger RNA expression (fold mRNA increase).

Corner Turn Test and Forelimb Placing Test

We used the corner turn test and forelimb placement test to assess the neurological function of the experimental rats 7 days following ICH as previously described (Hua et al., 2002; Krafft et al., 2014; Tan et al., 2017).

The corner turn test was conducted by two blinded observers. The experimental rats were allowed to proceed to a corner with an angle of 30°. We recorded the direction in which rats turned, and the process was repeated 10 times (60 s between trials); the percentage of right turns was calculated.

The forelimb placement test was conducted by two blinded observers. The rats were held by the torso, and all four limbs were allowed to hang freely in space. A trial was scored if a rat placed its forelimb on the edge of the countertop in response to vibrissae stimulation. Each forelimb was tested 10 times, and the percentage of successful scores was determined.

Morris Water Maze Test

The Morris water maze test was performed to measure the learning and spatial memory of rats as previously described (Dai et al., 2017). Twenty-three days following ICH, rats (n = 10, per group) were placed in a pool (200 cm in diameter, 50 cm in depth) in which they searched to find a platform (5 cm in diameter, top surface 1.5 cm below the surface of the water) within 120 s. The rats that failed the mission would be picked up and placed on the platform for 15 s to familiar with the situation. The rats were subjected to five consecutive days of trials. The latency time was recorded to assess spatial learning ability. The probe trial was performed on the sixth day by removing the platform, and each rat was allowed to swim freely (120 s). The number of times the target area (previous location of the platform) was

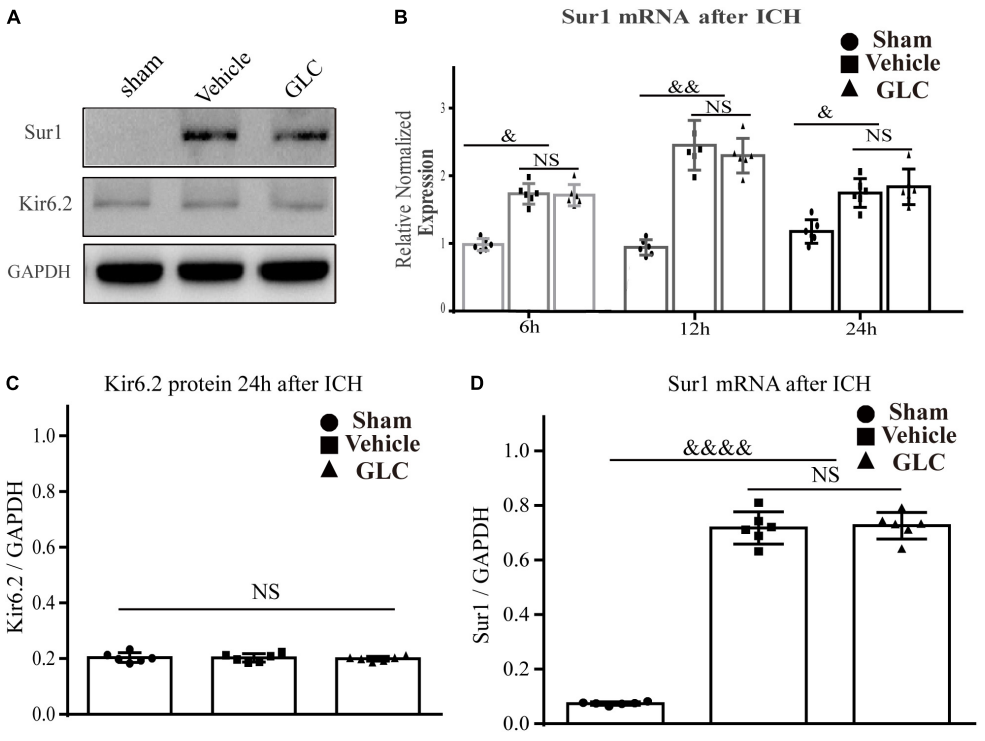

FIGURE 1 | Sulfonylurea receptor 1 (Sur1), but not Kir6.2, is upregulated in the aged rat model of intracerebral hemorrhage (ICH). **(A,C,D)** Western blot analysis and quantification of the mean density of the Sur1 (180 kDa) and Kir6.2 (45 kDa) bands surrounding the hematoma 24 h after ICH (six rats/group). The results of the mean band densities are presented as the mean ± SD, $^{\&\&\&\&}p < 0.0001$. **(B)** Relative normalized expression levels of Sur1 mRNA surrounding the hematoma at 6, 12, and 24 h after ICH, Sur1 mRNA expression increased and peaked at 12 h (six rats/group). The results are presented as the mean ± SD, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$.

crossed, the percent distance and percent time in the target quadrant were analyzed.

Statistical Analysis

The results of this study are expressed as the mean ± SD. Statistical analysis of the data was conducted using one-way analysis ANOVA, followed by Student–Newman–Keuls (SNK). Statistical significance was set as a p-value < 0.05.

RESULTS

Sur1 Is Upregulated After ICH in Aged Rats

The protein expression of Sur1 was significantly upregulated surrounding the hematoma after ICH in aged rats ($p < 0.0001$, **Figures 1A,D**), but no significant difference was observed between the rats in the GLC treatment group and the vehicle group ($p > 0.05$, **Figures 1A,D**). No difference was observed in KIR6.2 expression among the sham group, GLC treatment group, and vehicle group ($p > 0.05$, **Figure 1C**). The Sur1 mRNA expression was examined at 6, 12, and 24 h after ICH. Compared with the sham-treated rats, aged rats with ICH exhibited a remarkable increase in Sur1 mRNA ($p < 0.05$, $p < 0.01$, **Figure 1B**).

Glibenclamide Administration Improved Neurological Deficits

The corner turn score and forelimb placing score were used to measure the neurological function of the rats on day 7 after ICH. In these tests, the GLC-treated group had a lower corner turn score ($p < 0.05$, **Figure 2A**) and higher forelimb placing score ($p < 0.05$, **Figure 2B**) than the vehicle group.

Treatment With GLC Decreased Brain Water Content

The brain water content of the rats in the vehicle group was significantly increased 72 h after ICH, especially in the ipsilateral basal ganglia (Ipsi-BG: sham vs. vehicle, $p < 0.001$, **Figure 2C**). The GLC treatment remarkably decreased the brain water content in the ipsilateral basal ganglia (Ipsi-BG: vehicle vs. GLC, $p < 0.05$, **Figure 2C**).

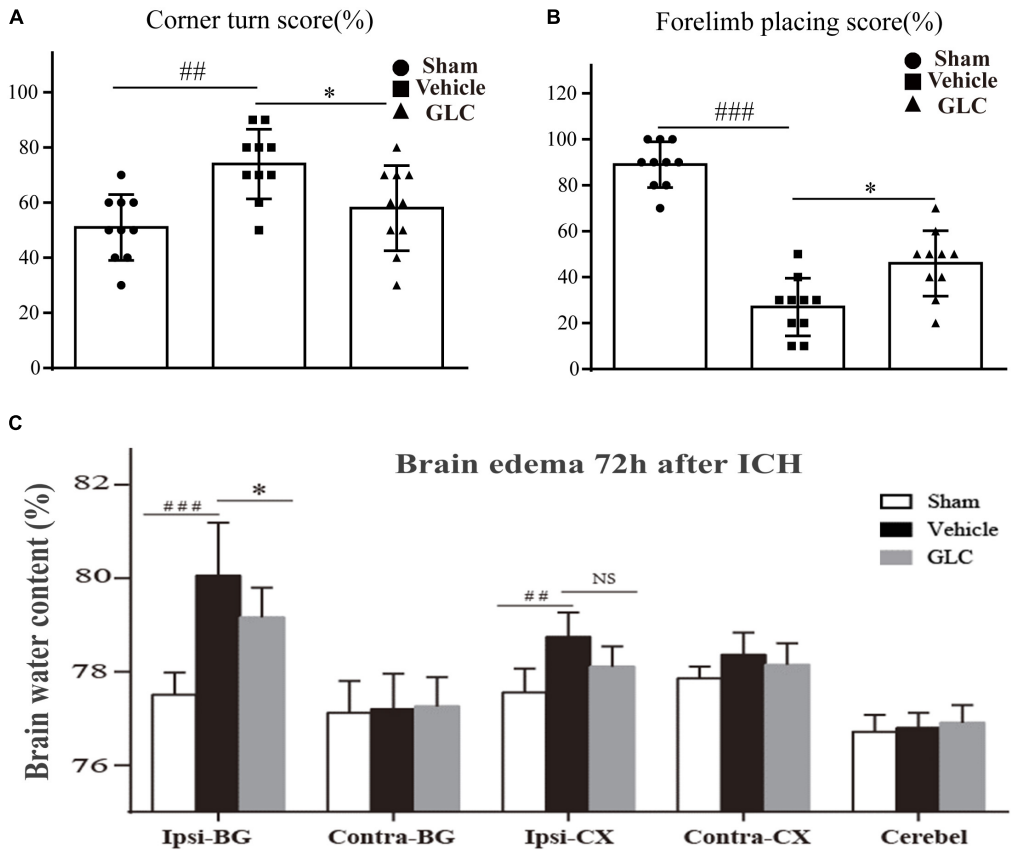

FIGURE 2 | (A) Results of the corner turn test on day 3 after intracerebral hemorrhage (ICH). **(B)** Results of the forelimb placement test on day 3 after ICH thrombolytic therapy. The results are presented as the mean ± SD. $^{*}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$. **(C)** Glibenclamide (GLC) significantly reduced ICH-induced brain edema in the ipsilateral basal ganglia (Ipsi-BG) and ipsilateral cortex (Ipsi-CX) 72 h after injury. The cerebellum (Cerebel) served as the internal control. The values are expressed as the mean ± SD, $n = 10$. Ipsi-BG: vehicle vs. sham $^{\#\#\#}p < 0.001$, vs. GLC $^{*}p < 0.05$; Ipsi-CX: vehicle vs. sham $^{\#\#}p < 0.01$, vs. GLC $p > 0.05$.

Glibenclamide Treatment Ameliorated the Performance of the Experimental Rats in the Morris Water Maze Test

The rats exhibited disadvantageous spatial learning and memory deficits following ICH. The latency of the rats in the GLC group was significantly shorter than that of the rats in the vehicle group beginning on the fourth of five consecutive days of acquisition training ($p < 0.05$, **Figure 3A**). The GLC-treated rats spent more time ($p < 0.05$, **Figure 3D**) and traveled a greater distance ($p < 0.05$, **Figure 3C**) in the target quadrat compared with the vehicle-treated rats. In addition, the rats in the vehicle group crossed the platform fewer times ($p < 0.001$ and $p < 0.05$, respectively, vs. sham and GLC, **Figure 3B**). The results demonstrated that GLC improved spatial learning and memory deficits following ICH.

Treatment With GLC Increased Brain-Derived Neurotropic Factor 72 h After ICH

Immunofluorescence staining suggested a significant increase in brain-derived neurotrophic factor (BDNF) co-localized with neurons in the GLC group compared with that in the vehicle group (**Figure 4A**). Studied regions were marked with "squares" (**Figure 4B**). Western blotting examination revealed that the GLC treatment significantly increased the protein expression of BDNF 72 h following ICH ($p < 0.01$, **Figures 4C,D**).

Treatment With GLC Decreased the Expression of NF-κB

We used western blotting and immunofluorescence staining to measure the expression of components of the NF-κB signaling pathway. Immunofluorescence staining demonstrated a remarkable decrease in Ikappa-B kinase (IKKβ) in the GLC group compared with the vehicle group (**Figure 5A**). The protein expression of NF-κB-p65 was significantly increased 24 h after ICH (sham vs. vehicle, $p < 0.001$, **Figures 5B,C**). The GLC treatment significantly decreased the protein expression of NF-κB p65 24 h after ICH (vehicle vs. GLC, $p < 0.01$, **Figures 5B,C**).

Glibenclamide Reduces ICH-Induced Neuroinflammation

The expression of inflammatory factors was examined using western blotting analysis. The results revealed that TNF-α, IL-1, and IL-6 expression was significantly increased 24 h after ICH (**Figure 6**). GLC significantly decreased the expression of TNF-α ($p < 0.01$, **Figure 6A,B**). GLC significantly reduced the expression of IL-1 ($p < 0.05$, **Figure 6A,C**). GLC decreased the

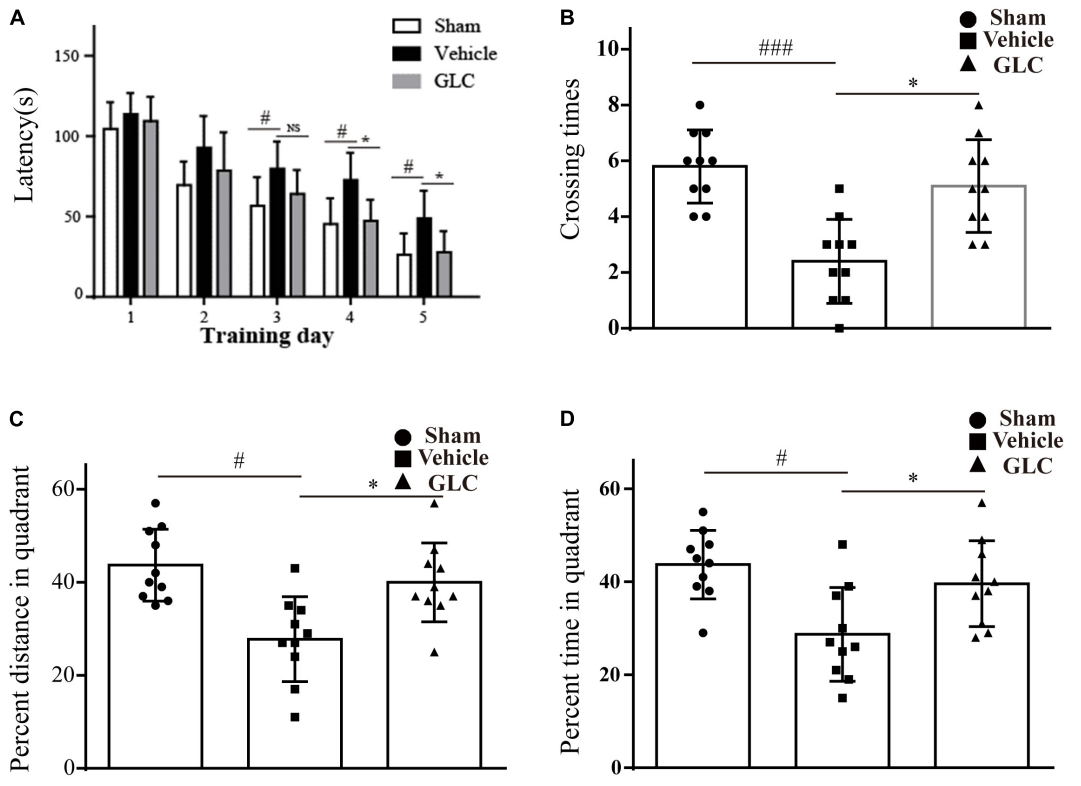

FIGURE 3 | Spatial learning and memory deficits were examined 4 weeks after intracerebral hemorrhage (ICH). The values are expressed as the mean ± SD, $n = 10$. **(A)** Escape latency in training trials: vehicle vs. sham $^{\#}p < 0.05$, vs. glibenclamide (GLC) $^{*}p < 0.05$. **(B)** Times that the platform was crossed in the probe trials: vehicle vs. sham $^{\#\#\#}p < 0.001$, vs. GLC $^{*}p < 0.05$. **(C)** Percent distance in the target quadrant in the probe trials: vehicle vs. sham $^{\#}p < 0.05$, vs. GLC $^{*}p < 0.05$. **(D)** Percent time in the target quadrant in the probe trials: sham $^{\#}p < 0.05$, vs. GLC $^{*}p < 0.05$.

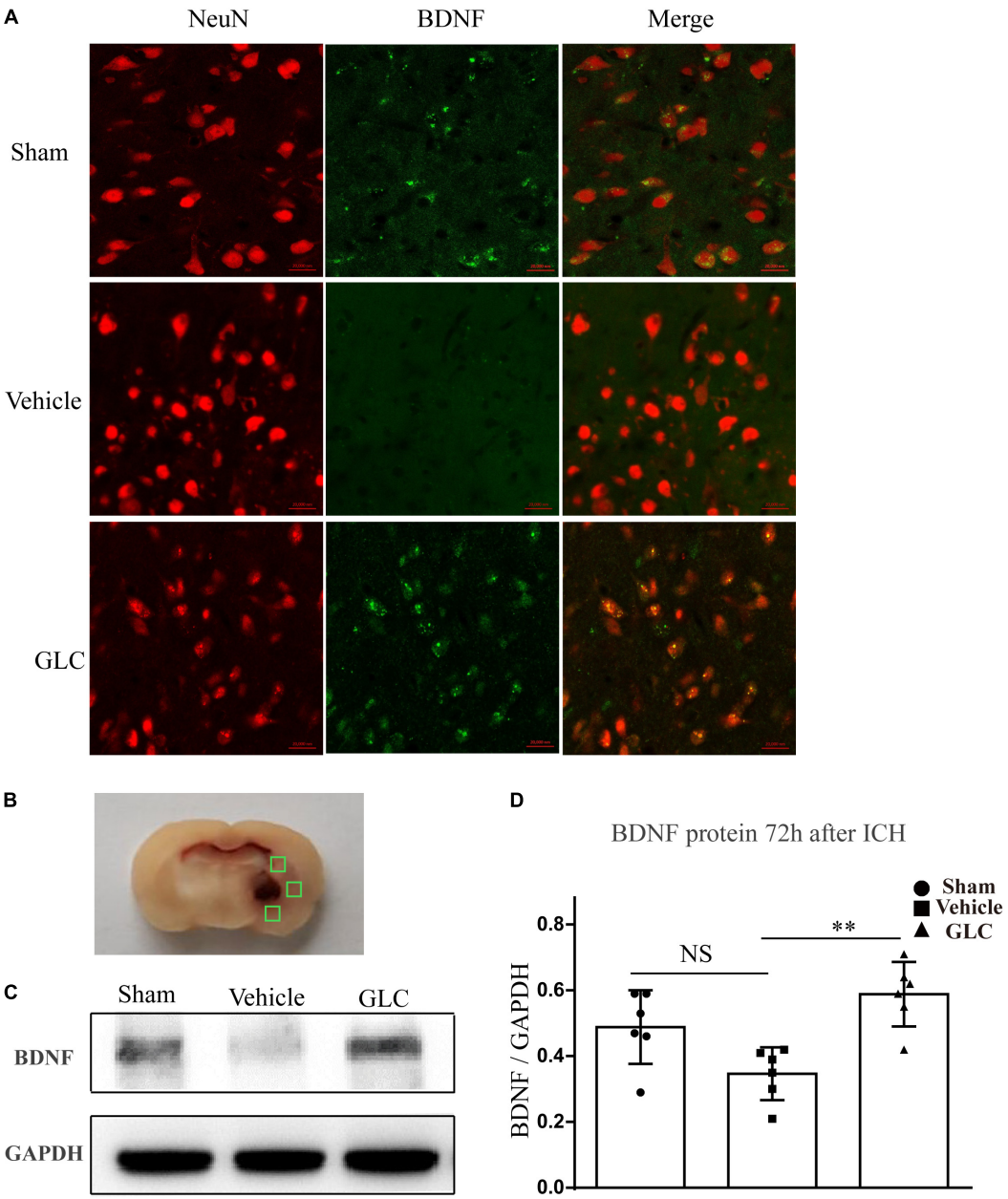

FIGURE 4 | Glibenclamide treatment significantly increased the expression of brain-derived neurotrophic factor (BDNF). **(A)** BDNF upregulation was observed in neuron cells surrounding the hematoma (six rats/group). Bar = 20 μm. **(B)** Studied regions were marked with " □". **(C,D)** The results of the mean band densities are presented as the mean ± SD, $^{**}p < 0.01$.

expression of IL-6, but no significant difference was observed ($p > 0.05$, **Figures 6A,D**).

DISCUSSION

Intracerebral hemorrhage is currently one of the most common diseases, particularly in the elderly population. With an aged rat model, we mimicked the pathophysiological processes observed in spontaneous ICH in elderly patients in the clinic. We detected an upregulation of Sur1 expression in an ICH model of an aged rat. Moreover, we suggested that the inhibition of Sur1 by GLC ameliorated neuroinflammation and improved neurological deficits.

Sulfonylurea receptor 1 forms two distinct ion channels: the Sur1-Trpm4 channel and Sur1-Kir6.2 channel (Tosun et al., 2013). Under normal conditions, Sur1 is constitutively expressed in some neurons of the CNS and exclusively forms Sur1-Kir6.2 channels (Simard et al., 2014). Previous study demonstrated, the expression increased in the Sur1-Trpm4 channel following

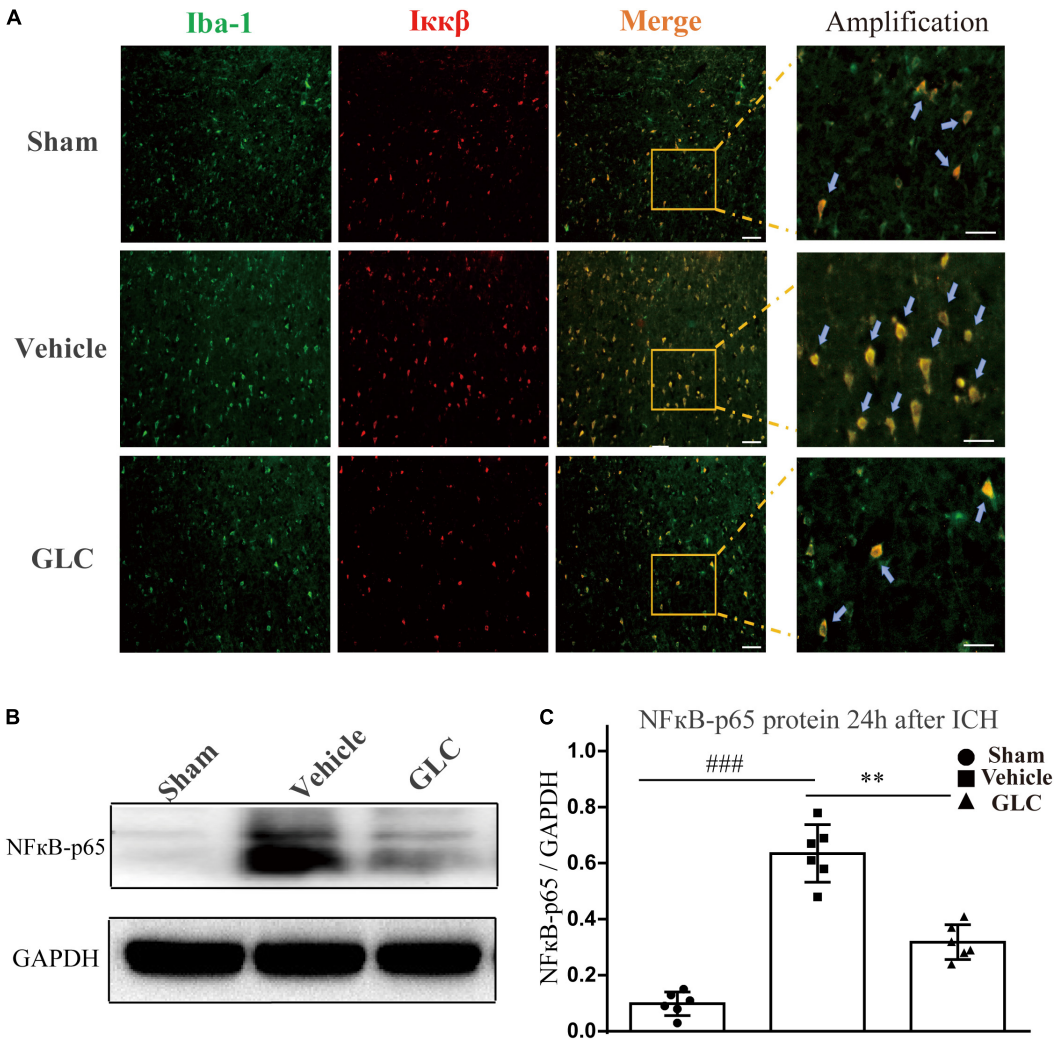

FIGURE 5 | The regulatory effect of glibenclamide (GLC) on the NF-κB signaling pathway after intracerebral hemorrhage (ICH). **(A)** GLC reduced microglial secretion of Ikappa-B kinase (IKKβ) (n = 6). Bar = 20 μm. **(B,C)** Western blot analysis and quantification of the mean density of the nuclear factor (NF)-κB-p65 (65 kDa) band surrounding the hematoma 24 h after ICH (six rats/group), vehicle vs. sham $^{###}p < 0.001$, vs. GLC $^{**}p < 0.01$. Amplified regions were marked with "squares". Iba-1 co-localized with Ikkβ were marked with "arrows".

ICH, which was not observed in uninjured brain tissues (Woo et al., 2013). We showed that the level of Sur1 was significantly increased 6 h following ICH in aged rats, and the increase continued until 12 h but decreased at 24 h. Interestingly, we found that the increased level and peak timepoint are somewhat inconsistent with the results of our previous study showing that Sur1 mRNA significantly increased 12 h after ICH in adult rats, and the increase was maintained until 24 h but decreased at 48 h. Several factors may explain this difference. One possible reason is related to the different measures of thrombin. Ibbotson et al. (1992) proved that coagulation rates in plasma are accelerated with age, suggesting that hematoma can produce much more thrombin in aged rats. Thrombin can induce the expression of matrix metalloproteinase (MMP)-9, and MMPs are involved in the expression of Sur1 (Caffes et al., 2015). The second possible explanation for our findings is related to Sur1 expression in the different microglial responses in adult and aged animals (Camacho et al., 2015). Microglia play an important role in inflammation after CNS injury, and inflammation may be a factor in the upregulation of Sur1 (Simard et al., 2009). Wasserman et al. (2008) reported a difference in microglial activation and macrophage distribution between young and aged rats following ICH. Therefore, it is possible that the timing of Sur1 upregulation after ICH might be different between young rats and aged rats. In this report, we demonstrate that the expression of Sur1 is upregulated in aged rats after ICH.

The present study shows that the Sur1-Trpm4 channel is implicated in ICH-induced inflammation. GLC administration significantly reduces the expression of the NF-κB, IL-1, TNF-α, and IL-6. Similarly, many studies have shown that GLC inhibits inflammation in animal models of SAH (Simard et al., 2009), experimental cardiac arrest (Nakayama et al., 2018), and cerebral

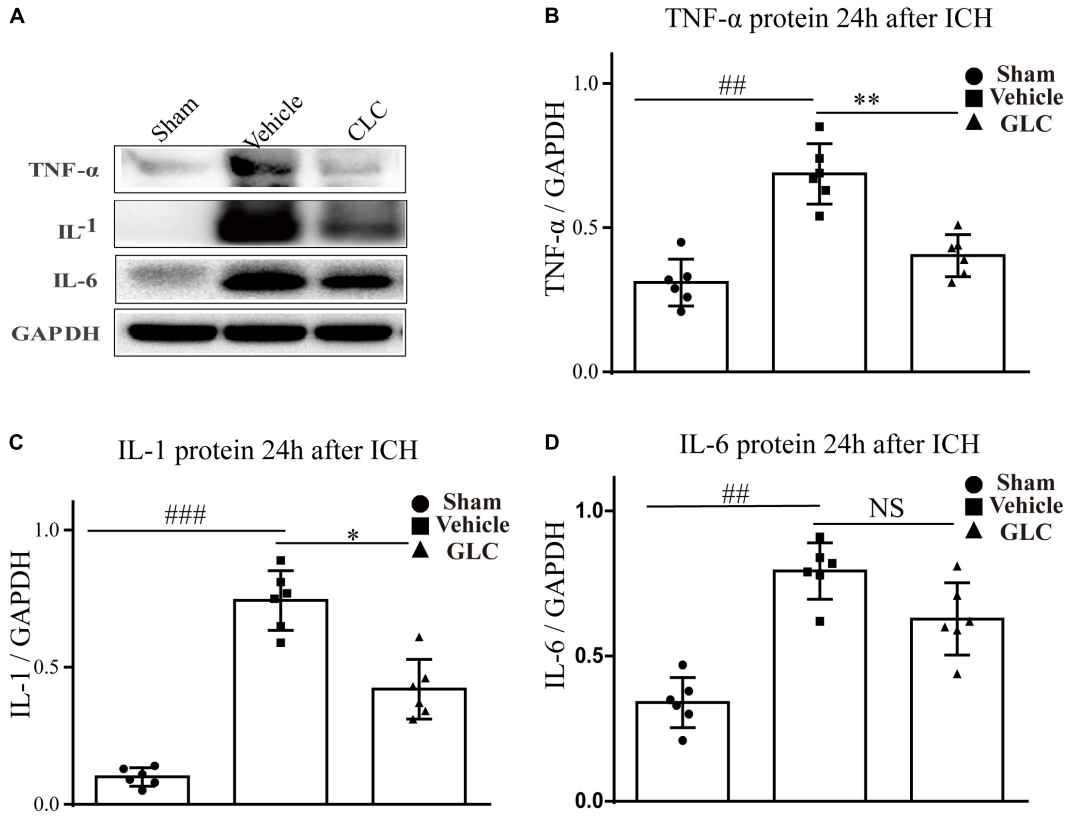

FIGURE 6 | Effect of glibenclamide (GLC) on neuroinflammation protein expression following intracerebral hemorrhage (ICH). The band intensity quantification is presented as the mean ± SD, **(A,B)** tumor necrosis factor (TNF)-α: vehicle vs. sham $^{\#\#}p < 0.01$, vs. GLC $^{**}p < 0.01$; **(A,C)** IL-1: vehicle vs. sham $^{\#\#\#}p < 0.001$, vs. GLC $^{*}p < 0.05$; **(A,D)** IL-6: vehicle vs. sham $^{\#\#}p < 0.01$, vs. GLC $p > 0.05$.

ischemic injury (Caffes et al., 2015). Our previous study determined that inhibition of Sur1 alleviated ICH-induced metalloproteinase (MMPs) expression. Many previous studies showed that MMPs play an important role in neuroinflammation (Vandooren et al., 2014; Hannocks et al., 2017; Mi et al., 2021). The increased activity of MMPs can affect the secretion of many types of inflammatory cytokines and the activation of inflammatory cells (Florczak-Rzepka et al., 2012).

This study found that treatment with GLC induced the expression of BDNF. Previous studies determined that BDNF stimulates neural progenitor cells to differentiate into mature neurons, and it exerts a neurotrophic effect at sites of injury (Shimotake et al., 2010; Deng et al., 2019). Previous studies have shown that neuroinflammation reduces the expression of BDNF and negatively affects many stages of neurogenesis (Hashimoto, 2015; Zhang et al., 2018; Zhong et al., 2020). Bi et al. (2016) proved that neuroinflammation attenuates the expression of BDNF by activating the NF-κB pathway. Therefore, a significant increase in BDNF may be involved in the activation of the NF-κB pathway.

Many factors are related to neurological deficits following ICH, including primary brain injury, edema, inflammation, and age (Keep et al., 2012; Guo et al., 2021). In this study, GLC improved neurological deficits, consistent with previous studies (Liew et al., 2012; Xu et al., 2015). Simard et al. (2006) proved that continuous subcutaneous infusion of GLC (75 ng/h) reached the peri-infarct regions of rats with cerebral ischemia, resulting in potential neuroprotection. Several recent clinical trials have shown that GLC is associated with improvements in midline shift, level of alertness, neurofunction deficits, and survival after large hemispheric infarction (Kimberly et al., 2018; Sheth et al., 2018; Vorasayan et al., 2019). Robert et al. (2020) concluded that inhibiting the ion channel Sur1-Trpm4 could be a valuable adjuvant to prevent and even reverse fluid accumulation in the brain parenchyma. Several clinical trials about the safety and efficacy of GLC in CNS diseases are ongoing, such as SE-GRACE and GASH. Within a few years, all these research findings make it possible to use GLC in clinical stroke treatment.

CONCLUSION

In summary, in the current study, we found that the expression of the Sur1-Trpm4 channel was significantly increased in perihematomal tissue following ICH in aged rats. We demonstrated that GLC ameliorated ICH-induced neuroinflammation and improved neurological outcomes,

and GLC may exert these effects in part by regulating the NF-κB signaling pathway through the Sur1-Trpm4 channel. Our findings may contribute to the further elucidation of the mechanism of action of CLC and assist in developing a novel therapeutic strategy for treating clinical stroke.

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 animal study was reviewed and approved by the Animal Care and Use Committee at Army Medical University.

AUTHOR CONTRIBUTIONS

BJ and YZ wrote the manuscript, analyzed the data, and designed and performed the experiments. YW, ZL, JT, and QC assisted with the experiments, prepared the figures, and performed the behavioral tests. GZ contributed to the conception of the review and gave final approval of the version to be published. All authors contributed to the article and approved the submitted version.

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REVIEW
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Vascular Dementia and Underlying Sex Differences

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Vascular dementia (VaD) is the second most common form of dementia after Alzheimer's disease (AD); where Alzheimer's accounts for 60–70% of cases of dementia and VaD accounts for 20% of all dementia cases. VaD is defined as a reduced or lack of blood flow to the brain that causes dementia. VaD is also known occasionally as vascular contributions to cognitive impairment and dementia (VCID) or multi-infarct dementia (MID). VCID is the condition arising from stroke and other vascular brain injuries that cause significant changes to memory, thinking, and behavior, and VaD is the most severe stage while MID is produced by the synergistic effects caused by multiple mini strokes in the brain irrespective of specific location or volume. There are also subtle differences in the presentation of VaD in males and females, but they are often overlooked. Since 1672 when the first case of VaD was reported until now, sex and gender differences have had little to no research done when it comes to the umbrella term of dementia in general. This review summarizes the fundamentals of VaD followed by a focus on the differences between sex and gender when an individual is diagnosed. In addition, we provide critical evidence concerning sex and gender differences with a few of the main risk factors of VaD including pre-existing health conditions and family history, gene variants, aging, hormone fluctuations, and environmental risk factors. Additionally, the pharmaceutical treatments and possible mitigation of risk factors is explored.

Keywords: vascular dementia, Alzheimer's disease, sex, gender, multi-infarct dementia

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INTRODUCTION

Vascular dementia (VaD), a heterogeneous group of brain disorders is the next most common form of dementia following Alzheimer's disease (AD) and accounts for at least 20% of dementia cases (Román, 2003; Korczyn et al., 2012). VaD is caused by a blocked or reduced blood flow to the brain which will deprive neurons of critical nutrients (Vijayan and Reddy, 2016). This deprivation eventually causes the neurons to die, and the brain tissue starts to shrink. Some common contributors to this kind of dementia include stroke, cardiovascular disease, diabetes, hyperlipidemia, and hypertension (Song et al., 2014).

Vascular cognitive impairment (VCI) is a term that encompasses a continuum of cognitive disorders with cerebrovascular pathology contribution, ranging from mild cognitive impairment to VaD (Nguyen et al., 2021). As a result, VCI and VaD constitute an intriguing junction of

cardiovascular disease (CVD), and neurodegenerative disorders, like AD, are a growing topic of research in recent years. Even though VCI and VaD research has identified a variety of causes and explanations for disease development, many aspects remain unknown, particularly sex differences in VCI (e.g., epidemiology), which are lacking in comparison to those available for CVD and AD.

History of Vascular Dementia (VaD)

The root meaning of the word dementia came from the Latin word demons which means "Without mind." The first case of VaD was reported in 1672 by Thomas Willis in his book De Anima Brutorum (Román, 2003). After the recognizing of the differences between hemorrhage and ischemic stroke, Bayle discovered that apoplexy was the obstruction of the arteries with effusion of blood in such small amounts because of the failure of the blood influx to the brain (Engelhardt, 2017). Apoplexy is unconsciousness and/or incapacitation resulting from a cerebral hemorrhage or stroke. Later, apoplexy was considered the cause of brain congestion or cerebral hyperemia in most of the 18th and early 19th centuries. The year 1894 is considered to be the beginning of the modern history of VaD due to Otto Binswanger and Alois Alzheimer, who were the first to separate the forms of dementia from neurosyphilis and recognize a heterogeneous VaD (Yang et al., 2016). In 1910 Emile Kraepelin closely observed the concept of Otto Binswanger and Alois Alzheimer with a different description for VaD as arteriosclerotic dementia or cerebral arteriosclerosis (Román, 2003). For 70 years after Kraepelin's description, the synonym for his description of VaD was called senile dementia. Hachinski et al. (1974) emphasized that vascular disease was responsible for dementia, via the occurrence of small or large cerebral infarcts; the term Multi Infract dementia (MID) was to be used synonymously with VaD. In 1995 a proposal was made to the National Institutes of Health that the broader term VaD could be changed to the new name Vascular Cognitive Impairment.

Symptoms, Risk Factors, Characteristic Features, and Subtypes of VaD

The symptoms of VaD differ depending on which part of the brain the vessels and blood flow are obstructed (Venkat et al., 2015). However, the common indications might appear as disorientation, difficulty thinking, understanding, inability to create new memories, agitation, or behavioral symptoms (Kalaria, 2016). Moreover, VaD symptoms may be most obvious when they happen soon after a major stroke, and they can gradually develop (Dichgans and Leys, 2017). **Figure 1** shows some of the risk factors, characteristic features, and subtypes associated with VaD.

The risk factors help perpetuate the occurrence of the VaD. Strokes that block a brain artery usually cause a range of symptoms like cognitive decline, neurological deficits, and further severe cardiovascular incidents that may include vascular dementia (Shabir et al., 2018). When VaD begins, it can be classified into various subtypes that are typically related to the root cause of the VaD (Rockwood et al., 1999). Stroke, multi-infarct, and subcortical dementia refer to the vascular events

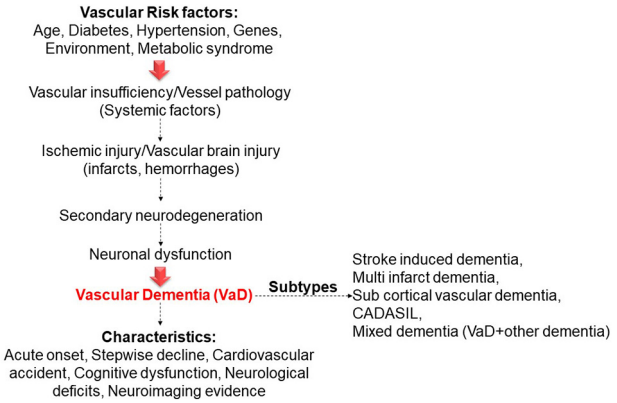

FIGURE 1 | Subtypes of VaD that are associated with some of the vascular risk factors and their characterized features.

leading to dementia (Kalaria and Erkinjuntti, 2006). CADASIL refers to a subtype of VaD, in which a specific gene variant must be present (Benisty et al., 2008). This variant whilst mixed with dementia causes a rare disease subtype that refers to the presence of multiple forms of dementia including the presence of vascular events (Benisty et al., 2008). In CADASIL, migraine with aura is more frequent in women and, stroke is more frequent in men before menopause (Gunda et al., 2012). After this age limit, the difference appears to vanish, however, men in the late stages of the disease may experience more cognitive impairment and cerebral atrophy.

Dementia is a consequence and a risk factor for stroke and VaD. Stroke is one of the primary causes of disability, and having a stroke doubles your risk of developing VaD (Vijayan and Reddy, 2016). Lower education, older age, diabetes mellitus, myocardial infarction, atrial fibrillation, epileptic seizures, sepsis, cardiac arrhythmias, congestive heart failure, global cerebral atrophy, and medial temporal lobe atrophy, and white matter changes have all been linked to an increased risk of dementia after a stroke (Pinkston et al., 2009). Post-stroke depression is another risk factor for VaD, which is more common in women than in men (Pendlebury and Rothwell, 2009). Female sex, medial temporal lobe atrophy, and a family history of dementia are all better predictors of pre-stroke dementia than post-stroke dementia (Podcasy and Epperson, 2016). Pre-stroke dementia could be a symptom or cause of a fundamental degenerative condition that makes vascular events more likely (Glader et al., 2003).

Multi-infarct dementia is a type of VaD, occurs when a series of minor strokes triggers a loss of brain function (Al-Qazzaz et al., 2014). A stroke, or brain infarct, occurs when the blood flow to any part of the brain is disrupted or blocked. Blood supplies oxygen to the brain, and brain tissue dies quickly without it. Memory and cognitive function loss can occur because of MID, as well as psychosocial issues. MID generally occurs in people aged 55 to 75 years and is more common in men than women (McKay and Counts, 2017). Medical conditions and lifestyle that increase the risk of MID include atrial fibrillation, rapid heartbeat, previous strokes, heart failure, cognitive decline before a stroke,

high blood pressure, diabetes, atherosclerosis, or hardening of the arteries, smoking, alcohol, a low level of education, a poor diet, little to no physical activity (Lechner and Bertha, 1991).

Subcortical VaD is the subtype that has attracted the most interest among all the potential VaD subtypes (Wallin et al., 2003). Lacunar infarcts and ischemic white-matter lesions with demyelination and axon loss are the most common brain lesions in this kind of VaD. The primary cause is an injury to the penetrating arteries vessel walls (Kalaria, 2016). Multiple vascular problems, such as arterial hypertension, diabetes, and ischemic heart disease, are common in patients with subcortical VaD. Impairment goal formulation, starting, planning, and organizing are among the clinical signs (O'Brien and Thomas, 2015). Cerebrovascular white matter lesions are associated with stroke, cognitive decline, dementia, and death in a meta-analysis of longitudinal studies (Debette and Markus, 2010). These lesions correlate with advancing age, female gender, and vascular risk factors, including hypertension, diabetes mellitus, smoking, and lower income (Tomimoto, 2011). Another study was unable to find significant differences in the role of sex in subcortical VaD cases (Choi et al., 2020).

Cognitive impairment caused by numerous central nervous system (CNS) disorders are referred to as mixed dementia. A combination of AD pathologies such as amyloid deposits and tau tangles—and vascular impairment, such as numerous microbleeds or infarcts, is the most common cause of this disease (Jellinger, 2004). Rendering to vascular theory, chronic diseases (hypertension, diabetes mellitus, cardiac disease, dyslipidemia, and obesity) and the sedentary lifestyle produce numerous vascular changes, which generate vascular atrophy of the vascular terminations, as well as a reduction in the number of terminal blood vessels (Zekry et al., 2002). These alterations affect the cerebral microvasculature and diminish the cerebral blood flow, which is mainly observed in untreated hypertensive patients and those treated sporadically or inadequately (Hanyu, 2012). It is generally accepted that vascular dementia and mixed dementias occur more frequently in males, with rates of 31 vs. 25% in females (Podcasy and Epperson, 2016).

Diagnosis, and Current Treatment

The diagnostic tools for VaD vary on the diagnosis of the patients. A person suspected of having VaD will generally have a brain scan to look for any changes that have taken place in the brain. A scan such as Computerized tomography (CT) or magnetic resonance imaging (MRI) is also common to visualize brain function and may rule out a blockage or build-up of fluid inside the brain (van Straaten et al., 2004). Carotid ultrasound is used to determine whether the carotid artery shows any signs of narrowing because of plaque deposits or structural issues causing reduced blood flow to the brain (Malojcic et al., 2017). Neurological exams for reflexes, muscle tone and strength, are used as well as qualitative exams on comparing strength on one side of the body with the other side, assessing ability to get up from a chair and walk across the room, the intensity of touch, sight, coordination, and balance. Neuropsychological tests are also used to examine the ability to speak, write and understand language, work with numbers, learn and remember information, develop a plan of attack/solve a problem, and respond effectively to hypothetical situations (Pimentel, 2009).

In terms of treatments, the medical field lacks actual drugs that are fully approved by the FDA. However, there are clinical trials for drugs that treat AD symptoms which can also be offered to patients with VaD as well (Huang et al., 2020). With regards to anti-dementia therapeutics, there are no specific drugs approved for VaD treatment. The cholinesterase inhibitors, and the NMDA (the N-methyl-D-aspartate receptor) antagonist, are the only medications currently licensed for AD treatment, have been found to show some cognitive improvements in mild to moderately advanced VaD (Kandiah et al., 2017; Yaowaluk et al., 2019; Jian et al., 2020). Memantine belongs to the aminoadamantane chemical class and is structurally like amantadine, an anti-Parkinson and antiviral drug. Memantine has been tested in a study that included 815 subjects with mild to moderately advanced VaD (Baskys and Hou, 2007). Treatment with 20 mg/day dose or placebo lasted 28 weeks. Data analysis showed a significant improvement in cognitive function, measured as ADAS-cog (Alzheimer's Disease Assessment Scale-cognitive subscale), from baseline, over placebo. In a recent study (Arrigo et al., 1990), 56 patients with dementia and cerebrovascular illness were randomized to receive either acetyl-L-carnitine (ALC) 1.5 g/day or placebo for 28 weeks in a multicenter, double-blind, placebo-controlled clinical trial. The individuals' scores on the Montreal Cognitive Assessment improved dramatically after taking ALC, particularly in the attention and language subitems. However, it's difficult to believe that the study was solely focused on VaD because the patients were already on donepezil, implying that a significant proportion of them may have been affected by AD or mixed dementia (Pennisi et al., 2020). A small but statistically significant improvement was found on the NOSGER (Nurses Observational Scale for Geriatric Patients) disturbing behavior scale. Additionally, there have been attempts to use similar medicines that treat underlying causes of VaD. For example, in a small study hypertension medicines had a positive correlation with VaD risk reduction compared to subjects with similar risk factors but were without the antihypertensive (Iulita and Girouard, 2017). These include tablets to reduce blood pressure, prevent blood clots and lower cholesterol. Sometimes if treated early blood vessels can repair and slow the rate at which VaD progresses and might prevent further development of symptoms (Lee, 2011). A 5-year follow-up study on a community sample of 1617 African Americans demonstrated that the use of medications that mediate vascular risk factors (antihypertensive drugs, anti-hyperlipidemic, and antidiabetic drugs) reduced the risk of incident dementia by 40% (Kennelly et al., 2009). Lastly, some research also suggests that after a big vascular event like a stroke, preventative measures are vital for mitigating the risk of developing VaD (Vijayan and Reddy, 2016; Hachinski et al., 2019).

CLINICAL PRESENTATION OF VaD

Since 2011, published studies are, not only in VaD but in some dementias and other cognitive disorders as well. However,

a critical review of the more recent trials and the effects of sex/gender on VaD is still lacking. The purpose of this clinical presentation is to provide an update on the existing studies dealing with sex and gender in VaD to obtain a clear and comprehensive overview of all clinical benefits, potential effects of sex/gender, limitations, and main outcomes in the management of these patients.

Data Source and Selection

A Medline (PubMed)-based literature review was performed by using the following search terms, in different combinations: "Vascular dementia," "Vascular cognitive impairment," "Vascular cognitive impairment and dementia," "Arteriosclerotic dementia," "multi-infarct dementia," "vascular contributions to cognitive impairment and dementia," "sex," and "gender." The studies had to include individuals with a clinical diagnosis of any type of Vascular dementia in relation to sex or gender and severity according to the internationally accepted guidelines or diagnostic criteria. Duplicated entries, studies on physiological brain aging or diseases different from VaD, VCI, or VCID, works on animals or cell cultures, studies not reporting the statistical analysis, non-English written papers, publications that are not research studies (i.e., abstracts, letters, commentaries, editorials, reviews, etc.), conference, meeting proceedings, or any other paper not published in international peer-reviewed journals, study protocols, personal communications, or unpublished data, as well as any other study that did not fit with the scope of this review were excluded. Articles listed in the references were also reviewed in search of more data.

Results

A total of 113 results were originally retrieved and screened. Of these, six publications were selected according to the inclusion and exclusion criteria. The examination of the references detected other five studies, whose analysis identified one additional paper fitting the purpose of this review. Therefore, a total of seven papers were eventually included in the qualitative synthesis (**Figure 2**), and the main findings are summarized in **Table 1** (Vinciguerra et al., 2020).

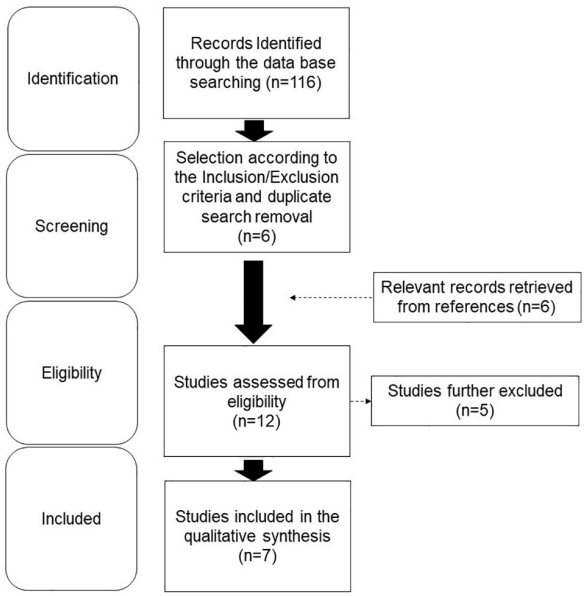

FIGURE 2 | Flow diagram showing the search strategy, the number of records identified, and the number of included/excluded studies.

GENERAL CAUSES AND RISKS FOR VaD

Generally, the common causes of VaD are environmental and pathophysiological that affecting the brain vessels, leading to a reduction of supplies like nutrition and oxygen to the brain (Kalaria, 2016; Killin et al., 2016; Caruso et al., 2019). Therefore, some of the risk factors for VaD coexist with the increased risk of stroke, heart disease, aging, atherosclerosis, high cholesterol, high blood pressure, diabetes, genetics, smoking, obesity, and atrial fibrillation. VaD progresses over time and targets cognitive abilities in the brain (Kalaria et al., 2016). This actively illustrates some of the neurological and behavioral changes that patients with VaD may experience such as memory impairment, loss of executive function (like decreased ability to plan), reasoning and organized thoughts and behaviors. Several regulatory mechanisms and cellular signaling are thought to play a role in VCI and VaD, including oxidative stress, neuroinflammation, endothelial dysfunction, hypoperfusion, blood-brain barrier (BBB) disruption, cortical hyperexcitability, and neurotransmitter imbalance (Vinciguerra et al., 2020). In addition, serological markers that can help in the diagnosis of VaD have been discovered in several investigations. Pro-inflammatory metabolites (such NO-related compounds), cytokines (including IL-1, TNF-α, IFN-γ, IL-4, IL-5, IL-8, G-CSF, and MIP-1b), and endothelial dysfunction indicators (like homocysteine) have all been reported to be elevated in VaD patients (Schmitz et al., 2015).

Moreover, common causes and risk factors of VaD change from one sex to another. For example, males have appeared to be slightly at a higher risk of getting VaD than females at a younger age, but women over the age of 85–90 have been shown to have a higher occurrence (Lucca et al., 2020). Therefore, females have high-risk factors that may be associated with preeclampsia, menopause and poorly timed hormone replacement therapy. Additionally, females have a higher risk of VaD with the presence of diabetes, obesity, and hypertension more than males (Gannon et al., 2019). On the other hand, males have a higher risk with the presence of hyperlipidemia, myocardial infarction, and heart disease like stroke, and heart attack (Skoog, 1994).

SEX-SPECIFIC DIFFERENCES IN VaD

Increasing evidence suggests that sex factors may play an important role in the pathogenesis of diseases, including cardiovascular disease and dementia. Sex differences in prevalence also depend on dementia subtypes, such as AD, VaD, Lewy body dementia (LBD), and Parkinson's dementia

TABLE 1 | Studies on vascular dementia (VaD) in relation with sex or gender.

Sex	Age in years (Average age)	Diagnostic criteria	Education and marital status	Medical risk factors	Subjects	Key findings	Adjusted variables	References
Male	≥55.0 (80.3)	MMSE, GMS-B, AGECAT, HAS, Katz's bADL's, IADL's, EURODEM, Risk Factors Questionnaire.	Primary school, High school or higher. Single, married or living as a couple, divorced, or separated, or widowed	Vascular disease (angina, myocardial infarct, and/or stroke), diabetes, hypertension, health status, depression, cognitive status, vascular risk factors (smoking, statin use, body mass index, and alcohol intake)	1,828	In men, but not in women, risk of VaD was higher among individuals with anxiety	No description	Santabárbara et al., 2020
Female	≥55.0 (79.8)				2,229			
Male	≥65.0 (no average age description about male)	MMSE, GMS, CAMCOG, DSM-III-R	–	Hypercholesterolemia, hypertension, or smoking, apolipoprotein E4 allele	12,270	There was no difference by sex in the cumulative risk of vascular dementia. The risk for a 65-year-old woman to develop vascular dementia at the age of 95 years was 0.040 compared with 0.041 for a man.	Age, the square term of age, dummy variables for study, smoking, education, self-reported myocardial infarction, and stroke.	Andersen et al., 1999
Female	≥65.0 (no average age description about female)				16,497			
Male	≥60.0 (70.1)	NINDS- AIREN, CASI, IQCODE	Less education (<7 Years), Higher education (≥7 Years)	Age, sex, obesity, hypertension, diabetes, stroke, drinking, smoking, education	845	Sex and a sex-age interaction showed significant effects with respect to probable VaD, but not to probable or possible AD or possible VaD.	No description	Yamada et al., 2008
Female	≥60.0 (72.2)				2,105			
Male	≥65.0 (no average age description about male)	NINDS, Association Internationale pour la Recherche et l'Enseignement en Neuroscience's criteria, CDR scale, DSM-III-R	Education (number of years of schooling)	Age, sex, education in years, apolipoprotein E4 allele, hypertension, high cholesterol, diabetes, Prevalence of cardiovascular and cerebrovascular factors, obesity, stroke, CABG, myocardial infarction	1,322	Vascular factors increase risks for AD and VaD differentially by sex.	Adjusted for all covariates listed in the medical risk factors list	Steinberg et al., 2014
Female	≥65.0 (74.0)				1,801			
Male	≥65.0 (no average age description about male)	MMSE, MRI-based evidence of lacunar state or ischemic WMLs, ADL, IADL, HDRS, Stroop T	Education (years)	Sex, hypertension, diabetes, hypercholesterolemia, coronary artery disease, tobacco smoking, atrial fibrillation, neurologic signs, family history, history of depression.	156	Moderate mocha coffee consumption was associated with higher cognition and mood status in non-demented elderly subjects with VCI. No description regarding relation of sex.	No description	Fisicaro et al., 2021

(Continued)

TABLE 1 | Continued

Sex	Age in years (Average age)	Diagnostic criteria	Education and marital status	Medical risk factors	Subjects	Key findings	Adjusted variables	References
Female	≥65.0 (no average age description about female)				144			
Male	16–102 (no average age description about male and/or female)	The Cross-Cultural Cognitive Examination and National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association Alzheimer's criteria	–	Sex, type 2 diabetes, lifestyle, cigarette smoking, and obesity	2,310,330 individuals, and 102,174 dementia case patients (no separate male and female number description)	Women with diabetes had a 19% greater risk for the development of vascular dementia than men.	Age, race, systolic blood pressure, self-report diabetes; total cholesterol, hypertension, alcohol.	Chatterjee et al., 2016
Female								
Male		STROBE, ARIC, CARDIA, CHS, FOS, NOMAS.	Education (≤8th grade, grades 9–11, completed high school, some college but no degree, ≥College graduate)	Age, race, education, alcoholic, cigarette smoking, any physical activity, BMI, waist circumference, history of arial fibrillation, LDL cholesterol, antihypertensive medication,	11 775	The results of this cohort study suggest that women may have greater cognitive reserve but faster cognitive decline than men, which could contribute to sex differences in late-life dementia.	No description	Levine et al., 2021
Female					14 313			

MMSE, Mini-Mental Status Examination; GMS-B, Geriatric Mental State B; AGECAT, Automated Geriatric Examination for Computer Assisted Taxonomy; HAS, History and Aetiology Schedule; bADL's, Katz's Index for basic activities of daily living; IADL's, the Lawton and Brody scale for instrumental activities of Daily Living Scale; EURODEM, European Studies of Dementia; GMS, Geriatric Mental State Examination; CAMCOG, Cambridge Examination of Mental Disorders Cognitive Test; CASI, Cognitive Abilities Screening Instrument; IQCODE, Informant Questionnaire on Cognitive Decline in the Elderly; CABG, Coronary Artery Bypass Graft surgery; NINDS, National Institute of Neurological Disorders and Stroke; CDR, Clinical Dementia Rating; WMLs, White Matter Lesions; ADL, Activities of Daily Living; IADL, Instrumental Activities of Daily Living; HDRS, Hamilton Depression Rating Scale; STROBE, Strengthening the Reporting of Observational Studies in Epidemiology; ARIC, Atherosclerosis Risk in Communities Study; CARDIA, Coronary Artery Risk Development in Young Adults Study, CHS, Cardiovascular Health Study; FOS, Framingham Offspring Study; NOMAS, Northern Manhattan Study.

(PD) (Kim et al., 2018). Therefore, studies are needed to investigate sex-specific differences, which can help understand the pathophysiology of dementia and identify potential therapeutic targets for both sexes. In response to variables that promote either positive or negative neuroplasticity, an individual's overall cognitive state might change over time. The physiological ability of the brain to establish and strengthen dendritic connections, cause good morphological changes, and boost cognitive reserve is referred to as positive neuroplasticity. Negative neuroplasticity refers to the same physiological ability of the brain to atrophy and weaken dendritic connections, produce detrimental morphological changes, and decrease cognitive reserve. Many factors promote positive or negative neuroplasticity including sex. Though women showing a greater risk of AD at later ages (Lin and Doraiswamy, 2014), it has been suggested that diagnosis of mild cognitive impairment (MCI) may be delayed in females, which may be responsible for the possible increased risk for MCI seen in males. Specifically, despite identical levels of neurodegeneration, females perform better in verbal memory than males (Sundermann et al., 2016a,b). This study shows that women have a cognitive reserve in this area, delaying the onset of abnormalities until higher levels of disease are present. Although the correlations are varied and multifaceted, there are sex differences in dementia. A study done in rats provides another example, showing that sex variations in neuroplasticity are the response of hippocampal neurogenesis to prolonged estradiol therapy (Barker and Galea, 2008).

With the prevalence of cardiovascular disease in recent years, the risk of VaD is also on the rise. Besides just simply having any form of cardiovascular disease, other factors can help contribute to developing VaD in some indirect ways. Sex can influence the occurrence of VaD, and there are trends that VaD follows

between the sexes. Some studies have shown a difference in how the prevalence of VaD is altered by a person's biological sex (Andersen et al., 1999; Podcasy and Epperson, 2016). The occurrence of dementia and the reasons for differences between the different sexes are complex. Like all dementia diagnoses, the symptoms can vary and are often caused by complex factors. There may not be a particular reason but a culmination of multiple factors at a personal level. For example, a woman experiencing menopause could also be experiencing symptoms of other health conditions that accumulate and lead to a higher risk of VaD (Robusto-Leitao and Ferreira, 2006; Yamada et al., 2008). Her risk is possibly higher than a male of similar age who only has hypertension. Additionally, another factor to consider besides age category risks is that females live 6+ years longer than males on average, so their occurrence of VaD is higher based on longevity (Robusto-Leitao and Ferreira, 2006). These factors only consider physical factors, there may be many more, including educational level and socioeconomic status, etc. These additional factors can be attributed to a more social environment aspect of VaD. For example, the opportunities available for women vary in the sense of a generational gap. Females were placed into a social role of caretaking the household and had less physically demanding work roles than men (Hörder et al., 2018). Therefore, the risk of VaD could increase for women. Also, the occurrence of smoking has changed with time, it was less socially acceptable for women to smoke tobacco and other products but in recent years the number of female smokers has increased. These factors lead to a higher vascular risk and could be directly related to higher dementia cases in women in the long run (Zhong et al., 2015). Lastly, the sex differences noted in dementia are not really prominent until after the age of 80, and that is only consistent in studies done in the United States (Podcasy and Epperson, 2016; Levine et al., 2021). Other studies in Europe show a sex difference in VaD early on, indicating that the occurrence of VaD can be influenced by external factors (Hasselgren et al., 2020). It is highly likely that each VaD diagnosis is unique and is a combination of both social and physical factors that affect an individual's neurobiology (**Table 2**).

Pre-existing Health Conditions/Family History Risk Factors for VaD

Perhaps one of the most common risk factors with any disease or condition is family history. A person's family history can indicate common trends and probabilities about a person's health, indicating what kind of lifestyle is best suited to everyone's needs. Indirectly a family history of cardiovascular disease can put a person, regardless of their sex, at a disadvantage for developing VaD because CVD is a hereditary risk factor that becomes a more significant risk factor for VaD (Treiber et al., 2008; Vijayan and Reddy, 2016). In terms of family history there are not many direct links of inherited conditions that can contribute directly to VaD. The only direct familial link to VaD occurs with gene variants and specific gene mutations (Lin et al., 2019). Additionally, the pre-existing health conditions that can contribute to VaD are smoking, obesity, hyperlipidemia, and old age (Tariq and Barber, 2018). Moreover, the factors mentioned are not specific to one sex but have been sorted by the sex in which the factor is more dominant (Podcasy and Epperson, 2016).

Risk Factors for Males and Females

The cardiovascular risk for VaD in women is less than that of men, but the prevalence varies (Steinberg et al., 2014). In the United States, men have a greater risk of having a heart attack and are twice more likely to have a heart attack than a women, yet the risk for CVD/heart disease is similar (Maas and Appelman, 2010). For smoking, worldwide, the trend has been consistent with men in developed countries smoking less than men in developing countries (Pampel, 2006). The use of tobacco products restricted to just the United States indicates that men smoke slightly more than women.

Researchers discovered a significant difference in age and education among the four groups of subjects based on their daily mocha coffee consumption. However, the General Regression Model revealed that the associations between coffee consumption and cognition and mood were independent of socio-demographic variables (Fisicaro et al., 2021). In terms of the sex-related effect, an earlier prospective study (Shinoda et al., 2015) of 455 participants (314 men) found that male drinkers had a lower incidence of small vessel disease than male non-drinkers and occasional drinkers, while female drinkers had a lower incidence of white matter lesions (WMLs) than female non-drinkers or occasional drinkers.

Obesity as a risk factor is prevalent in women, and overall globally, women tend to be more obese than men, but not in highly developed countries. The risk for smoking is also directly inverted for women. Women in developed countries, smoking more than currently developing countries. However, the gap within the United States of men and women smoking varies only by about 3% (Mackay and Amos, 2003). Lastly, in terms of hyperlipidemia (Davidson et al., 2002) and anxiety (Santabárbara et al., 2020) condition, even though men have a higher risk for VaD, and it is thought of as a man's disease, women are still at risk for high cholesterol, and the risk increases in women after menopause or early menopause. It is believed that younger women (or just generally those who have not reached menopause yet) are protected from more cardiovascular events than men due to the presence of high estrogen (El Khoudary et al., 2020).

Overlapping Risk Factors

As previously mentioned, there is no specific family history indicative of a high probability of developing VaD, besides CVD. However, there is not a significant difference between males and females in this area. The percentage of lifestyle-influenced risk factors that contribute to VaD in the United States is mentioned in **Table 3** (Whitmer et al., 2007; Gannon et al., 2019; Lewis et al., 2021). With these factors, other factors interrelated to family histories like gene variants and gene mutations can help contribute to VaD.

Gene Variants as a Risk Factor for VaD

Much work has been done on gene variants in AD, and the effects of these genes in VaD have been rendered. The leading players in VaD include a mutation in the Notch Receptor 3

TABLE 2 | General risk factors of VaD.

Vascular dementia common risk factors	
Genetics	Genetics can cause VaD depending on the presence of certain gene variants. This includes ApoE alleles and TDP43.
Obesity	Obesity leads to VaD due to higher blood pressure. Over time this can cause ventricular enlargement and atherosclerosis.
Hyperlipidemia	Hyperlipidemia blocks blood flow to the brain which overtime increases the chances of developing VaD.
Diabetes	Type II diabetes is highly associated with VaD because of abnormal blood flow to the brain. Type I is associated but not as highly as Type II.
Hypertension	Hypertension increases the risk of stroke which is a huge risk factor for VaD.
Stroke	Stroke impedes blood flow to areas of the brain. The oxygen deprivation destroys the brain tissue.
Atherosclerosis	Atherosclerosis prevents blood from reaching the brain fully. This deprives the tissue of the oxygen and nutrients.
Atrial Fibrillation	Atrial fibrillation carries and increased risk of stroke which is directly linked to vascular dementia.
Aging	Aging can increase the chance of VaD because there is a higher risk of atherosclerosis, heart attack and strokes.
Smoking	Smoking increases the risk of stroke but additionally toxins in cigarette smoke cause oxidative stress and inflammation, both associated with VaD.

All these factors can overlap and when combined the risk of developing VaD increases.

(NOTCH3) gene, which is directly responsible for one form of VaD, and Apolipoprotein E (APOE) variants (Skoog et al., 1998; Thomas et al., 2000; Cho et al., 2021). NOTCH3 is directly linked to cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy also known as CADASIL (Papakonstantinou et al., 2019). CADASIL is considered a very rare disease and has been recorded across all ethnic groups thus far (Kalaria et al., 2004). It is most often caused by a missense mutation but can alternatively be caused by null mutations or homozygous mutations. The symptoms of CADASIL include smaller cerebral vessels which in turn cause strokes, mood disturbances, and of course VaD (Dichgans, 2002). The other gene attributed as a risk factor for VaD is APOE and its variants (Skillbäck et al., 2018; Montagne et al., 2020). APOE has different variants denoted by e2 (APOE2), e3 (APOE3), and e4 (APOE4).

Astrocytes predominantly express APOE variants (APOE4) in the brain, cell types that are now recognized to play critical roles in largely lipid distribution, cerebrovascular function amyloid deposition, Tau phosphorylation, mitochondrial dysfunction (Fernandez et al., 2019), as depicted in **Figure 3**. Most of the research surrounding APOE has been centered around AD, however, the same relationships established between APOE and AD apply to VaD but in a lesser magnitude (Alam et al., 2014). For example, APOE4 has been the most prominent contributor to AD, and when two copies of the APOE4 allele are present the risk for AD increases 15-fold. This increase is directly analogous to VaD because the same APOE e4 variant is the same variant that influences the risk for VaD. However, the effect is weaker and does not increase the risk as dramatically as 15-fold (Rohn, 2014).

Risk Factors for Males and Females

Males have a decreased risk of VaD in carrying an APOE4 variant even if the male is homozygous, the link between APOE and AD/VaD is well established with females and not males (Rasmussen et al., 2018). This is such an important factor that even most studies involving tracing APOE to dementia are done with female mice (Adeosun et al., 2019). Most literature also supports that APOE2 and APOE3 are neutral factors in males developing VaD (Folin et al., 2004; Bell et al., 2012). In terms of the mutation for CADASIL, men have equal risk, but the symptoms of CADASIL affect men differently (Ruitenberg et al., 2001; Singhal et al., 2004). Men have more strokes on average than women at a younger age and retain cognitive ability better than women after a certain age (typically when comparing the sexes before menopause). APOE4 carries the highest risk for women developing dementia, especially for women who are homozygotes and carry two e4 alleles (Molero et al., 2001; Davidson et al., 2006). It is estimated that the risk increases 10-fold for VaD and an earlier onset of dementia if a person is homozygous. APOE2 is the least common allele but provides a protective effect against dementia and cognitive decline in women (Volgman et al., 2019). CADASIL affects women mainly by increasing the occurrence of frequent migraines accompanied by vision impairment (Rufa et al., 2004; Guey et al., 2016). This is most prevalent until the age of menopause and then cognitive impairment becomes more evident. The occurrence of psychiatric symptoms and migraines increase after menopause, but women, however. have a decreased rate of strokes in comparison to men (Bushnell et al., 2014).

TABLE 3 | Percentage of lifestyle influenced risk factors that contribute to VaD.

	Males	Females
Obesity	35%	40.4%
Hyperlipidemia	28.5%	8.9%
Smoking	16.7%	13.6%

Overlapping in Gene Variants

Overall, both males and females have an equivalent risk of having CADASIL. The symptoms do vary, and that aspect is dictated by the age of the male or female. There also was no inconsistency between sex and the type of mutations that caused CADASIL (Mizuno et al., 2020). When it comes to APOE as a contributor to VaD, however, there is a significant difference between males and females (Molero et al., 2001). Females have a higher risk than males when it comes to having variant APOE4 and females had a decreased risk when they carried APOE2. Males had a generally neutral experience when carrying alleles of APOE2 and APOE3. Lastly, APOE4 was not shown to be as much of an indicator in males for VaD as much as females (Molero et al., 2001; Li et al., 2017).

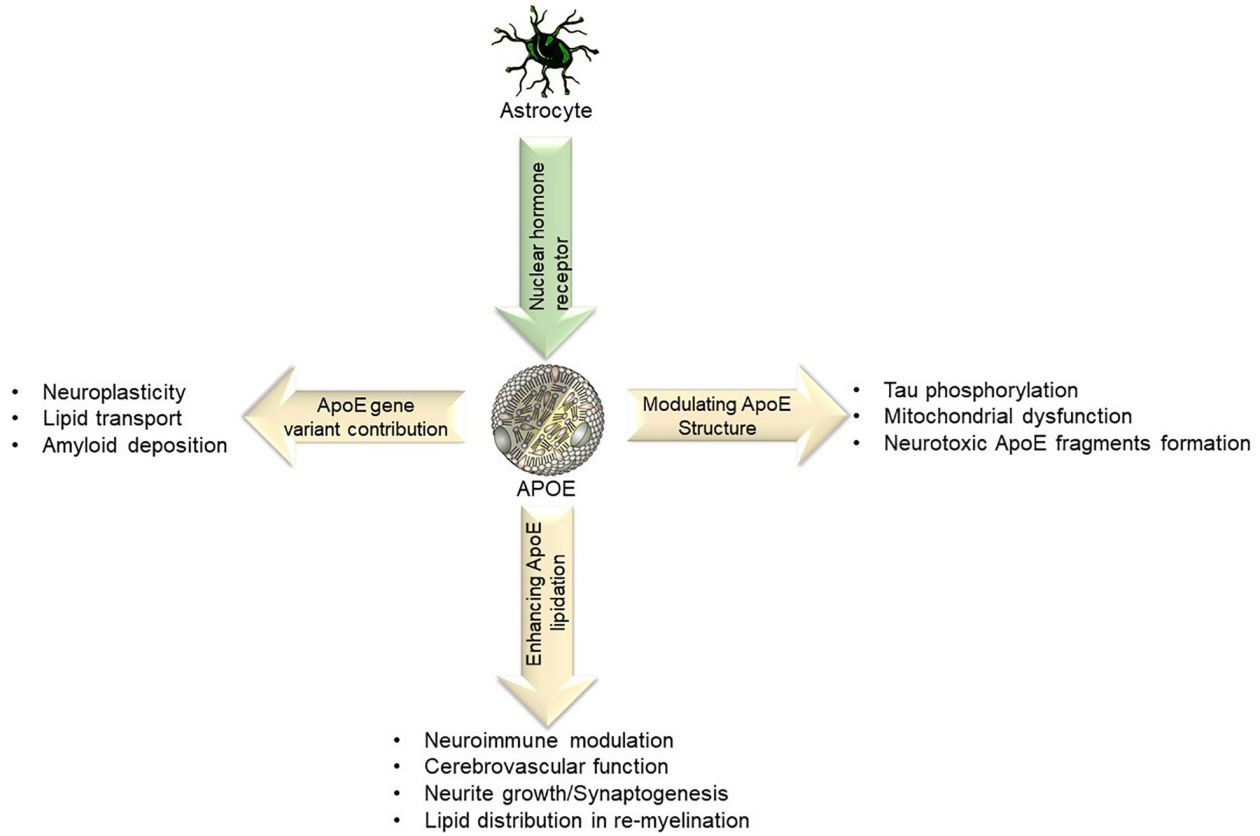

FIGURE 3 | Lipid transport refers to the ability of APOE to bind to LDL receptors and assist with lipid clearance. Certain APOE alleles also interact and affect amyloid deposition, neuroplasticity, neuroimmune modulation and in re-myelination processes.

Aging as a Risk Factor

Aging is one of the leading and highest risk factors of VaD because of the association of adiponectin (Song et al., 2014). Adiponectin is a protein hormone secreted by adipose tissue (fat cells). In aging, adiponectin plasma levels decrease, leading to an increased risk of CVD and diabetes, considered a risk factor of VaD (Song et al., 2014; Chatterjee et al., 2016; Chen et al., 2019). Additionally, adiponectin plays a massive role in cognitive dysfunction by controlling the insulin signal transduction in the brain (Rizzo et al., 2020). An epidemiology study implies that there is an exponential increase in dementia after the age of 65 by double around every 5 years. However, for participants aged 90 and older, dementia doubles every 5 years for females but not males (Corrada et al., 2010).

Vascular dementia and multi-infarct dementia are most common in males but have a higher gravity of impact in females. The study concluded that stroke prevalence, whether ischemic or hemorrhagic, was 44% greater in males than in females worldwide. Also, males experience their first strokes at a younger age than females (68.6 vs. 72.9 years) (Persky et al., 2010; Girijala et al., 2017). Although males' first stroke experience might be at a younger age than females, females have a higher risk of lifetime stroke because of their longer life expectancy, which also increases the risk of stroke with age (Reeves et al., 2008).

Hormone Fluctuations as a Risk Factor

Studies have been done to investigate the controversy between correlating estrogen changes to cognitive impairment, which causes VaD (Ali et al., 2018). The reason for this occurrence is the sudden downfall of estrogen in women during menopause; this happens to be one of the significant risk factors and the root prevalence of VaD in postmenopausal females. Furthermore, a few studies concluded that estrogen deficiency in postmenopausal women is indeed one of the risk factors, causing cognitive deficit that can lead to VaD (Robusto-Leitao and Ferreira, 2006). The four-core genotype (FCG) model is used to separate the contributions of sex hormones and sex chromosome complement (Bushnell et al., 2018). The sex determining SRY gene is relocated from the Y chromosome to an autosome in this scenario, allowing XX males (XXM) and XY females (XYF) to be produced. Because the created XXM have a hormonal status like that of XY males (and vice versa for XYF and XX females), a comparison between the two provides for a better understanding of chromosome vs. hormone impacts. In addition, sex chromosomes have received little consideration in connection to the pathogenesis of dementia (Rocca et al., 2014). Women have two copies of chromosome X, one from their mother and one from their father. The androgen receptor and many proteins involved in mitochondrial function, adipose tissue

distribution, apoptosis, and sensitivity to hypoxia are among the approximately 1,600 genes (about 155 million base pairs) on the X-chromosome (Wise et al., 2013). In female cells, most of the genes expressed on one of the two X-chromosomes are inactivated to avoid a genetic overload (Brown et al., 1991). As a result, X-chromosome inactivation patterns may provide a new viewpoint on the concept of laterality of brain activities in women vs. men.

Male vs. Female Hormonal Risk/Benefits

Females seem to be more susceptible overall to dementia than males. The association between sex hormones and VaD is still somewhat unknown in the scientific/medical field. While there is extended gratitude to consider sex as one of the factors of VaD, there are various factors that might distinctively impact females, such as pregnancy and reproductive history.

A study was focused on a comparison between serum sex hormone levels among VaD patients (males and females) and normal (controls) individuals to bridging the gap between sex hormones level and cognitive and neuropsychiatric manifestations of VaD (Xing et al., 2013). The results of the study showed that the testosterone and sex hormone-binding globulin (SHBG) levels were lower in male VaD patients, and the estradiol levels were found to be higher in female VaD patients in comparison to the controls. The study concluded that there were no correlations between hormone levels and neuropsychiatric symptoms in male VaD patients, whereas total estradiol (TE2) and testosterone (TT) levels were positively correlated in female VaD patients. Estrogens influence the function and pathophysiology of cerebral circulation. Estrogen decreases cerebral vascular tone and increases cerebral blood flow by enhancing endothelial-derived nitric oxide and prostacyclin pathways (Krause et al., 2006). In addition, however, blood vessels produce inflammatory factors that could contribute to VaD pathology. One study suggests that estrogens and their receptors may regulate the neuroinflammatory response, and in females, circulating estrogens may play a protective, anti-inflammatory role (Villa et al., 2016). Thus, estrogen preserves vascular function and neuroinflammatory response could be directly relevant to VaD.

Another study on animal models suggests that young adult male mice had worse pathological and functional outcomes following cerebral ischemia than females, which is consistent with clinical studies suggesting that high-androgen levels enhance stroke risk in younger populations (Abi-Ghanem et al., 2020). These sex differences could be due to sex hormones or sex chromosomal complement.

Hormone Therapy Risks/Benefits for VaD

Hormone replacement therapy (HRT) can be used for a variety of reasons, even to deal with symptoms of the menopause. As previously mentioned, women have protection against forms of dementia due to their high estrogen levels. Once menopause is reached, however, those estrogen levels deplete. In these cases, women can have added protection against VaD despite their initial lower estrogen. A few studies finding a correlation have analyzed the relationship between HRT, AD and VaD specifically. One study achieved that estradiol-based HRT was associated with a reduced risk of death both from VaD and AD, but the risk reduction was larger and appeared sooner in VaD than AD (Mikkola et al., 2017). In a few studies, the use of estrogen post-menopause decreased the user's risk for dementia and improved cognitive decline (Yaffe et al., 1998). Another study suggested that the prior use of estrogen was more effective in combating dementia and cognitive decline as opposed to using HRT during post-menopause (Henderson, 2008). Most types of HRT increase the risk of breast, ovarian and, womb cancer (D'Alonzo et al., 2019). But the risk is higher for those using combined HRT, which uses both estrogen and progestogen. The risk of cancer due to HRT can also vary from person to person. Things such as what age you are when you first start taking HRT, other medicines you may be taking, and your general health can impact the risk. People who begin HRT before or soon after menopause may have a bigger risk than those who start HRT later (Beral et al., 2011). In one such study, HRT was analyzed in postmenopausal women and the results indicated that HRT contributed to vascular risk factors including stroke and could not sufficiently demonstrate benefits for postmenopausal women regarding dementia (Hogervorst et al., 2000). The results are clearly divided for the use of HRT, but the main conclusion most studies come to is that HRT can be beneficial if used within a proper time frame to protect postmenopausal women from dementia forms.

Environmental Factors as a Risk Factor

The physical risk factors of VaD are only one aspect of the cause. VaD can arise due to many factors but one class of factors that are often overlooked are the environmental factors which often can dictate some of the other more tangible, direct factors (Killin et al., 2016). Some environmental risk factors include air quality elements, toxic heavy metals, trace elements, known occupational hazards, electrical/magnetic fields, and a few others (Killin et al., 2016; Bellou et al., 2017; Wang et al., 2019). These different environmental factors have been shown to have strong effects in propagating dementia (**Table 4**).

The factors listed in the table are the highest environmental factors that have a link to VaD, and they all are produced in different ways, some of which cannot be simply avoided. These factors can trickle down, and most likely do not directly affect the development of VaD but can exacerbate other existing risk factors. For example, the use of herbicides, pesticides, insecticides, and fertilizers are commonly used in farming practices all over the world, many of these are known endocrine disruptors that increased the risk of developing dementia (Kamel and Hoppin, 2004).

Other less obvious environmental factors can include weather patterns that govern a certain area as well as Vitamin D level deficiencies from lack of sunlight exposure (Sommer et al., 2017). The area in which a person lives can also influence the risk of VaD. For example, one study done in China indicated that in areas with less particulate that the people in those areas had a reduced occurrence of cerebrovascular events (Cai et al., 2018). Reduced risk of cerebrovascular events decreases the risk of VaD because if harmful vascular events do not occur, there is no disruption

TABLE 4 | Shows the various types of environmental factors and their uses.

Environmental factor	Factor	How it is produced/or used
Air Quality	Nitrous Oxides	Combustion, especially in areas of high motor vehicle use
	Ozone	UV Radiation reactions with oxygen and sometimes nitrogen oxides
Toxic Metals	Arsenic	Industrial use in mining and ore smelting
	Aluminum	Industrial mining and smelting
Trace Elements	Silicon/Silica	Industrial silicon and silica working introduces fine particles into the air
	Selenium	mining/oil refining
Work Hazards	Pesticides/Herbicides/ insecticides/fertilizers	General farming and crop production, can be endocrine disrupting
	Solvents/degreasing agents	Industrial grade chemical for various work trades

to brain tissue. Another study showed that with extremely low vitamin D levels patients were more likely to develop VaD and more likely to develop vascular events that perpetuated the occurrence of VaD (Chai et al., 2019). Another study indicated that warmer and wetter weather conditions provided for a better foundation for stroke patients to recover fully (Chen et al., 2013). This is important because stroke is one of the major contributors to VaD (Vijayan and Reddy, 2016). By making a full recovery from a stroke the risk of VaD is mitigated. Lastly, in one study air pollution and noise were linked to a progression of dementia in London, England (Carey et al., 2018). These results were more consistent with an AD diagnosis but there was a fair amount of VaD cases present as well.

ACCESSIBLE TREATMENTS FOR VaD

Pharmaceutical Treatments

Many drug categories have been used as a treatment for VaD patients. Some of these drug categories are vasodilators, calcium channel blockers, antiplatelet, etc. (Romàn, 2000; Chabriat and Bousser, 2006; Nimmrich and Eckert, 2013; **Table 5**). One of the most effective and standard treatment are cholinesterase inhibitors (Bullock, 2004). Cholinesterase inhibitors work by inhibiting acetylcholinesterase, which is accountable for clearing acetylcholine, a neurotransmitter responsible for muscle contractions, blood vessel dilation, and regulating heart rate. With the cholinesterase inhibited, the acetylcholine concentrations rise and lead to better communication between the nerve cells in the brain. An artificial increase in acetylcholine levels by physostigmine, an acetylcholinesterase inhibitor that increases the extracellular acetylcholine levels, impairs memory consolidation and rescue in rodent and human subjects (Haam and Yakel, 2017). Vasodilation drugs work mainly in preventing constriction of the blood vessels anywhere in the body and allowing greater blood flow. However, calcium channel blockers prevent calcium from entering the cells in the heart, vascular smooth muscle, and pancreas by lowering the blood pressure. Aspirin is a popular antiplatelet drug that works by blocking the movement of the cyclooxygenase chemical (COX) via the prostaglandin synthesis pathway (PGH2) (Warner et al., 2011; Ornelas et al., 2017).

The drugs that are mentioned in the table above are only the pharmaceutical treatments for VaD (Olivares et al., 2012; Wu et al., 2015). There have been a few studies indicating alternative treatments that have had success. The alternative and homeopathic treatments mostly treat the symptoms of the VaD but occasionally can treat the vascular underlying conditions (McCarney et al., 2003). A study done in 2018 concluded that acupuncture could in fact help to treat VaD and it generally worked by boosting the metabolism of glucose and oxygen, contributing to anti-oxidative stress reactions, and acted as an anti-apoptotic agent (Zhu et al., 2018). In another study, aromatherapy essential oils (rotated lemon, rosemary, lavender, and orange oils) were given to dementia patients topically throughout the day for a period of 28 days (Jimbo et al., 2009). After 28 days the aromatherapy was shown to have improved the patient's personal orientation and cognition. This improvement in the patients could be applied specifically to VaD and the treatment proved to be an efficacious non-pharmacological treatment. Music therapy was analyzed for its effect on dementia patients indicated that having at least 5 music therapy sessions a day reduced depression and generally improved the quality of life of dementia patients (van der Steen et al., 2018). The effects of music therapy on actual cognitive function were unknown, however. Lastly, art therapy was another means of homeopathic therapy, functioned similarly to music therapy in that it improved the patient's quality of life (Deshmukh et al., 2018). The actual cognitive function could not be measured but the therapy improved neuropsychiatric symptoms of dementia patients. While homeopathic remedies are a reasonable alternative, they tend to focus on the symptoms rather than the triggers. The causes of risk factors might be discussed in order to completely eliminate the risk of VaD. Thus far, there is no drug available related to sex/gender and therefore, the development of pharmaceutical treatment for VaD has become an essential but unmet need.

Elimination of Risk Factors

Eliminating a risk factor should zero in on a reduction of the major causes of VaD such as stroke, and CVD, with consideration regarding controlling of the other risk factors. One of the biggest risk factors for VaD is arterial hypertension (Paglieri et al., 2004). Arterial hypertension is the main cause of 50% of strokes regardless of the pathogenic mechanism involved. Also, lower blood pressure decreases the risk of VaD by 55% (Sierra, 2020). Indeed, the primary prevention focus is to decrease the prevalence of VaD by early and optimum treatment for stroke and CVD by targeting high-risk groups such as the elderly, hypertensive patients, diabetes patients, smokers, past transient ischemic attack or stroke survivors, hypercholesterolemia patients, and atrial fibrillation patients (Vijayan and Reddy, 2016).

TABLE 5 | Pharmacological treatment for VaD and their effects.

Drug classes	Drugs	Effects
Cholinesterase inhibitors	Donepezil, Galantamine, Rivastigmine	Safe and effective in reducing the progression of VaD in addressing the behavioral problems.
Vasodilators	Niacin (nicotinic acid), Cyclandelate, Papaverine, Isoxsuprine, Cinnarizine, Buflomedil, Naftidrofuryl, Ergoloid mesylates, Acetazolamide	Have shown to be less effective in treating VaD compared to cortical dementias like Alzheimer's.
Calcium channel blockers	Nimodipine	Moderately effective for short term in treating cerebrovascular disease and other types of dementia.
N-methyl-D-aspartate (NMDA) antagonists	Nicardipine	Improvement of cognitive deterioration
NMDA antagonists	Memantine	Improve cognition consistently across different cognitive scales, with at least no deterioration in global functioning and behavior.
Nootropic agents	Piracetam, Nicergoline, Oxiracetam Citicoline, Pentoxifylline	have beneficial effects for patients with dementia in prolonging their survival.
Antiplatelet agents	Aspirin, Triflusal, Ginkgo biloba	Reduce/prevent the occurrence of stroke.

The primary prevention aims to minimize dementia in the population by advising lifestyle changes such as the control of diabetes and hypertension (Eggink et al., 2019). The secondary prevention methods aim to mark stroke management and the prevention of recurrent stroke (Lip and Kalra, 2010). This has shown a 50% decrease in the risk of dementia in those with previous stroke experiences and a 16% decrease in those without stroke experiences in patients without cognitive impairment (Lo Coco et al., 2016).

The specific ways that dementia patients or dementia patient caregivers are prescribed to help reduce symptoms include lifestyle changes. These lifestyle changes are aimed at improving the vascular health of the body to reduce any kind of negative events like stroke or heart attack. The methods include diet changes, incorporating exercise either vigorous or more passive, cognitive therapy, and management of vascular issues like hypertension through medication. A research study published in 2016 demonstrated the effectiveness of integrating exercise into a stroke rehabilitation environment (Winstein et al., 2016). Stroke patients who included moderate aerobic activity and environmental enrichment in their treatment plans had higher recovery rates and, in some cases, spontaneous recovery. Another research published in 2018 found that incorporating passive exercise into a dementia patient's everyday routine resulted in substantial improvements in quality of life and activities of daily living (Zucchella et al., 2018). Most of the patients in this study improved their cognitive and physical functioning. A study done in Finland called the FINGER trial was aimed at improving the outcomes in VaD patients through a total reset of the patient's lifestyle (Kivipelto et al., 2020). With regular medical advice, intervention (diet, exercise, vascular risk management, lifestyle guidance, intellectual training, social activities, cognitive training, prevent head injury, stop smoking, reduce air pollution), and multi-domain intervention, cognitive function was partially restored. Another method to reduce symptoms of VaD includes cognitive therapy was done in 2019 aimed to test individuals with mild to moderate VaD and to view if there were any cognitive changes (Peng, 2019). There was no net improvement in the cognition of the dementia patients, but of those that had caregivers, the moods of the caregivers were improved and in turn led to a better mood and outlook for the dementia patients. As for medication, several drugs have been prescribed to treat VaD after the risk factors have been addressed. Since zinc deficiencies play a critical role in neurodegenerative diseases, a study published in 2018 found that carnosine and zinc, which are commonly used as a supplement to treat gastric ulcers, may be used as a future remedy for VaD (Kawahara et al., 2018). Lastly, in a study done in 2016, amlodipine, a common hypertension treatment was given to VaD patients (Fares et al., 2016). There was a slight improvement in the symptoms of VaD patients. Even though there is little research on non-traditional therapies, there is still a lot of importance in looking into the options with VaD since there is no standardized care. VaD is very challenging to treat, and this is partially due to a lack of understanding of the full pathophysiology.

CONCLUSION

With the rise of VaD cases across the globe, it is easy to see why researchers have taken an interest in its risk factors. An overlooked factor is often sex and gender. In this review, we explored the impact of sex/gender differences on VaD, including how they affect these risk factors: pre-existing health conditions and family history, gene variants, aging, hormone fluctuations, and environmental risk factors. Improvements in the following areas may help to reduce the prevalence of VaD: (1) stroke prevention in hypertensive patients, (2) appropriate treatment to prevent or slow atherosclerosis progression, (3) a better understanding of the relationship between menopause and hypertension control, and (4) efforts to reduce neurological complications associated with cardiac procedures, (5) avoiding poorly timed hormone replacement therapy (6) evading potentially dangerous drugs that impair memory and cognition, and (6) developing therapy targets based on a better understanding of the molecular function of

the apolipoprotein E4 genotype in dementia. These all play a significant role in developing VaD and additionally should be monitored to reduce the risk. When it comes to sex and gender there is not a fine line between how VaD presents itself. VaD is a multidimensional illness with many different moving pieces, and every case of VaD has its risk factors that can be attributed to a plethora of lifestyle factors and chance. Future research should be focused on treating VaD by assessing the occurrence of risk factors in sex and a variety of other lifestyle indications to create a more tailored treatment plan. If enough research is done, a strong connection between sex and VaD could be discovered, which may aid in improving current therapies and developing unique sex-based treatment plans. Additionally, more research should also be conducted on how socioeconomic factors impact a patient's physiology and how that impacts their ability to develop VaD. This could be important due to how social constructs can dictate health based on sex. For example, females up until recently had a more social role of refraining from smoking to maintain a feminine image. In addition, more research should be planned on possible gene therapies and how the occurrence of VaD affects the LGBTQ+ community and changes their physiology. This would be particularly salient in studying the occurrence of all types of dementia and VaD in the transgender population and for individuals that use hormone replacement therapies. Lastly, future trials must examine sex-specific differences to improve our understanding of how CVD affects cognitive impairment in women and men.

AUTHOR CONTRIBUTIONS

FA, AP, and YZ drafted the manuscript and figures. ZZ and DZ proofread and revised the manuscript. DZ gave the final proof for this submission. All authors contributed to the article and approved the submitted version.

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The Neutrophil to Lymphocyte Ratio Is Associated With the Risk of Subsequent Dementia in the Framingham Heart Study

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Objective: Active neutrophils are important contributors to Alzheimer's disease (AD) pathology through the formation of capillary stalls that compromise cerebral blood flow (CBF) and through aberrant neutrophil signaling that advances disease progression. The neutrophil to lymphocyte ratio (NLR) is a proxy of neutrophil-mediated inflammation, and higher NLR is found in persons diagnosed with clinical AD. The objective of this study was to investigate whether increased NLR in older adults is independently associated with the risk of subsequent dementia.

Methods: We examined associations of baseline NLR with incident dementia risk in the community-based Framingham Heart Study (FHS) longitudinal cohorts. The association between NLR and risk of dementia was evaluated using the cumulative incidence function (CIF) and inverse probability-weighted Cox proportional cause-specific hazards regression models, with adjustment for age, sex, body mass index (BMI), systolic and diastolic blood pressure, diabetes, current smoking status, low-density lipoprotein

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(LDH), high-density lipoprotein (LDL), total cholesterol, triglycerides, and history of cardiovascular disease (CVD). Random forest survival models were used to evaluate the relative predictive value of the model covariates on dementia risk.

Results: The final study sample included 1,648 participants with FHS (average age, 69 years; 56% women). During follow-up (median, 5.9 years), we observed 51 cases of incident dementia, of which 41 were AD cases. Results from weighted models suggested that the NLR was independently associated with incident dementia, and it was preceded in predictive value only by age, history of CVD, and blood pressure at baseline.

Conclusion: Our study shows that individuals with higher NLR are at a greater risk of subsequent dementia during a 5.9-year follow-up period. Further evaluating the role of neutrophil-mediated inflammation in AD progression may be warranted.

Keywords: Alzheimer's disease, dementia, Framingham, FHS, neutrophil to lymphocyte ratio, NLR, complete blood count (CBC), risk prediction

INTRODUCTION

The variability of amyloid-beta (Aβ) and tau pathology associated with clinical symptoms reflects that additional factors influence the progression of cognitive decline in Alzheimer's disease (AD). Vascular dysfunction and inflammation have been implicated in the pathogenesis of AD in the earliest stages of the disease prior to significant Aβ and tau accumulation (Iturria-Medina et al., 2016; Love and Miners, 2016; Nortley et al., 2019). In addition, clinical studies have indicated that the inflammatory status influences the rate of disease progression in patients with AD (Holmes et al., 2009; Cunningham and Hennessy, 2015).

Recently, neutrophils have been identified as potential key elements of innate immunity contribution to the disease, and a hyperactive neutrophil state has been found in patients with AD and has been associated with clinical progression (Dong et al., 2018). Neutrophils are the most abundant leukocyte type in the human blood, and although the best characterized function of neutrophils in the defense against infectious pathogens, neutrophils are implicated in the repair of both infectious and sterile injuries (Kruger et al., 2015). Results in animal models of AD have suggested that neutrophils may be implicated in the breakdown of the blood-brain barrier (BBB) and recruited in the brain parenchyma through the integrin LFA-1 predominantly in perivascular regions with Aβ deposition (Baik et al., 2014; Zenaro et al., 2015). A potential role of neutrophils in the hyperphosphorylation of tau has also been suggested (Zenaro et al., 2015; Nemeth et al., 2020).

Neutrophils are known to have a very short life cycle ranging from just a few hours to up to 5–6 days in circulation (Pillay et al., 2010). During normal inflammation, neutrophils are cleared from the circulation once the initial inflammatory insult is resolved. In aging, however, inaccurate neutrophil chemotaxis may lead to compromised clearance of neutrophils, potentially contributing to a chronic inflammation state with reduced formation of neutrophil extracellular traps (NET), phagocytic response, and reactive oxygen species (ROS) production (Tseng and Liu, 2014). These abnormalities may partially explain the contribution of neutrophil adhesion to brain capillaries in the formation of capillary stalls and reduction in cerebral blood flow (CBF) that is observed in animal models (Cruz Hernandez et al., 2019; El Amki et al., 2020), a mechanism that could occur at the early stages in aging and influence the pathological accumulation of Aβ and tau when sustained over years.

One of the most widely studied and available clinical markers of peripheral inflammation is the neutrophil to lymphocyte ratio (NLR), which has been associated with poor prognosis in several cancer outcomes (Walsh et al., 2005; Sarraf et al., 2009), diabetes (Imtiaz et al., 2012), and cardiovascular disease (CVD) (Bhat et al., 2013). Almost a decade ago, a higher NLR was first reported in patients with AD (Kuyumcu et al., 2012) and replicated in a study using longitudinal data and repetitive measures over time (Rembach et al., 2014). The higher NLR levels observed in patients with AD were associated with increased amyloid burden, but this association was no longer present after adjustment for age, sex, and ApoE4 status, thus potentially limiting the potential of NLR as a diagnostic biomarker in more advanced stages of the disease. Because of the hyperactive neutrophil state identified in patients with AD and the higher NLR ratios observed in AD versus controls, it is possible that higher NLR ratios in elderly adults may be independently associated with the risk of incident dementia, although, such an association has yet to be identified.

The aim of this study was to examine whether baseline NLR was independently associated with incident dementia in the Framingham Heart Study (FHS), a longitudinal, community-based cohort with rigorous and continuous surveillance for clinical dementia incidence and a large number of participants with NLR measures.

Abbreviations: AD, Alzheimer's disease; NLR, neutrophil to lymphocyte ratio; CI, confidence interval; CVD, cardiovascular disease; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders, 4th edition; FHS, Framingham Heart Study; HR, hazard ratio; MMSE, Mini-Mental State Examination; CBC, complete blood count.

MATERIALS AND METHODS

Standard Protocol Approvals, Registrations, and Patient Consents

All participants provided written informed consent. Study protocols and consent forms were approved by the institutional review board at the Boston University Medical Center.

The Framingham Heart Study

The FHS is one of the few historical active longitudinal cohort studies in the United States, initiated in 1948 and with over 70 years of follow-up evaluations. Following recruitment, the original cohort of 5,209 residents from Framingham, MA, underwent up to 32 examinations every 2 years, where various clinical and laboratory data were collected (Dawber et al., 1963). In 1971, a total of 5,124 children of the original cohort and their spouses were enrolled in the Offspring cohort who completed a total of nine examinations, with the latest examination performed in 2011–2014 (Kannel et al., 1979). *Offspring* cohort participants who attended the ninth examination cycle (2011–2014), during which a complete blood count (CBC) was obtained, were eligible for the present investigation.

Blood Collection and Complete Blood Count

Participants were asked to fast after 8:00 p.m., the evening before their clinic exam, and considered to be fasting after a minimum of a 10-h fast. Blood was drawn from participants in a supine position, using standard venipuncture technique, typically between 7:00 a.m. and 9:00 a.m. Hematology testing was performed using whole blood [Tyco Monoject, 15% ethylenediaminetetraacetic acid, EDTA, (K3)]. A CBC with differential was performed on EDTA whole blood using a Beckman Coulter HmX Hematology Analyzer as described previously (Sloan et al., 2015). Blood collection was performed following the exact same procedures in every subject. Hematology testing included estimation of intra-assay CV in ∼10% samples run in duplicate. The NLR was defined as the absolute neutrophil count by the absolute lymphocyte count from the CBC panel. Assessment of cognitive decline and dementia was performed by a panel blinded to the CBC/NLR results.

Dementia Characterization in the Framingham Heart Study

The main outcome of the present study was an incident clinical diagnosis of dementia using continuous surveillance until the conclusion of the follow-up period up to 2018. The diagnosis of dementia was based on criteria from the *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition (DSM-IV). The diagnosis of AD was based on criteria for possible, probable, or definite AD from the National Institute of Neurological and Communicative Disorders and Stroke and the AD and Related Disorders Association (NINCDS–ADRDA). Additional methods for continuous surveillance of dementia and AD in the FHS have been previously described (Seshadri et al., 1997, 2011; Au et al., 2012; Satizabal et al., 2016). In brief, the cognitive function of all participants was assessed using the Mini-Mental State Examination (MMSE) (Folstein et al., 1975) at every examination. The MMSE was used to flag participants for a dementia panel review based on their performance if any of the following occurred: (a) an absolute score <23 for all persons, (b) score <24 for persons with only high school completed, (c) score <26 for participants with a college education, (d) >3-point decline between successive examinations, or (e) >5-point decline from the highest obtained MMSE scores of the participants. Participants were also flagged for additional evaluation based on concerns from participants themselves, relatives, or other professionals. In additional visits, flagged participants had a full neuropsychological and neurological examination, which was also reviewed to refer for dementia evaluation by the dementia review panel. The panel, which includes neurologists (JS and SS) and neuropsychologists, used data from multiple sources to assess possible cognitive decline and dementia to determine whether a participant had dementia, the dementia subtype, and the date of diagnosis. After a participant died, a medical panel manually reviewed medical records up to the date of death to assess for the potential cognitive decline since the last examination of the participants. This medical panel referred any participants who may have presented with a cognitive decline for postmortem review by the dementia review panel [refer to Satizabal et al. (2016) – **Supplementary Material**, for a detailed description of dementia surveillance in the FHS]. The main outcome of the present study was incident dementia using continuous surveillance with clinician diagnosis at the conclusion of the follow-up period up to 2018.

Demographic and Clinical Covariates

Clinic examination corresponding to the CBC (examination cycle 9) was defined as the baseline. Smoking was defined based on smoking status prior to the year of baseline. We defined diabetes mellitus by fasting glucose levels above 126 mg/dl (7.0 mmol/L) and/or use of antidiabetic treatments. Levels of all cardiovascular risk variables, including body mass index (BMI), total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides, were determined from examination cycle 9. History of CVD events at the time of clinical examination included: reported history of coronary heart disease (CHD), congestive heart failure (CHF), myocardial infarct (MI), intermittent claudication (IC), ischemic stroke, intracerebral hemorrhage, or transient ischemic attack (TIA).

Statistical Analysis

R 3.6 and Python 3.7 were used for statistical analysis and visualization. The analysis involved the following variables: (a) the NLR; (b) outcome: clinical dementia diagnosis; and, (c) basic demographic and clinical parameters (continuous or categorical), which included age, sex, BMI, smoking, HDL, low-density lipoprotein (LDL), total cholesterol, triglycerides, systolic and diastolic blood pressure, diabetes, and history of CVD. Univariate analysis of demographic and clinical differences between study groups included *t*-tests for continuous variables and χ^2 tests for nominal variables. Kruskal–Wallis tests were applied to non-normal distributions. Spearman correlations were used to test the

association between continuous variables. Generally, we defined statistical significance by $p < 0.05$, and tendencies by $p \leq 0.1$.

Follow-up duration was calculated from the date of the CBC until the most recently available clinical diagnosis prior to the end of follow-up in 2018. To detect potentially independent associations between the NLR and incident dementia, we fit Cox proportional hazard models with inverse probability weighting (IPW). The IPW method weighted each subject by the inverse of the probability of their observed NLR using the median as a cutoff, adjusting for non-random selection of participants into high versus low NLR groups. A logistic regression model for high versus low NLR, adjusted for age, sex, BMI, diabetes, current smoking status, LDH, LDL, total cholesterol, triglycerides, systolic and diastolic blood pressure, and history of CVD was used to estimate these probabilities. The Cox models were then weighted by the inverse of the probabilities of the NLR, adjusted for the above covariates. The proportional hazards assumption was validated using the Schoenfeld residual test included in the cox.zph function of the coxph package (Grambsch, 1995; In and Lee, 2019). Next, using the propensity score model for low versus high NLR (split at the median) for all participants and the causalCmprsk package (Vakulenko-Lagun et al., 2020), we estimated the cumulative incidence function (CIF) for dementia for the two groups. Due to the competing risk of death, the models can be interpreted as cause-specific hazard models (i.e., for the risk of dementia among those still alive). Finally, in an exploratory approach, we applied the survival data implementation of Breiman's random forest models in the randomForestSRC (O'Brien et al., 2021) to estimate the relative importance of each of the covariates in dementia risk. Briefly, the variable importance of each predictor is estimated by using variable selection methods of random forest survival models. The variable selection method uses a prediction error approach by "noising-up" each variable in turn. The variable importance of a variable X_i is the difference in prediction error when X_i is randomly permuted, compared to the prediction error under the true values. The package ggRandomForests was used for visualization. Code is available upon reasonable request and for collaboration and reproducibility purposes. The data are available in the BioLINCC repositories.

RESULTS

Study Sample

The final study sample consisted of 1,648 FHS participants and included the combination of NLR and clinical and demographic covariates (**Figure 1**) described previously. **Table 1** summarizes baseline characteristics for all participants at the time of the CBC. The average age of participants in the study at baseline was 69 years [the interquartile range (IQR): 64, 76]; 55.8% were female (920).

Neutrophil to Lymphocyte Ratio

Intra-assay CVs were <2.5% for both the total neutrophil and lymphocyte counts. The absolute neutrophil and lymphocyte count and the NLR followed a unimodal distribution.

Although the participants with a diagnosis of dementia at baseline were excluded from the analysis, a brief comparison of the clinical labs at baseline indicated higher neutrophil counts and NLR in dementia cases (**Supplementary Figure 1**). In the final cohort (non-dementia) participants that showed a higher NLR (above median) were older and predominantly male; they had lower LDL, HDL, and total cholesterol and higher BMI, triglycerides, systolic blood pressure, and rates of CVD history at baseline ($p < 0.05$; **Table 1**).

Association of the Neutrophil to Lymphocyte Ratio and Incident Dementia

Of the 1,648 participants, 51 cases of incident dementia (41 confirmed AD cases) and 85 deaths without incident dementia were observed during a median follow-up of 5.9 years (IQR: 2.6, 6.9). Logistic models used to estimate IPW weights indicated strong associations between the NLR and age, male sex, BMI, and smoking status (**Supplementary Table 1**). Although the CIs are overlapping, and there is insufficient power to conclude that the incidence curves are different, CIF curves suggested that groups with a higher baseline NLR demonstrated a greater incidence of dementia (**Figure 2**). For example, the probability of dementia prior to death occurring within 5.9 years for those with low NLR at baseline is 2.73% [95% CI: 1.56, 4.43], while for those with high NLR it was 4.11% [95% CI: 3.05, 5.38]. Ranked feature importance of random forest competing for risk models assigned highest priority to age, systolic and diastolic blood pressure, and history of CVD, immediately followed by the NLR (**Figure 3**). Variables with lower importance than the NLR included total cholesterol, diabetes, BMI, LDL cholesterol, smoking status, sex, and triglycerides. Adjusted hazard ratios (HRs) for the IPW multivariate Cox proportional hazard models for incident dementia are summarized in **Supplementary Tables 2, 3**. Higher NLR at baseline was independently associated with incident dementia when included as a continuous variable (HR: 1.22; 95% CI: 1.05, 1.43; $p = 0.01$). Results indicated a 22% increase in dementia risk per unit increase in the NLR. Elevated HRs are

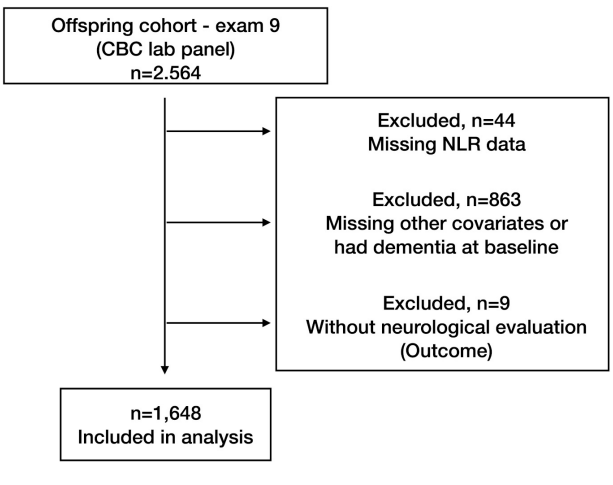

FIGURE 1 | Inclusion diagram.

TABLE 1 | Comparison of demographic and clinical characteristics by the neutrophil to lymphocyte ratio (NLR).

	Overall (*n* = 1,648)	NLR below median (*n* = 826)	NLR above median (*n* = 822)	*P*-value
Age, median [Q1, Q3]	69.0 [64.0, 76.0]	68.0 [63.0, 74.0]	71.0 [65.0, 77.0]	< 0.001
Female sex, *n* (%)	920 (55.8)	515 (62.3)	405 (49.3)	< 0.001
BMI, median [Q1, Q3]	27.7 [24.7, 30.9]	27.5 [24.3, 30.5]	28.0 [25.1, 31.4]	0.006
LDL cholesterol, median [Q1, Q3]	96.0 [78.0, 118.0]	101.5 [81.0, 122.0]	93.0 [75.0, 112.0]	< 0.001
HDL cholesterol, median [Q1, Q3]	60.0 [49.0, 74.0]	61.0 [50.0, 76.0]	58.0 [47.0, 71.0]	< 0.001
Total cholesterol, median [Q1, Q3]	183.0 [158.8, 207.0]	188.0 [163.2, 211.0]	177.0 [153.0, 202.0]	< 0.001
Triglycerides, median [Q1, Q3]	99.0 [74.0, 133.0]	96.0 [72.0, 132.0]	100.5 [76.0, 134.0]	0.041
Smoker, *n* (%)	78 (4.7)	32 (3.9)	46 (5.6)	0.126
Diabetes, *n* (%)	159 (9.6)	76 (9.2)	83 (10.1)	0.594
Systolic BP, median [Q1, Q3]	125.0 [115.0, 137.0]	125.0 [113.0, 136.0]	126.0 [116.0, 137.0]	0.011
Diastolic BP, median [Q1, Q3]	72.0 [65.0, 78.0]	72.0 [66.0, 78.0]	71.0 [65.0, 78.0]	0.176
CVD History, *n* (%)	295 (17.9)	122 (14.8)	173 (21.0)	0.001
NLR, median [Q1, Q3]	2.3 [1.8, 3.0]	1.8 [1.4, 2.0]	3.0 [2.6, 3.8]	< 0.001
Number of dementia cases, *n* (%)	51 (3.1)	16 (1.9)	35 (4.3)	0.01
Number of deaths, *n* (%)	92 (5.6)	33 (4.0)	59 (7.2)	0.007

BMI, body mass index; BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; Chol, cholesterol; CVD history, history of cardiovascular events. Definitions described in section "Materials and Methods."

also apparent with respect to categorical coding of NLR: above versus below the median (HR: 1.80; 95% CI: 0.96, 3.37; $p = 0.07$) in adjusted models. A sensibility analysis inspecting confirmed AD cases as an outcome similarly suggested elevated NLR was associated with higher rates of incident AD (**Supplementary Tables 4, 5** and **Supplementary Figure 2**).

DISCUSSION

Recently, neutrophils have been identified as potential innate immunity players contributing to AD pathology, and a hyperactive neutrophil state has been found in patients with AD and associated with AD progression. The NLR serves as a composite inflammatory biomarker, which incorporates information from two leukocyte subtypes. Exploration of the NLR as a proxy for neutrophil-mediated inflammation is of interest since the NLR can be obtained as part of the standard CBC panel, which is widely available in most hospitals. Our study in the community-based longitudinal FHS Offspring cohort demonstrated that individuals with elevated NLR are at a greater risk of dementia during a 5.9-year follow-up period. These findings persist after adjustment for a number of covariates that could play a confounding role in the association of NLR with dementia risk. Importantly, results from the sensitivity analysis suggested an association of the NLR and AD risk specifically.

The role of neutrophils in AD pathology and clinical progression has been previously suggested (Shad et al., 2013; Pietronigro et al., 2017; Dong et al., 2018; Stock et al., 2018; Bawa et al., 2020; Rossi et al., 2021). Although variations in the NLR have been associated with several clinical outcomes other than AD (Walsh et al., 2005; Sarraf et al., 2009; Imtiaz et al., 2012; Bhat et al., 2013), the interpretation of a change in the NLR remains controversial. Studies in animal models suggest that clearance of neutrophils may be reduced with aging, potentially compromising inflammation resolution following injury. The increased NLR with aging in humans may be indicative of such a process and whether abnormal neutrophil phenotypes are associated with the increased NLR should be studied in the future. Recent studies show that neutrophils may be involved in capillary stall formation and contribute to CBF reductions that when sustained over time may lead to oxidative stress, endothelial damage and influence the pathological accumulation of Aβ and

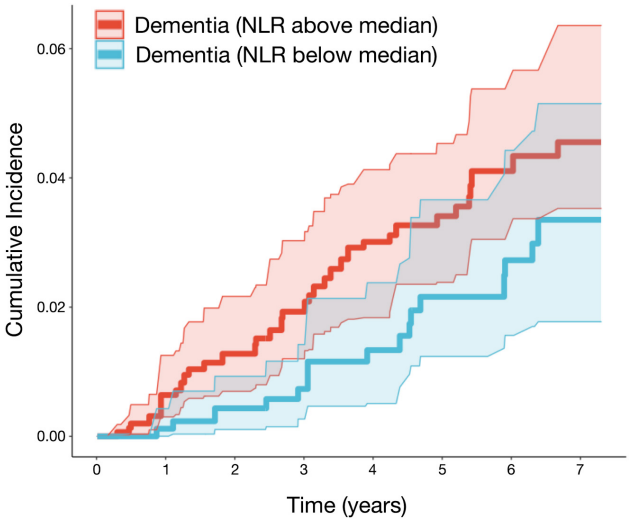

FIGURE 2 | The neutrophil to lymphocyte is associated with higher rates of incident dementia in the FHS. Adjusted cumulative incidence functions and 95% CIs for dementia for the NLR groups (defined as above/below median). Higher NLR at baseline was associated with a greater incidence of dementia. Models were adjusted for age, sex, BMI, systolic and diastolic blood pressure, diabetes, current smoking status, HDL, LDL, total cholesterol, triglycerides, and history of CVD ($n = 1,647$; events = 51; median follow-up = 5.9 years). BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; CVD, cardiovascular disease; FHS, Framingham Heart Study.

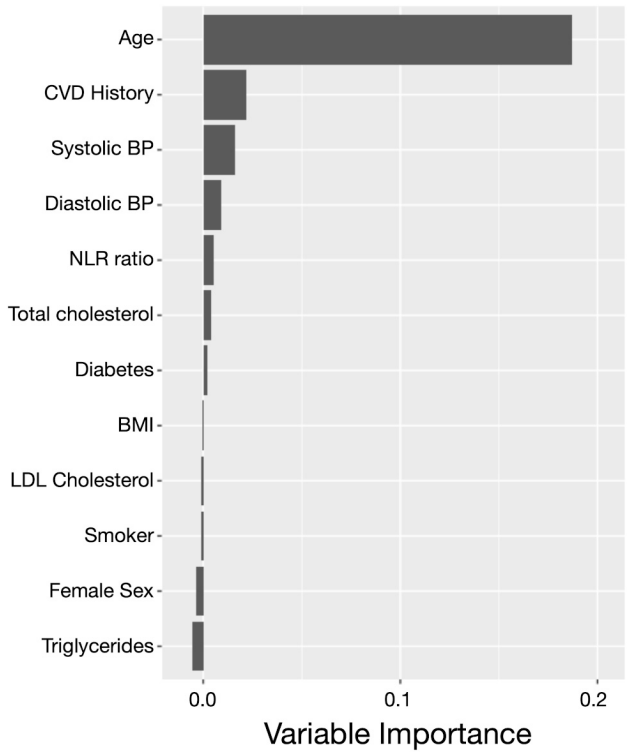

FIGURE 3 | The neutrophil to lymphocyte ratio plays an important role in incident dementia prediction. Ranked feature importance for random forest survival models for the prediction of incident dementia assigned a higher priority for the NLR (coded as continuous) over other clinical covariates. The highest priority was given to age, history of CVD, and hypertension, followed by the NLR and the remaining clinical and demographic covariates. Variable importance close to zero indicates the variable contributed nothing to predictive accuracy, and negative values indicate the predictive accuracy improved when the variable was misspecified and therefore was not informative. CVD, cardiovascular disease; FHS, Framingham Heart Study; NLR, neutrophil to lymphocyte ratio.

tau at the early stages of the disease (Cruz Hernandez et al., 2019; El Amki et al., 2020). Additionally, the vascular dysfunction state that is associated with AD may increase endothelial signaling facilitating neutrophil recruitment to the capillary vessel walls in a feed-forward loop. Studies need to evaluate whether the stall formations are associated with increased NLR.

Although our primary goal was to evaluate the association of the NLR with incident dementia, we also observed higher NLR in the dementia cases at baseline that was excluded from analysis when compared to dementia-free participants. The results are in agreement with previous research that identified higher NLR in patients with AD when compared to age-matched controls (Kuyumcu et al., 2012; Rembach et al., 2014). The NLR ratio has also been associated with conditions related to AD risk and influencing progressions, such as CVD (Angkananard et al., 2018; Kim et al., 2018; Haybar et al., 2019), obstructive sleep apnea (Altintas et al., 2015; Sunbul et al., 2015; Oyama et al., 2016; Rha et al., 2020), depression (Aydin Sunbul et al., 2016; Demircan et al., 2016; Arabska et al., 2018; Liang et al., 2019), and obesity (Rodriguez-Rodriguez et al., 2020). Our findings are therefore consistent with previous research that identified an association of the NLR with AD and related risk factors.

In observational studies like the FHS where there is no random assignment to treatment groups (or variable of interest like the NLR in this case), the unadjusted comparison between treatment groups may be misleading due to confounding. To adjust for measured confounders in our study, we used inverse probability of treatment weighting which is a robust approach for correcting for potential confounding (Neumann and Billionnet, 2016; Vakulenko-Lagun et al., 2020). In our study using weighted Cox models in elderly adults and a 5.9-year median follow-up suggested that the NLR was independently associated with incident dementia. Additionally, the variable importance results of random forest survival models suggested that the NLR had a considerable high predictive value when compared to other variables known to be relevant in dementia risks, such as age, sex, BMI, blood pressure, diabetes, smoking status, lipids, and history of CVD, suggesting that the NLR may add predictive value when incorporated in machine-learning models for dementia and this needs to be examined in future studies.

Our study has several limitations. First, although we adjusted for a relatively large number of covariates, we did not exclude/adjust for psychiatric and autoimmune disorders known to increase dementia risk that also to may influence the NLR ratios (e.g., depression) and future analysis should control for these conditions and look at each of the risk groups separately. Additionally, the number of dementia cases available for risk stratification in our study was relatively small and future studies should replicate our analysis if a larger number of incipient dementia cases are available. Second, in our primary analysis, we did not control for the number of years of education, the use of hypertensive drugs, and drugs potentially modulating the CBC results due to a considerable number of missing observations that limited the number of incipient dementia cases available for analysis. A sensitivity analysis also including the aforementioned variables showed associations with the same directionality of NLR associated with increased risk of incipient dementia. Additionally, our study did not account for the ApoE4 status, which is the main genetic risk factor for AD (Tsai et al., 1994; Kim et al., 2009; Liu et al., 2013) and previous research found that the ApoE4 status influences the NLR (Rembach et al., 2014). Cholesterol homeostasis has a pivotal function in regulating immune cells, and the role of ApoE in neutrophil-mediated inflammation has been suggested in experimental models (Terkeltaub et al., 1991; Barger and Harmon, 1997; Zhou et al., 2019; Doring et al., 2020).

CONCLUSION

Our study in 1,648 participants of a well-characterized community-based cohort shows an independent association of the NLR with future dementia, reinforcing the role of neutrophil-mediated inflammation in AD pathology and progression.

FUTURE PERSPECTIVES

Future studies need to determine whether the increased NLR observed in certain participants at higher risk of dementia is independent or mediated by the ApoE4 status and the role of other AD-risk factors in the association. Additionally, dementia is more frequent in women and some race and ethnic groups, and we were not powered to perform disaggregated analyses. Finally, whether the NLR is associated with other markers of vulnerability to cognitive decline (global cognition, MRI, PET, and AD-biofluid markers) in middle age needs to be further investigated.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://biolincc.nhlbi.nih.gov/home/.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Boston University Medical Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JR-C designed and conceptualized the study, analyzed the data, performed the statistical analysis, and drafted the manuscript. AJ, AB, SS, and JS interpreted the data, major role in the acquisition of data, and revised the manuscript for intellectual content. JB, NF, ND, CZ, ZK, OB, AP, AC, RB, and TW revised the manuscript for intellectual content. TW and RO designed and conceptualized study, interpreted the results, and revised the manuscript for intellectual content. 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.2021.773984/full#supplementary-material

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REVIEW
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The Interplay Between Brain Vascular Calcification and Microglia

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Vascular calcifications are characterized by the ectopic deposition of calcium and phosphate in the vascular lumen or wall. They are a common finding in computed tomography scans or during autopsy and are often directly related to a pathological condition. While the pathogenesis and functional consequences of vascular calcifications have been intensively studied in some peripheral organs, vascular calcification, and its pathogenesis in the central nervous system is poorly characterized and understood. Here, we review the occurrence of vessel calcifications in the brain in the context of aging and various brain diseases. We discuss the pathomechanism of brain vascular calcification in primary familial brain calcification as an example of brain vessel calcification. A particular focus is the response of microglia to the vessel calcification in the brain and their role in the clearance of calcifications.

Keywords: astrocyte, cerebrovascular calcification, ectopic calcification, microglia, mouse model, pericyte, primary familial brain calcification, vascular aging

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INTRODUCTION

Vascular calcification associated with vascular aging is prevalent in the elderly population. The presence of calcifications in blood vessels leads to vascular stiffness, loss of elasticity and decreased compliance causing impaired cardiovascular function and potential end-organ damage (Pescatore et al., 2019). Cerebral vascular calcification is considered a predictor of cardiovascular events such as heart attack as well as stroke (Rennenberg et al., 2009; Hermann et al., 2013). In addition, vascular calcification is a prevalent complication of diseases such as chronic kidney disease and diabetes and is associated with significant morbidity and mortality (Lanzer et al., 2021). Calcification of vessels also occurs in several genetic disorders or due to the systemic imbalance of phosphate metabolism (Takashi and Fukumoto, 2020; Rutsch et al., 2021).

Vasculature is divided into distinct zones based on their cellular and acellular composition, and function. Arteries are composed of lumen forming endothelial cells (*tunica intima*) separated from vascular smooth muscle cells (VSMC) (*tunica media*) by a basement membrane. Veins have incomplete VSMC coverage. Capillaries, the predominant vessel type in the brain, are positioned between arteries and veins. Capillaries are composed of endothelial cells and pericytes. In addition, the perivascular space along arteries and veins between the VSMC layer and astrocyte end-feet, which cover the entire vascular tree, contains several cell types, including perivascular macrophages and fibroblasts (**Figure 1A**).

Vascular calcifications can be distributed from the intimal layer (e.g., atherosclerosis) (**Figure 1B**) to the medial layer (**Figure 1C**) of the arterial wall, affecting different large and small arterial beds or capillaries (**Figure 1D**).

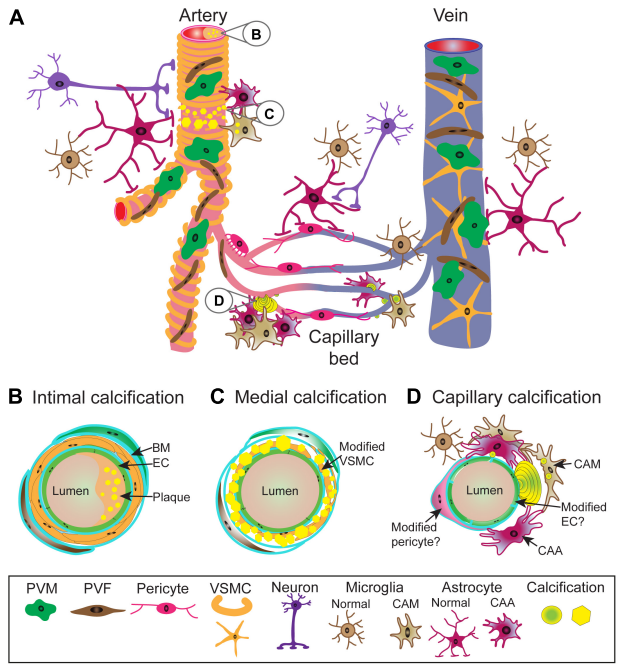

FIGURE 1 | Schematic representation of vascular calcifications along the central nervous system (CNS) vascular network. Cellular and structural diversity of the vascular network along the arterio-venous axis **(A)**. In arteries, lumen forming endothelial cells (EC) are separated from circumferentially wrapped vascular smooth muscle cells (VSMC) by a basement membrane (BM). Capillary endothelial cells are covered by pericytes (PC) with a prominent cell body and slender processes with which they share the basement membrane and are in direct contact. Capillaries transition into veins which have an incomplete mural cell coverage. The entire vasculature is covered by astrocyte end-feet and occasionally innervated by nerve fibers. Between VSMC layer and astrocyte end-feet reside perivascular fibroblasts (PVF) and perivascular macrophages (PVM). Arterial calcifications can be intimal **(B)**, resulting from atherosclerotic plaque in the lumen, as well as medial **(C)**, due to phenotypic alterations of VSMCs leading to mineralization of extracellular matrix. It is not known to what extent PVM and PVF become modified and contribute to the pathophysiology. Capillary calcifications **(D)** are characterized by the appearance of mineralized "beads" on the capillary wall, which protrude into brain parenchyma. While it is known that capillary calcifications elicit strong glial reactions, changes in pericytes and endothelial cells in calcified capillaries are poorly understood. Calcifications are depicted as yellow spheres or polygons. BM, basement membrane; CAA, calcification-associated astrocyte; CAM, calcification-associated microglia; EC, endothelium; PVF, perivascular fibroblast; PVM, perivascular macrophage; VSMC, vascular smooth muscle cell.

Unlike peripheral vascular calcification, intracranial calcifications (vascular or parenchymal) are less frequently reported and relatively poorly characterized. Intracranial calcifications are a common incidental finding in computed tomography (CT) imaging in the general population (Deng et al., 2015) and at autopsy. In addition to aging, brain calcifications occur due to metabolic alterations (e.g., chronic kidney disease, thyroid hormone imbalance), infections (e.g., viral encephalitis, neurocysticercosis, neurocryptococcosis), toxic injury (e.g., carbon monoxide poisoning, radio- and chemotherapy), genetic disorders (e.g., neurofibromatosis, tuberous sclerosis, primary familial brain calcification), brain tumors (e.g., oligodendrogliomas, meningiomas) as well as defective vascular morphogenesis during development (e.g., cavernomas, Sturge-Weber syndrome) (Nash et al., 2004; De La Torre et al., 2018; Donzuso et al., 2019; Saade et al., 2019). Parenchymal calcifications in the pineal gland, also referred to as "brain sand," are even used as an anatomical landmark in radiographic studies (Vígh et al., 1998). The pathogenic mechanisms involved in brain calcifications in these diverse conditions are not well-understood, except in the case of systemic calcium and phosphate imbalances accompanying diseases such as kidney failure, hypo- or hyperparathyroidism. Often, while reported, intracranial calcifications are not clearly distinguished as parenchymal, vascular or both. Nevertheless, intracranial vessel-associated calcifications have been reported to accompany sporadic and familial neurodegenerative [e.g., Parkinson's disease, Alzheimer's disease (AD)] and neuroinflammatory diseases (e.g., type I interferonopathies) (Fujita et al., 2003; Livingston et al., 2013; Ukai and Kosaka, 2016; Donzuso et al., 2019; Saade et al., 2019).

In children, the presence of intracranial calcifications is almost always associated with underlying pathology (Goncalves et al., 2020a,b), whereas in adults, these are viewed as part of the normal aging process. However, recent studies have shown that intracranial calcifications are strong predictors of adverse clinical outcomes (Bartstra et al., 2020). We, therefore, believe that a detailed description of intracranial vascular calcification and related pathomechanism can potentially assist in early prognosis and further our understanding for better therapeutic targeting of brain diseases.

In this review, we describe brain vascular calcifications with a focus on possible underlying pathomechanism and the role of microglia as modifiers of brain vascular calcification. We primarily focus on the pathophysiology of vascular calcification occurring in a neuropsychiatric disease – primary familial brain calcification (PFBC).

BONE IN THE BRAIN—AN OVERVIEW OF CEREBROVASCULAR CALCIFICATIONS

Calcification of large arteries such as the internal carotid artery, intracranial vertebrobasilar artery, middle cerebral artery, circle of Willis, as well as calcifications in the hippocampus and basal ganglia, are easily observed on CT images (Deng et al., 2015; Bartstra et al., 2020). It is estimated that 30% of aged individuals have brain calcifications (Nicolas et al., 2013a). However, due to relatively low resolution of CT imaging, additional histopathological analyses are required to identify calcification of microvasculature. Few case studies in aged human subjects have shown that both the calcification of the hippocampus and basal ganglia are vascular (Peters et al., 2018; de Brouwer et al., 2021). In the aged hippocampus, arteries, pre-capillaries and capillaries were calcified (Peters et al., 2018). Similar calcification of hippocampal vessels has been reported in AD patients (Wegiel et al., 2002; Schober et al., 2021). A recent study showed that the tunica media of arterioles in the globus pallidus is calcified

in aged individuals, confirmed both histologically and on CT. Calcification of the vascular tree showed a distribution pattern, starting in the ventral striatopallidum spreading posterolaterally into the external half of the globus pallidus (de Brouwer et al., 2021). There is differential involvement of the vascular tree (artery, capillary), which might be dependent on the anatomical region and underlying disease. For example, previous histopathological studies detected arterial calcification in aged subjects and AD patients compared to capillary bed calcification in the globus pallidus in Down syndrome patients (Wegiel et al., 2002; Schober et al., 2021).

PATHOGENESIS OF VASCULAR CALCIFICATION

In bone, hydroxyapatite or carbonated hydroxyapatite crystals are deposited in the extracellular matrix consisting mainly of collagen I fibers (Arnold et al., 2021). Mineral formation also depends on the presence of metabolic ions (citrate, lactate, carbonate) and proteins as well as glycans that can modify the mineralization process by stabilizing crystals or preventing crystal growth (Young, 2003; Davies et al., 2014). Bone consists of three cell types—(i) osteoblasts- tissue mineralizing cells, (ii) osteoclasts- mineralized tissue resorbing cells, and (iii) osteocytes- long-lived cells located deep inside the bone matrix (Salhotra et al., 2020). Ectopic calcification of the vessel wall is thought to be an active process that resembles the formation of bone with hardening of the tissue initiated by nucleation of calcium phosphate in a permissive matrix. Hydroxyapatite and carbonated hydroxyapatite are the most abundant calcium phosphate phases in both physiological and pathological calcification. Although the primary cause of vascular calcification initiation might be different, it leads to an osteogenic environment. After initiation, osteoblast- and osteoclast-like cells, and bone matrix proteins can be detected in these mineralized structures. Transcriptional programs and signaling pathways involved in normal bone formation are also identified along the calcifying vessel (Chen Y. et al., 2021). In intimal calcification, calcium phosphate deposition occurs in so-called atherosclerotic plaques residing inside the blood vessel lumen. The extent of plaque calcification correlates with plaque stability (Motoyama et al., 2007). Calcification of the middle layer of the blood vessel wall (medial calcification) is associated with aging, diabetes, kidney disease, and hereditary vascular calcification diseases. Although the deposition of hydroxyapatite is a common feature between intimal and medial calcification, initiation and propagation mechanisms differ (Lanzer et al., 2014). The processes that trigger atherosclerotic plaque formation, calcification and the role of innate immunity in these processes are not discussed in this review.

The prevalent view is that medial calcification is accompanied by a phenotypic change of resident vascular smooth muscle cells into cells that promote mineralization. Vascular calcification can be initiated by several triggers such as cellular senescence, inflammation, loss of anti-calcifying proteins and imbalance in extracellular phosphate (Pi) and pyrophosphate (PPi) levels (Pescatore et al., 2019). Cellular senescence and inflammation have been shown to induce trans-differentiation of human primary VSMC into osteoblast-like cells capable of mineralizing the matrix (Nakano-Kurimoto et al., 2009; Sorokin et al., 2020). In addition to VSMC, circulating progenitor cells, mesenchymal stem cells, endothelial cells, and fibroblasts have been shown to share the potential to trans-differentiate into osteoblast-like cells in mouse models of atherosclerosis (Jiang et al., 2021). How can extracellular matrix calcification be controlled by osteoblast and osteoblast-like cells? Poly(ADP)-ribose (PAR) generated in response to cell damage signaling (either physiological by osteoclasts or pathological as in VSMC) is involved in matrix calcification (Pescatore et al., 2019). A recent study in mice reported the VSMC and osteoblasts deposit PAR-calcium, which is localized in bone collagen fibrils at the site of formation of calcium phosphate crystals during bone formation (Chow et al., 2014), into the extracellular matrix in response to vascular calcification. This process likely provides calcium for the initiation of calcium-phosphate crystals (Muller et al., 2019). Currently, it is not clear how PAR-calcium is delivered to the extracellular matrix, but it might be concentrated in extracellular vesicles involved in matrix mineralization (Muller et al., 2019).

The calcification of blood vessels is also initiated by the loss of calcification inhibitors [e.g., matrix-Gla protein (MGP), osteoprotegerin] (Luo et al., 1997; Bucay et al., 1998) or an imbalance in Pi/PPi/purine metabolism leading to a favorable environment for calcium phosphate deposition (Rutsch et al., 2021). Analysis of mineral phases on calcified aortas of MGP-deficient mice ($Mgp^{-/-}$) using various spectroscopic and microscopic techniques suggest that calcification of elastin in the absence of MGP is reminiscent of multistep bone mineralization via amorphous calcium phosphate precursors to crystalline structures (hydroxyapatite) (Gourgas et al., 2018). Analysis of *Abcc6* and *Nt5e* knockout mice and mice lacking functional ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) has led to the hypothesis that reduced plasma PPi, an inhibitor of calcium phosphate crystal growth, leads to a pro-mineralizing environment (Shimada et al., 2021). ABCC6 (ATP-binding cassette subfamily C member 6) facilitates cellular ATP efflux and the cascade of extracellular ATP degradation to adenosine involves hydrolyzes ATP to AMP and PPi by ENPP1. AMP is further hydrolyzed by CD73, a membrane-bound ecto-5′-nucleotidase encoded by *NT5E,* to generate adenosine and Pi. PPi is further hydrolyzed by tissue non-specific alkaline phosphatase (TNAP encoded by *ALPL*) to extracellular Pi. Adenosine is an indirect inhibitor of calcification by inhibiting TNAP (St Hilaire et al., 2011).

Pathogenesis of Vascular Calcification in the Brain

Calcification of brain vessels is not specific to *Homo sapiens*, with calcification of cerebral arterioles, venules, and/or capillaries reported in laboratory mice, rats, domesticated animals (cats, dogs, cows, horses), and monkeys (Youssef et al., 2016). Only few studies have specifically characterized the pathomechanisms

underlying brain vascular calcification and its associated changes (**Table 1**). It is likely that causes described for peripheral vessels are similarly involved in the calcification of cerebral vessels. However, vasculature is organotypic and recent advances in single cell RNA sequencing technology have generated insights into molecular differences and similarities between various vascular beds in the body (Augustin and Koh, 2017). Blood vessels in the body show different susceptibility to calcification, even in genetic diseases. For example, patients with mutations in gene *NT5E*, encoding for CD73, show preferential calcification of arteries in the extremities (St Hilaire et al., 2011; Zhang Z. et al., 2015). The cellular composition and characteristics of cerebral vessels differ from systemic vasculature. Cerebral endothelial cells possess the blood-brain barrier (BBB), a multicomponent feature that tightly controls parenchymal protein, metabolite, and ionic composition (Keller, 2013). In addition, other vascular cells (e.g., pericytes) have been shown to present organ-specific characteristics, at least at the level of the transcriptome (Vanlandewijck et al., 2018). Furthermore, blood vessels in the brain possess another characteristic not found in peripheral vessels: they are ensheathed with astrocyte end-feet, slender astrocyte processes, enriched with water and potassium channels (Schaffenrath and Keller, 2021). Microglial processes and the cell body contact the vessel basement membrane directly along the vascular tree (Mathiisen et al., 2010; Joost et al., 2019), thereby regulating blood flow in a purinergic receptor P2Y12-dependent manner (Bisht et al., 2021). Furthermore, the composition of the extracellular matrix is different along the vascular tree. In the case of medial calcification, the prevalent ECM component is elastin and elastin haploinsufficiency impedes the progression of arterial calcification (Khavandgar et al., 2014). Thus, given differences in cellular and acellular composition, the pathophysiology of vascular mineralization likely differs between arteries and capillaries.

Vascular calcification increases with age but histopathological studies describing vascular calcifications in the aged human brain are rare (Fujita et al., 2003; Peters et al., 2018; de Brouwer et al., 2021). In general, arterial and capillary calcifications in aged brains have similar histological characteristics as described in various brain pathologies. In aged mice, thalamic blood vessels show the tendency to calcify (Fraser, 1968; Morgan et al., 1982; Yanai et al., 1984, 1987). A recent study showed that vessel-associated calcifications in the thalamus in old wild-type mice show an affinity for bone labeling dyes (Alizarin red and Osteosense). Additionally, aged endothelial cells showed increased TNAP (generates Pi) expression and collagen I deposition in the vascular basement membrane (Yang et al., 2020). The question of whether these matrix mineralization-associated proteins triggered thalamic vessel calcification was not investigated. Although no inflammatory reaction toward aging-related vessel calcifications was described using transmission electron microscopy (TEM) (Morgan et al., 1982), additional studies using other methods (e.g., immunohistochemistry) are needed to investigate whether age-related calcifications evoke a glial response. TEM studies on vascular calcifications in monkey and mouse brains have described early changes in the basement membrane with crystalline hydroxyapatite deposits coinciding with cellular debris or basement membrane degeneration (Morgan et al., 1982; Yanai et al., 1984, 1987, 1994). Alterations in the composition of the basement membrane (accumulation of osteopontin and the appearance of fibrotic collagen I) have also been reported to precede matrix mineralization in the case of hereditary cerebral hemorrhage with Dutch type -amyloidosis (Grand Moursel et al., 2019). Age is an independent

TABLE 1 | Alterations accompanying brain vascular calcifications.

Alteration/observation	Condition	Comment	References
Osteogenic environment	PFBC, AD, PD, aging	Accumulation of anti- and pro-calcification proteins in mineralized deposits on blood vessels	Fujita et al., 2003; Nahar et al., 2019; Zarb et al., 2019b
	PFBC	Cells expressing osteoblast markers around vascular calcifications.	Zarb et al., 2019b
	PFBC	Cells expressing osteoclast markers surrounding vascular calcifications. Osteoclast-like cells expressing cathepsin K are derived from microglia in a mouse model of PFBC.	Zarb et al., 2019b, 2021
	Zika virus infection	Differentiation of Zika virus infected pericytes into osteoblast-like cells *in vitro* in response to BMP2.	Chen W. et al., 2021
Changes in the basement membrane	Aging	Electron microscopy studies reveal degeneration of vascular basement membrane and the presence of hydroxyapatite crystals in aged mice.	Morgan et al., 1982; Yanai et al., 1984
	Aging	Increased deposition of collagen I in vascular basement membrane in aged mice.	Yang et al., 2020
	HCHWA-D	Vascular mineralization is preceded by accumulation of osteopontin and the appearance of fibrotic collagen I in autopsy samples.	Grand Moursel et al., 2019
Oxidative stress	PFBC	Accumulation of 2-ω-carboxyethylpyrrole CEP adducts in astrocytes surrounding vascular calcifications in a mouse model of PFBC.	Zarb et al., 2019b
Altered levels of inorganic phosphate	PFBC	Higher inorganic phosphate levels in the CSF in *Slc20a2*$^{-/-}$ mice and *SLC20A2* mutation carriers	Jensen et al., 2016; Paucar et al., 2017; Wallingford et al., 2017; Hozumi et al., 2018
	Aging	Endothelial cells of old mice are expressing higher levels of TNAP	Yang et al., 2020

AD, Alzheimer's disease; HCHWA-D, hereditary cerebral hemorrhage with amyloidosis-Dutch type; PD, Parkinson's disease; PFBC, primary familial brain calcification.

risk factor for both the development of cardiovascular disease and neurodegenerative disease (Yazdanyar and Newman, 2009; Hou et al., 2019), and thus, more studies are needed to characterize changes in aged blood vessels that lead to vascular calcifications in the brain.

In order to describe the pathophysiology of brain vessel calcification in detail, we will refer to studies investigating vascular calcification in PFBC.

Primary Familial Brain Calcification

As mentioned above, the list of diseases with brain calcification as a secondary manifestation is extensive and it has not been intensively investigated whether these calcifications are vascular and/or parenchymal. In the case of hereditary neuropsychiatric disease, primary familial brain calcification (PFBC), the presence of bilateral basal ganglia vascular calcifications is a diagnostic criterion (Balck et al., 2021). PFBC patients may present clinically with motor (e.g., parkinsonism, ataxia, speech disturbance) and/or non-motor (cognitive deficit, depression, psychosis) phenotypes or may even remain unaffected (Balck et al., 2021). In PFBC, the capillary bed is encrusted with the calcium phosphate deposits like "pearls on the string" in addition to the calcification of the medial layer of arteries and arterioles (Miklossy et al., 2005; Kimura et al., 2016). These small, mineralized deposits are located on the capillary wall adjacent to the parenchyma (Kobayashi et al., 1987) (**Figures 1A,D**).

Autosomal-dominant (AD)- PFBC is caused by mutations in the platelet-derived growth factor subunit B *(PDGFB)* (Keller et al., 2013) and its receptor–*PDGFRB* (Nicolas et al., 2013b), solute carrier family 20 member 2 *(SLC20A2)* (Wang et al., 2012), and xenotropic and polytropic retrovirus receptor 1 *(XPR1)* (Legati et al., 2015). SLC20A2 (also known as PiT2) is an inorganic phosphate importer (Kavanaugh et al., 1994) and XPR1 is the only known mammalian inorganic phosphate exporter (Giovannini et al., 2013). PDGFB and PDGFRB are growth factor and receptor, respectively, implicated in organ development (Andrae et al., 2008; Armulik et al., 2011). Autosomal-recessive (AR) form of PFBC is caused by mutations in myogenesis regulating glycosidase (*MYORG*) (Yao et al., 2018) and junctional-adhesion-molecule-2 (*JAM2*) (Cen et al., 2020). The cellular function of MYORG, an intracellular transmembrane protein belonging to glycosyl hydrolase family, is currently unknown. The knock-out of *Myorg* (alternative name in the mouse genome is AI464131) in mice does not lead to gross developmental defects (Yao et al., 2018). *JAM2* encodes for the transmembrane protein located to cell-cell adhesions and is implicated in trans-endothelial migration of leukocytes (Aurrand-Lions et al., 2001). The genes that cause both, AD-PFBC and AR-PFBC, are functionally different, and expressed by several cell types in the brain (Zarb et al., 2019a). XPR1 and SLC20A2 are ubiquitously expressed (Zeisel et al., 2018). PDGFRB is expressed by perivascular fibroblasts, vascular smooth muscle cells, pericytes and, astrocytes. PDGFB, the main ligand for PDGFRB, is expressed by endothelial cells and microglia, and by certain excitatory and cholinergic neurons (Armulik et al., 2011; Vanlandewijck et al., 2018; Zeisel et al., 2018). MYORG is expressed only by astrocytes (Yao et al., 2018) and JAM2 by perivascular fibroblasts, endothelial cells, and astrocytes (Vanlandewijck et al., 2018). Recent investigations using animal models and genetic analyses have led to a better understanding of the pathophysiology of vascular calcification in PFBC. Currently, three mouse models mimic the histopathological features of PFBC: *Slc20a2* (Jensen et al., 2013) and *Myorg* (Yao et al., 2018) knockouts, and PDGFB hypomorph ($Pdgfb^{ret/ret}$, retention motif knockout) (Keller et al., 2013). The retention motif knockout renders a biologically active PDGFB protein unable to bind extracellular heparan sulfate proteoglycans and thus, shape PDGFB gradients (Abramsson et al., 2003; Lindblom et al., 2003). In PFBC mutation carriers, calcification of the medial layer of arteries, as well as capillaries, is observed (Kozik and Kulczycki, 1978; Miklossy et al., 2005; Kimura et al., 2016). In addition, calcium precipitates have been reported on neurons and astrocytes (Kobayashi et al., 1987; Miklossy et al., 2005). Vascular calcifications are protein-rich and contain hydroxyapatite and trace elements (Zn, Fe) (Smeyers-Verbeke et al., 1975; Bouras et al., 1996). Vascular calcification is similar in mouse models of PFBC with single rounded or mulberry shaped lamellar structures deposited on the vessel wall of arterioles and capillaries and protruding into the parenchyma (Jensen et al., 2013; Keller et al., 2013) (**Figure 1**). Calcifications appear as nodules such that the entire capillary is occasionally surrounded by ring-like calcification. These calcifications contain calcium and phosphate as well as collagenous and non-collagenous bone proteins. They bind bisphosphonates, are of bone density and stain for histological dyes used to visualize bone (e.g., Alizarin red, Alcian blue) (Jensen et al., 2013, 2018; Keller et al., 2013; Yao et al., 2018; Zarb et al., 2019b). Mass spectrometry has been used to characterize calcifications in mice and has shown that they contain proteins that promote (e.g., secreted glycoprotein SPARCL1) or halt calcification (fetuin A, MGP, osteopontin) (Nahar et al., 2019). In addition, they contain amyloid precursor protein (APP), amyloid precursor-like protein 2 (APLP2), as well as secretogranin-1 (CHGB) and chromogranin A (CHGA), which are major constituents of large dense core vesicles involved in storing and delivering large neurotransmitters in neurons (Nahar et al., 2019). Accordingly, these calcifications can be visualized in tissue sections using antibodies against APP, APLP2, osteopontin, osteocalcin, collagen I (Nahar et al., 2019; Zarb et al., 2019b, 2021). Although calcifications contain APP, APLP2 and aggregated protein structures, these structures lack the β-pleated sheet conformation and structural regularity recognized by Thioflavin T or Congo red (Zarb et al., 2021). Chromogranins (CHGB, CHGA) are deposited in the amyloid plaques in AD patients and are deregulated in other brain disorders such as multiple sclerosis, amyotrophic lateral sclerosis, schizophrenia (Willis et al., 2011). Interestingly, a recent study identified a chromogranin A derived peptide from the adrenal gland, which inhibits the osteogenic trans-differentiation of VSMC *in vitro* (Orth-Alampour et al., 2021).

The underlying mechanism leading to vascular calcification and constituent cell types is currently unclear. The genes that cause both AD-PFBC and AR-PFBC are functionally different and expressed by several cell types at the neurovascular unit.

The pathogenesis of PFBC may be multifactorial with mutations potentially affecting multiple cell types in brain vessels and leading to the formation of vessel-associated calcifications. Cell type specific knockouts of involved genes should clarify the role of individual cell types. However, it has been demonstrated in mice that the endothelial expression of PDGFB is protective against brain calcifications (Keller et al., 2013), indicating that vessel-calcification might be caused by reduced PDGFB/PDGFRB signaling at the vessel wall. In summary, it remains to be discovered why mutations in structurally and functionally different protein families lead to the same disease and calcifications of cerebral arteries and capillaries.

Does Imbalance in Brain Phosphate Metabolism Cause Vascular Calcification?

One hypothesis is that vascular calcification in PFBC is caused by the locally altered phosphate levels in the cerebrospinal fluid (CSF) and perivascular spaces (Wallingford et al., 2017). There are no abnormalities in phosphate and calcium levels in the serum of PFBC patients (Manyam, 2005) and PFBC animal models (Keller et al., 2013; Jensen et al., 2016). In addition, analysis of serum calcification propensity of *Pdgfb*$^{ret/ret}$ mice did not point to alterations in the humoral anti-calcification defense (Zarb et al., 2019b). Studies on *Slc20a2*$^{-/-}$ mice suggest that loss of this phosphate importer increases local phosphate concentration in the cerebrospinal fluid, leading to calcium-phosphate deposition in the glymphatic space due to imbalanced phosphate metabolism and subsequent calcification of arterioles (Wallingford et al., 2017). Also, *SLC20A2* (but not *PDGFB*) mutation carriers show elevated levels of Pi in CSF (Jensen et al., 2016; Paucar et al., 2017; Wallingford et al., 2017; Hozumi et al., 2018). It was proposed that changes in Pi concentrations in the CSF could be caused by altered Pi absorption by choroid plexus epithelial cells (Wallingford et al., 2017). SLC20A2 belongs to the type III family of inorganic phosphate importers that consists of two proteins—SLC20A1 and SLC20A2 (frequently used protein name is PiT1 and PiT2, respectively). These proteins are known to maintain cellular phosphate homeostasis (Lederer and Miyamoto, 2012). Therefore, it is difficult to envision how *SLC20A2* mutations could lead to changes in the extracellular phosphate levels.

Cellular ATP and inorganic phosphate levels are sensed by cells by detecting changes in inositol pyrophosphate concentration (Azevedo and Saiardi, 2017). Previous studies have shown that XPR1 senses the intracellular phosphate level by binding to inositol pyrophosphates via its SPX domain (Wilson et al., 2019; Li et al., 2020). Proteins regulating various aspects of phosphate metabolism accomplish this via the SPX (named after yeast proteins Syg1, Pho81 and the mammalian Xpr1) domain. Yeast and plants have several SPX-domain-containing proteins but mammals have only one protein – XPR1 (Azevedo and Saiardi, 2017). A recent study identified crosstalk between SLC20A2 and XPR1 for maintaining constant intracellular phosphate and ATP levels, where XPR1 is a key inositol pyrophosphate-dependent regulator of this process (Lopez-Sanchez et al., 2020). An electron microscope study on *Slc20a2*$^{-/-}$ mice showed intracellular calcium phosphate crystals in pericytes and astrocytes (Jensen et al., 2018), indicating that the initial crystallization of calcium phosphate could occur intracellularly. Thus, the XPR1 and SLC20A2 mutation carriers could show altered intracellular phosphate metabolism. The consequences on vascular cells and how this leads to vascular calcification remain to be determined.

Blood-Brain Barrier Dysfunction–A Cause for Vascular Calcification in Primary Familial Brain Calcification?

Histopathological changes such as the extravasation of plasma proteins observed in the PFBC autopsy cases and the presence of vasogenic edema detected by magnetic resonance imaging indicate BBB dysfunction (Gomez et al., 1989; Miklossy et al., 2005). The initial interpretation of data suggested pericyte deficiency and BBB deficiency in PFBC as a potential link between the formation of brain calcifications (Keller et al., 2013). However, later studies on mouse models did not find evidence to support this view. The brain regions associated with vascular calcification (i.e., thalamus, mesencephalon, and dorsal pons) in *Pdgfb*$^{ret/ret}$ mice showed significantly lower BBB permeability (Vanlandewijck et al., 2015). It is likely that BBB permeability changes in PFBC occur independently and are not a direct cause of vascular calcification. No BBB disruption has been detected in *Slc20a2*$^{-/-}$ mice (Wallingford et al., 2017; Nahar et al., 2019). Interestingly, gene mutations in endothelial junctional proteins occludin, junctional adhesion molecule 3 (JAM3) and cerebral cavernous malformation (CCM) −1, −2, −3 lead to the BBB deficiency and brain calcification (Saitou et al., 2000; Mochida et al., 2010; O'driscoll et al., 2010; Fischer et al., 2013). In addition, biallelic-mutations in a gene encoding a cell-cell junction protein JAM2 causes PFBC (Cen et al., 2020; Schottlaender et al., 2020). This suggests that specific alterations in cell-cell adhesion at the vascular wall could lead to vascular calcification, which are not necessarily dependent on BBB permeability.

Phenotypic Change of Vascular Cells to Osteoblast-Like Cells in Primary Familial Brain Calcification?

The PDGFB/PDGFRB pathway is crucial for pericyte development (Armulik et al., 2011), which raises the question as to whether vascular calcification in PDGFB and PDGFRB mutation carriers is caused by the lack of pericytes. As discussed above, follow-up studies have not supported pericyte deficiency as a cause of vascular calcification in PDGFB mouse model (Vanlandewijck et al., 2015). In *Pdgfb*$^{ret/ret}$ mice, the vessels in deep brain regions had a higher pericyte coverage and less BBB leakage compared to cortical vessels, a region that does not calcify (Vanlandewijck et al., 2015). Thus, one might speculate that the presence of pericytes is needed for the development of calcifications. In addition, an alteration in pericyte numbers has not been observed in *Slc20a2*$^{-/-}$ mice (Wallingford et al., 2017; Nahar et al., 2019). Characterization of the extracellular environment surrounding vascular calcifications showed a so-called osteogenic environment in *Pdgfb*$^{ret/ret}$ mice and human PFBC cases (Zarb et al., 2019b). Calcifications were associated with cells expressing markers of osteoblasts, and osteocytes (Zarb et al., 2019b), but the cellular origin of these cells is not known. Cultured pericytes, depending on culture conditions, have the capacity to form extracellular calcifications

containing hydroxyapatite (Schor et al., 1990). Interestingly, *in vitro* infection of human fetal pericytes with Asian strain of Zika virus led to dedifferentiation of pericytes into bone forming cells via upregulation of bone morphogenetic protein 2 (BMP2) (Chen W. et al., 2021). Also, endothelial cells have the capacity to give rise to bone-forming cells, as was shown in fibrodysplasia ossificans progressiva, a disease characterized by extensive extraskeletal bone formation (Medici et al., 2010; Yao et al., 2013). Curiously, astrocytes have also been linked to the formation of chondrocytes in human gliomas in which a gradual morphologic change of astrocytes to cells indistinguishable from chondrocytes was observed (Kepes et al., 1984). Phenotypic alterations in pericytes in mouse models of PFBC have not been extensively investigated. Isolated brain vessels in PDGFB hypomorphs show upregulation of *Bmp2* and *Bmp4* expression (Armulik et al., 2010). Single cell RNA sequencing of brain endothelial cells showed that both *Bmp2* and *Bmp4* expression is elevated in $Pdgfb^{ret/ret}$ mice (Mae et al., 2021). *Bmps* are expressed by normal endothelial cells and pericytes. Altered *Bmp* expression in $Pdgfb^{ret/ret}$ mice could reflect alterations caused by disturbed pericyte-endothelial crosstalk due to reduced pericyte numbers (Armulik et al., 2010; Vanlandewijck et al., 2018; Mae et al., 2021). Upregulated BMP signaling induces proinflammatory effects in endothelial cells (Cai et al., 2012). Aortic calcification is known to be associated with inflammation and dependent on BMP2 signaling (Canet-Soulas et al., 2021). It remains to be investigated whether deregulated expression of *Bmp2* and *Bmp4* contributes to vascular inflammation and calcification in $Pdgfb^{ret/ret}$ mice (Torok et al., 2021).

What Is the Role of Astrocytes in Brain Vascular Calcification?

Vessel associated calcifications elicit a conspicuous glial response in mouse models and human PFBC cases (Chakrabarty et al., 2011; Keller et al., 2013; Zarb et al., 2019b) (**Figure 1**). TEM images reveal that calcifications in mouse models of PFBC are surrounded by glia – either astrocytes or microglia (Nahar et al., 2019). Microglia surrounding calcifications contain inclusions of phagocytosed material, whereas astrocytes surrounding calcifications show signs of degeneration and the accumulation of calcium crystals (Jensen et al., 2018; Nahar et al., 2019). Strongly glial fibrillary acidic protein (GFAP) positive astrocytes surrounding calcifications express podoplanin (Zarb et al., 2019b), a protein strongly expressed by a subset of reactive astrocytes in glioblastoma, and after ischemic and stab wound injuries (Kolar et al., 2015). In addition, astrocytes surrounding calcifications also express proteins like complement 3 and lipocalin 2, which are commonly upregulated during various insults (Bi et al., 2013). Astrocytes around calcifications could potentially lead to neuronal damage and modify the inflammatory response. In addition, GFAP-positive astrocytes around calcifications are positive for 2-ω-carboxyethylpyrrole (CEP) adducts, a peroxidation product of docosahexaenoic acid (Zarb et al., 2019b). It remains to be shown whether the CEP is produced in astrocytes surrounding calcifications or scavenged from the environment. Nevertheless, the accumulation of CEP is characteristic of inflammation-associated oxidative stress in the human brain (Xiong et al., 2021), which indicates the presence of oxidative stress, a well-characterized stimulator and accelerator of vascular calcification (Pescatore et al., 2019), in PFBC. Thus, accumulating evidence indicates that astrocytes could play a role in PFBC pathology. Interestingly, MYORG, a gene mutated in AR-PFBC, is exclusively expressed by S100β positive astrocytes in the mouse brain (Yao et al., 2018). This observation raises the question of whether cell-autonomous changes in astrocytes lead to vascular calcification. In fact, astrocytes are the only cell type at the neurovascular unit expressing all genes, except the PDGFB, causing PFBC.

Microgliopathies and Brain Calcification

Microglia, resident tissue macrophages in the brain, are essential for the proper central nervous system (CNS) development and recovery from injury (e.g., remyelination) (Li and Barres, 2018). In addition, microglia are responsible for sensing and removing self-injurious proteins such as aggregated Aβ, α-synuclein, and prions (Hickman et al., 2018). Many AD risk genes are expressed by microglia and are involved in modifying microglial phagocytosis among other pathways (Hansen et al., 2018). Brain calcifications comprise one of the pathological hallmarks of a wide range of brain diseases affecting microglia or causing autoinflammation. Patients with mutations in genes implicated in microglial development and function [e.g., interferon regulatory factor 8 (*IRF8*), colony stimulating factor 1 receptor (*CSF1R*), triggering receptor expressed on myeloid cells 2 (*TREM2*), negative regulator of reactive oxygen species (*NRROS*)] harbor intracerebral calcifications (Rademakers et al., 2011; Konno et al., 2017; Bigley et al., 2018; Dong et al., 2020; Chitu et al., 2021). Patients with Nasu-Hakola disease, a neurodegenerative disease caused by mutations in TYRO protein tyrosine kinase-binding protein (*TYROBP,* also known as DAP12) or *TREM2,* present with cerebrovascular changes and bilateral basal ganglia calcifications localized to the walls of blood vessels (Kalimo et al., 1994; Coomans et al., 2018). Of note, brain calcification has not yet been reported in various mouse *Csf1r* mutants or in mice deficient of *Trem2*, *Dap12*, *Lrrc33* (NRROS) and *Irf8* but it would be of great interest to investigate whether these mice develop calcifications during aging or along with existing pathologies. However, long-term elimination of microglia using the CSF1R inhibitor, PLX5622, in control and PFBC mice resulted in localized axonal damage to fiber tracts of the internal capsule and adjacent thalamic and striatal areas, characterized by bone protein containing axonal spheroids (Zarb et al., 2021). This indicates that the chronic absence of CSF1R signaling could also lead to white matter calcification in mice.

Dysregulation of the type I interferon pathway, an essential component of the brain's innate immune defense, also triggers brain calcification, which is considered a useful radiological imaging marker for these diseases (Mcglasson et al., 2015). Mutations in ubiquitin-specific peptidase 18 (*USP18*), a ubiquitin-specific protease, which negatively regulates type I interferon-signaling, lead to brain calcifications in humans (Meuwissen et al., 2016). Microglia specific deletion of *Usp18* in mice leads to the formation of calcifications

(Goldmann et al., 2015). On the other hand, a baseline type I interferon signaling in microglia is necessary to prevent calcification. Immunodeficiency caused by interferon-stimulated gene 15 (*IGS15*) mutations leads to intracranial calcifications in humans (Zhang X. et al., 2015).

Although brain calcification is a pathological hallmark of the above-mentioned diseases, the pathogenesis and the role of microglial involvement has not received much attention. One of the reasons could be that calcifications in brain tissue are generally considered clinically irrelevant due to their occurrence in aged individuals (Arnold and Kreel, 1991). Nevertheless, the presence of calcifications is an indication of altered homeostasis and diseases caused by a cell-autonomous defect in microglia are associated with brain calcification. Thus, microglial function seems to be critical in controlling brain calcification, either by removing apoptotic cells or controlling proteostasis of the ECM, and thereby nucleation of calcium phosphate, and/or removal of hydroxyapatite. Babies born with congenital Zika virus infection have intracerebral calcifications at birth (Moore et al., 2017), but show progressive clearance of intraparenchymal calcifications (Petribu et al., 2017), suggesting that calcifications can be reabsorbed. This raises the question of how and which cells are responsible for removing calcifications. It would not be farfetched to suggest that microglial activity plays a cardinal role.

Microglia and Vascular Calcifications in Primary Familial Brain Calcification

Microglia surround brain calcifications in human PFBC cases and PFBC mouse models (Miklossy et al., 2005; Nahar et al., 2019; Zarb et al., 2019b, 2021). A recent study using the PFBC mouse model (*Pdgfb*$^{ret/ret}$ mice) presented evidence that microglia play a protective role by halting the calcification of capillaries (Zarb et al., 2021). Transcriptomic analysis of calcified mouse brain tissue showed deregulation of inflammatory pathways associated with activated microglia. Microglia acquire various insult-dependent activation profiles, however, some of the signature genes are shared by different insults (Candlish and Hefendehl, 2021). While the expression of many proteins [e.g., lipoprotein lipase (LPL), CD68, integrin alpha X (ITGAX), cystatin F (CST7), C-Type Lectin Domain Containing 7A (CLEC7A or dectin-1)] by microglia surrounding calcifications or calcification-associated microglia (CAM) is shared with the previously identified core signature (Keren-Shaul et al., 2017), there are differences in the expression profile (e.g., expression of cathepsin K) (**Figure 2A**). Lineage tracing experiments have shown that cathepsin K expressing cells are derived from microglia (Zarb et al., 2021). In bone, cathepsin K, the principal collagen I degrading enzyme, is secreted by osteoclasts (Drake et al., 1996; Costa et al., 2011). In addition to cathepsin K, microglia surrounding calcifications express receptor activator of nuclear factor κ B (RANK or TNFRSF11A) (Zarb et al., 2021), a receptor known to regulate osteoclast differentiation (Li et al., 2000). Thus, microglia surrounding calcifications might acquire an osteoclast-like phenotype necessary for removing the calcified extracellular matrix. Interestingly, osteoclasts and brain macrophages share key signaling pathways for their development (e.g., TREM2/DAP12, CSF1) (Lee et al., 2021). During embryogenesis, osteoclasts originate from erythro-myeloid progenitors in the embryo and are subsequently maintained in adulthood by hematopoietic stem cells-derived blood leukocytes (Jacome-Galarza et al., 2019). Alternatively, microglia originate from erythro-myeloid precursors in the yolk sac (Ginhoux et al., 2010; Schulz et al., 2012; Goldmann et al., 2016; Utz et al., 2020) and are maintained by self-renewal in the adult. The brain harbors several macrophage populations: parenchymal macrophages (i.e., microglia) and non-parenchymal macrophages, which reside at CNS border regions including the meninges, choroid plexus and perivascular spaces (Kierdorf et al., 2019). Further studies using Cre-lines that distinguish microglia from perivascular macrophages, and peripheral myeloid cells are needed to clarify whether cathepsin K expressing cells surrounding vessel calcifications are derived exclusively from microglia. In addition, it remains to be investigated whether brain perivascular macrophages and/or peripheral monocytes modify cerebral vascular calcification.

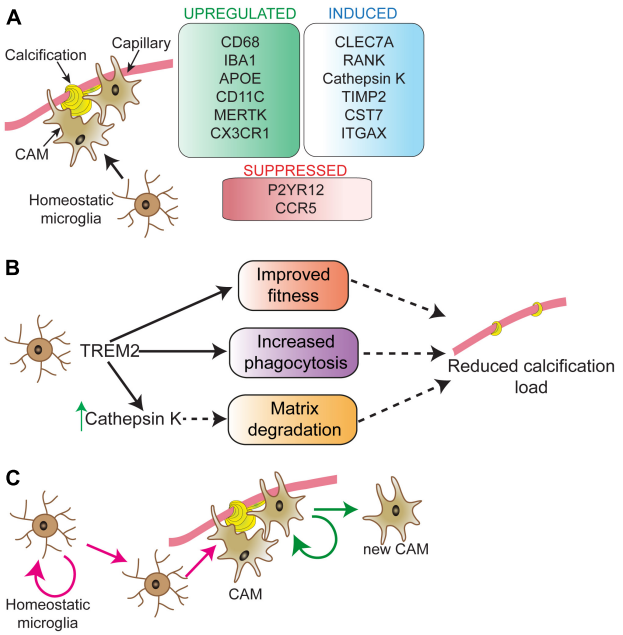

FIGURE 2 | Microglial phenotype in response to vascular calcifications. Calcification-associated microglia (CAM) show a distinct molecular signature **(A)**. Signature changes partially overlap with disease-associated microglia (DAM), except for RANK and cathepsin K expression, which are unique to CAM. Modifying microglial activity via TREM2 can play a direct role in controlling vessel calcification load **(B)**. The process through which TREM2 activity controls calcification load is still unknown. TREM2 could sustain microglial fitness and support microglial phagocytosis resulting in reduced calcification load. TREM2 activity is upstream of cathepsin K. Proliferating microglia surround vascular calcifications **(C)**. The nature of proliferating microglia in the near vicinity of calcifications and their fate is not well-understood. Depicted are two possibilities (pink and green arrows) for the origin and fate of proliferating microglia surrounding vessel calcifications. These possibilities are: expansion of calcification-associated microglia (green arrows) and/or replacement of parenchymal microglia in the vicinity of calcified vessels (pink arrows). CAM, calcification-associated microglia.

Atherosclerotic lesions contain several macrophage populations, which have different capacities to promote or halt lesion development (Willemsen and De Winther, 2020). Although macrophages in these lesions express osteoclast markers (tartrate-resistant acid phosphatase, cathepsin K, carbonic anhydrase 2), they have low mineral resorption potential due to altered nuclear factor of activated T cells type c-1 (NFATC1) signaling, resulting in a lower expression and activity of cathepsin K (Chinetti-Gbaguidi et al., 2017). Calcification-associated microglia specific knockout of cathepsin K should clarify its importance in degrading vascular calcifications in the PFBC (**Figure 2B**). Of note, there is also heterogeneity within calcification-associated microglia in PFBC. The expression of markers such as Ionized calcium binding adaptor molecule 1(IBA1) and CLEC7A are detected in all microglia surrounding calcifications, whereas expression of some markers (e.g., cathepsin K, ITGAX) are detected only in a subset of microglia (Zarb et al., 2021).

Long-term removal of microglia using the CSF1R inhibitor PLX5622 resulted in an increase in vessel-associated calcification load in *Pdgfb*$^{ret/ret}$ mice. Similar results were obtained in *Pdgfb*$^{ret/ret}$ mice lacking one or two functional alleles of *Trem2* (Zarb et al., 2021). The absence of cathepsin K expression by microglia surrounding calcifications in these mice indicates that TREM2 activity could be necessary to induce osteoclast-like phenotype in microglia (**Figure 2B**). Interestingly, single cell analysis of atherosclerotic lesions isolated from mouse models of atherosclerosis showed the presence of TREM2^{high} macrophages, which were enriched for genes highly expressed by osteoclasts, suggesting a role in lesion calcification (Cochain et al., 2018). This population also expressed markers for so-called disease-associated microglia described in aging and mouse models of AD (Keren-Shaul et al., 2017). It remains to be investigated whether the expression of osteoclast genes in TREM2^{high} macrophages is TREM2 dependent and whether these cells modify the calcification of atherosclerotic lesions.

Microglia turnover rate in the normal brain is relatively low, but under pathological conditions, the microglial turnover rate is increased (Prinz et al., 2019). Accordingly, conspicuous microglial proliferation can be detected in brain regions that develop vascular calcifications in a mouse model of PFBC (Zarb et al., 2021). There might be a higher microglial turnover rate in these regions since phagocytosis/degradation of calcifications by microglia could lead to exhaustion and death, similar to the microglial dynamics detected in the mouse model of AD (Prinz and Priller, 2017). It needs to be investigated whether there is a clonal expansion of CAM (**Figure 2C**, green arrows) and/or replacement of parenchymal microglia in the vicinity of calcified vessels which give rise to CAM (**Figure 2C**, pink arrows).

Microglia have emerged as disease modifiers in a wide range of neurodegenerative diseases (e.g., AD) (Hickman et al., 2018). Of six genes implicated in PFBC (*JAM2*, *PDGFB, PDGFRB, SLC20A2, XPR1, MYORG*), microglia express *JAM2, PDGFB, SLC20A2, XPR1*. Microglia do not express the receptor—PDGFRB, and thus, it is unlikely that *PDGFB* or *PDGFRB* haploinsufficiency in PFBC causes cell-autonomous microglial dysfunction. Nevertheless, cells of monocyte origin derived from PDGFB hypomorphs and PFBC patients carrying PDGFB mutation were reported to show defects in osteoclast-differentiation *in vitro* (Schiemenz et al., 2020). However, whether this finding also applies to brain macrophages was not investigated. Interestingly, *xpr1b*, an orthologue of *XPR1,* is crucial for the differentiation of tissue-resident macrophages and microglia in zebrafish (Meireles et al., 2014), indicating that PFBC genes could modify the development of tissue resident macrophages.

Currently, it is not known how microglia sense and degrade calcifications. Although microglia surround and phagocytose calcifications, there is currently no understanding of how they sense calcifications on vessels. Future studies should clarify how the calcified extracellular matrix on the vessel wall is sensed by microglia. In general, little is known how extracellular calcium phosphate crystals are recognized by macrophages (Mulay and Anders, 2016). It could involve various pattern-recognition receptors (PRRs) implicated in recognition damage- or pathogen-associated patterns. Recently, CLEC12A, a PRR belonging to the same class as CLEC7A, induced in CAM, was shown to recognize inflammation causing deposition of uric acid crystals in joints in gout (Neumann et al., 2014). However, this interaction was shown to be necessary to inhibit the immune response, as mice deficient in *Clec12a* exhibited hyperinflammation in response to uric acid crystals (Neumann et al., 2014). Another possibility is that the recognition/uptake of calcifications is receptor-independent and mediated via membrane lipids, similar to solid structure uptake in dendritic cells (Ng et al., 2008). Stiffness of vascular walls most likely differs between normal and calcified vessels such that microglia could sense and respond to altered tissue stiffness. Microglia have the capacity to respond to tissue stiffness as in the retina, where microglial phenotype and the control of vascular architecture was dependent on tissue stiffness and regulated by integrin signaling (Dudiki et al., 2020). As has been shown in other types of vascular injury, microglia could also respond to vascular calcifications by sensing alterations via purinergic receptor P2Y12 (Lou et al., 2016). Although it is not known whether vascular calcification in the brain involves alterations in purinergic signaling, altered Pi/PPi/purinergic signaling has been shown to cause vascular calcification in pseudoxanthoma elasticum (Rutsch et al., 2021). Additionally, it would be necessary to dissect signaling pathways that could trigger a coordinated degradation of proteins and hydroxyapatite by microglia. As discussed above, TREM2 activity is essential but most likely additional signaling pathways participate in this process. Since macrophages are capable of degrading phagocytosed hydroxyapatite (Hyvonen and Kowolik, 1992), it would be interesting to investigate whether microglia show differential capacity to degrade matrix depending on the Ca-phosphate phases (i.e., amorphous calcium phosphate vs hydroxyapatite).

OUTLOOK

Brain vessel calcifications that accompany aging are shared by diverse brain pathologies, and in some cases, comprise a diagnostic criterion. The brain possesses an energy reserve

limited to only a few minutes, and therefore, brain function is dependent on continuous blood flow delivered by the vasculature (Mergenthaler et al., 2013; Iadecola, 2017). Although vascular calcification is unlikely to be beneficial, insights into the consequences of vessel calcification on neurovascular coupling and brain function are needed. In addition, it is important to understand the vascular and parenchymal alterations preceding mineralization of the vessel wall as well as longitudinal changes in the composition of mineral phases and other components such as proteins and metabolic ions. Accumulating evidence suggests that dysfunction of resident brain macrophages leads to tissue calcification. Thus, removal of calcium phosphate precipitates in the brain parenchyma and vessel wall could be considered as a part of microglia parenchymal surveillance along with other well-described functions. However, the direct function of CAM is currently unclear and requires further study. Also, excellent questions for future research are how microglia recognize and degrade vascular calcifications and how to therapeutically target microglia in order to reduce vascular calcification.

AUTHOR CONTRIBUTIONS

AK wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Microvascular Changes in Parkinson's Disease- Focus on the Neurovascular Unit

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Vascular alterations emerge as a common denominator for several neurodegenerative diseases. In Parkinson's disease (PD), a number of observations have been made suggesting that the occurrence of vascular pathology is an important pathophysiological aspect of the disease. Specifically, pathological activation of pericytes, blood-brain barrier (BBB) disruption, pathological angiogenesis and vascular regression have been reported. This review summarizes the current evidence for the different vascular alterations in patients with PD and in animal models of PD. We suggest a possible sequence of vascular pathology in PD ranging from early pericyte activation and BBB leakage to an attempt for compensatory angiogenesis and finally vascular rarefication. We highlight different pathogenetic mechanisms that play a role in these vascular alterations including perivascular inflammation and concomitant metabolic disease. Awareness of the contribution of vascular events to the pathogenesis of PD may allow the identification of targets to modulate those mechanisms. In particular the BBB has for decades only been viewed as an obstacle for drug delivery, however, preservation of its integrity and/or modulation of the signaling at this interface between the blood and the brain may prove to be a new avenue to take in order to develop disease-modifying strategies for neurodegenerative disorders.

Keywords: Parkinson's disease, vasculature, pericytes, angiogenesis, blood-brain barrier, microglia

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BRAIN VASCULATURE

The brain is a highly oxygen consuming organ and, as a result, has developed a dense network of almost 650 km of microvessels (Pandey et al., 2016). The smallest entity is formed by capillaries that are in close contact with the surrounding parenchyma and allow the gas exchange. This close connection between blood and brain is termed the neurovascular unit (NVU). The NVU consists of endothelial cells, pericytes and the basal lamina forming the microcapillary wall, and cells in the immediate surrounding brain parenchyma including perivascular astrocytes, perivascular microglia and neurons.

Blood Vessels as Adaptors of Blood Flow

Capillaries of the brain are non-fenestrated vessels regulating the influx of nutrients and oxygen according to the changing demands of the brain (Iadecola, 2017).

The adaption to the brains requirements occurs by neurovascular coupling, matching the local blood supply to the neuronal demand by adjustment of the vascular intraluminal diameter (Carmignoto and Gomez-Gonzalo, 2010). Preservation of the highly balanced homeostasis of the brain's microenvironment, however, is guaranteed by the BBB.

Blood Vessels as Gate Keepers at the Blood-Brain Barrier

The BBB is formed by endothelial cells that require close contact with pericytes in order to form tight junctions, by the basal lamina and by astrocytic endfeet (Zhao et al., 2015). The integrity of the BBB is absolutely vital for normal neuronal function. A leaky BBB enables the uncontrolled entry of pathogens, toxins and inflammatory cells into the brain and leads to inflammatory and immune responses. BBB leakage, whether subtle or severe, ultimately leads to neuronal injury, neurodegeneration and accelerates disease progression (Bell et al., 2010; Sweeney et al., 2018a).

Blood Vessels as Communicators of Signals

As brain capillaries form the contact surface between the blood and the brain, cells at this interface are also the first sensors of systemic changes such as metabolic imbalances, systemic inflammation, circulating pathogens, changes in oxygen tension etc. In particular brain pericytes have been identified as first responders to systemic inflammation mediating signals from the blood onto the neighboring parenchyma cells (Duan et al., 2018). Vascular pathology and changes in cell signaling at and across the BBB may be the interface linking systemic risk factors (e.g., diabetes or chronic inflammation) to neuroinflammation and neurodegeneration (see section "Metabolic Disorders and Vascular Changes in Parkinson's Disease").

VASCULAR CHANGES IN PARKINSON'S DISEASE

Blood vessel alterations, BBB disruption and cerebral blood flow abnormalities are a common denominator of several neurodegenerative disorders and have been described in Alzheimer's disease (Sweeney et al., 2018b), amyotrophic lateral sclerosis (Zhong et al., 2008; Garbuzova-Davis and Sanberg, 2014; Winkler et al., 2014), Huntington's disease (Padel et al., 2018) and Parkinson's disease (PD). There is a growing appreciation that vascular alterations can contribute to disease onset and aggravate the neurodegenerative process as some vascular changes already occur before the onset of neuronal loss or behavioral deficits in animal models of the respective disease (Sagare et al., 2013; Winkler et al., 2014; Padel et al., 2016; Elabi O. et al., 2021).

Here we particularly outline the different histological vascular changes reported in patients with PD and summarize the evidence for vascular alterations from animal models of PD. This minireview does not cover the role of hypoperfusion or white matter lesions in the pathogenesis of PD.

Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder and one of the fastest growing neurological diseases. In 2015, PD affected 6.9 Million people worldwide, a number expected to double by 2040 due to the aging population (Dorsey and Bloem, 2018).

The progressive degeneration of the nigrostriatal system gives rise to the typical clinical symptoms rigidity, bradykinesia and resting tremor (Fearnley and Lees, 1991). In PD, dopaminergic neurons in the substantia nigra pars compacta (SNpc) are degenerating and the histopathological hallmark is the formation of Lewy bodies containing aggregated alpha-synuclein (α-syn) (Spillantini et al., 1997). Although PD is associated with these distinct histological changes, concomitant pathological alterations might sustain or aggravate the neuronal degeneration. This is particularly relevant as there is currently no therapy available that intervenes with the ongoing disease process. In this context, any contributor to the disease is important to elicit.

The Microvascular Environment in Parkinson's Disease

Almost 90 years ago, it was described that the capillary network in the SNpc is considerably denser than in the adjacent SN zona reticulata (Finley, 1936). Under normal conditions, a distinct tight pattern of neuron-capillary associations is observed in the SNpc. However, in PD, these close contacts between dopaminergic neurons and microvessels are lost leaving an "empty space" (Issidorides, 1971). Based on these early observations it was postulated that modifications of the vascular microenvironment of dopaminergic neurons may alter the availability of nutrients or lead to accumulation of toxic compounds in the immediate vicinity of these cells. Later, vascular alterations in PD were described more in detail ranging from signs of angiogenesis to BBB leakage and vascular regression (**Figure 1**).

Evidence for Angiogenesis in Parkinson's Disease

Angiogenesis refers to the formation of new blood vessels in adulthood. In the adult brain it usually occurs in response to hypoxia or inflammation (Tahergorabi and Khazaei, 2012). Angiogenesis can be recognized by an increase in vascular density and branching points, proliferation of vascular cells, expression of angiogenic markers or an increase in angiogenic molecules in the brain or cerebrospinal fluid (CSF).

Angiogenic Microvessels in Parkinson's Disease

First evidence for pathological angiogenesis in PD comes from studies in the 90's observing a 2.5-fold increase in the number of endothelial cells in PD brains (Faucheux et al., 1999) and an increased number of integrin αvβ3-positive vessels, an adhesion molecule that is present on angiogenic vessels (Brooks, 1996) in the SNpc, the locus coeruleus and the putamen, all regions which are affected in PD (Desai Bradaric et al., 2012).

Similar findings reporting angiogenesis in several brain regions were made in the 6-hydroxydopamine (6-OHDA) PD model in rats (Carvey et al., 2005) and mice (Elabi O. F. et al., 2021), in an experimental model of L-DOPA-induced dyskinesias (Westin et al., 2006) and in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) monkey model (Barcia et al., 2005).

Even though toxin-induced models are useful to study several aspects of PD, they do not reflect the slowly progressive nature

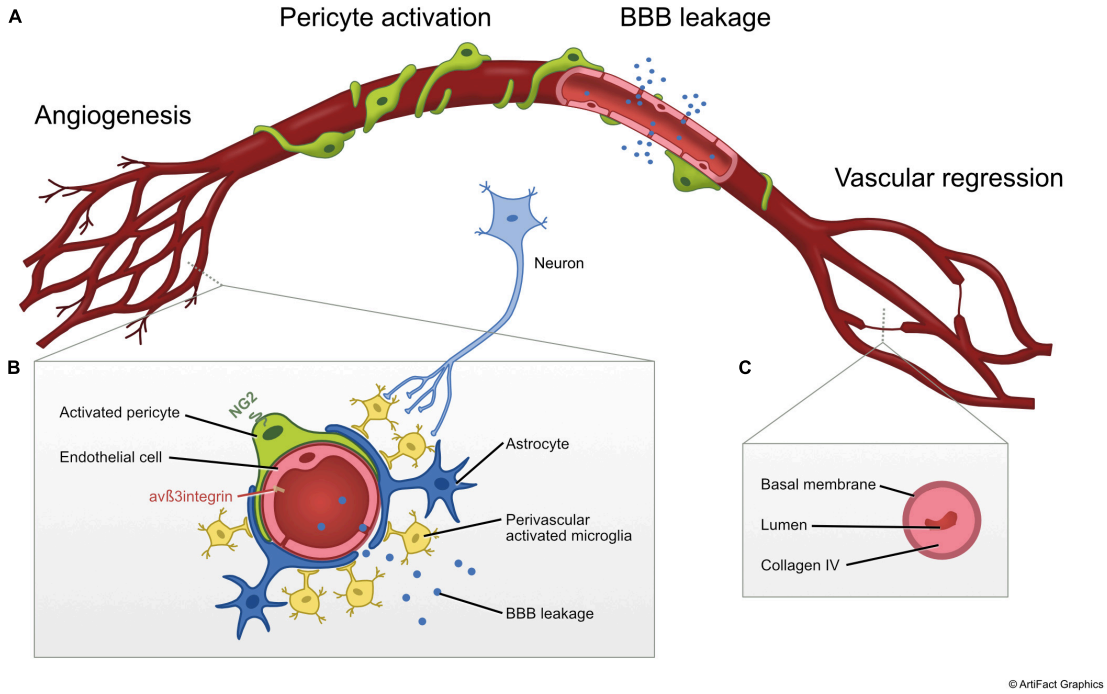

FIGURE 1 | Illustration of vascular changes observed in post mortem tissue and preclinical models of PD. **(A)** Simplified diagram illustrating the main vascular alterations in non-chronological order including (1) angiogenesis, (2) pericyte activation, (3) BBB leakage, and (4) vascular regression. **(B)** Cross-sectional diagram of the NVU during the angiogenic response. **(C)** Cross-sectional diagram of string vessel showing the collapsed basement membrane labeled with collagen-IV and absence of endothelial cells during vascular regression stage. The process of vascular regression includes loss of pericytes and signaling between pericytes and endothelial cells, causing destabilization of endothelial cells and endothelial cell death. NG2, neuron-glial antigen 2.

of PD pathology. Using a human α-syn overexpression mouse model that recapitulates the progressive aggregation of human α-syn (Hansen et al., 2013), we confirmed an increase in vessel density indicating angiogenesis at the moderate stage of the animal model. In late-stage animals, however, the vessel density was significantly reduced suggesting dynamic and stage-dependent vascular changes in PD (Elabi O. et al., 2021).

Pathological Pericyte Activation

Interestingly, at the early stage of α-syn-aggregation, we observed an activation of pericytes that was preceding changes in vessel density and behavioral deficits (Elabi O. et al., 2021). Pericytes line the entire microvasculature of the brain and have an important function in angiogenesis (Stapor et al., 2014). Activation of pericytes leads to changes in morphology and marker expression. Capillary pericytes generally have a flat cell soma with extensive longitudinal and thin processes (Dore-Duffy and Cleary, 2011). However, under pathological conditions, pericytes acquire a more bulging cell soma with shorter processes, typical of activated and migratory pericytes (Dore-Duffy and Cleary, 2011; Ozen et al., 2014). This pattern is predominantly seen following injury and during the early stages of angiogenesis and often associated with expression of markers such as NG2 and/or RGS5 (Ozerdem and Stallcup, 2004; Berger et al., 2005). Angiogenesis requires first pericyte detachment from the vessel wall, allowing endothelial sprouting and then subsequent pericyte recruitment for stabilization and maturation of the vasculature (Kamouchi et al., 2012). We have previously shown that pericytes are activated in the 6-OHDA PD model (Padel et al., 2016), and that pathological pericyte activation is a feature of also other neurodegenerative disorders (Padel et al., 2018).

Pericytes are one of the first responders to brain hypoxia (Gonul et al., 2002; Duz et al., 2007) and to systemic inflammation (Duan et al., 2018). Importantly, pericytes alter their signaling toward a pro-inflammatory secretome when activated (Rustenhoven et al., 2017; Gaceb and Paul, 2018; Gaceb et al., 2018). Interestingly, a direct observation that α-syn can activate pericytes comes from an *in vitro* study where exposure to monomeric α-syn leads to secretion of high amounts of pro-inflammatory molecules in pericytes that in turn mediated hyperpermeability in endothelial cells resulting in BBB leakage (Dohgu et al., 2019).

Thus, it is conceivable that pericyte activation may form the starting point of vascular alterations and a cascade of pathological signaling events in the NVU in PD.

Angiogenic Molecules

Findings indicating angiogenesis in PD are supported by reports showing an upregulation of the pro-angiogenic molecule Vascular Endothelial Growth Factor (VEGF) in the SNpc of PD patients (Wada et al., 2006; Yasuda et al., 2007; Lan et al., 2021) and non-human primates (Barcia et al., 2005). Increased levels of soluble VEGF receptor-2 and placental growth factor, and lower levels of angiopoietin 2 (an anti-angiogenic molecule) were detected in the CSF of PD patients (Janelidze et al., 2015). In

this study, angiogenesis markers in the CSF were associated with microbleeds and white matter lesions on imaging, suggesting abnormal angiogenesis in PD (Janelidze et al., 2015). Further strengthening these findings, a recent study demonstrated CSF changes in miRNAs regulating pathways of angiogenesis and BBB components in PD patients with moderate disease, implying impairment of these pathways as part of the progression of PD (Fowler et al., 2021).

Blood-Brain Barrier Dysfunction in Parkinson's Disease

Blood-Brain Barrier Dysfunction in Parkinson's Disease Animal Models

Angiogenesis is a double-edged sword as newly formed vessels are immature and can lead to BBB leakage, especially when pericyte recruitment is impaired.

Indeed, a dysfunctional BBB has been demonstrated in a number of different PD models showing leakage of albumin and other tracers into the brain parenchyma (Carvey et al., 2005, 2009; Westin et al., 2006; Zhao et al., 2007; Chen et al., 2008), increased entry of drugs (Carta et al., 2006; Westin et al., 2006) and infiltration of peripheral immune cells otherwise are prevented from crossing the BBB (Benner et al., 2008; Brochard et al., 2009; Reynolds et al., 2010). Few studies have not been able to confirm BBB leakage, likely due to the methods used (Astradsson et al., 2009; Elabi O. F. et al., 2021).

A study using the A53T PD mouse model showed that the expression of tight junction-related proteins at the BBB decreased leading to increased vascular permeability (Lan et al., 2021). When we examined the temporal dynamics of BBB leakage in another progressive α-syn-PD mouse model (Elabi O. et al., 2021), we detected significant extravascular fibrinogen accumulation already at the early stage preceding behavioral deficits (Elabi O. et al., 2021).

Blood-Brain Barrier Dysfunction in Parkinson's Disease Post Mortem Tissue

The evidence of an impaired BBB in PD from animal models is validated by compelling post mortem studies in PD using a variety of different methods. Gray and Woulfe (2015) found a sevenfold increase in extravasated erythrocytes, a threefold increase in hemosiderin depositions (often grouped around capillaries), a significant perivascular deposition of hemoglobin (8.6-fold increase) and a 9.4-fold increase in extrasudated fibrinogen in the striatum of PD patients compared to controls. Greater fibrinogen accumulation (Yang et al., 2015), higher IgG leakage and loss of tight junction proteins (Pienaar et al., 2015) were also reported in other autopsy studies.

Similarly, a 10-fold increase of extravascular $CD4^+$ and $CD8^+$ lymphocytes has been shown particularly in the SNpc in post mortem PD brain tissue (Brochard et al., 2009) demonstrating pathological immune cell infiltration across the BBB.

Blood-Brain Barrier Dysfunction Examined in the Cerebrospinal Fluid

In line with post mortem findings, CSF studies have shown BBB leakage as indicated by increased levels of CSF albumin in PD correlating with the severity of the disease (Pisani et al., 2012), or with the level of angiogenic factors in the CSF (Janelidze et al., 2015).

Blood-Brain Barrier Dysfunction Using *in vivo*-Imaging

Blood-brain barrier dysfunction in PD patients *in vivo* is more difficult to study. Using positron-emission tomography (PET), progressive BBB impairment has been shown in the midbrain of PD patients as seen by an increased uptake of the tracer ^{11}C-verapamil indicating impairment of the BBB efflux pump P-glycoprotein (Kortekaas et al., 2005; Bartels et al., 2008) and analysis of dynamic contrast-enhanced magnetic resonance images revealed higher BBB leakage in PD patients (Al-Bachari et al., 2020), whereas a study using rubidium-82-PET could not detect BBB leakage in PD patients (Fujita et al., 2021).

Vascular Regression

Angiogenesis and BBB leakage are not the only vascular pathology that has been observed in PD. Signs indicating vascular regression come from reports showing endothelial degeneration, decrease in vessel length and number of branching points and increase in vessel diameter in the SN of PD patients compared to age-matched controls (Guan et al., 2013; Yang et al., 2015). In addition, PD patients had higher numbers, density and total length of "string vessels" when compared to controls (Yang et al., 2015). String vessels are linked to endothelial cell degeneration leaving empty collapsed basal membrane tubes that do not take part in perfusion (Brown, 2010). Using electron microscopy, Farkas et al. (2000) also demonstrated basal membrane thickening in cerebral capillaries in PD.

Vascular regression likely indicates a later stage of vascular pathology in PD. When studying the temporal dynamics of vascular changes, we noted that early pericyte activation and BBB leakage were followed by angiogenesis, whereas vascular rarefication did not occur until the late stage of the PD model (Elabi O. et al., 2021). Thus, the microvasculature in PD might undergo both, an angiogenic and pruning vascular response, whereby occurrence of BBB leakage could be an early event, followed by the attempt for neovascularization at the moderate stage of the disease and vascular degeneration as a sign of late-stage disease. In the α-syn-PD mouse model we observed colocalization of α-syn and phosphorylated α-syn with endothelial cells at all stages, which suggests a direct involvement of α-syn in the vascular pathological mechanism in addition to a pathological stimulation of pericytes (Elabi O. et al., 2021).

Inflammation and Vascular Pathology

Microglia

In PD, neuroinflammation is a well-known pathology as documented by increased numbers of activated microglia in PD brains (Mcgeer et al., 1988; Croisier et al., 2005; Zhang et al., 2005; Whitton, 2007; McGeer and McGeer, 2008) and increased levels of pro-inflammatory molecules in the CSF of PD (Blum-Degen et al., 1995).

Several studies demonstrating an increased activation of microglia also reported BBB disruption in these PD models

(Carvey et al., 2005; Zhao et al., 2007; Elabi O. et al., 2021). The interactions between microglia and blood vessels are complex: Microglia are likely activated by the parenchymal leakage of plasma proteins (Merlini et al., 2019), on the other hand, activated microglia may also induce angiogenesis and vascular leakage *via* the release of inflammatory and pro-angiogenic molecules (Naldini and Carraro, 2005; Ritzel et al., 2015; Haddick et al., 2017; Salter and Stevens, 2017; Chen et al., 2019). The proinflammatory cytokines cause a reduction in the expression of tight junction proteins and increase matrix metalloproteinase-3 and −9, which affect the BBB integrity (Raymond et al., 2016; Bonetti et al., 2019; Edwards et al., 2020).

Recently, we have observed that activated microglia are highly localized particularly in the perivascular space in PD (Elabi O. F. et al., 2021). It has been suggested that perivascular microglia have a dual effect on vessels, maintaining vascular integrity under normal conditions, but phagocytosing the vessel and impairing BBB integrity under prolonged inflammation (Haruwaka et al., 2019). The reason for this increase in activated perivascular microglia in PD is not known, however, we noted the level of perivascular microglia to be strongly associated with the number of pericytes (Elabi O. F. et al., 2021), suggesting a possible interaction of these two cell types at the vascular border.

Other Inflammatory Cell Types

Within the NVU, also astrocytes can release pro-inflammatory cytokines and angiogenic molecules that can affect vascular function (Lee et al., 2010; Barcia et al., 2011; Kam et al., 2020). Several studies have highlighted the role of astrocytes in the control of vascular function, *via* e.g., cross-talk with microglia (Wang et al., 2014; Ni et al., 2018).

Similarly, pericytes can produce a variety of inflammatory and angiogenic molecules (Gaceb and Paul, 2018; Gaceb et al., 2018). Activation of pericytes specifically *via* α-syn stimulates release of cytokines and increases expression MMP9 that lead to an increased EC permeability (Dohgu et al., 2019), placing pericytes as mediators between α-syn and BBB disruption.

Metabolic Disorders and Vascular Changes in Parkinson's Disease

An increasing number of studies suggest an association between neurodegeneration and metabolic diseases. Epidemiological evidence indicates that diabetes is also a risk factor and a negative prognostic factor for PD (Cereda et al., 2011; Pagano et al., 2018; Heinzel et al., 2019; Sergi et al., 2019; Chohan et al., 2021). The link between metabolic dysfunction and neurodegeneration in PD is further strengthened by studies demonstrating a beneficial effect of anti-diabetic medication in PD and PD models (Foltynie and Athauda, 2020). In particular, Exenatide, a glucagon-like peptide-1 (GLP-1) receptor agonist, has shown neuroprotective effects in preclinical models of PD and entered clinical trials (Athauda et al., 2017).

Even though a number of hypotheses has been put forward to what is leading to this aggravation of PD in the presence of diabetes (Sergi et al., 2019), not much attention has been paid to the fact that diabetes and PD both share pathological microvascular alterations in the brain. Similar to the retinal and renal complications, diabetes is associated with signs of cerebral vascular proliferation and progressive BBB disruption (Starr et al., 2003; Huber et al., 2006; Salameh et al., 2016; Machida et al., 2017; Takechi et al., 2017; Rom et al., 2019; Yamamoto et al., 2019). We have examined the interactions of type 2 diabetes (DMT2) and PD at the microvascular interface and shown that DMT2 in combination with a PD lesion resulted in a significant depletion of pericytes, and reduced interactions between microvessels and perivascular microglia which was associated with a lack of the angiogenic response seen in toxin-induced models (Elabi O. F. et al., 2021). It is conceivable that the diabetic state inhibits the attempt of compensatory angiogenesis seen in PD and accelerates the vessel changes toward a later stage of vascular regression.

DISCUSSION AND OUTLOOK

Current evidence points to a dynamic evolution of multiple vascular changes in PD (**Figure 1**). These changes might start with pathological pericyte activation and subtle BBB leakage, continue with compensatory angiogenesis that then fails and cumulates in vascular regression. What constitutes the initiator of these events still remains unknown, but their occurrence is certainly contributing to a disturbed neuronal microenvironment. Interventions stabilizing the vasculature and preventing the progression of BBB dysfunction are clearly indicated. Treatment with platelet-derived growth factor (PDGF-BB), a growth factor required for pericyte recruitment and vessel maturation (Jain and Booth, 2003), not only induced neurorestoration and behavioral recovery in PD animal models (Zachrisson et al., 2011; Padel et al., 2016), but also normalized the number of activated pericytes (Padel et al., 2016) and changed the inflammatory secretome of pericytes toward a trophic factor pattern *in vitro* (Gaceb et al., 2018). PDGF-BB has shown safety and tolerability in a phase I/IIa clinical trial in PD patients (Paul et al., 2015; Paul and Sullivan, 2019). It now remains to be seen whether approaches targeting vascular pathology, pericyte activation and vascular signaling at the BBB can modify the progression of the disease.

AUTHOR CONTRIBUTIONS

GP and OE wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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Aging-Related Vascular Inflammation: Giant Cell Arteritis and Neurological Disorders

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Aging is characterized by the functional decline of the immune system and constitutes the primary risk factor for infectious diseases, cardiovascular disorders, cancer, and neurodegenerative disorders. Blood vessels are immune-privileged sites and consist of endothelial cells, vascular smooth muscle cells, macrophages, dendritic cells, fibroblasts, and pericytes, among others. Aging also termed senescence inevitably affects blood vessels, making them vulnerable to inflammation. Atherosclerosis causes low-grade inflammation from the endothelial side; whereas giant cell arteritis (GCA) causes intense inflammation from the adventitial side. GCA is the most common autoimmune vasculitis in the elderly characterized by the formation of granulomas composed of T cells and macrophages in medium- and large-sized vessels. Recent studies explored the pathophysiology of GCA at unprecedented resolutions, and shed new light on cellular signaling pathways and metabolic fitness in wall-destructive T cells and macrophages. Moreover, recent reports have revealed that not only can cerebrovascular disorders, such as stroke and ischemic optic neuropathy, be initial or coexistent manifestations of GCA, but the same is true for dementia and neurodegenerative disorders. In this review, we first outline how aging affects vascular homeostasis. Subsequently, we review the updated pathophysiology of GCA and explain the similarities and differences between vascular aging and GCA. Then, we introduce the possible link between T cell aging, neurological aging, and GCA. Finally, we discuss therapeutic strategies targeting both senescence and vascular inflammation.

Keywords: giant cell arteritis, inflammation, neurological aging, vascular aging, vasculitis

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INTRODUCTION

Aging is a multifactorial phenomenon that affects virtually every organ system in the human body and is characterized by the progressive functional decline of those organs (Borgoni et al., 2021). Not only infectious diseases like COVID-19, shingles, and pneumonia, but also malignant neoplasms and vascular diseases increase in frequency with aging. Atherosclerosis is a prototypical form of vascular aging (Ungvari et al., 2018; Tyrrell and Goldstein, 2021), and recent studies have revealed the involvement of immune cells and low-grade inflammation in atherosclerosis, known as "inflammaging" (Ferrucci and Fabbri, 2018; Franceschi et al., 2018).

On the other hand, many autoimmune diseases are common in young to middle-aged women, with an exception that is exclusively found in the elderly, giant cell arteritis (GCA)

(Weyand and Goronzy, 2014). GCA is classified as large vessel vasculitis (LVV) and affects the aorta and its major branches (Pugh et al., 2022). Large vessel involvement can be complicated by aortic dissection and aneurysm formation, while inflammation of medium-sized arteries causes headache, jaw claudication, loss of vision, and stroke. Moreover, extravascular manifestations —such as fever, malaise, weight loss, polymyalgia rheumatica—also frequently occur (Buttgereit et al., 2016; Yamaguchi et al., 2022). The currently available treatments for GCA include glucocorticoids and tocilizumab (TCZ), an IL-6 receptor inhibitor. TCZ reduces flare-up of GCA and has steroid-sparing effect (Stone et al., 2017); however, discontinuation of TCZ almost inevitably leads to flare-up of GCA, suggesting that TCZ alleviates symptoms, but it does not cure the disease (Quinn et al., 2021). Therefore, further elucidation of the immunopathogenesis of GCA is essential.

This mini-review summarizes the pathological mechanism of vascular aging. Then, the updated immunopathogenesis of GCA is presented and the similarities and differences between vascular aging and GCA are discussed. Then, we introduce the link between T cell aging, neurological aging, and GCA. Finally, possible therapeutic strategies targeting senescence and vascular inflammation are discussed.

VASCULAR AGING AND GIANT CELL ARTERITIS

Vascular Aging

Vascular aging refers to the cellular and functional changes that occur in the vasculature during aging, and accounts for most of the morbidity and mortality in the elderly (**Figure 1A**). Aging-induced functional and structural alterations of the vasculature contribute to not only cardiovascular disease, but also a wide range of age-related diseases, such as cognitive impairment, Alzheimer's disease, sarcopenia, and kidney dysfunction (Ungvari et al., 2018). A variety of pathophysiological mechanisms drive vascular aging: reactive oxygen species (ROS), mitochondrial dysfunction, inflammation, cellular senescence, genomic instability, increased apoptosis, epigenetic alterations, and clonal hematopoiesis of indeterminate potential (CHIP) (Jaiswal et al., 2017; Ungvari et al., 2018). Among these, mitochondrial dysfunction may play a pivotal role in the vascular aging process (Ungvari et al., 2018; Tyrrell et al., 2020). Impaired mitochondrial biogenesis associated with excess ROS production promotes cellular senescence in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs). Senescent ECs and VSMCs have an increased pro-inflammatory secretome called senescence-associated secretory phenotype (SASP), and this further enhances pathological remodeling of the extracellular matrix and disrupts barrier function in ECs.

CHIP is a relatively new concept in atherosclerosis. With aging, the risk of somatic mutations increases in hematopoietic stem cells residing in bone marrow (Jaiswal et al., 2017). An expansion of hematopoietic clones carrying somatic mutations — most frequently loss-of-function alleles in the genes *DNMT3A*, *TET2*, and *ASXL1*—in the absence of any other hematologic abnormalities is defined as CHIP (Jaiswal et al., 2017). During aging, monocytes that carry such mutations are recruited from the lumen of the blood vessel to the atherosclerotic plaque and there produce excess IL-6, IL-1β, and chemokines (Tyrrell and Goldstein, 2021).

Metabolic reprogramming has also been shown to be involved in vascular aging (Shirai et al., 2016). Macrophages from patients with coronary artery disease (CAD) have an enhanced glycolytic flux as well as increased activity of the tricarboxylic acid cycle, leading to an overproduction of mitochondria-derived ROS. This, in turn, promotes dimerization of the glycolytic enzyme pyruvate kinase M2 and enables its nuclear translocation, boosting IL-6 production (Shirai et al., 2016). At the same time, such metabolically reprogrammed macrophages from CAD patients show increased expression of PD-L1, an immunoinhibitory checkpoint molecule, making CAD patients vulnerable to herpes zoster (Watanabe et al., 2017b, 2018a). PD-L1 expression is regulated by pyruvate, an intermediate metabolite of glycolysis. Immunostaining performed in humans demonstrated that PD-L1 is highly expressed in macrophages infiltrating the vessel wall from the early stage of atherosclerosis (Watanabe et al., 2017b). Thus, metabolic reprogramming in CAD macrophages exacerbates vascular inflammation via IL-6 production, while exerting an immunosuppressive function through PD-L1 expression.

A recent single-cell analysis of human carotid atherosclerotic plaque demonstrated multiple cellular activation—such as ECs, VSMCs, T cells, B cells, and myeloid cells— and mutual activation between cell types (Depuydt et al., 2020). This study not only identified EC subsets with angiogenic capacity and endothelial to mesenchymal transition, but also revealed 2 populations of macrophages; pro-inflammatory macrophages with excess production of IL-1β and tumor necrosis factor and fibrosis-promoting macrophages. Thus, the complex interaction of a wide variety of immune cells and vascular cells shapes the pathogenesis of vascular aging.

Giant Cell Arteritis

In recent years, great progress has also been made in the understanding of the pathophysiology of GCA. Research has identified the pivotal roles of vascular dendritic cells (DCs) and microvascular ECs in the adventitia in initiating and exacerbating vascular inflammation (Watanabe et al., 2017c; Wen et al., 2017; Zhang et al., 2017). Studies have also elucidated cellular signaling pathways and metabolic fitness in vasculitogenic T cells and macrophages (Watanabe et al., 2018a,b; Zhang et al., 2018, 2019).

The vasculitogenic response is initiated by vascular DCs which reside in the media-adventitial border (**Figure 1B**). When vascular DCs receive an external danger signal via Toll-like receptor, they upregulate costimulatory molecules, such as CD80 and CD86, and release chemokines. This, in turn, recruits monocytes and $CD4^+$ T cells mainly from vasa vasorum located in the adventitia (Ma-Krupa et al., 2004). The monocytes differentiate into tissue macrophages, phagocyte cell debris, and release cytokines, chemokines, and growth factors—such as vascular endothelial growth factor (VEGF)—to further enhance inflammation and neoangiogenesis (Kaiser et al., 1999). At the

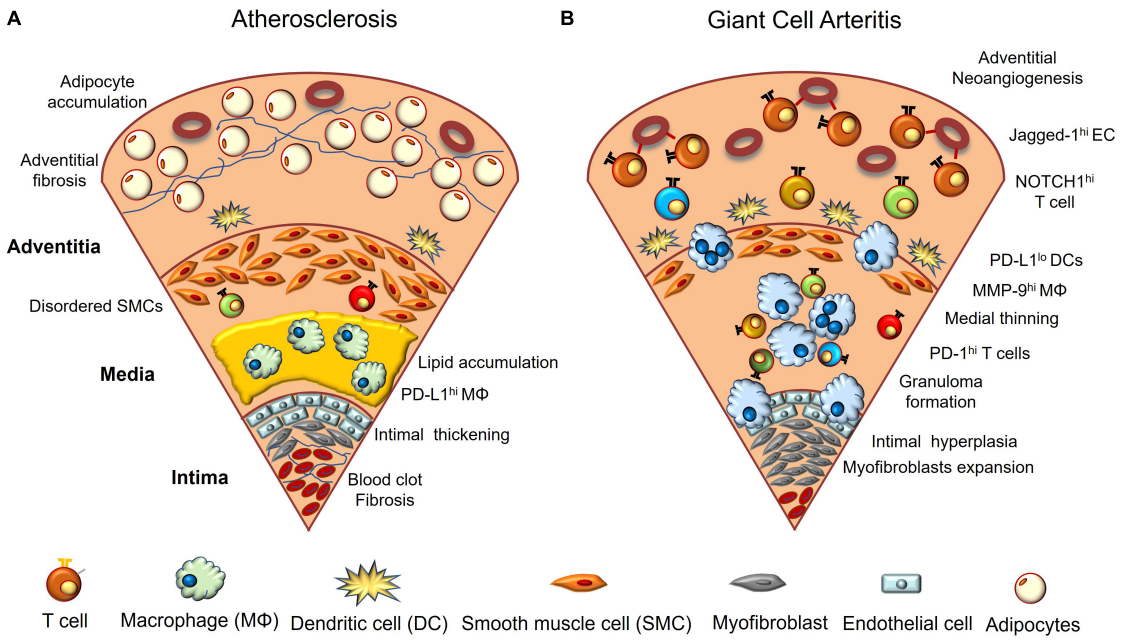

FIGURE 1 | Vascular aging and giant cell arteritis. **(A)** In aged vasculature, lipids accumulate between the intima and media, and macrophages and foam cells phagocytose them. Vascular endothelial cells are multilayered, and blood clots composed of red blood cells and platelets adhere to the luminal surface of the intima. Fibrosis and calcification are often observed. The arrangement of vascular smooth muscle cells becomes irregular and adipocytes accumulate in the adventitial layer. **(B)** GCA-affected arteries are characterized by infiltration of macrophages and activated $CD4^+$ T cells. NOTCH1 ligand (Jagged-1)-expressing endothelial cells interact with NOTCH1 expressed on $CD4^+$ T cells in the adventitia and polarize T cell differentiation toward Th1 and TH17. Macrophages and multinucleated giant cells release excess proteolytic enzymes such as matrix metalloprotease-9 (MMP-9), disrupting the external elastic membrane, and infiltrated T cells also release various cytokines. As a result, the media becomes thin and the inner elastic membrane collapses. In the intima, myofibroblasts overgrow and block the lumen of blood vessels. Angiogenesis is prominent in the adventitia. Dendritic cells present at the border of the adventitia and media have decreased PD-L1 expression; however, activated T cells highly express PD-1.

same time, they form multinucleated giant cells and digest extracellular matrix by releasing proteolytic enzymes, such as matrix metalloproteinase (MMP)-2 and MMP-9, disrupting the elastic membranes (Rodriguez-Pla et al., 2005; Watanabe et al., 2018b; Weyand et al., 2019). Such tissue-destructive macrophages enable activated T cells to infiltrate into otherwise immune-privileged sites and form granulomas. Activated $CD4^+$ T cells are dependent on an increased activity of Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Zhang et al., 2018; Vieira et al., 2022) and release multiple cytokines, including IFN-γ, IL-17, IL-21, IL-9, and IL-22 (Deng et al., 2010; Ciccia et al., 2015; Watanabe et al., 2017a; Zerbini et al., 2018). Such a cytokine milieu transforms ECs, VSMCs, and fibroblasts into myofibroblasts, which leads to the occlusion of the blood vessels (Weyand and Goronzy, 2013; Parreau et al., 2021).

The innermost ECs of vasa vasorum control the entry of immune cells. VEGF, which is primarily derived from macrophages and enriched in GCA plasma (Baldini et al., 2012), not only promotes neoangiogenesis in the adventitial layer, but also upregulates the expression of the NOTCH1 ligand on microvascular ECs (Wen et al., 2017). The NOTCH1 ligand stimulates NOTCH1 receptor, expressed on GCA $CD4^+$ T cells, and this shifts T cell differentiation toward Th1 and Th17 via activation of mammalian target of rapamycin (mTOR). Thus, microvascular ECs play an unexpected role in instructing T cell differentiation in GCA. Therefore, inhibition of NOTCH signaling or mTOR activation could be a new therapeutic strategy for this disease. High mTOR activity in GCA $CD4^+$ T cells is, in part, regulated by CD28 signaling, a "second signal" from antigen-presenting cells (Zhang et al., 2019). For this reason, blocking CD28 signaling could serve as an alternative option to suppress vasculitis.

The next noteworthy research revealed the lack of a system that suppresses aberrant immune activation in vascular lesions. Particularly, deficient expression of PD-L1 is a hallmark feature of vascular DCs present in temporal arteries (Watanabe et al., 2017c; Zhang et al., 2017). Surprisingly, vascular DCs residing in the temporal arteries and monocyte-derived DCs share the feature. PD-L1-deficient DCs have increased capacity for activating $CD4^+$ T cells as well as skew naïve $CD4^+$ T cell differentiation toward inflammatory phenotypes, such as Th1, Th17, and IL-21-producing T cells. Inhibition of the PD-1/PD-L1 interaction in a preclinical vasculitis mouse model exacerbated the pathology of GCA and recapitulated the intimal hyperplasia and neoangiogenesis in the adventitia, implicating this inhibitory mechanism as an essential regulator of vascular remodeling (Watanabe et al., 2017c; Zhang et al., 2017). Another group confirmed the decreased PD-L1 expression on vascular DCs during vascular inflammation using a mouse

model (Sun et al., 2022). Further elucidation of the regulatory mechanism of PD-L1 expression on DCs is necessary.

Recently, attention has been focused on the role of neutrophils in GCA. Neutrophils phagocytose and subsequently kill microorganisms efficiently (Burn et al., 2021). In addition, they unleash their cellular contents into the extracellular space, even post-mortem. In GCA, apoptosis-resistant immature neutrophils produce high levels of ROS, disrupting the endothelial barrier and increasing vascular permeability (Wang et al., 2020).

Similarities and Differences Between Vascular Aging and Giant Cell Arteritis

Although the disease mechanisms of GCA and CAD may differ, there are significant overlaps between the two. In both diseases, increasing age is the strongest risk factor, and $CD4^+$ T cells and macrophages produce excessive cytokines and chemokines in the vascular lesion, resulting in neoangiogenesis in the adventitia and intimal hyperplasia (**Figure 1**). However, GCA is more tissue-destructive, with macrophages and multinucleated giant cells destroying vascular structures. As a result, the VSMCs undergo cell death and the medial layer becomes thinner. In contrast, in atherosclerosis, the VSMCs proliferate and become irregularly arranged. Arterial stiffness increases due to the accompanied calcification and fibrosis, and adipocyte accumulation is more prominent in atherosclerosis (Tyrrell and Goldstein, 2021).

The direct link between these two diseases is missing. Rather, epidemiological studies have demonstrated that the incidence of GCA was inversely correlated with cardiovascular risk factors, such as obesity, smoking, hyperglycemia, and hypercholesterolemia (Tomasson et al., 2019; Wadstrom et al., 2020). This is consistent with our clinical experience that GCA-positive temporal artery biopsies show little atherosclerosis; while GCA-negative temporal artery biopsies often show atherosclerosis. The exact mechanism behind how vascular aging protects against GCA development remains unclear. One possibility is that, since high blood glucose upregulates PD-L1 expression on macrophages (Watanabe et al., 2018a), both tissue macrophages in atherosclerotic plaque and monocyte-derived macrophages from CAD patients overexpress PD-L1 and fail to support clonal expansion of $CD4^+$ T cells (Watanabe et al., 2017b). Impaired T cell immunity due to hyperglycemia may suppress the excess inflammatory response, as seen in GCA.

T Cell Aging and Giant Cell Arteritis

In contrast to vascular aging, T cell aging may participate in the pathogenesis of GCA (**Figure 2**). With aging, thymic involution accelerates homeostatic proliferation of naïve T cells (**Figure 2A**); however, naïve T cells, particularly naïve $CD8^+$ T cells, fail to maintain their absolute number (**Figure 2B**). Variation of T cell receptors is also reduced, while clonal sizes increase (**Figure 2C**). In addition, aging-related T cells—such as senescent T cells, exhausted T cells, and T effector memory CD45 RA (TEMRA) cells—accumulate (**Figure 2D**). Senescent T cells show irreversible cell cycle arrest but have SASP and release pro-inflammatory cytokines. Exhausted T cells express programmed death-1 (PD-1), TIM3, and LAG-3, and their effector functions are defective. TEMRA cells have short telomeres and show cell cycle arrest while maintaining high effector function (Goronzy and Weyand, 2017).

Although the direct evidence showing T cell aging in GCA is lacking, some indirect evidence does exist. Firstly, the T cell repertoire in GCA vascular lesions is restricted, while clonal sizes are expanded (Weyand et al., 1994). Secondly, $CD4^+$ T cells have an increased capacity for cytokine production. Thirdly, granulomatous inflammation in Takayasu arteritis—another type of LVV seen in young women—is composed of macrophages, $CD4^+$ T cells, and $CD8^+$ T cells; while $CD8^+$ T cells are rarely seen in GCA (Watanabe et al., 2020a,b). Further studies are needed to determine whether T cell aging accelerates vascular pathology in GCA.

NEUROLOGICAL AGING AND GIANT CELL ARTERITIS

Neurological Aging

Dementia and neurodegenerative disorders are now a major problem in public health. In 2018, nearly 50 million people were affected by Alzheimer's disease and other dementias all over the world (Grande et al., 2020). Dementia and neurodegenerative disorders are prototypes of neurological aging, with aging being the strongest risk factor for these diseases (Hou et al., 2019). Pathways implicated in the development and progression of these disorders include brain resilience, vascular damage, neuroinflammation, oxidative stress, dysfunctional autophagy, apolipoprotein homeostasis, and cellular senescence (Baker and Petersen, 2018; Grande et al., 2020; Montagne et al., 2020). Evidence is accumulating that various cell types in the neural network, including neurons, oligodendrocytes, astrocytes, microglial cells, and endothelial cells show senescent phenotypes with age. Commonly observed features of senescent cells include cell cycle arrest, telomere shortening, resistance to apoptosis, and the SASP, just like in peripheral tissues (Baker and Petersen, 2018).

Neurological Aging and Giant Cell Arteritis

Well-known neurological complications of GCA include stroke, cerebral infarction, and ischemic optic neuropathy. Cerebrovascular accident (CVA) is not specific to GCA, and does not have high diagnostic accuracy in GCA (van der Geest et al., 2020). However, patients with GCA are more likely to develop CVAs than age- and sex-matched controls (Robson et al., 2016; Tomasson et al., 2019), particularly within 1 year from diagnosis. This may be explained by strong vascular inflammation and high-dose glucocorticoids. The risk factors for CVA include increasing age, male, and social deprivation in GCA (Robson et al., 2016).

In addition, the evidence is accumulating that other forms of neurological disorders, such as dementia and neurodegenerative disorders, can be initial or coexistent manifestations of GCA (Pascuzzi et al., 1989; Caselli, 1990; Caselli and Hunder, 1993;

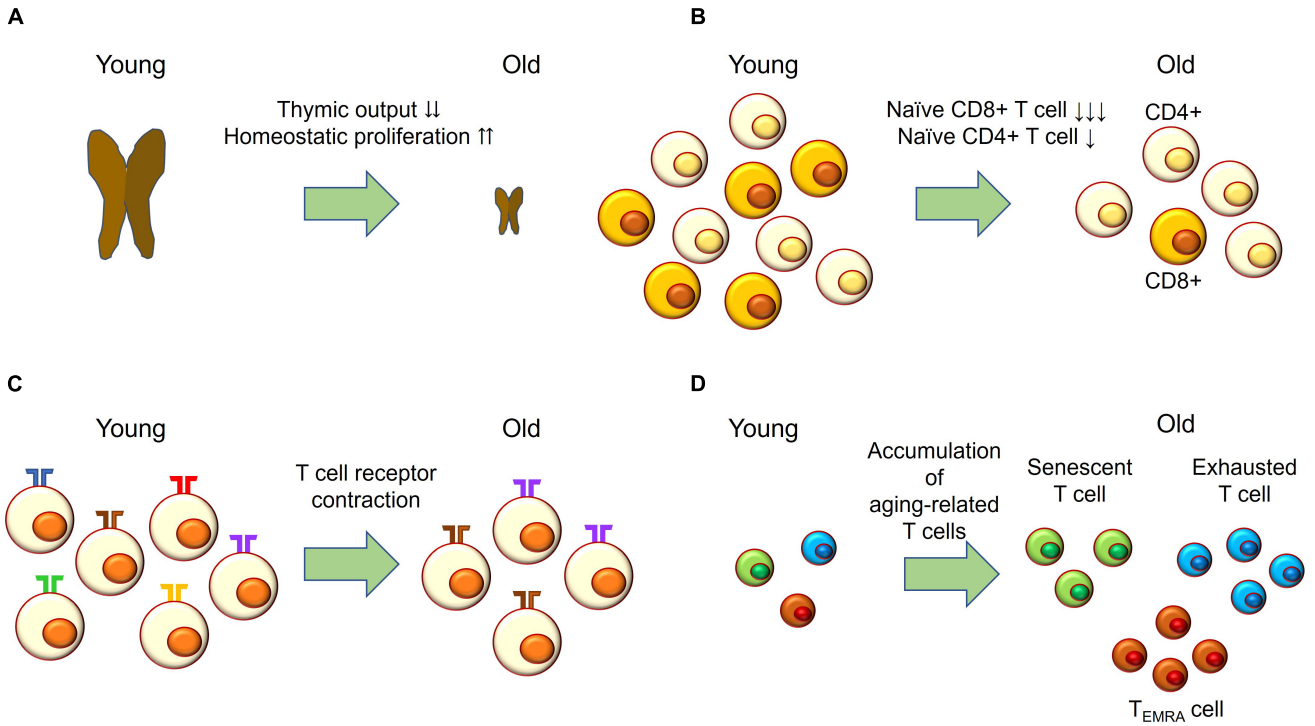

FIGURE 2 | T cell aging. **(A)** With age, thymic output drastically decreases and the naïve T cell compartment is maintained by homeostatic proliferation. **(B)** Naïve $CD4^+$ T cells slightly decrease in absolute number with age; while naïve $CD8^+$ T cells drastically decrease with age. **(C)** The number of T cell receptors—also called the T cell repertoire—declines with age, while clonal sizes increase with age. This process is called T cell receptor contraction. **(D)** T effector memory CD45 RA (TEMRA) cells; exhausted T cells expressing PD-1, TIM-3, and LAG-3; and senescent T cells accumulating with age.

Ely, 1998; Incalzi et al., 2005; Alisky, 2008; Solans-Laqué et al., 2008; Kushida et al., 2011; Lahaye et al., 2020). It is noteworthy that some of these cases showed improvement after GCA treatment, indicating that GCA could manifest as a "treatable" neurological disorder. Blood sampling and imaging may be useful in diagnosing GCA in patients with dementia, who have difficulty in reporting symptoms (Kushida et al., 2011).

More recently, an epidemiological study has demonstrated that neurodegenerative disorders accounted for 11% of deaths in GCA patients (Chazal et al., 2018). This suggests that the prevalence of neurodegenerative disorders in patients with GCA could be much higher. Such manifestations had been underestimated or considered rare, but this may be no longer the case for patients with GCA. Glucocorticoids and other treatments may not be sufficient to control neurological aging due to vascular damage from GCA or that patients with GCA are able to live longer than before and develop neurodegenerative diseases during the disease process.

DISCUSSION

We have focused on the relationship between aging in various organ systems and GCA. Is there a way to suppress both senescence and vascular inflammation? As described earlier, since vascular aging is characterized by low-grade inflammation, several attempts have been made to prevent the recurrence of cardiovascular events by controlling inflammation. A large clinical study showed that controlling inflammation by blocking IL-1β could suppress atherosclerosis and reduce cardiovascular events (Ridker et al., 2017); however, subsequent research revealed that IL-1β has atheroprotective effects in mice (Gomez et al., 2018), which suspended further clinical research.

Are there any ways to inhibit cellular senescence? Three strategies for targeting cellular senescence have been proposed (Ovadya and Krizhanovsky, 2018). The leading option is to induce apoptosis selectively in senescent cells with senolytic drugs. The second approach is to potentiate an immune response against senescent cells, leading to apoptotic cell death. The third one is selective blockade of SASP (Ovadya and Krizhanovsky, 2018). Among those therapies, inhibitors of JAK-STAT pathway are not only expected to be effective against cellular senescence by suppressing excessive cytokine signaling, but also expected for treating GCA (Zhang et al., 2018; Rathore et al., 2022; Vieira et al., 2022). In addition, recent research has shown that the complement C3a receptor, expressed on ECs, promotes dysfunction of blood brain barrier as well as vascular inflammation during aging, leading to neurodegenerative disease (Propson et al., 2021). Targeting complement activation could also be a novel therapeutic approach for the treatment of brain aging, neurodegenerative disorder, and vascular inflammation.

In conclusion, GCA is not just vascular aging, but vascular inflammation with the involvement of T-cell and neurological aging. Drugs that could simultaneously inhibit cellular senescence and vascular inflammation may be useful.

AUTHOR CONTRIBUTIONS

RW drafted the manuscript. MH revised and finalized the manuscript. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Pathophysiological Mechanisms Underlying Idiopathic Normal Pressure Hydrocephalus: A Review of Recent Insights

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The pathophysiologic mechanisms underpinning idiopathic normal pressure hydrocephalus (iNPH), a clinically diagnosed dementia-causing disorder, continue to be explored. An increasing body of evidence implicates multiple systems in the pathogenesis of this condition, though a unifying causative etiology remains elusive. Increased knowledge of the aberrations involved has shed light on the iNPH phenotype and has helped to guide prognostication for treatment with cerebrospinal fluid diversion. In this review, we highlight the central role of the cerebrovasculature in pathogenesis, from hydrocephalus formation to cerebral blood flow derangements, blood-brain barrier breakdown, and glymphatic pathway dysfunction. We offer potential avenues for increasing our understanding of how this disease occurs.

Keywords: idiopathic normal pressure hydrocephalus (iNPH), glymphatic circulation, ventriculoperitoneal (VP) shunt, cerebral blood flow, dementia, communicating hydrocephalus, blood brain barrier (BBB) breakdown

INTRODUCTION

Idiopathic normal pressure hydrocephalus (iNPH) is a common dementia-causing neurological disorder seen in the elderly, with 120 new cases per year per 100,000 population greater than 70 years (Iseki et al., 2014; Martín-Láez et al., 2015). iNPH classically presents with the clinical triad of gait disturbance, urinary incontinence, and dementia, with gait disturbance typically presenting first and cognitive manifestations arising later. The hallmark of the disease is an enlarged ventricular system without an increase in intracranial pressure (ICP). Despite progress in characterizing iNPH and its natural history, its pathophysiology has not been clearly defined.

Treatment for iNPH is centered around cerebrospinal fluid (CSF) shunting, which leads to improvement in symptoms, including dementia, in many patients (Toma et al., 2013). This distinguishes iNPH from other causes of dementia which are largely irreversible and hence represents an opportunity to characterize mechanisms contributing to cognitive impairment.

Ultimately, clinical responses to shunting are varied, and long-term outcomes indicate that while shunting improves the natural history, the durability of treatment is less than with other etiologies of hydrocephalus (Junkkari et al., 2021). Taken together, these data suggest that ventriculomegaly alone does not account for the natural history of iNPH, challenging the traditional role of CSF dynamics and appealing for a better understanding of iNPH's underlying pathogenesis.

iNPH has been examined from many view points, ranging from intracranial pressure dynamics, traditional radiographic parameters, advanced neuroimaging modalities, analysis of regional and global cerebral perfusion, and changes at the cellular and molecular levels, including the activity of the glymphatic pathways and the blood-brain barrier. Conclusions to be drawn from this disparate body of work are unsettled. In this manuscript, we review recent evidence related to the pathophysiology of iNPH in the context of current theories, noting areas of interest for future study. In particular, we focus on changes involving the cerebrovasculature, which may be central to pathogenesis.

CLINICAL FEATURES

The diagnosis of iNPH involves a combination of clinical symptoms, radiologic findings, and results of diagnostic evaluations (**Table 1**; Relkin et al., 2005; Nakajima et al., 2021). The criteria for probable iNPH differ somewhat between American/European and Japanese guidelines and are listed separately in **Table 1**. While both guidelines are accepted and used in practice, the broader radiographic criteria in the American/European guidelines may lead to a greater proportion of patients classified as probable iNPH (Andersson et al., 2017). Confounding the work-up of iNPH is the lack of specificity in diagnostic criteria and overlapping features shared with other neurodegenerative conditions including Alzheimer's disease and other movement disorders including Parkinsonism. Gait disturbance is the most common feature and typically is the first symptom to arise (Hebb and Cusimano, 2001).

Diagnostic radiographic features include ventricular enlargement with an Evans index of 0.3 or greater (**Figure 1**; Jacobs and Kinkel, 1976; George et al., 1995). Other common findings on brain imaging include a callosal angle of 90 degrees or less (Borzage et al., 2021), periventricular hyperintensities, and enlargement of the temporal horns (Relkin et al., 2005). A trial of CSF drainage is often undertaken to aid in the diagnosis, commonly *via* the lumbar subarachnoid space. Clinical benefit after temporary CSF drainage is strongly predictive of improvement in at least one symptom after shunting (Marmarou et al., 2005; Toma et al., 2013). In patients unable or unwilling to receive a shunt, serial lumbar punctures may be a treatment option (Isik et al., 2019).

Frustrating the iNPH clinical picture, there is not a clear neuroanatomic basis for the condition's manifestations. It was originally held that stretching of white matter tracts from ventricular dilation led to iNPH's clinical findings (Hakim and Adams, 1965), but there are several limitations with this hypothesis. First, it is not immediately obvious which white matter tracts would be causative. While the corticospinal tracts are clearly at risk for stretch, corticospinal tract-related gait disorders tend to differ from the hypokinesia and disequilibrium typical of iNPH (Rubino, 2002; Bugalho and Guimarães, 2007; Baker, 2018). Second, ventricular volume prior to CSF diversion-a surrogate for white matter tract stretching-does not correlate with the degree of symptomatology (Neikter et al., 2020). Third, other causes of ventriculomegaly both pathological (e.g., obstructive hydrocephalus) and physiological (e.g., ex vacuo hydrocephalus), do not typically cause gait disturbance as a primary manifestation. Fourth, the actual decrease in ventricular size after shunting is fairly modest in clinical responders, typically resulting in less than a 10% reduction in Evan's index (Neikter et al., 2020). Thus, while changes in the corticospinal tracts and corpus callosum correlating with iNPH have been noted in some studies using tractography and transcranial magnetic stimulation (Röricht et al., 1998; Mataró et al., 2007; Siasios et al., 2016; Sarica et al., 2021), it is difficult to correlate symptomatic improvement following CSF drainage with decreased stretching of white matter tracts alone, and these changes may ultimately be epiphenomena.

Some authors have hypothesized that dysfunction of the cortico-basal ganglia-thalamo-cortical (CBGTC) is involved in iNPH (Curran and Lang, 1994; Lenfeldt et al., 2008), though the CBGTC loop may not explain dementia and urinary incontinence. Recent investigations have implicated striatal dopamine reuptake transporter density in iNPH gait impairment, offering a potential mechanism for basal ganglia dysfunction (Pozzi et al., 2021; Todisco et al., 2021). Other research has implicated frontal lobe dysfunction in not only the cognitive and urinary disturbances, but also gait disorder as well (Bugalho and Guimarães, 2007). As multiple lesional effects are suggested in iNPH, a fruitful approach to understanding the neuroanatomic basis may involve network-level investigations of functional connectivity (Griffa et al., 2020).

THEORIES ON PATHOGENESIS

Given shared features with other neurodegenerative conditions, iNPH is defined as a unique entity by ventricular enlargement. Our efforts to understand the etiology of the disease are thus inextricably tied to understanding how hydrocephalus develops in iNPH patients.

Generally speaking, communicating hydrocephalus in iNPH and other etiologies results from an imbalance between CSF formation and removal. This is thought to be due to impairment in the return of CSF to the circulation in most cases, through scarring or obstruction of the arachnoid granulations (Chen et al., 2017). A compelling anecdote to challenge this trad itional model is the observation that subarachnoid spaces are not universally dilated in communicating hydrocephalus (Egnor et al., 2002; Greitz, 2004). It warrants mention that in chronic states of hydrocephalus, CSF absorption is coupled to production; that is, while hydrocephalus may initially occur because of impaired absorption of CSF, the imbalance is a transient phenomenon, and the compensated state implies that a new equilibrium between absorption and production is reached. Theoretically, the new equilibrium may be reached through

TABLE 1 | American/European (Relkin et al., 2005) and Japanese (Nakajima et al., 2021) criteria for probable idiopathic normal pressure hydrocephalus.

	American/European	*Japanese*
Clinical	Gait/balance disturbance One impairment involving either cognition or urination	More than one symptom in the clinical triad: gait disturbance, cognitive impairment, and urinary incontinence
Historical	Insidious onset of symptoms with progression over time Age >40 years at symptom onset Symptom duration for at least 3–6 months No previous insult which could lead to secondary hydrocephalus No other neurologic, psychiatric, or medical cause for symptoms	Age ≥60 years No obvious preceding diseases causing ventricular dilation (e.g., subarachnoid hemorrhage, meningitis, head injury) Clinical symptoms not completely explained by other neurological or non-neurological disease.
Investigational	Ventricular enlargement without macroscopic obstruction with Evans Index >0.3 At least one of the following features: 1. Enlargement of temporal horns without hippocampal atrophy 2. Callosal angle of ∼90 degrees or less 3. Evidence of altered brain water content including periventricular signal changes 4. Aqueductal or fourth ventricular flow void seen on MRI CSF opening pressure on lumbar puncture between 5–18 mmHg (70–245 mmH_2O)	Ventricular enlargement with Evans Index >0.3 CSF opening pressure ≤200 mm H_2O, normal CSF content One of the following two features: 1. Neuroimaging features of narrowing of the sulci and subarachnoid space over the high convexity/midline surface (DESH) with gait disturbance: small stride, shuffle, instability during walking, and increase in instability on turning 2. Improvement of symptoms after CSF tap test and/or drainage test

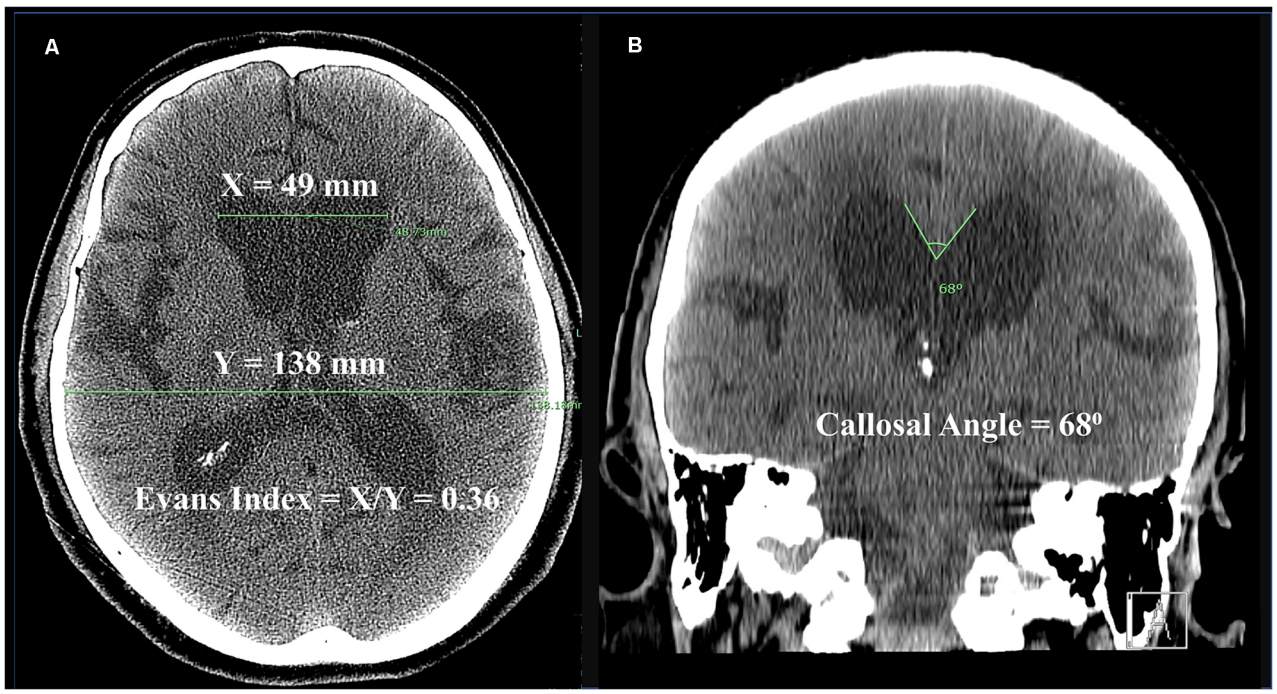

FIGURE 1 | Computed tomography (CT) scan from a 70-year-old man with iNPH. **(A)** Axial image demonstrating Evans Index, which is the ratio of the maximum bifrontal horn width (X) to the maximum biparietal internal diameter of the skull (Y). An Evans Index >0.3 is present in iNPH. **(B)** Coronal image demonstrating callosal angle, here measured to 68 degrees. Normal callosal angles are greater than 90 degrees, while acute callosal angles occur in iNPH. Disproportionately enlarged subarachnoid space hydrocephalus (DESH) is apparent, as the Sylvian fissures are dilated out of proportion to sulci near the convexity.

increased CSF absorption through other means, or by decreased CSF production.

Unlike other types of communicating hydrocephalus, such as subarachnoid hemorrhage, a lesion causing impaired CSF outflow is not apparent in iNPH. An alternative explanation of communicating hydrocephalus, however, invokes arterial pulsations. In this theory, homeostasis of the CSF spaces including the ventricles relies on the normal propagation through the cerebrovasculature of pulsations delivered through the cardiac cycle. In cases in which cerebral arteries lose compliance, the additional pulse pressure is delivered distally to the capillaries and veins, which may alter CSF dynamics in such a way to produce ventriculomegaly (Egnor et al., 2002; Greitz, 2004). Preliminary evidence suggests that CSF drainage may improve vascular compliance and, subsequently, CBF (Bateman, 2000). Hence, as there is not a lesion to block CSF egress at the

level of the arachnoid granulations in the traditional model of communicating hydrocephalus, impaired vascular compliance may be sufficient to produce iNPH.

Pulsations transferred through the elastic arterial system cause movement of CSF back and forth through the aqueduct during the cardiac cycle (Marmarou et al., 1978; Linninger et al., 2005; Kahlon et al., 2007; Scollato et al., 2008; Ringstad et al., 2015; Yamada et al., 2020). In healthy adults the net movement of ventricular CSF is craniocaudal, however, in iNPH patients, the net movement is typically reversed, towards the third and lateral ventricles (Kim et al., 1999; Penn et al., 2011; Ringstad et al., 2016), which results in transependymal flow of ventricular CSF into the interstitial space (Ringstad et al., 2017). The flow pattern often reverts to anterograde flow after shunting (Ringstad et al., 2016). Clinical study of CSF dynamics in iNPH indicates elevated resistance to CSF outflow and increased CSF pulsatility. These features predict treatment response after CSF diversion, indicating normalization of CSF dynamics and a more physiologic state (Eide and Sorteberg, 2008, 2010; Malm et al., 2011; Qvarlander et al., 2013; Jacobsson et al., 2018).

That iNPH may fundamentally represent a vascular disorder is intriguing, given the high incidence of vascular risk factors including hypertension and diabetes in iNPH patients (Eide and Pripp, 2014; Jaraj et al., 2016; Israelsson et al., 2017). Supporting this notion is the near-ubiquitous finding of deep white matter and periventricular lesions in iNPH (Krauss et al., 1997), hallmarks of small vessel disease. Variations in regional hypoperfusion and degree of hypoxic changes may help explain the clinical heterogeneity of iNPH and poor responses to shunting. Our view is that iNPH is fundamentally a cerebrovasculature disorder. Impaired compliance triggers a cascade of events culminating in the development of hydrocephalus, which subsequently begins a cycle that unchecked eventually progresses to irreversible dementia and neurologic injury. Shunting reverses some of the clinical manifestations, although even with treatment the disease is associated with progressive morbidity, which suggests a component of irreversible small vessel disease. Below we discuss three interrelated systems that may be central to the development and progression of iNPH: cerebral blood flow, the glymphatic system, and the blood-brain barrier. A flow diagram demonstrating possible pathogenic relationships is depicted in **Figure 2**.

DEFICIENT CEREBRAL BLOOD FLOW (CBF)

Our understanding of the role of cerebral blood flow (CBF) in iNPH has evolved considerably in the last two decades. In early work, consistent patterns could not be drawn between the association between various CBF changes and: (1) the diagnosis of iNPH, (2) disease severity, or (3) improvement after shunting, which may have been due in part to disparate imaging protocols, inconsistent diagnostic criteria, and small sample sizes (Owler and Pickard, 2001). More recently, several studies reported regional hypoperfusion in critical areas, suggesting that vascular insufficiency is relevant to iNPH.

A number of studies have found regional CBF deficits in iNPH patients compared to age-matched healthy controls, including deficits in the periventricular white matter (Momjian et al., 2004; Ziegelitz et al., 2014, 2016; Virhammar et al., 2017), lentiform nucleus (Owler et al., 2004a; Ziegelitz et al., 2014, 2016; Virhammar et al., 2017), thalamus (Owler et al., 2004a; Virhammar et al., 2017), caudate (Owler et al., 2004a), and basal medial frontal cortex (Ziegelitz et al., 2014). Global reductions have been identified as well (Momjian et al., 2004; Owler et al., 2004a; Ziegelitz et al., 2014, 2016). One study noted inverse correlations between thalamic and putaminal CBF and severity of iNPH (Owler et al., 2004a), but most studies found no association between the magnitude of global or regional CBF values and severity of iNPH.

Clinical improvements after CSF drainage have been associated with improvements in CBF in the lateral and frontal white matter regions (Virhammar et al., 2014), periventricular white matter (Ziegelitz et al., 2015, 2016; Satow et al., 2017), periventricular thalamus (Ziegelitz et al., 2015), medial frontal region (Klinge et al., 2008), supplemental motor area (Lenfeldt et al., 2008), brainstem (Agerskov et al., 2020), and globally (Chang et al., 2009). Relating to prognostic variables prior to treatment, one study found decreased preoperative CBF in basal frontal lobes and anterior cingulate region in iNPH patients who responded to shunting compared to non-responders (Murakami et al., 2007). However, in most studies, no associations were found between preoperative regional CBF values and clinical response to shunting.

In light of this body of work, it is useful to consider the clinical findings in iNPH as the result of hypoperfusion. Depending on the extent of the CBF deficit, hypoperfusion may account fo rany or all findings of iNPH. Bladder dysfunction in iNPH is typically referable to detrusor overactivity, which may occur through effects on the frontal lobe or basal ganglia (Andersson, 2004; Sakakibara et al., 2008, 2012). Similarly, both the frontal lobe and basal ganglia have been implicated in gait disturbances that characterize iNPH (Bugalho and Guimarães, 2007). While cognitive impairment may be considered a diffuse lesion, some evidence suggests early frontal involvement in iNPH, consisting of psychomotor slowing and impaired attention rather than memory deficits, before progressing to more profound impairment (Iddon et al., 1999; Ogino et al., 2006; Picascia et al., 2015). With improved perfusion after shunting, symptoms may regress unless infarcts have already occurred.

The mechanism of impaired perfusion of deep gray matter and periventricular white matter is a subject of debate. Hypoperfusion may result from compression of the deep vascularity, compression of superficial venous outflow, impaired autoregulation, changes related to transependymal flow, or some combination of these factors (Momjian et al., 2004; Owler et al., 2004b; Bateman, 2008; Scollato et al., 2008; Chang et al., 2009). While a shunt would potentially improve any of these factors, the manner by which improvement in CBF occurs after shunting has not been well characterized. The effect of CSF drainage on cerebral perfusion pressure (CPP) through decreased ICP is limited as the ICP is not elevated, raising questions as to whether improved CPP

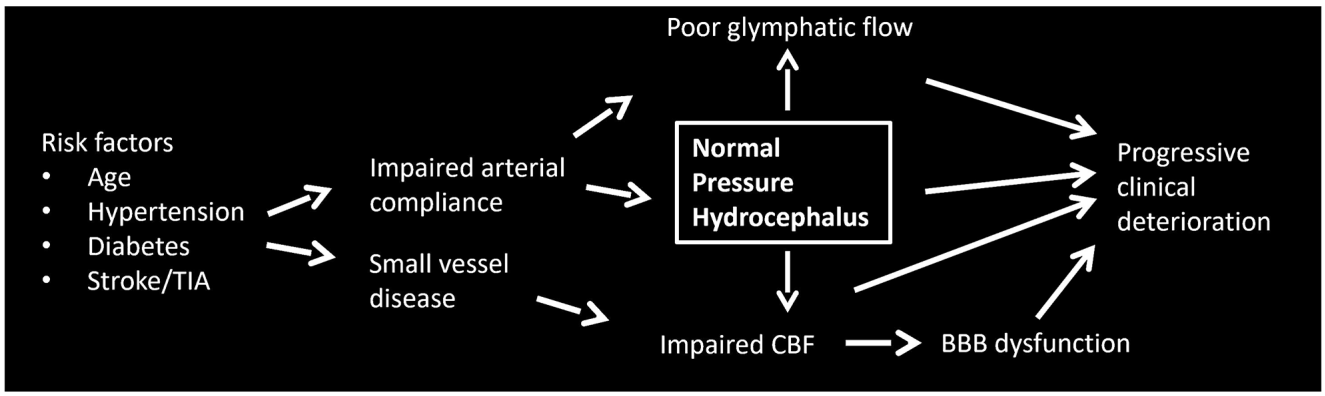

FIGURE 2 | Flow diagram demonstrating possible pathogenic relationships in iNPH.

alone may explain CBF changes (Eide and Sorteberg, 2010). One explanation may involve the CSF pulsatility curve, in which a relatively small change in ICP leads to decreased CSF pulsatility which may have downstream effects on CBF (Qvarlander et al., 2013).

IMPAIRED GLYMPHATIC CIRCULATION

The glymphatic (glial-lymphatic) system is a recently discovered homeostatic mechanism by which fluid moves through the brain parenchyma, acting as a waste disposal mechanism for brain tissue (**Figure 3**; Iliff et al., 2012; Nedergaard, 2013). In brief, subarachnoid CSF is pumped along periarterial channels by arterial pulsations (Iliff et al., 2013; Mestre et al., 2018) and enters the interstitial compartment through aquaporin-4 transporters within astrocyte endfeet (Plog and Nedergaard, 2018). CSF joins interstitial fluid and moves through the interstitial space towards perivenous and perineural channels through which it is removed from the brain. These efflux pathways include perisinusal lymphatic vessels that drain into extracranial lymphatics (Louveau et al., 2015; Ahn et al., 2019). Glymphatic circulation constitutes a primary role in the clearance of toxoids and waste products from the brain parenchyma, akin to the lymphatic function of other organs (Jessen et al., 2015), and is most active during sleep (Kang et al., 2009; Shokri-Kojori et al., 2018).

Mounting evidence suggests impairment of the glymphatic system by multiple mechanisms contributes to neurodegenerative diseases (Rasmussen et al., 2018). Much of the advances in understanding these pathways hail from the recognition that dysfunction of the glymphatic system contributes to amyloid-beta buildup in Alzheimer's disease (Rasmussen et al., 2018; Mestre et al., 2020). Other conditions associated with glymphatic impairment relevant to iNPH include aging (Zhou et al., 2020), diabetes (Jiang et al., 2017), and hypertension (Mestre et al., 2018).

Clinical evaluation of iNPH patients demonstrates sluggish glymphatic flow (Ringstad et al., 2017; Eide and Ringstad, 2019; Bae et al., 2021). Lumbar intrathecal gadobutrol injection in iNPH patients resulted in delayed enhancement of subarachnoid spaces and cortical surfaces, compared to younger patients receiving gadobutrol for workup of intracranial hypotension (Ringstad et al., 2017). The age difference between the two patient groups somewhat limits the study's conclusions, as even healthy older people have impaired glymphatic function (Zhou et al., 2020). Given that glymphatic dysfunction also is involved in Alzheimer's disease (Tarasoff-Conway et al., 2015), this may represent a common pathway for cognitive decline in the two

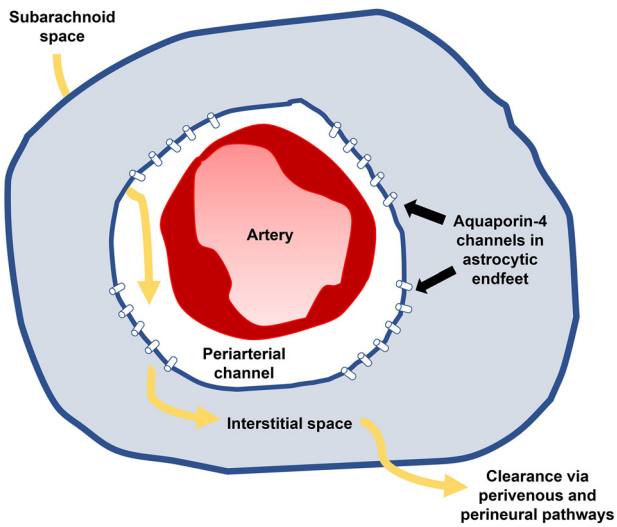

FIGURE 3 | Glymphatic influx (yellow arrows) occurs along periarterial channels within subarachnoid spaces (white) and enters the parenchyma (blue) through aquaporin-4 transporters on astrocytic endfeet. Subarachnoid CSF joins interstitial fluid and passes through the brain, delivering substances to and from the parenchyma before being absorbed along perivenous and perineural channels. Impaired glymphatic circulation may result in part from impaired influx through poor arterial compliance and results in progressive neurotoxicity contributing to iNPH's clinical manifestations.

conditions (Reeves et al., 2020), but may not necessarily be related to urinary incontinence and gait in iNPH.

One theory posited to explain glymphatic impairment in iNPH is loss of arterial compliance. As vessels become increasingly stiff, the pump driving glymphatic influx is weakened, resulting in the buildup of waste substances in the interstitial fluid. This may in part explain the retrograde movement of subarachnoid CSF into the ventricular system, as the outflow resistance increases along glymphatic pathways. It is possible that the improvement in CSF dynamics after shunting (Ringstad et al., 2016) improves the glymphatic flow and thus cognition. However, that this could represent a primary insult in iNPH has been called into question (Gallina et al., 2020).

Another potential mechanism for glymphatic impairment is through reduced expression of aquaporin-4 channels, which has been demonstrated in iNPH patients (Hasan-Olive et al., 2019). In Alzheimer's disease, decreased expression of aquaporin-4 leads to impaired clearance of misfolded proteins, which results in neurotoxicity and cognitive decline (Xu et al., 2015; Zeppenfeld et al., 2017). Glymphatic impairment in iNPH may lead to a similar buildup of waste products with resultant neurotoxicity that leads to cognitive dysfunction. Improvement in the glymphatic flow after shunting iNPH and subsequent clearance of accumulated interstitial substances may improve neuronal function and hence cognition after shunting, though this is untested.

BLOOD-BRAIN BARRIER BREAKDOWN

The blood-brain barrier (BBB), the brain's unique microvascular interface consisting of endothelial tight junctions, pericytes, and astrocytic endfeet, plays a critical role in maintaining the optimal conditions for proper neuronal functioning by acting as a selective barrier between the blood and brain (**Figure 4**; Bradbury et al., 1963; Bernacki et al., 2008; Abbott et al., 2010). The BBB prevents the entry of toxins while facilitating the transportation of metabolites and nutrients into the CNS. Given its role in homeostasis and neuroprotection, the BBB has been investigated in a host of neurological disorders (Sweeney et al., 2018; Profaci et al., 2020).

Several investigations have demonstrated pathology related to the BBB in iNPH patients. Distorted and thickened basement membranes and degenerated pericyte processes were demonstrated in biopsy specimens from iNPH patients (Eidsvaag et al., 2017; Eide and Hansson, 2020). Pericytes are essential components of the BBB and play important roles in induction, maintenance, and selective permeability (Abbott, 2002; Armulik et al., 2010). Pericyte degeneration has been shown to cause increased permeability to water and both low- and high-molecular-weight tracers, creating a leaky BBB (Armulik et al., 2010).

Additional evidence for compromise of the BBB was identified in iNPH patients through the extravasation of fibrin, a blood coagulation protein, in frontal biopsy specimens (Eide and Hansson, 2020). In this study, scattered fibrin staining around capillaries within cortical layers was seen in all iNPH patients compared to fewer than 30% of patients with other

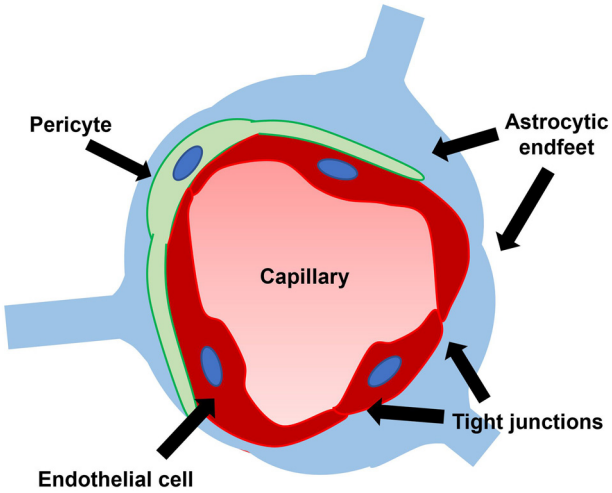

FIGURE 4 | The blood-brain barrier consists of endothelial tight junctions, pericytes, and astrocytic endfeet. A leaky blood-brain barrier through dysfunction of one or multiple of these constituent parts has been demonstrated in iNPH.

neurological diseases (Eide and Hansson, 2020). Increased fibrin deposition in the brain parenchyma in NPH biopsies correlated with increased levels of glial fibrillary acidic protein (GFAP), a marker of reactive astrogliosis (Eide and Hansson, 2020). Astrogliosis decreases compliance, which may in turn contribute to altered CSF dynamics in NPH (Lu et al., 2011; Fattahi et al., 2016). Both the degree of fibrin extravasation and astrogliosis correlated with reduced expression of aquaporin-4 transporters on perivascular astrocytic endfeet in biopsies of NPH patients, suggesting a link with glymphatic function (Eide and Hansson, 2018, 2020).

BBB dysfunction in iNPH may be related to deficient CBF. In a murine model of chronic cerebral hypoperfusion, hypoxia-induced injury to pericytes lead to BBB disruption (Liu et al., 2019). Though not tested in iNPH, a similar mechanism may explain pericyte injury and subsequent loss of BBB integrity. Pericyte dysfunction and other BBB insults have been demonstrated through means such as inflammation, hyperglycemia, and ischemia in Alzheimer's disease, traumatic brain injury, and other disorders (Erickson and Banks, 2013).

CONCLUSIONS

From the initial insult leading to hydrocephalus and onset of clinical manifestations to the irreversible changes occurring later in untreated cases, the cerebrovasculature is closely tied to the pathogenesis of iNPH. Relevant mechanisms include diminished CBF, glymphatic disruption, and changes to the BBB. Additional work is needed to further characterize how these pathophysiologic mechanisms inter-relate. Further, future studies should address how these pathologic features are reversed with shunting, which will provide insights into both iNPH and other neurodegenerative conditions. Answers to these questions

will shed light on improving clinical responses and enhancing the durability of shunting.

AUTHOR CONTRIBUTIONS

PB, RB, KW, WC, and AK: investigation, manuscript writing, and editing. BO and XS: manuscript editing. ZZ, MB, and DW: conception and manuscript editing. CL and DL: conception, manuscript editing, and supervision. All authors contributed to the article and approved the submitted version.

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The role of neutrophils in the dysfunction of central nervous system barriers

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Leukocyte migration into the central nervous system (CNS) represents a central process in the development of neurological diseases with a detrimental inflammatory component. Infiltrating neutrophils have been detected inside the brain of patients with several neuroinflammatory disorders, including stroke, multiple sclerosis and Alzheimer's disease. During inflammatory responses, these highly reactive innate immune cells can rapidly extravasate and release a plethora of pro-inflammatory and cytotoxic factors, potentially inducing significant collateral tissue damage. Indeed, several studies have shown that neutrophils promote blood-brain barrier damage and increased vascular permeability during neuroinflammatory diseases. Recent studies have shown that neutrophils migrate into the meninges and choroid plexus, suggesting these cells can also damage the blood-cerebrospinal fluid barrier (BCSFB). In this review, we discuss the emerging role of neutrophils in the dysfunction of brain barriers across different neuroinflammatory conditions and describe the molecular basis and cellular interplays involved in neutrophil-mediated injury of the CNS borders.

KEYWORDS

neutrophils, blood-brain barrier, blood-cerebrospinal fluid barrier (BCSFB), neurodegenerative diseases, neuroinflammation

Introduction

The central nervous system (CNS) is physically separated from the peripheral milieu by specialized barriers ensuring a constant state of homeostasis and efficient neuronal function. It was previously considered an immune-privileged site, and this view was attributed to the low number of CNS immune cells and the presence of physical barriers limiting the communication between the systemic circulation and neural tissue (Bechmann et al., 2007; Galea et al., 2007). Over the past decades, this view has been challenged by several studies showing the presence of immune cells, including T and B lymphocytes, in the healthy brain (Anthony et al., 2003; Smolders et al., 2018). Additionally, recent reports describing CNS lymphatics, meningeal tissue

and skull bone marrow as active immune hubs contributed to a broader vision of the immune cell dynamics within the healthy CNS (Louveau et al., 2015; Ribeiro et al., 2019; Alves de Lima et al., 2020; Brioschi et al., 2021; Cugurra et al., 2021; Herz et al., 2021; Rustenhoven et al., 2021). Particularly, under physiological conditions, meningeal immunity plays a key role in modulating cognition and behavior in a cytokine-dependent manner, suggesting that brain borders play an active role in brain functions (Filiano et al., 2016; Ribeiro et al., 2019; Alves de Lima et al., 2020; Herz et al., 2021). During CNS inflammatory pathologies, peripheral immune cells migrate into the brain and meninges and play a pathogenic role in several neuropathological disorders (Prinz and Priller, 2017; Rossi et al., 2021). Neutrophils constitute the most abundant immune cell population in the human peripheral blood (Juul et al., 1984), and their role in disease development has been shown in several CNS disorders such as stroke, multiple sclerosis (MS) and Alzheimer's disease (AD) (Rossi et al., 2020, 2021). Their vast granule storage of enzymes potentially inducing collateral tissue damage, the capacity to rapidly extravasate and increase vascular permeability and the ability to favor tissue remodeling, suggest neutrophils may have a key role in the development CNS inflammatory disorders (Rossi et al., 2020; Fischer et al., 2022). This review summarizes recent data on the contribution of neutrophils to brain injury focusing on pathological mechanisms occurring at CNS borders. We discuss what is known about the involvement of neutrophils in the induction of endothelial injury, increased vascular permeability and degradation of extracellular matrix promoting neuroinflammation and neurodegeneration.

Overview of the central nervous system barriers

The CNS is physically separated from the peripheral milieu by two main barriers: the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) (**Figure 1**). Although both barriers are characterized by high expression of junctional proteins and selective permeability capacities, they also have unique anatomical and molecular characteristics. The BBB is an endothelial structure of brain microvessels, which limits the passage of plasma molecules and blood cells into the brain and maintains the chemical composition of the neuronal "milieu" (Zlokovic, 2008). On the other hand, the BCSFB separates the blood from the CSF and was classically associated with the epithelium of the choroid plexus (ChP) and arachnoid matter (Brøchner et al., 2015; Engelhardt et al., 2017) (**Figure 1**). Overall, the CNS barriers not only protect the parenchyma, restricting the diffusion of pathogens and harmful blood molecules, but also play a critical role in the uptake of nutrients and excretion of byproducts of brain metabolism (Ballabh et al., 2004). The BBB consists of a monolayer of tightly packed and specialized non-fenestrated endothelial cells (ECs) brought together by junctional complexes, covering brain capillaries and postcapillary venules (**Figure 1**). It is fenced by the glia limitans formed by perivascular astrocyte endfeet and parenchymal basement membrane, which further restricts BBB permeability. This specialized and dynamic structure, together with the surveying microglia, astrocytes, pericytes, neuronal branches, and the acellular basement membrane, form the neurovascular unit (NVU) (Villabona-Rueda et al., 2019). Furthermore, the BBB represents a high-resistance electrical barrier, expressing transporters and efflux pumps for the regulation of ion fluctuations and solutes, and limiting the paracellular and transcellular movement of molecules. The inter-endothelial junctional structures are responsible for the "gate" function of ECs and consist of tight junction proteins (TJ), adherens junctions (AJs) and gap junctions (Chow and Gu, 2015; Tietz and Engelhardt, 2015; Sweeney et al., 2019; Saint-Pol et al., 2020). Brain endothelial TJs are the most apical cell-cell junctional complexes and constitute the core elements of the BBB with a crucial role in regulating paracellular permeability and maintaining cell polarity. They contain transmembrane proteins, including occludin claudins and junctional adhesion molecules, anchored to intracellular scaffolding proteins of the membrane-associated guanylate kinase family, also known as zonula occludens (ZO-1, -2, -3) (Zlokovic, 2008). The integral membrane proteins provide support to the TJs structure, with occludin contributing to the regulation and stabilization of the junctional structures (Cummins, 2012), whereas claudins are critical sealing determinants of paracellular "tightness" between adjacent ECs (Alahmari, 2021). Particularly, claudin-5, the most abundant transmembrane TJ protein at the BBB, plays a prominent role in maintaining the structural integrity of the vasculature and preventing the diffusion of small molecules through the intercellular space (**Figure 2**; Greene et al., 2019). Reduced expression of occludin, claudin-5, and ZO-1 is considered a sensitive indicator of BBB alterations associated with increased permeability during CNS diseases (Zlokovic, 2008; Cummins, 2012; Greene et al., 2019). Among the junctional complexes between the cerebral ECs, vascular endothelial (VE)-cadherin represents the major AJ transmembrane protein. VE-cadherin is linked to the cytoskeleton via catenins such as p120, β-catenin, plakoglobin, and γ-catenin, ensuring AJ assembly and BBB stability (Zlokovic, 2008; Li et al., 2018). Besides the organized junctional complexes, additional independent molecules are present at ECs junctions, including platelet-endothelial cell adhesion molecule-1 (PECAM-1 or CD31) and CD99. These proteins have homophilic and heterophilic binding capacity and have a role in the context of leukocyte transmigration during inflammatory responses (Lou et al., 2007; Zlokovic, 2008; Sullivan and Muller, 2014). Alterations in junctional molecules facilitate leukocyte transmigration during brain inflammation.

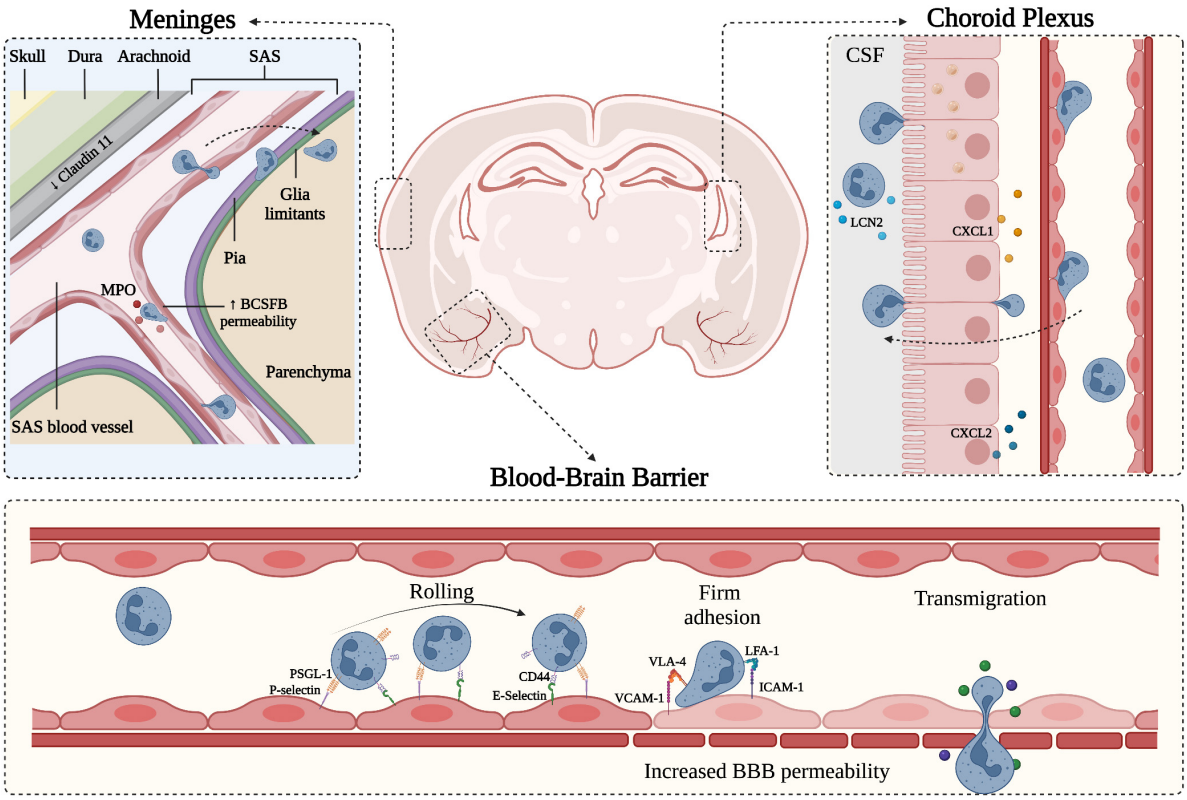

FIGURE 1
Schematic representation of neutrophil migration through the CNS barriers. CNS barriers limit the accessibility of circulating cells and molecules and can be divided into two main components: the Blood-Brain-Barrier (BBB) situated within the brain parenchyma and the blood-CSF-barrier (BCSFB) present in the choroid plexus (ChP) and in the meninges. Starting from the outer part of the CNS (depicted on the top left), the meninges represent a series of connective layers surrounding the brain, including dura mater and leptomeninges (arachnoid and pia mater). During CNS inflammatory diseases, neutrophils adhere in pial vessels and migrate into the SAS; these cells may then cross pia matter and migrate into the underlying brain parenchyma. Within the leptomeningeal vessels, neutrophils release MPO, potentially damaging endothelial cells and the extracellular matrix. On the top right, a representation of the choroid plexus shows the production of neutrophil chemoattractants CXCL1 and CXCL2 by the epithelial cells leading to neutrophil infiltration across the BCSFB. In both ChP stroma and within the CSF, neutrophils have been shown to produce LCN2. On the lower panel, a simplified model of the BBB is depicted, along with the classical neutrophil migration cascade. Inflamed BBB endothelial cells express adhesion molecules such as P- and E-selectins mediating capture and rolling through interaction with neutrophil PSGL-1 and CD44. Endothelial ICAM-1 and VCAM-1 mediate the arrest phase by binding neutrophil integrins LFA-1 and VLA-4, respectively. Firm adhesion and transmigration may contribute to increased BBB permeability (created with BioRender.com).

Indeed, the binding of α4β1 integrin expressed on lymphocytes to the endothelial vascular adhesion molecule (VCAM-1) induces the phosphorylation of VE-cadherin and leads to the opening of AJ contacts, facilitating leukocyte transmigration (Vockel and Vestweber, 2013).

The BBB together with the BCSFB are considered part of the neurovascular system, providing active immune surveillance and supporting coordinated immune responses (Engelhardt et al., 2017; Mastorakos and McGavern, 2019). Even though the BCSFB is more accessible and permeable than the BBB, the latter has received more attention for its involvement in CNS pathologies (Felgenhauer, 1995; Tumani et al., 2017). Nevertheless, growing evidence suggests the BCSFB also plays a significant role in the communication between the periphery and the CNS during inflammatory neurological disorders. The BCSFB was mainly associated with two areas of the CNS: the ChP and the arachnoid membrane covering the subarachnoid space (SAS). The ChP is located in the lateral and fourth ventricles and is an epithelial tissue surrounding fenestrated vessels. It represents a major site of CSF production in the brain and strictly regulates the exchange of substances between the blood and the CSF (Lun et al., 2015). ChP capillaries do not have TJs, allowing the free movement of molecules through fenestrations and intercellular gaps. However, the choroidal BCSFB is characterized by the presence of junctional molecules in the epithelial lining of ChP, allowing selective permeability of blood components. Despite functional similarities between the BBB and the BSCFB, the choroidal BCSFB shows mainly the expression of claudins 1–3, 9, 12 and 22 and almost no expression of claudin-5, suggesting morphological differences that may lead to a more permeable barrier to small molecules, macromolecules and circulating

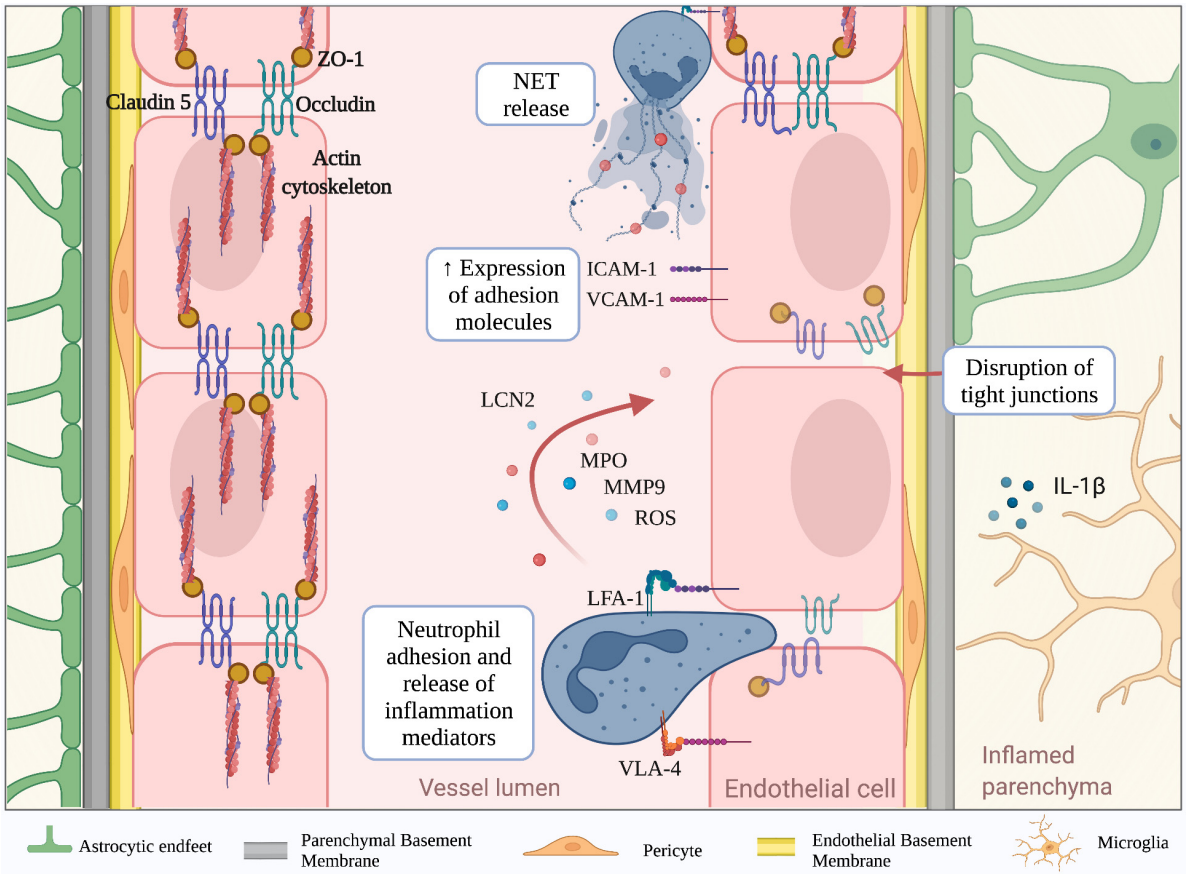

FIGURE 2
Molecular mechanisms mediating neutrophil-dependent BBB dysfunction. The BBB is a complex structure mainly composed of endothelial cells tightly bound together by junctional molecules, astrocytic endfeet and pericytes. During neuroinflammation, microglia and astrocytes produce IL-1b, which is a potent inducer of endothelial ICAM-1 and VCAM-1 and promotor of neutrophil infiltration. Engagement of neutrophil integrins as well as other stimuli, lead to the production of neutrophil extracellular traps (NETs) and release of inflammatory mediators including reactive oxygen species (ROS), lipocalin 2 (LCN2), myeloperoxidase (MPO) and matrix metalloproteinase 9 (MMP-9). These neutrophil-derived factors contribute to the reduction of tight junction proteins (claudin-5, occludin and ZO-1) and BBB breakdown. (Created with BioRender.com).

immune cells (Kratzer et al., 2012; Tietz and Engelhardt, 2015). The pial microvessel blood-CSF barrier across pial microvessels is also considered part of the BCSFB (Brøchner et al., 2015). Endothelial cells isolated from the pial vessels show similarities with the BBB for some general ultrastructural features and transendothelial electrical resistance (Allt and Lawrenson, 1997). However, pial microvessels show some differences in the junctional systems compared to the BBB and lack astrocyte and NVU proximity, suggesting these vessels are more permeable and easier to cross by leukocytes during inflammatory responses (Allt and Lawrenson, 1997; Cassella et al., 1997).

The meningeal tissue is now considered an active and complex immune hub (Alves De Lima et al., 2020). From a structural point of view, the meninges are divided into three main layers: dura, arachnoid and pia. Dura mater is the outermost layer and is securely attached to the periosteum of the skull (Protasoni et al., 2011). It is composed of a dense and thick fibrous tissue supplied with lymphatic vessels, meningeal arteries and veins (Protasoni et al., 2011). The arachnoid mater is an avascular, thin and translucent membrane forming a barrier between the dura mater and the CSF flowing in the SAS (Yasuda et al., 2013). This space includes trabeculae and collagen bundles generated by fibroblast-like cells, which connect the arachnoid to the pia mater (Alcolado et al., 1988; Saboori and Sadegh, 2015). The deepest layer of the meninges is the pia mater, a thin and transparent membrane composed of connective tissue permeable to solutes and containing a capillary network that nourishes the brain (Adeeb et al., 2013). The pia mater is anchored on the brain surface by astrocyte processes and together with the arachnoid matter forms the leptomeninges (Derk et al., 2021). In addition to their protective role, the meninges also constitute a route of drainage for brain interstitial fluid to lymphatic vessels and deep cervical lymph nodes (Natale et al., 2021), providing communication between the CNS and the immune system. The bona fide barriers from the meningeal tissue can be subdivided into three main interfaces: (1) the

BCSFB associated with the arachnoid layer separating the dura and its fenestrated vessels from the CSF; (2) the BCSFB at the level of SAS pial microvessels; and (3) the pial surface layer, which together with glia limitans is considered a brain-CSF barrier (Brøchner et al., 2015). The junctional components of the meningeal barriers are less studied, but recent work identified claudin-11 as a marker for the arachnoid BCSFB (Brøchner et al., 2015). However, further studies are needed to better understand the structural and molecular features of meningeal barriers and how they regulate brain function during health and disease.

Blood-brain barrier dysfunction during neuroinflammation

Neuroinflammation is a well-defined pathological feature of several neurodegenerative disorders and associates with BBB structural changes and increased vascular permeability (Zlokovic, 2008; Rossi et al., 2011; Zenaro et al., 2017; Liebner et al., 2018). For example, studies performed in stroke models and experimental autoimmune encephalomyelitis (EAE), the most common model of MS, have shown increased BBB permeability due to a reduction of claudin-5, occludin, and ZO-1 levels in the mouse brain (Jiao et al., 2011; Wang et al., 2016). Similar results were obtained in cerebral amyloid-beta angiopathy in AD post-mortem brains, suggesting the reduction of TJ molecules represents a general feature of BBB dysfunction across several brain diseases (Carrano et al., 2012). The redistribution of inter-endothelial junctional proteins during BBB dysfunction depends on their phosphorylation state, which can be affected by growth factors and inflammatory cytokines (Van Itallie and Anderson, 2018). Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that induces rapid phosphorylation of occludin and ZO-1, disrupting the TJs organization and promoting endothelial permeability (Antonetti et al., 1999; Wang et al., 2001; Storkebaum and Carmeliet, 2004; Murakami et al., 2009). Moreover, pro-inflammatory cytokines, including interferon (IFN)-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-1β, also affect the molecular mechanisms associated with TJs integrity (Capaldo and Nusrat, 2009). Particularly, IFN-γ can directly modify the barrier function of TJs in cultured brain ECs by inducing Rho kinase activity, leading to actin cytoskeletal contractions and junctional disruption (Bonney et al., 2019). In addition, IL-1β reduces the expression of ZO-1 and transendothelial electrical resistance, increasing the paracellular permeability, the expression of adhesion molecules, and leukocyte migration across BBB models *in vitro* (Labus et al., 2014). During neuroinflammation, the release of IL-1β by activated microglia increases the BBB leakage and suppresses the capacity of astrocytes to maintain BBB integrity. Furthermore, IL-1β stimulates astrocytes to produce pro-inflammatory cytokines, promoting BBB breakdown and increased leukocyte migration (Wang et al., 2014). Exposure of brain ECs to pro-inflammatory cytokines also triggers the expression of adhesion molecules, such as P- and E- selectins, VCAM-1 and intercellular adhesion molecule-1 (ICAM-1), which may bind their counter-ligands on activated circulating immune cells, allowing leukocyte extravasation in the inflamed brain (Wong and Dorovini-Zis, 1992; Rahman et al., 2000; Hauptmann et al., 2020). Despite being constitutively expressed on brain ECs, ICAM-1 is further up-regulated in response to pro-inflammatory mediators, and its engagement activates signal transduction pathways leading to increased BBB permeability (Adamson et al., 2002). BBB integrity can also be affected by oxidative stress, particularly by the imbalance between the generation of reactive oxygen species (ROS) and their elimination by scavenger systems. High levels of ROS trigger intracellular pathways leading to TJ rearrangement and matrix metalloproteinase (MMP) activation, which directly compromise BBB integrity. *In vitro* studies demonstrated that H_2O_2 induces cytoskeleton dysfunction and alters the localization of occludin and ZO-1, increasing paracellular permeability of brain endothelial cells (Lee et al., 2004; Anasooya Shaji et al., 2019). ROS also enhances the release of MMPs by brain endothelial cells, acting directly on TJ proteins and degrading the extracellular matrix (Aexander et al., 2002; Harada et al., 2012). Large amounts of ROS can be released by parenchymal-resident cells, such as activated microglia, leading to a loss of BBB integrity. Particularly, the microglial NADPH oxidase system is the main producer of superoxide anion (O_2^-), which damages ECs and increases BBB permeability (Kahles et al., 2007; Rojo et al., 2014). The principal source of ROS from CNS infiltrating peripheral immune system cells is represented by activated neutrophils, which are professional phagocytes with a strong NADPH oxidase machinery and may also contribute to BBB damage. Neutrophil adhesion to inflamed cerebral vasculature engages integrins leading to ROS production and release of both granule enzymes and complex web-like structures of decondensed DNA fibers and antimicrobial molecules, called neutrophil extracellular traps (NET) (**Figure 2**; Pietronigro et al., 2017). Thus, migrating neutrophils across the CNS barriers release inflammatory and cytotoxic factors promoting endothelial injury and vascular permeability.

Neutrophils-blood-brain barrier interplay

Leukocytes receive a variety of signals upon interaction with inflamed endothelial cells, facilitating a cascade of adhesive events, including tethering and rolling, integrin activation, arrest (or firm adhesion), luminal crawling and diapedesis (also called transmigration) (Ley et al., 2007; Vestweber, 2015). Tethering and rolling of circulating leukocytes in inflamed

CNS venules are mainly mediated by endothelial P- and E-selectin and their counter-ligands P-selectin glycoprotein ligand-1 (PSGL-1), TIM (T cell immunoglobulin and mucin domain)-1 glycoprotein and CD44 (Siegelman et al., 2000; Piccio et al., 2002, 2005; Battistini et al., 2003; Fabene et al., 2008; Rossi et al., 2011; Angiari and Constantin, 2014; Angiari et al., 2014; Pietronigro et al., 2016; Zenaro et al., 2017; **Figure 1**). Leukocyte arrest in CNS venules is mediated by the VLA (very late antigen)-4 integrin binding to VCAM-1 and LFA (lymphocyte functional antigen)-1 and Mac-1 integrins binding to ICAM-1 and ICAM-2 (**Figure 1**; Engelhardt and Ransohoff, 2005; Rossi et al., 2011). Whereas activated T cells mainly rely on VLA-4 (α4β1 integrin) for their intravascular arrest, neutrophils highly depend on β2 integrins (LFA-1 and Mac-1) to arrest and crawl on inflamed brain endothelium (Rossi et al., 2011; Gorina et al., 2014; Zenaro et al., 2015; Pietronigro et al., 2019). However, VLA-4 integrin represents an alternative pathway for neutrophil adhesion, whereas VCAM-1 is a marker of CNS vascular inflammation, suggesting that VLA-4-VCAM-1 interactions may also control neutrophil adhesion during CNS inflammatory diseases (Johnston and Kubes, 1999).

The capacity of neutrophils to increase vascular permeability has been known for four decades, and the interplay between neutrophil adhesion and vascular damage has been studied in several pathological conditions, including brain diseases (Wedmore and Williams, 1981; Wang and Doerschuk, 2002; DiStasi and Ley, 2009; Rossi et al., 2020). In stroke models, neutrophils adhere to cerebral microvessels one hour after stroke induction, suggesting they are early contributors to vascular dysfunction (Hallenbeck et al., 1986; Kataoka et al., 2004; Sienel et al., 2022). Neutrophils can also rapidly adhere in cerebral vessels after status epilepticus induction and contribute to endothelial damage promoting chronic recurrent seizures (Fabene et al., 2008). Interestingly, in both focal ischemia and epilepsy models, it has been suggested that intravascular neutrophil adhesion *per se* without transmigration is sufficient to induce BBB damage and neuronal dysfunction and death, pointing to neutrophil adhesion mechanisms as drug targets for CNS diseases (Fabene et al., 2008; Sienel et al., 2022).

Studies in EAE models also demonstrated the pathogenic role of neutrophils and the contribution of these cells to blood-spinal cord barrier (BSCB) disruption (Wu et al., 2010; Aubé et al., 2014). These data were confirmed by the analysis of postmortem CNS samples of MS patients showing infiltrated neutrophils in areas of BBB or BSCB leakage. However, whereas ICAM-1 and VCAM-1 have been shown to be upregulated on CNS microvascular endothelial cells during EAE, the molecular mechanisms controlling neutrophil adhesive interactions with the BBB or BSCB are still unclear (Steffen et al., 1994). A role for neutrophils has also been shown in AD, and our group has demonstrated that LFA-1 integrin mediates neutrophil interaction with brain ECs expressing ICAM-1 in transgenic mice with AD-like disease (Zenaro et al., 2015). In addition to the adhesion capacity on the vascular wall, neutrophils can also stall in brain capillaries, obstructing blood flow. Indeed, neutrophil depletion induces capillary reperfusion and reduces brain damage in experimental models of stroke and AD (Cruz Hernández et al., 2019; El Amki et al., 2020). Together, these studies suggest a complex detrimental role for neutrophils in CNS inflammatory diseases by increasing vascular permeability following adhesion to the ECs and inducing ischemic phenomena by plugging cerebral capillaries.

During inflammatory responses, neutrophils can release a plethora of factors potentially contributing to tissue injury. Furthermore, it has been known that adhesion on vascular endothelium *via* engagement of β2 integrins leads to neutrophil activation and consequent secretion of inflammation mediators such as ROS and cytotoxic granule enzymes (**Figure 2**; Richter et al., 1990; Cheung et al., 1993; DiStasi and Ley, 2009). Indeed, in animal models of stroke, NET formation and release of neutrophil elastase (NE) have been reported to induce increased BBB permeability, and the inhibition of these two factors reduces brain injury and promotes recovery (Kang et al., 2020). Neutrophils can also produce MMPs, which have been previously shown to represent a key factor in the induction of vascular damage (Aexander et al., 2002; Kolaczkowska and Kubes, 2013). MMPs were shown to be increased in the CNS during several neuroinflammatory conditions, including MS, stroke, epilepsy, and AD, although their presence was not clearly associated to neutrophil infiltration (Backstrom et al., 1996; Clark et al., 1997; Kieseier et al., 1998; Nygårdas and Hinkkanen, 2002; Solé et al., 2004; Wilczynski et al., 2008; Takács et al., 2010; Quirico-Santos et al., 2013; Py et al., 2014). However, among all MMPs produced by neutrophils, MMP-9 was highly correlated with vascular damage during diseases such as tuberculosis, myocardial infarction and cystic fibrosis (Kurihara et al., 2012; Halade et al., 2013; Garratt et al., 2015; Ong et al., 2017; Gelzo et al., 2022). MMP-9 was also associated with BBB dysfunction, and its expression is involved in the disruption of BBB TJs and degradation of basal lamina (**Figure 2**; Mun-bryce et al., 1998; Asahi et al., 2001; Moxon-Emre and Schlichter, 2011; Li et al., 2013). Furthermore, MMP-9 expression was clearly correlated with the presence of granulocytes in models of stroke and ischemia-reperfusion injury, in which neutrophils were considered the main source of MMP-9 (Romanic et al., 1998; Gidday et al., 2005; Turner and Sharp, 2016). In addition, by using MMP-9$^{-/-}$ mice, several studies confirmed the critical role of this enzyme in the induction of BBB permeability in stroke models and EAE (Dubois et al., 1999; Asahi et al., 2001; Agrawal et al., 2006; Svedin et al., 2007). Finally, MMP-9-positive neutrophils have been found surrounding brain microvessels with severe type IV collagen degradation and BBB breakdown in patients with stroke, clearly showing a strong association between neutrophils, MMP-9 and BBB damage also during human CNS disease (Rosell et al., 2008). Interestingly, the C-1562T polymorphism in the MMP-9 gene, which is

associated with an elevated MMP-9 expression, increases the susceptibility to neuropsychiatric conditions, suggesting that neutrophil MMP-9 may represent a pathogenic factor also in mental disorders (Rybakowski et al., 2009a,b).

Human MMP-9 is covalently linked to lipocalin (LCN2), which increases its stability and confers protection from proteolytic degradation (Tschesche et al., 2001). LCN2 expression is increased during aging and several neuroinflammatory conditions, including AD, MS and stroke suggesting a role for LCN2 in CNS disorders (Anwaar et al., 1998; Marques et al., 2012; Chou et al., 2015; Weng and Chou, 2015; Al Nimer et al., 2016; Dekens et al., 2017). However, whereas *in vitro* data suggested a possible role for LCN2 in preserving BBB integrity, other studies clearly showed that LCN2 expression was found in cerebral endothelial cells and neutrophils in a mouse model of stroke, and its inhibition significantly reduced BBB leakage *in vivo* (Du et al., 2019; Wang et al., 2020). In support of these data, LCN2 was increased in the CNS and CSF in patients with vascular dementia, correlating with reduced expression of TJ proteins and increased BBB permeability, thus suggesting a role for LCN2 in BBB dysfunction (Kim et al., 2017; Llorens et al., 2020).

Myeloperoxidase (MPO) is the most abundant protein stored in neutrophil azurophilic granules. It can be released upon β2 integrin cross-linking in neutrophils and its expression was associated with areas of myeloid cell infiltration in stroke and MS (**Figure 2**; Matsuo et al., 1994; Walzog et al., 1994; Breckwoldt et al., 2008; Chen et al., 2008; Sajad et al., 2009; Forghani et al., 2012, 2015; Pulli et al., 2015). The link between MPO secretion and endothelial damage has also been reported. Indeed, pharmacological inhibition of MPO attenuates endothelial dysfunction in a mouse model of atherosclerosis (Cheng et al., 2019). Notably, MPO-deficient mice showed decreased leakage in LPS-induced BBB inflammation compared to wild-type littermates (Üllen et al., 2013). These results were supported by *in vitro* data showing that the MPO-H_2O_2-Cl_2 system causes barrier dysfunction in primary brain microvascular endothelial cells by inducing alterations of TJs and AJs (Üllen et al., 2013). Using the MPO inhibitor 4-aminobenzoic acid hydrazide, BBB dysfunction was partially rescued, demonstrating a direct link between BBB increased permeability and MPO release (Üllen et al., 2013). Moreover, in EAE and stroke mouse models, inhibition of MPO with N-acetyl lysyltyrosylcysteine amide prevented BBB breakdown and reduced disease severity, further demonstrating a role for MPO in CNS barrier breakdown (Zhang et al., 2016; Yu et al., 2018). Of note, high expression of MPO was associated with ischemic stroke, and MPO inhibition had beneficial effects, suggesting that MPO may represent a therapeutic target in stroke (Malle et al., 2007; Kim et al., 2016; Kim H. J. et al., 2019; Wang et al., 2022). Interestingly, epidemiological studies associated MPO polymorphisms with an increased risk of neurodegenerative diseases (Reynolds et al., 1999; Crawford et al., 2001; Combarros et al., 2002; Zappia et al., 2004; Pope et al., 2006). Moreover, MPO^+ cells (probably neutrophils) were increased in the brain in subjects with AD and Parkinson's disease (PD) (Zenaro et al., 2015; Gellhaar et al., 2017), thus supporting a role for this enzyme in neurodegeneration. Importantly, recent studies by Smyth et al. have shown that MPO staining is mainly associated with infiltrating neutrophils in the AD brain, further providing evidence of a role for neutrophil-derived inflammatory factors in BBB dysfunction (Smyth et al., 2022).

Choroid plexus: A gateway for neutrophil infiltration

The ChP constitutively expresses adhesion molecules and cytokines supporting continuous immune surveillance of the CNS (Steffen et al., 1996; Meeker et al., 2012; Kunis et al., 2013; Lun et al., 2015; Strominger et al., 2018). Indeed, the stromal compartment of the ChP is populated by several types of cells, including immune cells of peripheral origin (Marques et al., 2012; Schmitt et al., 2012; Szmydynger-Chodobska et al., 2012; Marques and Sousa, 2015). During inflammatory responses, the ChP epithelium and blood vessels further upregulate adhesion receptors and chemokines, facilitating the migration of blood leukocytes into the CSF (Steffen et al., 1994; Vercellino et al., 2008; Marques et al., 2009, 2012; Kunis et al., 2013). In addition to immune cell migration, structural and functional changes at the level of ChP are present during various brain disorders, and BCSFB breakdown may precede clinical symptoms (Schwartz and Baruch, 2014; Alicioglu et al., 2017; Saul et al., 2020; Gião et al., 2022; Mold and Exley, 2022). During AD, the dysfunctional ChP is characterized by alterations of secretory, barrier, transport, and immune functions. Moreover, several imaging studies in AD subjects showed premature ChP aging with increased lipofuscin vacuoles and Biondi bodies compared to age-matched healthy controls (Miklossy et al., 1998; Wen et al., 1999; Serot et al., 2000; Krzyzanowska and Carro, 2012; Tadayon et al., 2020). In addition, the ChP of AD patients have a lower rate of CSF production (Silverberg et al., 2001), show an increase of several inflammatory transcripts and downregulation of claudin-5, claudin-11, and claudin-18, pointing to a role for ChP breakdown in disease development (Bergen et al., 2015; Kant et al., 2018). Expression of TJ proteins in ChP epithelial cells has also been studied in MS. Although the specific functions of individual TJ proteins are unclear, the analysis of post-mortem brain tissues from MS patients showed a selective loss of claudin-3 compared to control tissues. Claudin-3 displays ChP selectivity and is expressed at the apical parts of epithelial cells, acting as a sealant in a similar manner as claudin-5 at the BBB level (Steinemann et al., 2016). Confirming these data, mice lacking claudin-3 showed impaired BCSFB

function and a faster onset and increased EAE severity, clearly indicating a role for claudin-3 in ChP breakdown in CNS autoimmune diseases (Kooij et al., 2014).

Neutrophils can migrate into the CSF through a dysfunctional ChP in several CNS inflammatory conditions. Peripheral administration of the TLR2 ligand PAM3CSK4, a prototypic Gram-positive bacterial lipopeptide, induces the migration of neutrophils through the ChP barrier (Mottahedin et al., 2017). Neutrophils can also infiltrate the ChP in EAE mice and represent a source of CSF LCN2 during early disease phases (**Figure 1**; Marques et al., 2012). Supporting these data, neutrophils migrate into the ChP stroma in MS patients, and this process is probably favored by a high expression of adhesion molecules on ChP blood vessels (Vercellino et al., 2008; Rodríguez-Lorenzo et al., 2020). Similarly, in models of traumatic brain injury (TBI), the choroidal epithelium produces CXCL1 and CXCL2 chemokines, which promote neutrophil migration, supporting the view that ChP represents an entry point for neutrophils invasion of the damaged brain (**Figure 1**; Szmydynger-Chodobska et al., 2009). Also, neutrophils migrate in the ChP in stroke models, further showing that ChP is a key site for neutrophil infiltration (Otxoa-De-Amezaga et al., 2019). Despite a clear demonstration of neutrophil migration into the ChP during CNS diseases, the molecular mechanisms promoting neutrophil-dependent ChP damage are unclear. However, previous studies have shown a correlation between ChP dysfunction and increased expression of MMP-8 and MMP-9 (Batra et al., 2010). These two enzymes have been shown to mediate neutrophil-dependent damage in other pathological contexts, suggesting this may be the case also during CNS inflammatory conditions. Collectively, these data suggest that ChP is a gateway for neutrophil entry into the brain, and further studies are needed to better understand neutrophil contribution to BCSFB breakdown.

Neutrophil contribution to meningeal inflammation

The meninges also serve as an important route for immune cell entry into the CNS (Ransohoff et al., 2003; Derk et al., 2021). Indeed, dural venous sinuses were recently shown to be active sites of immune cell trafficking and contain both adaptive and innate immune cells (Mrdjen et al., 2018; Jordão et al., 2019; Van Hove et al., 2019; Rustenhoven et al., 2021). Particularly, recent results have shown that dural sinuses regulate T cell trafficking and contain APCs presenting CSF antigens to patrolling T cells, demonstrating a role for dura in CNS immune surveillance (Rustenhoven et al., 2021). Furthermore, by releasing cytokines, meningeal immune cells regulate several brain functions, including spatial learning (Derecki et al., 2010; Radjavi et al., 2014; Brombacher et al., 2021), short-term memory (Ribeiro et al., 2019), sensory responses (Oetjen et al., 2017) and adult hippocampal neurogenesis (Wolf et al., 2009). Notably, recent studies described meningeal-bone marrow channels directly feeding the meninges with immune cells (Herisson et al., 2018; Brioschi et al., 2021; Cugurra et al., 2021), suggesting that a dysfunctional meningeal immunity may contribute to CNS disorders, such as MS, AD, and PD (Russi and Brown, 2015; Silva and Ferrari, 2019; Zou et al., 2019; Mentis et al., 2021). The meningeal BSCFB is more permissive to the entry of immune cells and large molecules compared to the highly restrictive BBB (Ransohoff et al., 2003). The permeability changes of the arachnoid barrier were less studied in CNS diseases. However, recent studies showed that claudin 11, a TJ protein enriched in the arachnoid barrier, is downregulated in EAE mice (Uchida et al., 2019), indicating a disruption of the arachnoid BCSFB. Increased permeability of the leptomeningeal vessels has also been shown in MRI studies in several brain disorders, including stroke, CNS infections and cerebral amyloid angiopathy (Ineichen et al., 2022). Leptomeninges are now recognized as key players in the development of MS and EAE (Pikor et al., 2015; Russi and Brown, 2015; Rua and McGavern, 2018; Wicken et al., 2018). Indeed, subpial lesions are considered the most common type of lesions in MS patients and autoreactive effector T cells have been shown to infiltrate the leptomeninges via pial microvessels, demonstrating a fundamental immunological role for this vascular district in CNS autoimmunity (Bartholomäus et al., 2009; Schläger et al., 2016; Filippi et al., 2018). Accordingly, reports of gadolinium enhancement in the leptomeningeal compartment have been described in MS, confirming a role for meningeal inflammation in MS (Absinta et al., 2015).

Several studies performed in animal models of AD, MS, TBI, stroke and systemic inflammation have shown increased expression of the adhesion molecules, such as ICAM-1, VCAM-1 and P-selectin, as well as consequent leukocyte adhesion in pial microvasculature (Kerfoot and Kubes, 2002; Piccio et al., 2002; Mel'nikova, 2009; Zhou et al., 2009; Zenaro et al., 2015; Szmydynger-Chodobska et al., 2016; Dusi et al., 2019; Lodygin et al., 2019; Pietronigro et al., 2019). These findings support the idea that meningeal tissue represents a gateway for immune cell access into the CNS and open the possibility of a neutrophil contribution to BCSFB dysfunction (Walker-Caulfield et al., 2015). Indeed, studies performed in patients with stroke and its animal model demonstrated the presence of extravasated neutrophils in the leptomeninges, suggesting a role for neutrophils in meningeal inflammation (Perez-de-Puig et al., 2015; Kim S. W. et al., 2019). Also, in a mouse model of subarachnoid hemorrhage, neutrophils infiltrated the meninges and were associated with neuronal damage (Coulibaly et al., 2021). Notably, mice deficient for MPO lacked neuronal dysfunction suggesting this lysosomal enzyme represents a putative mechanism of neutrophil-dependent damage to the underlying brain tissue in subarachnoid hemorrhage

(Coulibaly et al., 2021). Furthermore, studies performed by our group in mouse models of AD showed that neutrophils migrate in pial vessels and brain parenchyma during early disease stages *via* an LFA-1-dependent mechanism promoting cognitive deficit and neuropathological hallmarks of AD (Zenaro et al., 2015). Furthermore, it has been suggested that neutrophil influx in the leptomeninges of EAE mice during pre-clinical stages of the disease promotes early BCSFB breakdown facilitating the access of other immune cells into the CNS (Christy et al., 2013). Overall, although current data provide evidence of neutrophil accumulation in the leptomeninges in models of CNS diseases, how neutrophils promote BCSFB dysfunction and neuronal damage is still largely unknown.

Conclusion and future directions

The role of neutrophils in the induction of BBB damage is now well documented in animal models of neuroinflammation and patients with CNS diseases. Although less investigated, the contribution of neutrophils to BSCFB breakdown is now emerging from recent studies on stroke, AD and MS. Neutrophil-mediated damage to CNS barriers since early disease stages may pave the way for the recruitment of other leukocyte populations during brain inflammatory diseases, and further studies are needed to clarify this interesting aspect (Christy et al., 2013; Rossi et al., 2020, 2021). However, whereas neutrophil accumulation in the meninges and ChP was clearly demonstrated, the intravascular adhesion mechanisms mediating neutrophil-endothelial interactions and the interplay between neutrophils and other barrier cells, such as ChP epithelial cells, are still unknown. Indeed, *in vivo* tracking of migrating neutrophils in animal models of CNS disorders using advanced microscopy together with non-invasive imaging of radioactive leukocyte tracers may improve our future understanding of neutrophil interaction with CNS borders (Mel'nikova, 2009; Zhou et al., 2009; Szmydynger-Chodobska et al., 2016; Lodygin et al., 2019; Pietronigro et al., 2019). Neutrophils release their arsenal of cytotoxic molecules during inflammatory responses, potentially causing significant collateral tissue damage (Kolaczkowska and Kubes, 2013). However, the molecular mechanisms contributing to neutrophil-dependent inflammation of CNS barriers leading to neuronal and glial cell dysfunction are largely unknown and may represent a new field of investigation. Finally, the elucidation of the molecular pathways underlying neutrophil contribution to CNS diseases may help design new therapies for neurological disorders such as stroke, MS and AD.

Author contributions

All authors contributed to the literature search and writing the review.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The stress hyperglycemia ratio is associated with the development of cerebral edema and poor functional outcome in patients with acute cerebral infarction

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Background and purpose: Absolute hyperglycemia at admission has been shown to be associated with the development of cerebral edema (CED) after acute cerebral infarction. Stress hyperglycemia is a more objective reflection of hyperglycemic state than absolute hyperglycemia. However, studies on the associations between stress hyperglycemia and CED are limited. We aimed to explore the associations of stress hyperglycemia, measured by stress hyperglycemia ratio (SHR), with the development of CED and poor functional outcome of acute cerebral infarction.

Methods: Patients with acute middle artery cerebral infarction admitted to the Department of Neurology, West China Hospital of Sichuan University, within 24 h of symptom onset from January 2017 to March 2021 were included. Stress hyperglycemia was assessed by the SHR: admission fasting plasma glucose (FPG)/hemoglobin A1c (HbA1c). The primary outcome was the degree of CED evaluated on brain image. The secondary outcomes were moderate-to-severe CED, poor functional outcome (modified Rankin Scale score > 2), and death at 90 days. The associations between the SHR and outcomes were assessed with multivariate logistic regression analyses. We further compared the predictive value of the SHR, admission random plasma glucose (RPG), and admission FPG for outcomes in the training dataset and validation dataset.

Results: 638 patients were enrolled. Each 0.1-point increase in the SHR was independently associated with a 1.31-fold increased risk of a higher degree of CED [odds ratio (OR): 1.31 (95% confidence interval (CI): 1.20–1.42), $P < 0.001$]. The SHR was independently associated with moderate-to-severe CED [per 0.1-point increase: OR: 1.39 (95% CI: 1.24–1.57), $P < 0.001$], poor functional outcome [per 0.1-point increase: OR: 1.25 (95% CI: 1.12–1.40), $P < 0.001$], and death [per 0.1-point increase: OR: 1.13 (95% CI: 1.03–1.25), $P < 0.05$]. The predictive value of the SHR (as a continuous variable), exhibited by the area

under the curve in receiver operating characteristic analysis, was higher than that of the RPG and FPG for moderate-to-severe CED and poor functional outcome ($P < 0.05$).

Conclusion: The SHR is independently associated with the severity of CED, poor functional outcome, and death after acute cerebral infarction, and the SHR (as a continuous variable) has a better predictive value for moderate-to-severe CED and poor functional outcome than the RPG and FPG.

KEYWORDS

stress hyperglycemia ratio, glucose, cerebral edema, functional outcome, death

Introduction

Cerebral edema (CED) is a pathophysiological process that occurs after acute cerebral infarction. Notably, the degree of CED often influences the prognosis of patients with acute cerebral infarction (Ferro et al., 2021). The mortality related to space-occupying infarcts by progressive CED within the first days after stroke onset can reach up to 80% (Berrouschot et al., 1998; Broocks et al., 2018). Recently, there has not been considerable progress in treatments to alleviate the development of CED. Therefore, identifying the related risk factors associated with progressive CED is important because they could guide early intervention.

Hyperglycemia is frequently observed in acute cerebral infarction, and it was estimated that 39–83% of diabetic patients and 8–63% of non-diabetic patients have elevated plasma glucose levels at admission (Kruyt et al., 2010). Stress reactions in acute severe illness or preexisting abnormalities in glucose metabolism have been proposed to account for hyperglycemia (Kruyt et al., 2010). Pathophysiologically, hyperglycemia can contribute to the damage of the blood–brain barrier (BBB) in acute ischemic stroke, which can then lead to brain edema or hemorrhagic transformation (Huang et al., 2013; Spronk et al., 2021). The absolute elevation of plasma glucose levels was found to be associated with malignant CED after large hemispheric infarction in previous studies (Shimoyama et al., 2014; Ong et al., 2017). Different from absolute hyperglycemia, stress hyperglycemia is a relative elevation of blood plasma glucose levels adjusted for background glucose, which is a more objective reflection of acute hyperglycemic state (Dungan et al., 2009; Roberts et al., 2015; Chen et al., 2022). Stress hyperglycemia is often evaluated by the stress hyperglycemia ratio (SHR) (Su et al., 2017; Zhu et al., 2019). The SHR is often defined as plasma glucose divided by hemoglobin A1c (HbA1c) and represents real transient hyperglycemia controlled for background plasma glucose (Su et al., 2017; Zhu et al., 2019). Stress hyperglycemia has been found to be associated with stroke recurrence, hemorrhagic transformation, neurological deficits, and mortality after acute ischemic stroke in many previous studies (Pan et al., 2017; Zhu et al., 2019; Li et al., 2020; Yuan et al., 2021). In addition, the SHR was shown to be a better prognostic indicator than the random plasma glucose (RPG) and fasting plasma glucose (FPG) for poor functional outcome in patients with acute cerebral infarction (Chen et al., 2022). However, to our knowledge, the number of previous studies on the association of stress hyperglycemia with the development of CED and its prognosis after acute cerebral infarction is limited, and a comparison of the predictive values of plasma glucose and the SHR for worse CED has not been reported.

The aim of our study was to explore the association of the SHR with the development of CED and its prognosis in patients with acute cerebral infarction and to compare the predictive values of the SHR, admission RPG, and FPG for worse CED and poor functional outcome.

Materials and methods

Study participants

We analyzed data from a prospectively collected database between January 2017 and March 2021, the Chengdu Stroke Registry described previously in Liu's research (Liu et al., 2019), in which the patients were consecutively recruited at the Department of Neurology, West China Hospital, Sichuan University. To assess the predictive value of the SHR for moderate-to-severe CED and poor functional outcome, data from 2017 to 2019 formed the training dataset and those from 2020 to 2021 formed the validation dataset. The inclusion criteria were as follows: (1) age $\geq$ 18 years, (2) admission within 24 h after symptom onset, and (3) involvement of the middle cerebral artery (MCA) territory of infarction, with or without the involvement of the adjacent territories. The exclusion criteria were as follows: (1) absence of the FPG data within 48 h after symptom onset or the HbA1c data during their hospital stay, (2) absence of the admission RPG data upon arrival at the hospital, (3) absence of brain imaging within 24–120 h after symptom onset, (4) venous blood samples for testing

FPG or RPG that were drawn after performing brain imaging, (5) parenchymal hemorrhage (PH) type 2 occurring before or at the same time as the head imaging to evaluate the degree of CED —the definition of PH type 2 is hemorrhage > 30% of the infarct area according to the European Cooperative Acute Stroke Study criteria (Hacke et al., 1995), and (6) bilateral cerebral infarction. This study was approved by the Biomedical Research Ethics Committee of West China Hospital, Sichuan University.

Data collection

We collected information on demographics; time from onset; history of diabetes mellitus and other vascular risk factors; presence of symptomatic occlusion of major cerebral arteries relevant to acute ischemic lesions on computed tomography angiography [carotid artery or middle cerebral artery (MCA) M1-M2], magnetic resonance angiography or digital subtraction angiography; admission RPG, FPG, and other laboratory tests (if FPG was tested more than once during hospitalization, we chose the earliest one); HbA1c; and key treatments during hospitalization (acute endovascular treatment, intravenous thrombolysis, antihypertensive therapy, insulin, oral hypoglycemic agents, antiplatelet therapy, statin). Stroke severity was evaluated with the National Institutes of Health Stroke Scale (NIHSS) score on admission by well-trained neurologists. Stroke etiology was classified based on the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) criteria (Adams et al., 1993). Diabetes mellitus was defined as physician-diagnosed diabetes mellitus. For patients who received acute endovascular treatment, successful [modified thrombolysis in cerebral infarction (mTICI) 2b–3] or unsuccessful reperfusion was also recorded (Dargazanli et al., 2018).

Assessment of stress hyperglycemia

The SHR was calculated to assess stress hyperglycemia according to the following formula: FPG (mmol/L)/HbA1c (%) (Zhu et al., 2019). Fasting venous blood samples were drawn to measure FPG within 48 h after symptom onset during the morning hours (range: 04:00–12:00) after an overnight fast. HbA1c was measured during hospitalization. FPG was tested by an enzymatic method, and HbA1c was measured by high-performance liquid chromatography analysis in the Department of Laboratory Medicine, West China Hospital.

Outcome measurements

The primary outcome was the CED grade on brain CT or brain MRI at 24–120 h after onset. The CED grade was classified according to the Safe Implementation of Thrombolysis in Stroke—Monitoring Study (SITS-MOST) protocol: CED-0: no CED; CED-1: focal brain swelling ≤ 1/3 of the hemisphere; CED-2: focal brain swelling > 1/3 of the hemisphere; and CED-3: brain swelling with midline shift (Wahlgren et al., 2007; Strbian et al., 2013). In patients who underwent brain imaging more than once, the most severe CED was chosen for assessment. Two well-trained neurologists reviewed the brain images. If there was disagreement, a decision was made with the help of a third neurologist. The secondary outcomes were moderate-to-severe CED (CED-2–3), 90-day poor functional outcome [modified Rankin Scale (mRS) score > 2 within 90 days after stroke], and 90-day death (death within 90 days regardless of causes). All included patients were followed up by telephone interviews 90 days after stroke.

Statistical analysis

The associations between the SHR and CED grades were assessed using ordinal logistic regression after verification of the proportional odds assumption across all CED degrees. The associations of the SHR with moderate-to-severe CED, poor functional outcome, and death at 90 days were assessed using a binary logistic regression model. The variables with $P < 0.05$ in univariate analysis that changed the odds ratio of the SHR by at least 10 percent in a multivariate logistic regression model were included in the model (Kernan et al., 2000). Some vital clinical variables that might be associated with outcomes were also included in the multivariate logistic regression model. Subgroup analysis was preset to validate the robustness of the results. The following modifiers were included in the subgroup analysis: age (≥ 65 vs. < 65 years), baseline NIHSS score (≥15 vs. <15), diabetes mellitus, acute endovascular treatment, and intravenous thrombolysis. Receiver operator characteristic (ROC) curves were used to calculate the predictive values of SHR, FPG, and RPG as both continuous and binary variables for predicting moderate-to-severe CED, poor functional outcome, and death at 90 days in the training cohort and validation cohort. As a binary variable, FPG was dichotomized to ≥ 7 mmol/L and < 7 mmol/L and RPG was dichotomized to ≥ 10 mmol/L and < 10 mmol/L based on previous studies (Zhang et al., 2021; Mac Grory et al., 2022). For the SHR, the ROC curve in the training cohort was applied to identify an optimized cutoff value (the maximum value of the Youden index) of the SHR to predict the onset of moderate-to-severe CED. The predictive value of that cutoff value for outcomes was assessed in both the training cohort and the validation cohort. The DeLong test package in MedCalc was used to compare the area under the curve (AUC) values of the SHR, FPG, and RPG. All statistical analyses were performed using R version 4.0.4 (R Foundation for Statistical Computing, Vienna, Austria), MedCalc 20.027 (MedCalc, Belgium), and SPSS version 25.0 (IBM Corp., Armonk, NY, United States). A two-tailed $P < 0.05$ was considered statistically significant.

Results

Baseline characteristics

From January 2017 to March 2021, 1795 patients who were diagnosed with acute middle cerebral artery infarction within 24 h after stroke onset were screened. Among them, 638 patients met the inclusion criteria, but not the exclusion criteria, for the final analysis (training dataset: n = 407; validation dataset: n = 231) (Figure 1). Baseline characteristics were well balanced between the included and excluded patients except for hyperlipidemia, previous ischemic stroke/TIA, and valvular heart disease (Supplementary Table 1). The baseline characteristics of the training and validation datasets are shown in Table 1. In the training dataset, the median [interquartile range (IQR)] age was 70 (61–79) years, 235 patients (57.7%) were male, and the median (IQR) NIHSS score was 10 (3–16). In the validation dataset, the median (IQR) age was 70 (62–78) years, 143 patients (61.9%) were male, and the median (IQR) NIHSS score was 10 (4–15). Compared with patients in the training dataset, those in the validation dataset were more likely to have hypertension (P = 0.019), hyperlipidemia (P = 0.001), diabetes mellitus (P = 0.029), large artery atherosclerosis and small artery occlusion as TOAST subtypes (P = 0.022), large artery occlusion (carotid artery or MCA M1-M2) (P = 0.004), statin treatment (P = 0.046), and endovascular treatment (P < 0.001). Patients in the validation dataset had a longer onset to admission time (P = 0.004) and higher FPG (P = 0.002) and SHR (P = 0.004) values. Fewer patients in the validation dataset had a history of smoking (P = 0.018) and previous stroke/transient ischemic stroke (TIA) (P = 0.009). In the training dataset, blood samples for testing FPG were collected at a median (IQR) time of 23.4 (16.8–41.6) h from onset, and the median (IQR) time from onset to brain imaging to assess the degree of CED was 56.1 (41.8–76.9) h. In the validation dataset, blood samples for testing FPG were collected at a median (IQR) time of 25.0 (17.8–41.9) h from onset, and the median time from onset to brain imaging to assess the degree of CED was 57.9 (45.0–78.1) h. The median (IQR) SHR value was 1.00 (0.88–1.16) in the training dataset and 1.06 (0.89–1.30) in the validation dataset. There were 20 patients lost to follow-up at 90 days in the training dataset (4.9%) and 21 patients in the validation dataset (9.1%).

Association between the stress hyperglycemia ratio and cerebral edema

In univariate analysis, the SHR; sex; atrial fibrillation/atrial flutter; onset to admission time; baseline NIHSS score; diabetes mellitus; large artery atherosclerosis, cardioembolism, and other etiologies of the TOAST classification; large artery occlusion [carotid occlusion or MCA occlusion (M1–M2)]; endovascular treatment; intravenous thrombolysis; oral hypoglycemic agents; and antiplatelet therapy were found to be associated with a worse CED grade (Table 2). In ordinal logistic regression (Table 2), the correlation with the SHR was significant [per 0.1-unit increase: odds ratio (OR): 1.31, 95% confidence interval (CI): 1.20–1.42, P < 0.001] after adjusting for potential covariates from the univariate analysis and some variables of clinical significance, such as age and RPG.

Eighty-five patients in the training dataset developed moderate-to-severe CED (20.9%). A ROC curve showed that the optimized cutoff value of the SHR to predict moderate-to-severe CED was 1.25 (Figure 2A). The SHR was dichotomized into groups of high (≥ 1.25) and low (< 1.25) SHR. In the training dataset, a higher SHR (≥ 1.25) remained independently associated with moderate-to-severe CED in multivariate logistic regression (OR: 6.87, 95% CI: 3.46–13.63, P < 0.001) (Supplementary Table 2).

Association between the stress hyperglycemia ratio and 90-day poor functional outcome

Two hundred and two patients in the training dataset had poor functional outcome (52.2%). In univariate analysis, the SHR; age; sex; atrial fibrillation/atrial flutter; previous ischemic stroke/TIA; baseline NIHSS score; large artery atherosclerosis, cardioembolism, and other etiologies of the TOAST classification; and large artery occlusion [carotid occlusion or MCA occlusion (M1–M2)] were associated with 90-day poor functional outcome (Table 3). The SHR was independently associated with the 90-day poor functional outcome after adjustment for potential confounders in model 1 (per 0.1-point increases: OR: 1.25, 95% CI: 1.12–1.40, P < 0.001) (Table 3). In model 2, compared with patients with a lower SHR, those with a higher SHR (≥ 1.25) had a 3.73-fold higher risk of poor functional outcome at 90 days (OR: 3.73, 95% CI: 1.74–7.97, P < 0.001) (Table 3).

Association between the stress hyperglycemia ratio and 90-day death

Sixty-six patients in the training dataset died (17.1%). In univariate analysis, the SHR; age; baseline NIHSS score; large artery atherosclerosis and cardioembolism of the TOAST classification; carotid occlusion; antiplatelet use; and statin use were associated with 90-day death (Supplementary Table 3). The SHR was independently associated with 90-day death with adjustment for potential confounders in model 1 (per 0.1-point increases: OR: 1.13, 95% CI: 1.03–1.25, P = 0.01). In model 2,

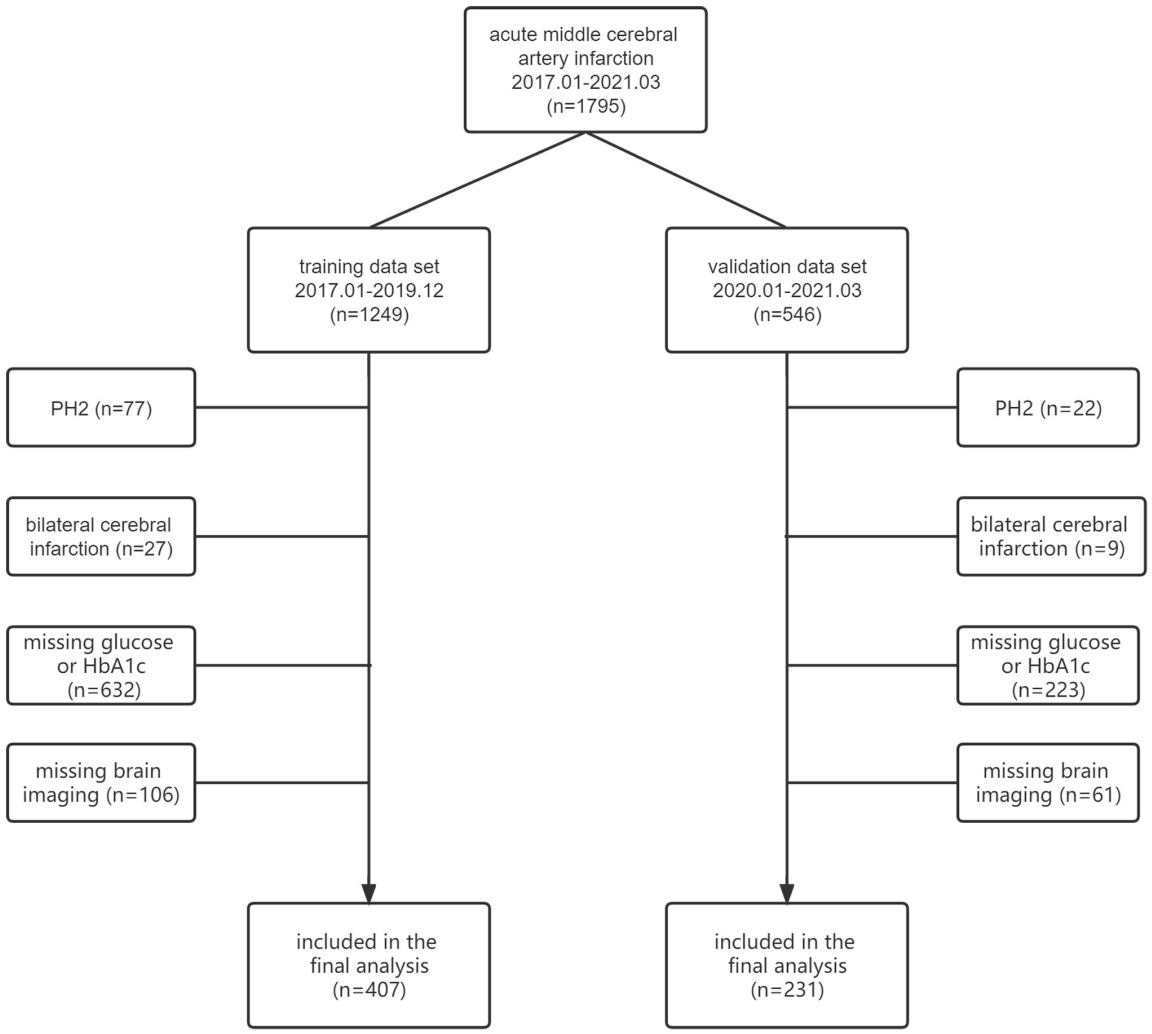

FIGURE 1
Flowchart of the screening of patients. PH, Parenchymal hemorrhage; HbA1c, hemoglobin A1c.

compared with patients with a lower SHR, those with a higher SHR (≥ 1.25) had a 2.79-fold higher risk of death at 90 days (OR: 2.79, 95% CI: 1.42–5.49, $P = 0.003$).

Subgroup analysis

Subgroup analysis was performed by age (≥ 65 vs. < 65 years), baseline NIHSS score (≥ 15 vs. < 15), diabetes mellitus, endovascular therapy, and intravenous thrombolysis (**Supplementary Figures 1–3**). There was a stronger association of a higher SHR with 90-day poor functional outcome (OR: 2.34 vs. 23.07; $P = 0.027$) in patients who received acute endovascular therapy compared with those who did not receive acute endovascular therapy (**Supplementary Figure 2**). In other subgroup analyses, no significant interaction between the SHR and stratified variables was observed ($P > 0.05$).

Receiver operator characteristic analysis

ROC analysis was applied to assess the performance of the SHR in predicting moderate-to-severe CED, 90-day poor functional outcome, and 90-day death (**Figure 2**). In the training dataset, compared with the RPG and FPG, the SHR had a significantly higher AUC value in the prediction of moderate-to-severe CED (AUC: 0.76, 95% CI: 0.72–0.80, $P < 0.001$) ($P < 0.01$). For the 90-day poor functional outcome, the predictive value of the SHR, as indicated by the AUC, was 0.67 (95% CI: 0.63–0.72, $P < 0.01$), which was significantly higher than that of the RPG ($P < 0.01$) and FPG ($P = 0.049$). For 90-day death, the predictive value of the SHR exhibited by the AUC was 0.64 (95% CI: 0.59–0.69, $P = 0.0011$), which was not significantly different from that of RPG or FPG ($P > 0.05$). In the validation

TABLE 1 Baseline characteristics of the training dataset and validation dataset.

Variables	Training dataset (n = 407)	Validation dataset (n = 231)	P-value
Age (year), median (IQR)	70 (61–79)	70 (62–78)	0.959
Male, n (%)	235 (57.7)	143 (61.9)	0.303
Onset to admission time (hour), median (IQR)	5 (3–24)	9 (4–24)	0.004
Vascular risk factors, n (%)			
Hypertension, n (%)	220 (54.1)	147 (63.6)	0.019
Hyperlipidemia, n (%)	19 (4.7)	27 (11.7)	0.001
Diabetes mellitus, n (%)	90 (22.1)	69 (29.9)	0.029
Atrial fibrillation/Atrial flutter, n (%)	146 (35.9)	73 (31.6)	0.275
Valvular heart disease, n (%)	43 (10.6)	16 (6.9)	0.127
Previous ischemic stroke/TIA, n (%)	49 (12.0)	13 (5.6)	0.009
Smoking, n (%)	131 (32.2)	54 (23.4)	0.018
Alcohol consumption, n (%)	63 (15.5)	50 (21.6)	0.05
Baseline NIHSS, median (IQR)	10 (3–16)	10 (4–15)	0.679
TOAST subtypes, n (%)			0.022
Large artery atherosclerosis	124 (30.5)	91 (39.4)	
Small artery occlusion	63 (15.5)	47 (20.3)	
Cardioembolism	133 (32.7)	57 (24.7)	
Other etiology	6 (1.5)	2 (0.9)	
Undetermined	81 (19.9)	34 (14.7)	
Occlusion site, n (%)			0.004
Carotid occlusion	75 (18.4)	60 (26.0)	
MCA occlusion (M1–M2)	130 (31.9)	87 (37.7)	
No record or other	202 (49.6)	84 (36.4)	
Treatment during hospitalization, n (%)			
Endovascular treatment	68 (16.7)	86 (37.2)	<0.001
Intravenous thrombolysis	57 (14.0)	41 (17.7)	0.211
Antihypertensive therapy	151 (37.1)	95 (41.1)	0.315
Insulin	54 (13.3)	34 (14.7)	0.61
Oral hypoglycemic agents	54 (13.3)	39 (16.9)	0.214
Antiplatelet	357 (87.7)	205 (88.7)	0.7
Statin	350 (86.0)	211 (91.3)	0.046
mTICI 2b-3*, n (%)	60 (88.2)	79 (91.9)	0.451
RPG (mmol/l), median (IQR)	7.36 (6.27–9.00)	7.26 (6.34–9.13)	0.488
FPG (mmol/l), median (IQR)	5.94 (5.19–7.32)	6.48 (5.28–8.34)	0.002
HbA1c (%), median (IQR)	6.00 (5.60–6.40)	5.90 (5.60–6.60)	0.557
SHR, median (IQR)	1.00 (0.88–1.16)	1.06 (0.89–1.30)	0.004

IQR, interquartile range; TIA, transient ischemic attack; NIHSS, the National Institutes of Health Stroke Scale; TOAST, Trial of Org 10172 in Acute Stroke Treatment; MCA, middle cerebral artery; mTICI, modified Thrombolysis in Cerebral Infarction; RPG, random plasma glucose; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; SHR, stress hyperglycemia ratio.

*The proportion of the patients receiving endovascular treatment.

dataset, for moderate-to-severe CED, the predictive value of the SHR (AUC: 0.72, 95% CI: 0.66–0.78, $P < 0.001$) was higher than that of the RPG and FPG ($P < 0.01$). For the 90-day poor functional outcome, the predictive value of the SHR (AUC: 0.70, 95% CI: 0.63–0.76, $P < 0.001$) was also higher than that of the RPG and FPG ($P < 0.05$). In the prediction of 90-day death, the SHR had a significantly higher AUC (AUC: 0.63, 95% CI: 0.56–0.70, $P = 0.017$) than the FPG ($p < 0.01$), while there was no significant difference compared with the RPG ($P = 0.31$).

As binary variables, for moderate-to-severe CED, in the training dataset, the predictive value of the SHR ($\geq$ 1.25 vs. $<$ 1.25) (AUC: 0.7, 95% CI: 0.66–0.75, $P < 0.01$) was higher than that of the RPG ($\geq$ 10 vs. $<$ 10) and FPG ($\geq$ 7 vs. $<$ 7) ($P < 0.01$) (Figure 3). For the 90-day poor functional outcome, the SHR (AUC: 0.61, 95% CI: 0.56–0.66, $P < 0.01$) had a significantly higher AUC value than the RPG ($P < 0.01$), but there was no significant difference between the AUCs of the SHR and FPG ($P = 0.23$). For 90-day death, the predictive value of the SHR (AUC: 0.64, 95% CI: 0.59–0.69) was higher than that of the RPG ($P < 0.01$), while there was no significant difference in the AUC between the SHR and FPG ($P = 0.41$). In the validation dataset, for moderate-to-severe CED and 90-day death, there was no significant difference in AUCs between the SHR and FPG, or RPG ($P > 0.05$). For the 90-day poor functional outcome, the predictive value of the SHR (AUC: 0.62, 95% CI: 0.55–0.69, $P < 0.01$) was higher than that of the RPG ($P = 0.0069$), while there was no significant difference in the AUCs between the SHR and FPG ($P = 0.82$). The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the SHR in the prediction of outcomes are presented in Supplementary Table 4.

TABLE 2 Associated factors for the development of CED on training dataset.

Variables	Univariate analysis, OR (95%CI)	*P*-value	Multivariate analysis, OR (95%CI)	*P*-value
Age	1.01 (1.00–1.03)	0.063	1.01 (0.99–1.02)	0.44
Male	0.65 (0.45–0.94)	0.023		
Hypertension	0.97 (0.67–1.39)	0.861		
Hyperlipidemia	0.99 (0.42–2.32)	0.976		
Atrial fibrillation/Atrial flutter	2.70 (1.84–3.97)	<0.001		
Previous ischemic stroke/TIA	0.66 (0.37–1.16)	0.146		
Valvular heart disease	1.00 (0.56–1.79)	0.992		
Smoking	0.70 (0.48–1.04)	0.075		
Alcohol consumption	0.67 (0.41–1.12)	0.128		
Diabetes mellitus	0.51 (0.32–0.80)	0.003	0.36 (0.19–0.69)	0.002
Onset to admission time	0.96 (0.94–0.98)	<0.001		
Baseline NIHSS	1.16 (1.13–1.19)	<0.001	1.10 (1.06–1.13)	<0.001
TOAST classification				
Large artery atherosclerosis	13.95 (6.14–31.74)	<0.001	3.4 (1.36–8.49)	0.009
Cardioembolism	17.31 (7.63–39.28)	<0.001	3.19 (1.26–8.03)	0.014
Others	12.72 (5.45–29.71)	<0.001	5.44 (2.18–13.54)	<0.001
Small-artery occlusion	Reference		Reference	
Occlusion site				
Carotid occlusion	14.68 (8.40–25.66)	<0.001	6.85 (3.63–12.94)	<0.001
MCA occlusion (M1–M2)	5.89 (3.75–9.26)	<0.001	3.83 (2.19–6.69)	<0.001
No record or other	Reference		Reference	
Endovascular treatment	2.28 (1.41–3.70)	0.001	0.41 (0.22–0.77)	0.005
Intravenous thrombolysis	1.70 (1.02–2.85)	0.044	1.19 (0.67–2.11)	0.56
Antihypertensive therapy	0.83 (0.57–1.21)	0.332		
Insulin	0.81 (0.47–1.38)	0.43		
Oral hypoglycemic agents	0.46 (0.26–0.80)	0.006		
Antiplatelet	0.40 (0.23–0.70)	0.001		
Statin	0.73 (0.43–1.22)	0.224		
mTICI (2b/3)	0.29 (0.07–1.19)	0.087		
RPG	0.98 (0.93–1.04)	0.535	1.03 (0.95–1.12)	0.52
SHR (per 0.1-point increases)	1.33 (1.24–1.43)	0.001	1.31 (1.20–1.42)	<0.001

OR, odds ratio; CI, confidence interval; TIA, transient ischemic attack; NIHSS, the National Institutes of Health Stroke Scale; TOAST, Trial of Org 10172 in Acute Stroke Treatment; MCA, middle cerebral artery; mTICI, modified Thrombolysis in Cerebral Infarction; RPG, random plasma glucose; SHR, stress hyperglycemia ratio.

Discussion

The main finding of our study is that the SHR is independently associated with the development of CED, poor functional outcome, and death at 90 days. Moreover, the SHR (as a continuous variable) has a better predictive value for moderate-to-severe CED and 90-day poor functional outcome than the RPG and FPG.

To our knowledge, few studies have focused on the SHR with CED (Cannarsa et al., 2022) and there have been no studies comparing the SHR with the FPG or RPG in the prediction of CED. Previous literature has mostly focused on the prognosis of mild stroke or a cohort of different acute ischemic stroke types (Pan et al., 2017; Tziomalos et al., 2017; Merlino et al., 2020; Yuan et al., 2021). In those studies, stress hyperglycemia was shown to be associated with a high risk of stroke recurrence, hemorrhagic transformation, or a worse long-term outcome. Moreover, stress hyperglycemia was found to be associated with acute cerebrovascular events because it might aggravate oxidative stress and endothelial dysfunction (Pan et al., 2017).

Stress hyperglycemia, measured by the SHR, which is defined as FPG/HbA1c, is a reliable reflection of a transient increase in blood glucose levels adjusting for the background blood glucose level (Su et al., 2017; Zhu et al., 2019). Compared with the chronic status of hyperglycemia, acute hyperglycemia is associated with greater oxidative stress, an increase in inflammatory factor levels, and neurohormonal derangements such as excessive elevations of glucagon, epinephrine, cortisol, tumor necrosis factor-α (TNF-α), and interleukin-1 levels (Dungan et al., 2009). The acute elevation of blood glucose levels in turn exacerbates these inflammatory factors, which might form a vicious cycle (Dungan et al., 2009). Hyperglycemia plays a critical role in the destruction of BBB integrity mediated by inflammatory factors and oxidative stress (Spronk et al., 2021). In addition, acute glucose fluctuation is harmful to the intact endothelium and promotes a stronger oxidative stress response (Dungan et al., 2009). Hyperglycemia was shown to aggravate

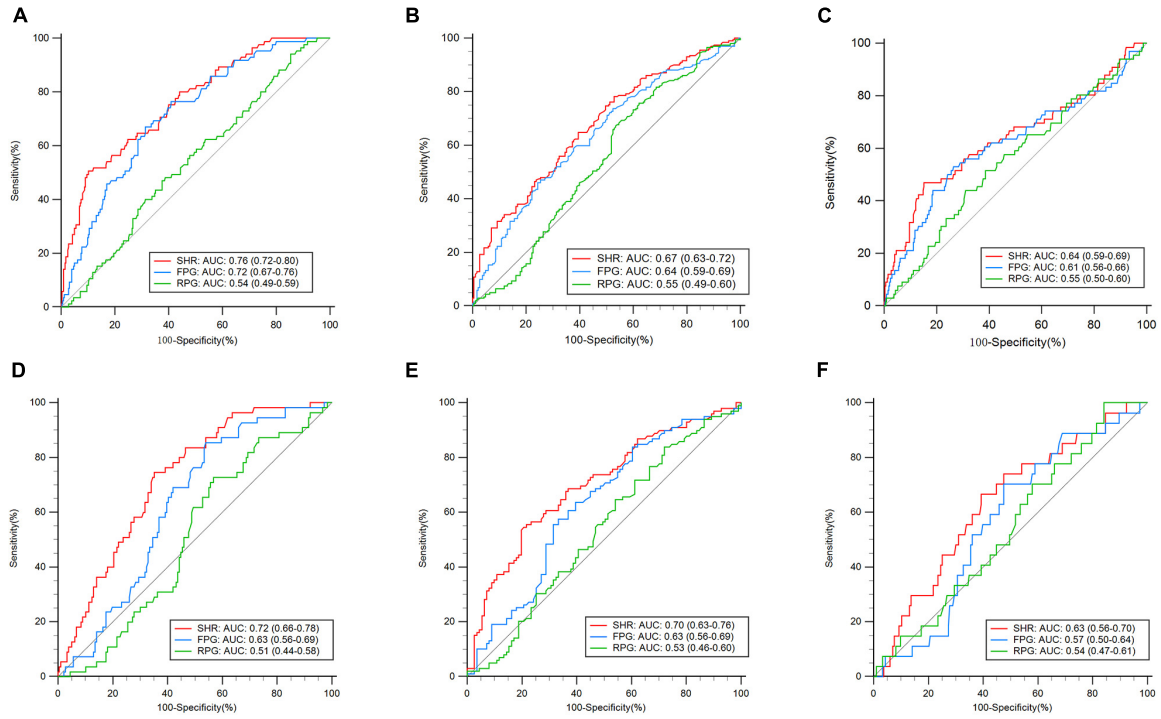

FIGURE 2
Receiver operating characteristic curve (ROC) analyses of the stress hyperglycemia ratio (SHR), admission fasting plasma glucose (FPG), and admission random plasma glucose (RPG) for the prediction of outcomes. Training dataset: **(A)** moderate-to-severe cerebral edema (CED), **(B)** 90-day poor functional outcome, and **(C)** 90-day death. Validation dataset: **(D)** moderate-to-severe CED, **(E)** 90-day poor functional outcome, and **(F)** 90-day death.

CED by disruption of the BBB in animal studies (Huang et al., 2013; Yuen et al., 2019). Our findings provide further evidence for the association between the SHR and the severity of CED after stroke.

For moderate-to-severe CED, 90-day poor functional outcome, and 90-day death, there was no significant interaction between the admission NIHSS score and the higher SHR ($\geq$ 1.25) with respect to the outcomes. While a previous study found that stress hyperglycemia only reflected stroke severity rather than having a direct association with adverse outcomes, the researchers found that patients with stress hyperglycemia had more severe stroke; however, when including the NIHSS score, stress hyperglycemia was not significantly associated with adverse outcomes (Tziomalos et al., 2017). Thus, the researchers believed that the SHR was a marker of the stress response mediated by cortisol levels, which was not directly associated with adverse outcomes (Tziomalos et al., 2017). In our study, when the NIHSS score was included in the multivariate model, the SHR was still independently associated with the outcomes. Our findings suggested that the SHR was directly associated with moderate-to-severe CED and poor functional outcome and was more than a marker of the stress response. Similar to our findings, previous literature suggested that the acute elevation of glucose levels in acute illness might promote a stronger inflammatory response, which can contribute to the disruption of the BBB (Spronk et al., 2021). Disruption of the BBB can lead to the development of CED or hemorrhagic transformation.

The definition of the stress hyperglycemia has varied in previous studies, and many studies focused on a stroke population without a history of diabetes mellitus, which might be due to the lack of a consensus definition of a cutoff value for stress hyperglycemia for patients with preexisting diabetes mellitus (Dungan et al., 2009; Yoon et al., 2016; Tziomalos et al., 2017; Zhu et al., 2019). However, considering patients with diagnosed diabetes mellitus is necessary because stress hyperglycemia can also occur in patients with diabetes mellitus. In our study, multivariate logistic regression showed that the SHR was independently positively associated with moderate-to-severe CED, 90-day poor functional outcome, and 90-day death, and further subgroup analysis showed no significant interaction between diabetes mellitus status and the higher SHR ($\geq$ 1.25) for the outcomes. Our result was similar to previous literature which demonstrated that elevated plasma glucose levels were prominently associated with poor outcomes regardless of a history of diabetes mellitus in acute ischemic stroke or acute myocardial infarction (Ishihara et al., 2007; Tsivgoulis et al., 2019).

TABLE 3 Predictive factors for the development of 90-day poor outcome (mRS > 2) on training dataset.

Variables	Univariate analysis, OR (95%CI)	*P*-value	Multivariate analysis OR (95%CI), model 1	*P*-value	Multivariate analysis OR (95%CI), model 2	*P*-value
Age	1.04 (1.02–1.06)	<0.001	1.04 (1.02, 1.06)	<0.001	1.04 (1.02, 1.06)	<0.001
Male	0.58 (0.39–0.87)	0.009				
Hypertension	1.18 (0.79–1.76)	0.43				
Hyperlipidemia	1.46 (0.56–3.86)	0.441				
Atrial fibrillation/Atrial flutter	2.35 (1.53–3.60)	<0.001				
Previous ischemic stroke/TIA	1.99 (1.05–3.76)	0.034	2.37 (1.08, 5.17)	0.031	2.30 (1.07, 4.94)	0.033
Valvular heart disease	1.19 (0.62–2.29)	0.597				
Smoking	0.90 (0.59–1.39)	0.636				
Alcohol consumption	0.59 (0.33–1.05)	0.074				
Diabetes mellitus	0.79 (0.49–1.28)	0.342	0.54 (0.25, 1.13)	0.102	0.64 (0.31, 1.31)	0.221
Onset to admission time	1.00 (0.98–1.02)	0.979				
Baseline NIHSS	1.14 (1.10–1.18)	<0.001	1.14 (1.09, 1.19)	<0.001	1.14 (1.10, 1.19)	<0.001
TOAST classification						
Large artery atherosclerosis	3.44 (1.71–6.89)	0.001				
Cardioembolism	4.18 (2.10–8.34)	<0.001				
Others	2.54 (1.22–5.29)	0.013				
Small-artery occlusion	Reference		Reference		Reference	
Occlusion site						
Carotid occlusion	5.11 (2.73–9.58)	<0.001	3.10 (1.41, 6.81)	0.005	3.05 (1.40, 6.64)	0.005
MCA occlusion (M1–M2)	1.76 (1.12–2.78)	0.014	1.13 (0.61, 2.09)	0.695	1.14 (0.62, 2.09)	0.676
No record or other	Reference		Reference		Reference	
Endovascular treatment	1.34 (0.79–2.29)	0.28	0.45 (0.21, 0.94)	0.035	0.54 (0.26, 1.13)	0.101
Intravenous thrombolysis	0.67 (0.38–1.19)	0.172	0.41 (0.20, 0.82)	0.012	0.40 (0.20, 0.80)	0.01
Antihypertensive therapy	0.95 (0.63–1.44)	0.807				
Insulin	1.19 (0.66–2.17)	0.564				
Oral hypoglycemic agents	0.76 (0.42–1.36)	0.349				
Antiplatelet	0.54 (0.29–1.03)	0.062				
Statin	0.63 (0.35–1.14)	0.125				
mTICI (2b/3)	0.42 (0.079–2.27)	0.316				
RPG	1.00 (0.94–1.07)	0.913	1.03 (0.94, 1.13)	0.5	1.04 (0.95, 1.13)	0.368
SHR (per 0.1-point increases)	1.32 (1.20–1.45)	<0.001	1.25 (1.12, 1.40)	<0.001		
SHR (≥ 1.25)	5.46 (2.88–10.35)	<0.001			3.73 (1.74, 7.97)	0.001

OR, odds ratio; CI, confidence interval; TIA, transient ischemic attack; NIHSS, National Institutes of Health Stroke Scale; TOAST, Trial of Org 10172 in Acute Stroke Treatment; MCA, middle cerebral artery; mTICI, modified Thrombolysis in Cerebral Infarction; RPG: random plasma glucose; SHR, stress hyperglycemia ratio.

For the 90-day poor functional outcome, we found that the positive effect of the SHR was more significant in patients who received acute endovascular therapy than in those who did not, with interactions between the SHR level and endovascular treatment status. Cannarsa suggested that the SHR was associated with malignant CED, intracranial hemorrhage, and poor functional outcome after mechanical thrombectomy, and the blood sample for testing plasma glucose was collected before mechanical thrombectomy (Cannarsa et al., 2022). In our study, the blood sample of testing FPG, which was used to evaluate the SHR, was obtained after acute endovascular treatment. Merlino showed stress hyperglycemia was associated with 90-day poor outcomes in patients undergoing mechanical thrombectomy, and the time of testing FPG was after mechanical thrombectomy as well (Merlino et al., 2021). Thus, it is necessary to be concerned about plasma glucose levels after acute endovascular treatment. Similar to our findings, a previous meta-analysis showed that there was a significant interaction between plasma glucose levels and endovascular treatment status (Chamorro et al., 2019). It was proposed that the redox-mediated harmful effects of glucose are common in endovascular treatment when successful reperfusion is achieved (Chamorro et al., 2019).

Admission plasma glucose has been demonstrated to be a risk factor associated with CED after acute ischemic stroke in previous studies (Shimoyama et al., 2014; Cheng et al., 2020; Dowlati et al., 2021). However, our findings showed that the RPG was not associated with this outcome, while the SHR was independently associated with a higher degree of CED, 90-day

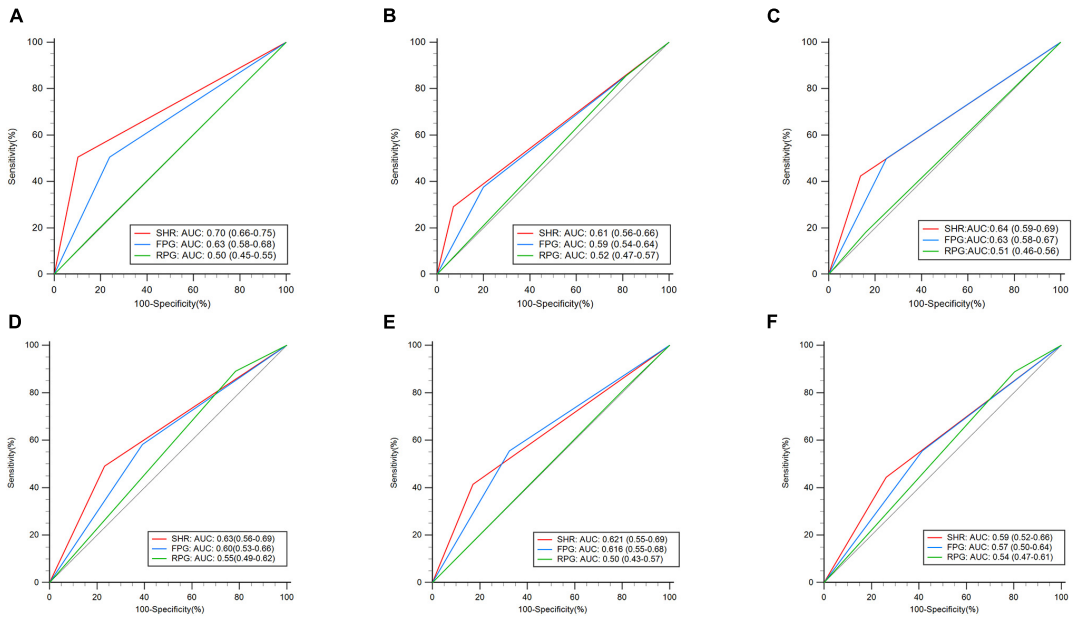

FIGURE 3
Receiver operating characteristic curve (ROC) analyses of the stress hyperglycemia ratio (SHR), admission fasting plasma glucose (FPG), and admission random plasma glucose (RPG) as binary variables for the prediction of outcomes. Training dataset: **(A)** moderate-to-severe cerebral edema (CED), **(B)** 90-day poor functional outcome, and **(C)** 90-day death. Validation dataset: **(D)** moderate-to-severe CED, **(E)** 90-day poor functional outcome, and **(F)** 90-day death.

poor functional outcome, and 90-day death, and the predictive value of the SHR (as a continuous variable) for moderate-to-severe CED and 90-day poor functional outcome, indicated by the AUC, was better than that of the RPG or FPG. Previous literature showed that the SHR was more strongly associated with outcomes than glucose levels: A previous study showed that stress hyperglycemia was associated with poor outcomes of acute cerebral infarction, such as stroke exacerbation, inpatient mortality, or functional deficits at discharge, while glucose levels were not (Roberts et al., 2021). In another study, glucose levels were not associated with critical illness (in-hospital death or critical care) in multivariate logistic regression analysis, while the SHR maintained a significant association. The definition of the SHR in that article was glucose divided by estimated glucose derived from HbA1c, which is similar to the concept of the SHR in our study (Roberts et al., 2015). Similarly, the SHR, rather than glucose, was considered to be a risk factor associated with in-hospital mortality in patients with acute myocardial infarction (Chen et al., 2021). Su's study showed that the SHR had an AUC value of 0.67, while glucose had an AUC value of only 0.52 in the prediction of 90-day all-cause mortality in acute illness (Su et al., 2017). It was proposed that the elevation of absolute glucose levels did not accurately reflect the real stress hyperglycemia state during acute illness without considering the impact of background plasma glucose. To the best of our knowledge, there is still a lack of research comparing the predictive value of admission plasma glucose with the SHR for CED. Previous predictive models of malignant CED, such as the Enhanced Detection of Edema in Malignant Anterior Circulation Stroke (EDEMA) score and DASH score, included admission plasma glucose as an item (Shimoyama et al., 2014; Ong et al., 2017). However, according to our study and previous literature, it was suggested to further compare the predictive power of the SHR and RPG in those models.

The baseline characteristics between the training dataset and the validation dataset were not totally similar. The main reason might be the development of acute endovascular treatment in our hospital in recent years, which led to the increasing number of patients with large cerebral artery occlusion and large artery atherosclerosis as TOAST subtypes. Notably, our results showed the SHR had the highest AUC value for outcomes in both the training dataset and the validation dataset, which reflected the transportability of our model.

The strength of this study is that the comparison of the predictive values of the RPG, FPG, and SHR for worse CED in acute cerebral infarction has not been explored before, to our knowledge. However, there were some limitations of our study: First, patients were excluded due to the lack of RPG, FPG, HbA1c, and brain imaging data, which might cause selection bias, and we compared the baseline information between the excluded and included patients to compensate for this. Second, our study was a single-center study and the results of our study should be validated by multicenter studies in future. Third, our studies only focused on patients with acute middle cerebral

infarction with or without the involvement of the adjacent territories, not for other types.

Conclusion

In our study, the SHR, measured by the FPG/HbA1c ratio, is independently associated with CED, poor functional outcome, and death after acute cerebral infarction. The SHR (as a continuous variable) appears to have a better predictive value for moderate-to-severe CED and poor functional outcome than the RPG and FPG. In future studies, exploring whether the predictive value of the model consisting of traditional risk factors for the development of CED will be improved by adding the SHR is significant.

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 Biomedical Research Ethics Committee of West China Hospital, Sichuan University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

YD contributed to the study design and data analysis and wrote the manuscript. MiL and SZ contributed to the study design and data analysis and edited the manuscript. SW, JL, and JW assembled the collected data. MeL and LW contributed to the study design and discussion. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2022.936862/full#supplementary-material

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