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Multimorbidity in the context of neurodegenerative disorders

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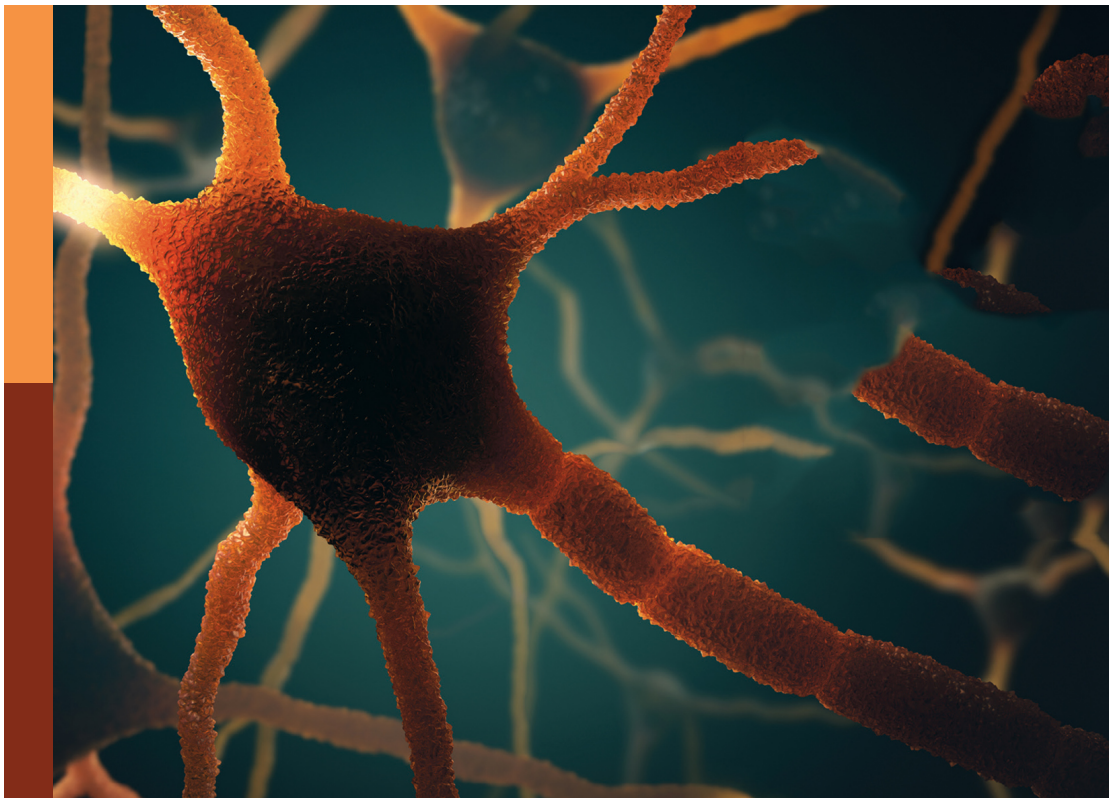

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Multimorbidity in the context of neurodegenerative disorders

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Editorial: Multimorbidity in the context of neurodegenerative disorders

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Editorial on the Research Topic
Multimorbidity in the context of neurodegenerative disorders

The term *multimorbidity*, when applied to one person, is defined as the co-occurrence of two or more medical conditions, none of which qualifies as an *index* disorder (Boyd and Fortin, 2010; Skou et al., 2022). Such a combination is found among either transient or long-term physical, infectious, and/or mental health conditions. Mechanisms underlying the development of multimorbidity are complex; for example, Skou et al. summarized these mechanisms in three areas, i.e., relating to aging and inflammation; socioeconomic, psychosocial and behavioral determinants of health; and medication-related (Skou et al., 2022). Systemic multimorbidity is highly prevalent in older people (Marengoni et al., 2011; Skou et al., 2022), which is also of concern due to the progressive increase in human longevity (Oeppen, 2019).

Multimorbidity often includes Neurodegenerative Disorders (NDs), most of which are incapacitating, incurable and lack disease-modifying treatments. It may deceive early diagnosis, especially in low- and middle-income countries, crippled by a shortage of advanced imaging equipment (Hricak et al., 2021) and poor capacity. Nonetheless, an increasing number of studies worldwide report multimorbidity in patients previously diagnosed with a single neurological disease (Habek et al., 2020; Borm et al., 2022). Such findings may explain the failure of many clinical trials, which follow the usual rule that a prospective patient of a clinical trial be rendered ineligible in the presence of unrelated comorbidities (Weiss et al., 2014; Marrie et al., 2016; Unger et al., 2019). Remarkably, experiments with organotypic brain slices *in vitro* suggest that the progressive growth of an area of silent pathology in the brain may synergize with the leading edge of a distinct subliminal pathology, and such a combination may react in unexpected ways to certain drugs currently prescribed for either such NDs (Simões-Pires et al., 2021).

Near-exponential increase of publications in the past 30 years highlights the progressive awareness of Multi- and/or Comorbidity (https://pubmed.ncbi.nlm.nih.gov/?term=Multimorbidity$+$OR$+$Comorbidity&sort=date),

inclusive among the hitherto small numbers of publications involving NDs (https://pubmed.ncbi.nlm.nih.gov/?term=%28%28Multimorbidity%29$+$OR$+$%28Comorbidity%29%29$+$AND$+$%28%28Neurodegeneration%29$+$OR$+$%28Neurodegenerative%29%29&sort=date), and both the interest and investment in this broad scientific field are recognized as of utmost importance (Navickas et al., 2016). Along these lines, the present Frontiers Research Topic aimed at novel approaches to multimorbidity in the context of Neurodegeneration, the contents of which are summarized here.

Santiago and Potashkin, from the Rosalind Franklin University of Medicine and Science, in Chicago, USA, offered an overview of the association of diabetes, cardiovascular disease, depression, and the gut microbiome, upon Alzheimer's disease (AD), and highlighted a variety of biological pathways involved in such comorbidities. Remarkably, inflammation stands out as a common dysregulated pathway shared by most of those comorbidities, and associated with increased risk of AD. In addition, certain drugs commonly prescribed for either diabetes or cardiovascular disease also show promising results in AD patients. The authors discussed the possible roles of both common dysregulated pathways, as well as genetic factors, in comorbidities associated with AD.

Luo et al., from the Medical Informatics Center at Peking University, in China, developed a novel multimorbidity index that incorporates disease combinations, as compared with individual diseases only, to predict 5-year mortality risk based on 13 chronic conditions among almost 12,000 community-dwelling older adults aged 65–84 years. The authors reported that the multimorbidity index incorporating disease combinations showed a better performance in predicting mortality among community-dwelling older adults. These findings strengthen the need to consider significant disease combinations to capture synergistic effects when evaluating multimorbidity in medical research and clinical practice.

Zhang et al., from Sichuan University, in China, tackled the problem that, despite abundant evidence that vascular risk factors (VRF) associate with cognitive impairment (CI), such association had not been studied in patients with multiple systems atrophy (MSA). The authors evaluated for CI a total of 658 patients with MSA only, MSA with predominant parkinsonism (MSA-P), and MSA with predominant cerebellar ataxia (MSA-C). All 3 groups had similar prevalences of CI, however, patients with more than one vascular risk factors were significantly more likely to have CI in both the MSA and MSA-P groups. Their findings that multiple VRF had a cumulative impact on CI in MSA patients highlights the need for comprehensive management of VRF in MSA.

El Idrissi and Alonso, from The City University of New York, USA, used a mouse model (PH-Tau-Tg) of Tau-induced neurodegeneration similar to that observed in Alzheimer disease, to test for interaction between pathological human Tau and Insulin signaling. The study showed that insulin signal transduction is altered in PH-Tau-Tg mice, and that injection of exogenous insulin reduces the excitability of cortical neuronal circuits. The authors propose that abnormal Tau may potentiate the toxic environment by interfering with the insulin signaling cascade and inducing insulin resistance in the brain of patients with Alzheimer's disease. The results favor the hypothesis that alterations in insulin signal transduction pathway may play a causative role in AD.

The article by the team of Stanisavljevic et al., of the University of Rhode Island, USA, aimed at testing the effects of Hypertension upon Cerebral Amyloid Angiopathy (CAA), a common comorbidity of Alzheimer's disease (AD). To that effect, the authors bred rTg-DI transgenic rats, a model of CAA, with spontaneously hypertensive stroke prone (SHR-SP) rats, producing bigenic rTg-DI/SHR-SP and non-transgenic SHR-SP littermates. The experiments showed, for example, that non-pharmacological hypertension in rTg-DI rats causes a redistribution of vascular amyloid and altered the size and distribution of thalamic occluded vessels. In addition, bigenic rTg-DI/SHR-SP rats provides a non-pharmacological model to further study hypertension and CAA as co-morbidities for CSVD and VCID.

Aiming at contributing to unveil the controversial relationship between vascular disease and Parkinson's disease, Ma et al. (a), from the Institute of Geriatric Medicine in Beijing, China, compared the total burden of cerebral small vessel disease (CSVD) in patients at either early or advanced Hoehn and Yahr (H&Y) scale stages of Parkinson's disease (PD), as well as in normal controls (NC). A total CSVD score was calculated for each participant, based on lacunes, high-grade white matter hyperintensities, enlarged perivascular spaces, and cerebral microbleeds. After adjusting for multiple variables, the data showed that higher H&Y stage correlated with increased total CSVD score. The overall analysis indicates that CSVD may play a critical role in patients with PD, and the total CSVD score is a potential neuroimaging marker for monitoring the progression of PD.

An article of the same group above, from the Institute of Geriatric Medicine in Beijing, China, used the diffusion tensor image analysis along the perivascular space (DTI-ALPS), to study the glymphatic system activity in patients with either early or late Parkinson's disease (PD) as compared with normal controls [NCs; Ma et al. (b)]. Patients with late, but no early, PD had lower ALPS index than NCs. Together with other results, the authors concluded that impairment of the glymphatic system is involved in PD, and that DTI-ALPS index may be a promising biomarker of the glymphatic system in PD patients.

In an article led by Butler et al., from Roche in Basel, Switzerland, in a case-control study data from a total of over 186,000 individuals, half of whom were diagnosed with Alzheimer's disease (AD), were examined to compare the prevalence of comorbidities between AD cases and individually-matched controls during the 5 years prior to diagnosis (or

index date for controls). Comorbidities were also identified with a differential time-dependent prevalence trajectory prior to AD diagnosis. The authors found a greater comorbidity burden among those who later developed AD than in controls, and identified five main comorbidity clusters three of which contained comorbidities that increased in frequency over time in AD cases but not in controls during the 5-year period before AD diagnosis. These clusters may help in distinguishing AD cases and non-cases.

Kim et al., from the Department of Neurosurgery of the Hallym University College of Medicine, in Anyang, South Korea, examined the Korean National Health Insurance Database to investigate whether patients with Alzheimer's disease (AD) or Parkinson's disease (PD) were more likely to contract COVID-19 and experience worse outcomes. The study showed that patients with COVID-19 infection were more likely to have a pre-existing AD diagnosis or a PD diagnosis (although the association did not reach significance for PD). In addition, having AD (but not PD) was associated with higher COVID-19-related mortality and both diseases were associated with higher odds of severe COVID-19 infection. The findings underscore the importance of COVID-19 prevention measures and the need for further research in the risk and outcomes of COVID-19 infections in patients with AD and PD.

Soon et al. sought to determine the extent to which an imaging technique could potentially help guide management for patients undergoing investigation for normal pressure hydrocephalus. Decisions about the appropriateness of a ventricular shunt placement are often based on the extent to which the symptoms of gait impairment, incontinence, and cognitive impairment, respond to external lumbar drainage. Novel 3-dimensional linear indices describe both the directional expansion of the ventricles and describes the distribution of fluids across compartments. Using a validated modified Frailty Index, the authors determined the frailty status in 21 individuals with probable normal pressure hydrocephalus. The morphological 3-directional morphological indices derived from MR imaging were not adequate to predict which patients responded to CSF drainage. The study reinforces the importance that neither frailty status nor MR findings should be used to predict clinical responsiveness to shunting. However, there may be utility in using this imaging to differentiate patients with NPH from those with Alzheimer's and health controls.

The article by She et al. examined the prevalence and pattern of comorbidity among Chinese patients with first-ever ischemic stroke within 2 weeks of admission to the University Hospitals in Shandong, China. Comorbidity was present in more than 90% of the stroke- patients; and was significantly higher among women. These comorbidities clustered into three patterns namely degenerative-cardiopulmonary disease, heart-gastrointestinal-psychiatric disease; and metabolic-kidney disease. On further investigating the association of comorbidities and physical dependence and cognitive functions among acute phase stroke patients, She et al. identified that a higher number of comorbidities was associated with a greater likelihood of physical dependence and cognitive impairment. Almost all the disease clusters were positively associated with the physical dependence and poor cognition among these patients. The findings from the study highlight the high burden of co-morbidity among stroke patients and the relevance of identifying these diseases which are associated with the physical and cognitive function of the patients. The authors reinforce the need for longitudinal studies to generate stronger evidence for this association.

In a longitudinal study among Japanese older adults without Cardio Vascular Disease (CVD), Makino et al. examined the prospective associations of absolute CVD risk using the WHO region-specific risk estimation charts, with the incidence of cognitive impairment (CI). Within a median follow up period of 48 $\pm$ 2 months, the incidence of cognitive impairment in any domain was 13% in this population. Higher level of CVD risk at baseline was significantly associated with higher risk of any type of cognitive impairment. However, this association was different between the subtypes of CI. While the baseline CVD risk predicted the incidence of non-amnestic CI, it was not associated with amnestic CI. Through this article the authors demonstrate that CVD risk level -which can be easily obtained in clinical practice- is a valuable tool for subtype specific screening of dementia.

We expect that the series of articles published in the current Research Topic will further our understanding of multimorbidity within the Nervous System, and stimulate new experimental models to better understand the interaction of two or more simultaneous conditions, as well as the design of novel therapeutic approaches to Neurodegenerative Diseases.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

MV received funding from F. Hoffmann-La Roche Ltd. and Biogen and consulted for F. Hoffmann-La Roche Ltd., outside of this study; MV has equity ownership in Abbott Laboratories, Johnson and Johnson, Medtronic, Merck, AbbVie and Amgen.

The remaining 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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REVIEW
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The Impact of Disease Comorbidities in Alzheimer's Disease

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A wide range of comorbid diseases is associated with Alzheimer's disease (AD), the most common neurodegenerative disease worldwide. Evidence from clinical and molecular studies suggest that chronic diseases, including diabetes, cardiovascular disease, depression, and inflammatory bowel disease, may be associated with an increased risk of AD in different populations. Disruption in several shared biological pathways has been proposed as the underlying mechanism for the association between AD and these comorbidities. Notably, inflammation is a common dysregulated pathway shared by most of the comorbidities associated with AD. Some drugs commonly prescribed to patients with diabetes and cardiovascular disease have shown promising results in AD patients. Systems-based biology studies have identified common genetic factors and dysregulated pathways that may explain the relationship of comorbid disorders in AD. Nonetheless, the precise mechanisms for the occurrence of disease comorbidities in AD are not entirely understood. Here, we discuss the impact of the most common comorbidities in the clinical management of AD patients.

Keywords: Alzheimer's disease, cardiovascular disease, comorbidities, depression, gut microbiome, inflammation

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INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disease affecting around 50 million people worldwide (Alzheimer's Association, 2016). Accumulation of extracellular amyloid beta plaques and intraneuronal neurofibrillary tangles are hallmark features of the disease (Bloom, 2014). Although several causative genetic factors have been identified, the vast majority of the cases are sporadic. Indeed, environmental factors and lifestyle choices appear to be the main determinants of the disease.

For several decades, AD patients have been classified according to several clinical measurement scales that primarily determine cognitive impairment status in patients. AD patients are staged into three main clinical categories that include pre-clinical AD, mild cognitive impairment (MCI), and overt AD (Albert et al., 2011). The current classification system does not consider important disease prognostic factors, such as the presence of coexisting disease conditions. Comorbid diseases may occur before or concomitantly with AD and may affect the disease's overall clinical status and progression. Several lines of evidence have established associations between AD and other chronic diseases, including diabetes, cardiovascular disease, depression, and inflammatory bowel disease (Casserly and Topol, 2004; Chatterjee and Mudher, 2018) (Ownby et al., 2006; Zhou et al., 2015) (Fu et al., 2020) (**Figure 1**). In addition to these diseases, neuropathological investigations have revealed an increasing frequency of overlapping co-pathologies, including co-aggregates of TDP-43 in AD patients' brains that could lead to faster progression and atypical clinical presentation

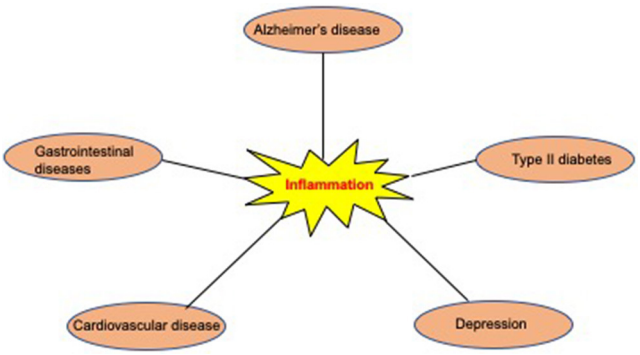

FIGURE 1 | Most common comorbidities associated with AD. Evidence from epidemiological and molecular studies suggest that several conditions including type 2 diabetes, cardiovascular diseases, depression, and gastrointestinal diseases may be associated with an increased risk for AD. Inflammation may be a central mechanism underlying the association between AD and most of its comorbidities.

(Matej et al., 2019). The presence of coexisting disease conditions may ultimately have a detrimental impact on AD patients' disease management. Understanding the biological mechanisms leading to comorbid diseases in AD may provide novel routes for therapeutic interventions. To this end, herein, we discuss the most prevalent disease comorbidities in AD and their impact on the clinical management of AD patients.

TABLE 1 | Epidemiological studies investigating the association between AD, dementia, cognitive impairment, and diabetes.

Study	Study design	Main results
Alzheimer's disease		
Janson et al. (2004)	Cohort study	Diabetes or impaired fasting glucose was present in 81% of AD patients
Cheng et al. (2012)	Meta-analysis	Diabetes associated with a higher risk of AD
Dementia		
Fontbonne et al. (2001)	Cohort study	Diabetes associated with a 2–3-fold increase risk of dementia
Biessels et al. (2006)	Systematic review	The incidence of dementia was higher in diabetes compared to non-diabetic patients
Cognitive impairment and dementia		
Rawlings et al. (2014)	Cohort study	Diabetes in midlife was associated with a 19% greater cognitive decline in 20 years
Geijselaers et al. (2017)	Cross-sectional	Diabetes associated with cognitive decline
Zheng et al. (2018)	Cohort study	Diabetes and HbAc1 levels associated with cognitive decline in 10 years follow up. Prediabetes associated with an increased risk of dementia
Xue et al. (2019)	Meta-analysis	Diabetes associated with a 1.25–1.9-fold increase in cognitive impairment and dementia
Marseglia et al. (2019)	Cohort study	Diabetes and prediabetes associated with accelerated cognitive impairment

ALZHEIMER'S DISEASE AND DIABETES

According to the World Health Organization, type 2 diabetes (T2D) is the most prevalent metabolic disease affecting 422 million people worldwide. Hyperglycemia and insulin resistance are characteristic features of the disease. Numerous lines of evidence support the association between T2D and AD. T2D is a well-established risk factor for AD, and AD is sometimes referred to as diabetes type 3 (Kandimalla et al., 2017; de la Monte, 2019). Substantial evidence from epidemiological studies indicates T2D is associated with an increased risk of AD in several populations. A systematic review of 14 longitudinal studies revealed a high risk of AD and vascular dementia among T2D patients (Biessels et al., 2006). This study suggested that vascular disease complications, alterations in insulin, glucose, and amyloid metabolism may underlie the association between both diseases (Biessels et al., 2006). Another study found a significantly lower cognitive performance among diabetic patients compared to healthy controls after 4 years follow-up period (Fontbonne et al., 2001). This study found a 2–3-fold increase in developing dementia in diabetes patients. Similarly, a cross-sectional study found that subjects with T2D performed worse in all cognitive domains than those with normal glucose metabolism (Geijselaers et al., 2017). Consistent with these findings, a recent meta-analysis of 144 prospective studies identified a 1.25–1.9-fold increase for cognitive impairment and dementia in patients with diabetes (Xue et al., 2019).

Glycated hemoglobin levels are an indicator of diabetes used by most clinicians. In this regard, a study identified significant longitudinal associations between hemoglobin A1c (HbAc1) levels, diabetes status, and accelerated cognitive decline over 10 years of follow-up (Zheng et al., 2018). Patients with prediabetes also displayed an increased risk for dementia, suggesting that even early alterations in glucose metabolism can trigger neurodegeneration. In support of these findings, a population-based cohort study showed that diabetes and prediabetes are associated with accelerated cognitive decline (Marseglia et al., 2019). A prospective cohort study found that diabetes in midlife was associated with more significant cognitive decline over 20 years compared to non-diabetic patients (Rawlings et al., 2014). In another study, 81% of AD patients exhibited impaired fasting glucose and diabetes (Janson et al., 2004), thereby demonstrating the high prevalence of diabetes among AD patients. The same study also identified islet amyloid, a pathological hallmark of diabetes, in AD patients compared to normal subjects. More extensive studies have corroborated these results. For example, a meta-analysis of 19 studies, including over 6,000 subjects with diabetes, showed that individuals with diabetes had a higher risk for AD than healthy controls (Cheng et al., 2012). Contrary to these findings, several studies have reported no association between diabetes and AD (Hassing et al., 2002; MacKnight et al., 2002; Akomolafe et al., 2006). A summary of the main findings of epidemiological studies addressing the relationship between diabetes and AD and cognitive decline is presented in **Table 1**.

Despite the numerous lines of evidence linking T2D and AD, this association's underlying mechanism remains poorly understood. Several mechanisms for this linkage have been postulated, including impaired glucose metabolism, vascular abnormalities, impaired insulin signaling, amyloidosis, and inflammation (Chatterjee and Mudher, 2018). For example, HbAc1, a measure of average blood glucose level, has been positively associated with the increased risk of cognitive decline and dementia in several studies (Yaffe et al., 2006; Rawlings et al., 2017; Zheng et al., 2018). Decreased brain glucose metabolism has been documented in subjects with MCI and T2D compared to those with MCI but not T2D suggesting that T2D may accelerate cognitive impairment (Li W. et al., 2016). In the context of vascular abnormalities, patients with T2D had a higher risk of cerebral amyloid angiopathy (Peila et al., 2002), a condition associated with brain infarcts and AD (Merlini et al., 2016; Noguchi-Shinohara et al., 2017).

Another potential mechanism linking AD and T2D is hyperglycemia. One notable example was illustrated using a murine model of AD wherein the induction of acute hyperglycemia increased amyloid beta in hippocampal interstitial fluid in young animals and prominent amyloid beta plaques in aged mice (Macauley et al., 2015). Nonetheless, more mechanistic studies are needed to determine whether these results can be recapitulated in humans.

Impaired insulin signaling is one of the most supported hypotheses linking T2D with cognitive decline and dementia. Alterations in the phosphatidylinositol 3-kinase and protein kinase B/Akt PI3K-AKT pathway in both T2D and AD patients have been observed by numerous studies suggesting this pathway may play a critical role in the development of AD among T2D patients (Liu et al., 2011; Gabbouj et al., 2019; Santiago et al., 2019). For example, decreased activity of several components of the PI3K-AKT pathway was found in the frontal cortex of both T2D and AD patients postmortem (Liu et al., 2011). Another study identified increased levels of insulin-like growth factor receptor (IGF-1R) and decreased levels of insulin receptor binding protein-2 (IGBP-2) in the temporal cortex of AD patients (Moloney et al., 2010). These findings provided evidence for the presence of insulin resistance in the brain of AD patients.

Amyloidosis is also a common shared pathological feature in T2D and AD. Accumulation of amylin polypeptide in pancreatic islets is present in 95% of T2D patients, and it has been demonstrated to impair islet function (Cooper et al., 1987). Furthermore, both amyloid β and amylin accumulate in tissues in response to innate immune responses or bacterial infections (Miklossy and McGeer, 2016). These findings support the hypothesis that T2D, like AD, may result from a protein misfolding mechanism (Mukherjee et al., 2015).

The immune system has been shown to play a pivotal role in the development of AD and T2D. Increased proinflammatory cytokines in both diseases is one of the most common findings identified in numerous studies. For instance, elevated cytokines and chemokines have been found in T2D (Boni-Schnetzler et al., 2008) and AD patients (Lai et al., 2017). Furthermore, increased levels of peripheral inflammatory markers are associated with disease progression in AD (Italiani et al., 2018). The proinflammatory cytokine tumor necrosis factor (TNF) is known to trigger insulin resistance (Hotamisligil et al., 1993) and exacerbate the accumulation of amyloid beta in AD models (Blasko et al., 1999; Liao et al., 2004). Therefore, targeting TNF signaling is being investigated as a potential therapeutic for AD (Decourt et al., 2017).

Diseases that share common dysregulated pathways are likely to share some of the same therapeutic targets. In this regard, drugs commonly prescribed for treating T2D have shown some promise in AD patients. Antidiabetic medications such as metformin and glucagon-like peptide 1 receptor agonists (GLP-1) have been investigated as potential AD therapies. For example, long-term and high dose metformin use was associated with a lower risk of incident AD in T2D patients (Sluggett et al., 2020). Similarly, a meta-analysis of 14 studies showed the use of metformin was associated with a reduced risk of dementia in T2D patients (Campbell et al., 2018). A small pilot study showed metformin associated with improved executive function, memory, and attention in a group of non-diabetic patients with MCI and AD (Koenig et al., 2017). Contrary to these findings, the potential neuroprotective effect of metformin has been challenged by other investigations. For instance, a population-based case-control study, including more than 7,000 individuals, found that long-term use of metformin associated with a greater risk of developing AD (Imfeld et al., 2012). Likewise, a cohort study including 4,651 elderly patients with T2D found that metformin's long-term usage increased the risk of developing PD, AD, and vascular dementia (Kuan et al., 2017). Therefore, additional larger prospective and randomized controlled trials are required to evaluate metformin as a potential drug for preventing AD.

Another promising group of antidiabetic drugs, the dual glucagon-like peptide and glucose-dependent insulinotropic peptide (GLP-1/GIP) receptor agonists, have shown neuroprotective effects in animal models of AD (Holscher, 2018; Zhang and Holscher, 2020). Exendin-4 (exenatide), a GLP-1 receptor agonist, has been shown to improve motor symptoms in PD clinical trials (Aviles-Olmos et al., 2013; Athauda et al., 2017). Recently, a double-blinded placebo-controlled trial found that exenatide was safe and well-tolerated in AD patients and lowered Aβ42 levels in extracellular vesicles (Mullins et al., 2019). However, exenatide treatment did not produce significant changes in cognitive measures and biomarkers in CSF. Notwithstanding, it is essential to note that the study evaluated a small number of subjects ($N = 21$) from a single center for 18 months. The small sample size and the early termination of the trial may explain the negative outcomes. A randomized placebo-controlled trial with 38 AD patients showed that liraglutide, another GLP-1 agonist, increased the blood-brain glucose transport capacity in the AD treated group compared to placebo (Gejl et al., 2017). This finding is promising in light of the several studies that suggest that reduction in the glucose transporters in the brain and impaired glucose metabolism may be early pathogenic events that exacerbate neurodegeneration in AD (Guo et al., 2005; Liu et al., 2008, 2009; Winkler et al., 2015). Future evaluation in more extensive and well-characterized clinical

TABLE 2 | Studies investigating the association between cardiovascular risk factors, dementia and AD.

Study	Study design	Main results
Alzheimer's disease		
Petrovitch et al. (2000)	Longitudinal cohort study	Elevated blood pressure in midlife associated with the development of neuritic plaques and neurofibrillary tangles in AD
Khachaturian et al. (2006)	Population-based cohort study	Use of antihypertensive drugs associated with a lower incidence of AD
Dementia		
Guo et al. (1999)	Community-based cohort study	Use of antihypertensive drugs associated with a decreased risk for dementia
van Dijk et al. (2004)	Community-based cohort study	Hypertension associated with severe white matter lesions in non-demented individuals
Peila et al. (2006)	Population-based cohort study	Use of antihypertensive drugs associated with a reduced risk for dementia and cognitive decline in men

trials will be valuable to determine their therapeutic potential for AD.

ALZHEIMER'S DISEASE AND CARDIOVASCULAR DISEASE

Cardiovascular risk factors have long been recognized as closely related to the development of AD. The impact of cardiovascular risk factors in AD has been documented at clinical and pathological levels (**Table 2**). The first studies that recognized a potential link between cardiovascular disease and AD correlated the presence of brain infarcts with greater cognitive decline and dementia compared to those without brain lesions (Snowdon et al., 1997). Concurrent cerebrovascular disease was documented to be more commonly observed in AD than in other neurodegenerative diseases (Toledo et al., 2013).

ALZHEIMER'S DISEASE AND STROKE

Cardiovascular diseases, including stroke, atrial fibrillation, and coronary heart disease, have been linked to AD. Lacunar strokes, also known as silent brain infarcts, are the most common type of ischemic stroke and results from the occlusion of blood vessels responsible for supplying deep brain structures. Several studies have shown that lacunar strokes greatly increase the risk of cognitive decline and AD. An earlier prospective study showed that the presence of silent brain infarcts at baseline more than doubled the risk of dementia (Vermeer et al., 2003). Similarly, silent brain infarcts are associated with brain atrophy and increased risk of cognitive impairment and dementia (Thong et al., 2013). These findings have been supported by larger studies. For example, a meta-analysis of 7 cohort studies and 2 nested case-control studies showed that stroke increased risk for AD (Zhou et al., 2015). Furthermore, white matter lesions, characteristic of ischemic stroke, are associated with cognitive decline and AD (Prins et al., 2004; Inaba et al., 2011). Another study found that increased fibrinogen associated with a greater increased in dementia in older subjects with white matter lesions (Hainsworth et al., 2017). This study suggested that some degree of blood-brain barrier dysfunction in older people may be related to risk for dementia. The main results from epidemiological studies investigating the association between AD and cardiovascular disease are presented in **Table 3**.

TABLE 3 | Epidemiological studies investigating the association between AD, dementia, cognitive impairment and cardiovascular disease.

Study	Study design	Main results
Alzheimer's disease		
Hofman et al. (1997)	Population-based study	Atherosclerosis associated with a higher risk for AD and vascular dementia
Bunch et al. (2010)	Prospective cohort study	Atrial fibrillation associated with senile, vascular, and Alzheimer's dementia
Inaba et al. (2011)	Cohort study	White matter lesions associated with cognitive decline and AD
Zhou et al. (2015)	Meta-analysis	Stroke increased the risk of AD
Dementia		
Vermeer et al. (2003)	Cohort study	The presence of silent brain infarcts more than double the risk of dementia
Newman et al. (2005)	Longitudinal cohort study	Coronary heart disease and peripheral artery disease associated with an increased risk for dementia
van Oijen et al. (2007)	Population-based, prospective cohort study	Atherosclerosis associated with an increased risk for dementia
Ikram et al. (2008)	Population-based cohort study	Men who suffered from myocardial infarction had an increased risk of dementia
Deckers et al. (2017)	Meta-analysis	Coronary heart disease associated with an increased risk for cognitive impairment and dementia
Cognitive impairment		
Ott et al. (1997)	Cross-sectional, population-based study	Atrial fibrillation associated with cognitive impairment and dementia
Knecht et al. (2008)	Cross-sectional	Atrial fibrillation associated with cognitive impairment and hippocampal atrophy
Roberts et al. (2010)	Population-based cohort study	Coronary heart disease associated positively with non-amnestic mild cognitive impairment
Marzona et al. (2012)	Randomized controlled trial	Atrial fibrillation associated with an increased risk of cognitive decline in the absence of overt stroke
Thong et al. (2013)	Cohort study	Silent brain infarcts associated with cognitive impairment

ALZHEIMER'S DISEASE AND ATRIAL FIBRILLATION

Similarly, atrial fibrillation is another cardiovascular disease associated with an increased risk for AD. Atrial fibrillation is characterized by an irregular often rapid heart rate resulting in poor blood flow. This condition could lead to blood clots, stroke, heart failure, and other cardiovascular diseases. A diagnosis of atrial fibrillation correlated positively with cognitive impairment and dementia, with a stronger association in women, in a large cross-sectional, population-based study (Hainsworth et al., 2017). Interestingly, the association was stronger for AD with cerebrovascular disease than for vascular dementia (Ott et al., 1997). A meta-analysis of 14 studies identified a positive association between atrial fibrillation and dementia (Kwok et al., 2011). However, further analysis with patient stratification showed the association was significant in studies focusing solely on stroke (Kwok et al., 2011). Nevertheless, even in the absence of stroke, atrial fibrillation has been associated with cognitive decline and hippocampal atrophy (Knecht et al., 2008). These results were confirmed by other large studies wherein cognitive and functional decline was positively associated with atrial fibrillation in the absence of overt stroke (Bunch et al., 2010; Marzona et al., 2012).

The underlying mechanism by which atrial fibrillation is linked to AD is unknown. It has been proposed that cerebral hypoperfusion and low cardiac output resulting from atrial fibrillation cause damage to the nerve cells contributing to neurodegeneration in AD (de Bruijn and Ikram, 2014). However, it remains unknown whether atrial fibrillation contributes to neurofibrillary tangles and amyloid plaques, pathological hallmarks of AD. One study found that atrial fibrillation is associated with large ischemic lesions but not AD neuropathology (Dublin et al., 2014). The same study, however, documented that neuropathological changes associated with AD were more common in people with permanent atrial fibrillation (Dublin et al., 2014). Therefore, the evidence linking atrial fibrillation with AD neuropathology is scarce and more studies are needed to understand the underlying mechanism. The main results from epidemiological studies investigating the association between AD and atrial fibrillation are presented in **Table 3**.

ALZHEIMER'S DISEASE AND CORONARY HEART DISEASE

Coronary heart disease (CHD) is another condition within the cardiovascular disease spectrum that has been implicated in AD. CHD is the most common heart disease and one of the leading causes of death worldwide. There is evidence that CHD increases the risk of cognitive impairment and dementia but there are some discrepancies among the studies. For example, a longitudinal cohort study revealed that the incidence of dementia was higher in subjects with CHD, particularly in those with peripheral arterial disease, compared to normal subjects (Newman et al., 2005). This result remained significant after the exclusion of vascular dementia (Newman et al., 2005). A population-based cohort study found a positive association between CHD and non-amnestic MCI but not amnestic MCI (Roberts et al., 2010). Nevertheless, some studies have found no association between CHD and AD or dementia. A population-based case-control study including 557 dementia cases suggested that coronary artery bypass grafting was not associated with dementia or AD (Knopman et al., 2005). Similarly, a larger population-based study including 3,734 Japanese-American men failed to find a significant association between coronary artery bypass surgery and permanent cognitive impairment (Petrovitch et al., 1998). Several factors including sample size, methods, patient selection, population genetics and environmental factors may explain the differences among the studies.

The association between CHD and AD has been reinforced by larger epidemiological studies. For instance, a larger population-based cohort showed that men with unrecognized myocardial infarction had an increased risk of dementia (Ikram et al., 2008). A meta-analysis of 10 prospective cohort studies showed that CHD increased the risk of cognitive impairment and dementia (Deckers et al., 2017). Consistent with these findings, a more recent and larger meta-analysis including 16 CHD studies (1,309,483 individuals), and seven heart failure studies (1,958,702 individuals), showed a 27 and 60% increased risk of dementia among CDH and heart failure patients, respectively (Wolters et al., 2018).

Although there are some discrepancies among epidemiological studies, most of the studies suggest CHD is a risk factor for cognitive impairment and dementia. Interestingly, atherosclerosis has been suggested as the underlying mechanism linking CHD to dementia. For example, neuropathological examination in 1,000 subjects revealed that more than 77% of AD subjects had apparent circle of Willis atherosclerosis (Yarchoan et al., 2012). In addition to intracranial vessels, atherosclerosis in extracranial vessels has been linked to AD. For instance, subjects with severe carotid and femoral atherosclerosis showed a 3-fold increase risk of dementia (Hofman et al., 1997). This positive association was even stronger in subjects with both atherosclerosis and apolipoprotein epsilon 4 (APOEε4) genotype (Hofman et al., 1997). Another prospective cohort study also found a positive association between atherosclerosis and dementia but failed to identify differences among APOEε genotypes (van Oijen et al., 2007). The linkage between atherosclerosis and AD may be related to alterations in cholesterol homeostasis and inflammatory processes. Elevated serum cholesterol levels and inflammation are two main determinants in the pathogenesis of atherosclerosis and these are intimately associated with AD (Notkola et al., 1998; Casserly and Topol, 2004; Liu et al., 2020). A summary of the main findings of epidemiological studies addressing the relationship between AD and CHD is presented in **Table 3**.

ALZHEIMER'S DISEASE AND CARDIOVASCULAR RISK FACTORS

In addition to cardiovascular diseases *per se*, risk factors for cardiovascular diseases including hypertension,

hypercholesterolemia, and obesity have been associated with an increased risk for AD. For example, non-demented individuals with hypertension had a higher risk of severe white matter lesions compared to healthy subjects (van Dijk et al., 2004). Moreover, in a longitudinal study with 36 years of follow up, elevated systolic blood pressure in mid-life is associated with the development of neuritic plaques and neurofibrillary tangles, characteristic of AD (Petrovitch et al., 2000).

Given the link between hypertension and cognitive decline, antihypertensive drugs have been investigated as potential therapeutics for dementia. Most of the longitudinal studies have found an inverse relationship between the use of antihypertensive drugs and dementia. For example, a longitudinal study including 1,810 individuals showed that non-demented subjects taking antihypertensive drugs had a lower risk of dementia (Guo et al., 1999). Similarly, the use of any antihypertensive drug was associated with a lower incidence of AD (Khachaturian et al., 2006). Further analysis revealed that the use of potassium-sparing diuretics is associated with a greater reduction in the risk of AD (Khachaturian et al., 2006). Interestingly, another study revealed that for each year of antihypertensive treatment there was a reduction in the incidence rate of dementia compared to subjects never treated with antihypertensive drugs (Peila et al., 2006). Nonetheless, some studies showed no benefit in the use of antihypertensive drugs for cognitive decline and dementia (Morris et al., 2001; Lindsay et al., 2002; Yasar et al., 2005).

ALZHEIMER'S DISEASE AND DEPRESSION

A history of depression has been associated with an increased risk of developing AD later in life. Depression is very common among the elderly and it is characterized by the loss of appetite, sleep disturbances, loss of energy, and fatigue, among many other symptoms (Ownby et al., 2006). Depression is linked to cognitive impairment and overall functional capacity in AD patients (Espiritu et al., 2001). Both depression and AD have a great impact on the quality of life and daily activities of patients. For example, a diagnosis of depression and biomarkers for AD is associated with a decline in driving performance on a road test suggesting patients with these conditions present with significant challenges when driving (Babulal et al., 2018). Earlier epidemiological studies suggested a positive association between depression and AD (Kokmen et al., 1991; Speck et al., 1995). Indeed, a case-control study claimed that depression may appear 10 years before the onset of dementia (Speck et al., 1995). These results were further supported by a later case-control study that found that the first signs of depression may appear 25 years earlier before the onset of dementia (Green et al., 2003). Collectively, these earlier studies suggested depression may be one of the earliest signs of dementia. Nevertheless, other case-control studies did not find a significant association between both diseases (French et al., 1985; Broe et al., 1990).

Likewise, cohort studies identified a significant association between depression and a greater risk of dementia. A retrospective cohort study including 19,000 patients supported the hypothesis that depression is a predictor of future dementia (Buntinx et al., 1996). Similarly, another study showed a positive association between depressed mood and risk for dementia (Devanand et al., 1996). Furthermore, a systematic review and meta-analysis of both case controls and cohort studies identified a positive association between depression and increased risk for AD (Ownby et al., 2006). Of note, this study indicated that depression, rather than a prodromal symptom, may be a risk factor for AD. The main results from epidemiological studies investigating the association between AD and depression are presented in **Table 4**.

TABLE 4 | Studies investigating the association between depression, dementia, and AD.

Study	Study design	Main results
Alzheimer's disease		
Kokmen et al. (1991)	Population-based case-control study	Episodic depression associated positively with AD
Devanand et al. (1996)	Prospective longitudinal study	Depressed mood moderately increased the risk for AD
Green et al. (2003)	Cross-sectional, case-control study	Depression symptoms may occur 25 years before the onset of AD
Ownby et al. (2006)	Systematic review, meta-analysis	Depression associated with an increased risk of AD
Dementia		
Speck et al. (1995)	Case-control study	Depression may occur 10 years before the onset of dementia
Buntinx et al. (1996)	Retrospective cohort study	Old age depression may be a predictor of subsequent dementia

The mechanisms underlying the association between depression and AD are not well-understood. Very few studies have identified shared genetic risk factors between both diseases including APOEε (Stewart et al., 2001) and complement receptor 1 (CR1) (Hamilton et al., 2012), however, there are some discrepancies (Zubenko et al., 1996; Mauricio et al., 2000). Recently, a larger genome-wide association study did not identify shared genetic variants between depression and AD (Gibson et al., 2017). These findings suggest that the underlying mechanism explaining the association between depression and AD may not be explained by shared genetic factors.

Another potential mechanism linking both depression and AD may be related to inflammation and vascular disease. In this regard, higher levels of TNF and apoptotic signaling ligand FAS have been documented in patients with depression and heart disease (Parissis et al., 2004). Moreover, the upregulation of proinflammatory cytokines associated with depression, atherosclerosis, and subsequent coronary heart disease in women (Suarez et al., 2004). The connection between depression and inflammation has been further reinforced by the finding that treatment with antidepressants resulted in the alteration of pro and anti-inflammatory cytokines (Castanon et al., 2002). Conversely, treatment with anti-inflammatory drugs and cytokine inhibitors have elicited antidepressant effects (Kohler

et al., 2016). Collectively, these results suggest that inflammatory processes may be intimately related to the development of depression and the use of anti-inflammatories may be a potential therapeutic strategy against depression.

ALZHEIMER'S DISEASE AND THE GUT MICROBIOME

The human gastrointestinal tract is home to trillions of microorganisms collectively called the gut microbiome. Dysbiosis of the human gut microbiome has been linked to numerous diseases including respiratory, metabolic, autoimmune, and neurodegenerative diseases (Lynch and Pedersen, 2016; Dinan and Cryan, 2017). This is not surprising since the gut microbiome influences not only nutrient metabolism but it is also intimately related to the immune system and brain development. Normal flora contributes to the production of neuroactive molecules including serotonin, GABA, acetylcholine, histamine, tryptophan, and catecholamines (Dinan and Cryan, 2017). For example, alterations in tryptophan metabolism through the kynurenine pathway have been linked to AD (Giil et al., 2017). Because the gut microbiome is known to play a role in autoimmunity, neuroinflammation, and neurogenesis in the brain, a gut-brain axis of neurodegeneration has been implicated in the pathogenesis of AD and other neurodegenerative diseases (Fung et al., 2017). Biochemical studies showed that *Escherichia coli* can produce amyloid fibers and regulate amyloidosis (Chapman et al., 2002). Also, disturbances to the microbiome homeostasis by drugs and diet may increase pathogen susceptibility and inflammation. For example, a systematic review suggested that antibiotic use was associated with severe dementia (van der Maaden et al., 2015). Interestingly, a randomized double-blind and controlled clinical trial showed the efficacy of probiotic treatment in improving cognitive function in AD patients (Akbari et al., 2016). Nevertheless, because of the small sample size used in this trial, further studies are needed to verify these findings.

Accumulating evidence from epidemiological studies suggests that inflammatory bowel disease (IBD) is associated with an increased risk of dementia. A population-based study including 32,298 patients with irritable bowel syndrome showed an increased risk of dementia in patients older than 50 years (Chen et al., 2016). Furthermore, a cohort study of 1,742 patients with IBD showed a significant positive association between IBD and subsequent development of dementia (Zhang et al., 2020). These findings are supported by a recent meta-analysis that found a positive association between IBD and subsequent development of AD (Fu et al., 2020).

Crohn's disease is another gastrointestinal disease that has been implicated in AD. One study identified a common genetic factor between Crohn's' disease and AD. A genetic variant near the IPMK gene, associated with Crohn's disease (O'Donnell et al., 2019), was found to increase the risk of AD (Yokoyama et al., 2016). The genetic overlap between these diseases and AD is not substantial and thus unlikely to explain the comorbidity between AD and inflammatory gut diseases. The exact mechanisms by which gastrointestinal diseases are linked to AD are unknown, but the consensus among the studies suggest that disruption in the gut microbiome can lead to the production of toxic metabolites that can infiltrate through the blood-brain barrier and cause widespread neuroinflammation. The main results from the studies investigating the association between AD and gastrointestinal diseases are presented in **Table 5**.

TABLE 5 | Studies investigating the association between the gut microbiome, dementia and AD.

Study	Study design	Main results
Alzheimer's disease		
Akbari et al. (2016)	Randomized double-blind controlled trial	Probiotic treatment improved cognitive function in AD patients
Yokoyama et al. (2016)	Genome-wide association study	A genetic variant near IMPK is shared between Crohn's disease and AD
Giil et al. (2017)	Case-control	Plasma levels of several kynurenines were lower in AD compared to controls
Fu et al. (2020)	Systematic review, meta-analysis	Inflammatory bowel disease associated with an increased risk for AD
Dementia		
van der Maaden et al. (2015)	Systematic review	The use of antibiotics may be associated with dementia
Chen et al. (2016)	Population-based study	Inflammatory bowel disease associated with an increased risk for dementia
Zhang et al. (2020)	Longitudinal cohort study	Inflammatory bowel disease associated with an increased risk for dementia

BIOINFORMATIC APPROACHES TO UNDERSTANDING COMORBIDITIES IN AD

Bioinformatic-based studies have laid the groundwork for the discovery of dysregulated biological pathways, therapeutic targets, and biomarkers, in neurodegenerative diseases (Santiago et al., 2017). Network biology approaches have been useful in identifying shared and unique pathways between AD and other diseases. In the context of diabetes, network analysis of transcriptomic data from AD and T2D brains revealed a central role for autophagy in the molecular linking of both diseases (Caberlotto et al., 2019). Another study using blood transcriptomic data showed that shared networks between MCI and T2D were related to inflammation whereas those networks shared between advanced AD and T2D were associated with the impairment in insulin signaling and defective cardiovascular system (Santiago et al., 2019).

In regards to cardiovascular and gut-related diseases, some bioinformatic studies have explored the connection with dementia. For example, one study showed that genes associated with AD such as apolipoprotein E (*APOE)*, alpha 2

macroglobulin (*A2M)*, paraoxonase 2 (*PON2)*, and microtubule-associated protein 4 (*MAP4)*, were closely related to genes associated with cardiovascular disease, including catechol-O-methyltransferase (*COMT)*, cystathionine beta synthase (*CBS)*, and WNK lysine deficient protein kinase 1 (WNK1) suggesting both diseases are linked through shared molecular networks (Ray et al., 2008). Weighted gene coexpression network analysis of proteomic data from over 400 postmortem brains with AD identified 23 shared proteins between AD and cerebral atherosclerosis and suggested that cerebral atherosclerosis contributed to dementia risk through decreased synaptic signaling and regulation and increased myelination (https://doi.org/10.1101/793349). In the context of gastrointestinal diseases, an integrative meta-analysis of 3 microarrays from patients with Crohn's disease identified ELAV-like RNA binding protein 1 (ELAVL1) and APP as the most significantly, upregulated and downregulated, respectively, in the blood of patients with Crohn's disease (Li et al., 2020). Interestingly, both genes have been linked to AD. For instance, APP is central to the pathogenesis of AD (O'Brien and Wong, 2011) and mutations in *APP* are known to cause familial AD (Weggen and Beher, 2012). Understanding APP metabolism and processing has been key to better understand the pathogenesis of AD (O'Brien and Wong, 2011). *ELAVL1* has been reported in AD, and its alteration may be related to APP processing (Amadio et al., 2009). To the best of our knowledge, bioinformatic-based studies exploring the association between inflammatory bowel disease and AD are not currently available. Future studies investigating the shared molecular networks between inflammatory bowel disease and AD will be important for identifying potential mechanisms and therapeutic targets.

CONCLUSIONS

Several comorbidities associated with AD may be involved in the disease pathogenesis and progression and thus, may have important clinical implications in the management of patients. For example, disease comorbidities like T2D and depression are associated with poor prognosis in AD patients (Li J. Q. et al., 2016). Therefore, it is important to carefully address the presence of comorbid diseases in AD in order to provide personalized treatment.

Despite the substantial evidence provided by epidemiological studies regarding the linkage between AD and some of its comorbidities, the precise mechanism explaining their coexistence with AD is still poorly understood. Epidemiological studies are useful in providing the basis for understanding disease risk factors and comorbidities but causal relationships are more difficult to disentangle. Because of the shared genetics, environmental factors, and strong competing risk of death, the mechanisms underlying the association between AD, dementia, and other diseases remain a challenging task for scientists and clinicians. Integrative bioinformatic approaches combining epidemiological, genetic, transcriptomic, proteomic, and metabolomic data may be key to better understand comorbidities in AD (Santiago and Potashkin, 2014).

Inflammation appears to be a central mechanism linking AD with other chronic diseases, however, it remains unclear whether inflammation plays a causative role or it is a consequence of neurodegeneration (Pugazhenthi et al., 2017). Most of the research indicates a bidirectional association between inflammation and AD (Newcombe et al., 2018). Several studies have suggested that neuroinflammation is fundamental in the pathogenesis of AD and contributes as much as do Aβ plaques and NFT (Heneka et al., 2015). Furthermore, the presence of AD pathological features in cognitively normal individuals suggest that multiple factors are required for the progression to AD (29876101). Neuroinflammation has been proposed as one of the earliest events preceding AD (20160456). For example, elevated levels of inflammatory cytokines IL-18, TNFα, and IFNγ have been shown to increase Aβ production in AD cellular models (Blasko et al., 1999; Sutinen et al., 2012). These inflammatory cytokines activate microglia, the resident phagocytes of the brain, which aid in the clearance of Aβ (Paresce et al., 1996; Bamberger et al., 2003). In this context, persistent neuroinflammation results in a decrease in the microglia phagocytic capacity leading to the accumulation of toxic Aβ (Krabbe et al., 2013). These studies reinforce the hypothesis that neuroinflammation may be an initial trigger in the neurodegenerative cascade in AD.

While some genetic factors are strongly connected with the development of late-onset AD, genetics alone does not explain the vast majority of the AD cases. The fact that many of the comorbidities associated with AD are related to dysregulated metabolic pathways implies that lifestyle factors play a role in the disease pathogenesis. In this regard, lifestyle modifications including exercise and diet may interact with genetic susceptibility genes and improve cognitive abilities in AD patients (Liang et al., 2018; Jensen et al., 2019). For example, physical exercise elicited a greater positive effect in cognitive function in AD patients who were APOEε carriers compared to non-carriers (Jensen et al., 2019). Moreover, APOEε carriers with a sedentary lifestyle showed a greater amyloid beta deposition compared to non-carriers (Head et al., 2012).

In the context of diet, adherence to a Mediterranean diet has been associated with a decreased risk for AD (van den Brink et al., 2019). Similar to physical exercise, genetic-diet interactions have been documented in AD patients. For example, a Mediterranean diet showed beneficial effects in cognitive function in AD patients that were carriers of several genetic variants in genes including *CRI*, *CLU,* and *PICALM* but not in APOEε carriers (Martinez-Lapiscina et al., 2014). Also, a ketogenic diet has been investigated as a potential therapeutic strategy in AD patients. Several clinical trials on ketogenic diets have shown promising results in improving cognitive function in AD patients (Henderson et al., 2009; Taylor et al., 2018). Interestingly, APOEε carriers were less responsive to a ketogenic diet compared to non-carriers (Reger et al., 2004). Collectively, these results illustrate the close interaction between genetic and environmental factors in modifying an individual disease risk for AD. While some lifestyle modifications such as exercise and diet, may be beneficial for AD patients, other factors including comorbidities and genetic profiles should be taken into consideration when evaluating treatments.

AUTHOR CONTRIBUTIONS

JS and JP wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Total Cerebral Small Vessel Score Association With Hoehn and Yahr Stage in Parkinson's Disease

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Background: This study aimed to evaluate the total cerebral small vessel disease (CSVD) score in patients with Parkinson's disease (PD) at different stages and related factors.

Methods: A 100 and seven patients with idiopathic PD and 62 normal controls (NCs) who underwent brain magnetic resonance imaging (MRI) were enrolled. PD patients were divided into two groups: early PD [(Hoehn and Yahr (H&Y) 1–1.5, *n* = 36)] and advanced PD (H&Y 2–4, *n* = 71) groups. We calculated the total CSVD score for each participant based on lacunes, high-grade white matter hyperintensities (WMH), enlarged perivascular spaces (EPVS), and cerebral microbleeds (CMBs). Differences in total CSVD score between the PD and NCs and between the two subgroups were compared. In addition, a multivariate logistic regression analysis was conducted to investigate the association between CSVD markers and clinical variables in PD.

Results: Lacunes were found in 9.3% of patients with PD, periventricular WMH (PVWMH) in 89.7%, deep WMH (DWMH) in 81.3%, EPVS in 85%, and CMBs in 2.8%. Compared with NCs, patients with PD showed higher PVWMH and DWMH scores. Advanced PD patients exhibited greater PVWMH (P = 0.041), DWMH (P = 0.046), and total CSVD score (P = 0.044) than the early PD group. After adjusting for multiple variables, higher H&Y stage was independently correlated with increased total CSVD score (OR = 2.667, 95% CI 1.154–2.266) and PVWMH score (OR = 2.237, 95% CI 1.084–1.696).

Conclusions: CSVD may play a critical role in patients with PD. The total CSVD score is a potential neuroimaging marker for monitoring the progression of PD.

Keywords: Parkinson's disease, cerebral small vessel disease, lacunes, white matter hyperintensities, enlarged perivascular spaces, cerebral microbleeds

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INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disease, with so far an unclear underlying mechanism. The contribution of vascular pathology to PD is receiving increasing attention. However, there were controversial reports on the relationship between vascular disease and PD. Cerebral small vessel disease (CSVD) comprises a group of disorders of various etiologies that affect the small arteries, arterioles, venules, and capillaries in the brain (Pantoni, 2010). On brain

MRI, CSVD can present as lacunes, white matter hyperintensities (WMH), enlarged perivascular spaces (EPVS), and cerebral microbleeds (CMBs; Pantoni, 2010). The total CSVD score has been used to assess neuroimaging markers in CSVD, including lacunes, high-grade WMH, EPVS in the basal ganglia, and CMBs, which might be better than separately measuring only one or two features (Klarenbeek et al., 2013; Staals et al., 2014). The total CSVD score, is, therefore, a more complete estimate of the full impact of CSVD on the brain (Staals et al., 2014).

CSVD has been shown to contribute to motor and cognitive functions in PD (Linortner et al., 2020). Previous work has also demonstrated that WMH is correlated with motor dysfunction and several non-motor symptoms in PD (Lee et al., 2018, 2020; Huang et al., 2020). However, other CSVD markers have received little attention. Only three studies calculated the total CSVD burden in patients with PD. Their findings indicated that CSVD burden was related to motor symptoms (especially gait/postural instability), cognitive impairment, and affective disorders (Shibata et al., 2019; Chen et al., 2020, 2021). Another autopsy study also revealed the severity of SVD pathology characterized by globus pallidus interna pallor associated with Hoehn and Yahr (H&Y) stage. However, the interaction effect between CSVD burden and H&Y stage in PD has not yet been reported. It is still unclear whether comorbid CSVD exacerbates the progression of PD.

The H&Y stage is a widely used scale for evaluating disease progression in PD (Hoehn and Yahr, 1967; Goetz et al., 2004), while the H&Y transition time is also considered a useful measure of disease progression in PD (Zhao et al., 2010). Several neuroimaging studies have shown that the H&Y stage correlates with progressive nigrostriatal terminal dysfunction (Vingerhoets et al., 1994; Staffen et al., 2000). These findings support the usefulness of the H&Y stage for categorizing patients with PD and capturing disease progression.

In our study, we aimed to investigate the total CSVD burden in patients with PD at different stages based on the H&Y scale. We also examined factors related to the total CSVD score and other CSVD markers in PD. This study may help elucidate the relationship between CSVD and PD and identify potential neuroimaging markers for diagnosing and monitoring PD progression.

MATERIALS AND METHODS

Subjects and Clinical Assessments

Patients with idiopathic PD (n = 107, mean age: 66.20 ± 8.69 years) and age-and sex-matched normal controls (NCs; n = 62, mean age: 65.69 ± 6.45 years) were recruited. All PD patients were diagnosed based on the UK Parkinson's Disease Society Brain Bank Clinical Diagnostic Criteria. All participants were right-handed Chinese natives. We excluded patients whose PD was induced by cerebrovascular disease, medications, trauma, encephalitis, poisoning, and other neurodegenerative diseases. Vascular risk factors were recorded, including hypertension, diabetes mellitus, hyperlipidemia, coronary heart disease, atrial fibrillation, and smoking status. Neurological examinations were evaluated using the Mini-Mental State Examination (MMSE), Unified Parkinson's Disease Rating Scale part III score (UPDRS-III), H&Y Stage, Hamilton Rating Scale for Depression (HAMD), Hamilton Rating Scale for Anxiety (HAMA), Parkinson's disease questionnaire-39 (PDQ-39), and non-motor symptoms questionnaire (NMSQ). Patients with PD receiving dopaminergic medications were examined in a clinically defined "OFF" state. All neuropsychological scales were completed by a neurologist blinded to clinical diagnosis. Patients with obvious cognitive deficits were excluded (MMSE score ≤24). Patients were classified into the early (H&Y 1–1.5) and advanced PD groups (H&Y 2–4), based on the H&Y stage. This study was approved by a local ethics committee, and written informed consent was obtained from each participant after a detailed description of the study was provided.

MR Image Acquisition

All MRI examinations were performed using a 3.0 T MRI scanner (Philips, Achieva TX, 8-channel high-resolution head coil). Sequences consisted of high-resolution T1-weighted 3D [repetition time/echo time (TR/TE) = 7.4/3 ms, flip angle (FA) = 8°, field of view (FOV) = 24 cm × 24 cm, matrix = 256 × 256, and 1.2 mm slice thickness without slice gap], T2-weighted (T2WI, TR/TE = 2,500/100 ms; FOV = 24 cm × 24 cm, matrix = 256 × 256, 5 mm slice thickness, and 1.5 mm slice gap), fluid-attenuated inversion recovery (FLAIR; TR/TE = 8,000/140 ms; TI = 2,400 ms; FOV = 24 cm × 24 cm, matrix = 256 × 228, and 4 mm slice thickness without slice gap), diffusion-weighted imaging (DWI; TR/TE = 5,000/76.4 ms; matrix = 128 × 128, and 5 mm slice thickness), susceptibility-weighted imaging (SWI; TR/TE = 16/22 ms; FOV = 24 cm × 24 cm, matrix = 240 × 240, and 2.8 mm slice thickness without slice gap).

MRI Analysis

CSVD markers include lacunes, WMH, EPVS, and CMBs (**Figure 1**). Lacunes were defined as round or ovoid cerebrospinal fluid-filled cavities in the basal ganglia or white matter, usually 3–15 mm, with low signal on T1WI and DWI, and high signal on T2WI (Wardlaw, 2008; Wardlaw et al., 2013). Periventricular WMH (PVWMH) and deep WMH (DWMH) lesions were investigated using the Fazekas scale from 0 to 3 (Fazekas et al., 1987). PVWMH was defined as 0 = absence, 1 = "caps" or pencil-thin lining, 2 = smooth "halo" and 3 = irregular PVWMH extending into the deep white matter. DWMH refers to 0 = absence, 1 = punctate foci, 2 = beginning confluence of foci, 3 = large confluent areas)Fazekas et al., 1987). EPVS were defined as punctate hyperintensities on T2WI in the basal ganglia, usually <3 mm in diameter, based on a previous study (Doubal et al., 2010). Isolated single large invaginations of cerebrospinal fluid round perforating vessels were not counted. EPVS were rated as follows: 0 = no EPVS, 1 = <10 EPVS, 2 = 11–20 EPVS, 3 = 21–40 EPVS, and 4 = >40 EPVS. If there was an asymmetry between the sides, the hemisphere most affected was calculated (Doubal et al., 2010). CMBs are well-defined, round hypointensities, ≤10 mm on SWI images (Wardlaw et al., 2013).

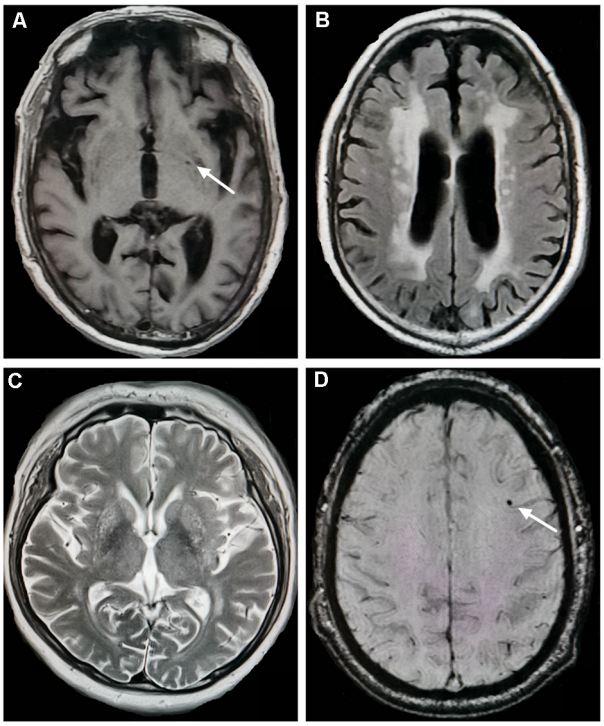

FIGURE 1 | Neuroimaging markers of cerebral small vessel disease. **(A)** Lacunes (white arrow) in the left basal ganglia (T1-weighted imaging). **(B)** Severe periventricular white matter hyperintensities (WMH; fluid-attenuated inversion recovery). **(C)** Enlarged perivascular spaces in the basal ganglia (T2-weighted imaging). **(D)** Cerebral microbleeds (white arrow) in the left frontal lobe (susceptibility-weighted imaging).

All MRI lesions were assessed by two trained neurologists blinded to the participants' clinical information.

Total CSVD Burden/Score

Based on the description by Staals et al., we calculated the total CSVD score on an ordinal scale from 0 to 4. One point on the CSVD score was awarded for each of the following: ≥1 lacunes, ≥1 CMBs, high-grade WMH (Fazekas score = 3 in PVWMH or ≥2 in DWMH), and moderate-to-severe EPVS (>10 in the basal ganglia; Staals et al., 2014).

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) software (version 19.0) was used to analyze clinical and demographic variables. A two-sample *t*-test and Chi-square test were conducted to examine the clinical differences between continuous and categorical variables, respectively. The Mann–Whitney *U* test was used to compare the differences in H&Y stage and CSVD markers between groups. Multivariate ordered logistic regression analysis was performed between the total CSVD score and the clinical variables in PD. The total CSVD score was the dependent variable, and clinical factors were independent variables, including age, sex, vascular risk factors, years of education, disease duration, LED, UPDRS-III, H&Y stage, MMSE, HAMD, and HAMA score. Factors related to each CSVD marker (lacunes, WMH, EPVS, and CMBs) were further analyzed. Statistically significant was set at $p < 0.05$.

TABLE 1 | Demographic and clinical data of the subjects.

	PD (*n* = 107)	NCs (*n* = 62)	t/x^2	*P*
Age (year)	66.20 ± 8.69	65.69 ± 6.45	−0.429	0.669
Sex (M/F)	56/51	29/33	0.486	0.486
Hypertension (%)	35 (32.7)	28 (45.2)	2.603	0.107
Diabetes mellitus (%)	12 (11.2)	23 (37.1)	18.193	0.000*
Hyperlipidemia (%)	32 (29.9)	32 (51.6)	7.861	0.005*
Coronary heart disease (%)	17 (15.9)	19 (30.6)	5.099	0.024*
Atrial fibrillation (%)	1 (0.9)	0 (0.0)	0.583	0.445
Current smoking (%)	3 (2.8)	7 (11.3)	5.078	0.024*

**Chi-square tests, P < 0.05.*

RESULTS

In this study, we recruited 107 patients with PD and 62 NCs. Demographic and clinical data are presented in **Table 1**. NCs had a higher proportion of diabetes mellitus, hypercholesterolemia, coronary heart disease, and current smoking. The two groups did not differ in terms of age, sex, prevalence of hypertension, and atrial fibrillation. In the PD group, lacunes were present in 9.3%, PVWMH in 89.7%, DWMH in 81.3%, EPVS in 85%, and CMBs in 2.8% patients. Compared with NCs, PD patients showed higher PVWMH (U = 2,720.50, P = 0.039) and DWMH scores (U = 2,658.50, P = 0.011). However, there were no significant differences in lacunes, BG-EPVS, CMBs, and total CSVD score between patients with PD and controls.

Compared with the early PD group, advanced PD patients showed higher levodopa equivalent dose (LED), higher UPDRS-III, and UPDRS total score, longer disease duration, higher PDQ-39 score, and lower smoking rate (**Table 2**). The two groups did not differ in age, sex ratio, education years, and vascular risk factors except smoking status, MMSE, HAMD, HAMA, and NMSQ scores. **Figure 2** shows the percentages of different CSVD scores in the two subgroups. Patients with advanced PD exhibited greater PVWMH, DWMH, and total CSVD scores than the early PD group (**Table 2**). After adjusting for smoking status, advanced PD patients still showed greater PVWMH (F = 4.935, P = 0.028), DWMH (F = 5.824, P = 0.018), and total CSVD score (F = 5.121, P = 0.026) than the early PD group. However, there was no significant difference in lacunes, EPVS, and CMBs between the two subgroups.

In multivariate ordered logistic regression analysis, the higher H&Y stage was independently correlated with increased total CSVD (OR = 2.667, 95% CI 1.154–2.266, P = 0.022) and PVWMH score (OR = 2.237, 95% CI 1.084–1.696, P = 0.029; **Table 3**). However, there was no significant association between the total CSVD score and sex, years of education, disease duration, LED, UPDRS-III, MMSE, HAMD, HAMA, PDQ-39, NMSQ, and vascular risk factors such as hypertension, hyperlipidemia, smoking, and cardiovascular disease. No marked relationships were demonstrated between the H&Y stage and lacunes, DWMH, EPVS, and CMBs scores. In

TABLE 2 | Demographic and total CSVD score in the early and advanced PD groups.

	ePD (*n* =36)	aPD (*n* =71)	$t/x^2/U$	*P*
Age (year)	66.36 ± 9.26	66.11 ± 8.45	0.139	0.890
Sex (M/F)	19/17	37/34	0.004	0.948
Hypertension (%)	13 (36.1)	22 (31.0)	0.285	0.593
Diabetes mellitus (%)	5 (13.9)	7 (9.8)	0.390	0.533
Hypercholesterolemia (%)	7 (19.4)	25 (35.2)	2.833	0.092
Coronary heart disease (%)	5 (13.9)	12 (16.9)	0.162	0.687
Atrial fibrillation (%)	0 (0)	1 (1.4)	0.512	0.474
Current smoking (%)	3 (8.3)	0 (0)	6.087	0.036[b]
LED (mg/day)	287.85 ± 247.81	474.36 ± 305.12	−3.173	0.002[a]
UPDRS-III	15.97 ± 6.29	31.63 ± 11.74	−8.980	0.000[a]
UPDRS total	23.44 ± 8.00	37.34 ± 13.31	−6.722	0.000[a]
H&Y stage	1.2 (1-1.5)	2.5 (2-4)	−17.183	0.000[a]
Education	13.67 ± 3.36	13.21 ± 2.78	0.746	0.457
Duration	5.11 ± 4.35	8.48 ± 4.39	−3.761	0.000[a]
MMSE	28.69 ± 1.60	28.13 ± 1.63	1.713	0.090
HAMD	7.31 ± 4.74	9.01 ± 5.21	−1.650	0.102
HAMA	9.00 ± 5.53	9.92 ± 5.64	−0.798	0.426
PDQ-39	16.61 ± 12.31	28.97 ± 19.31	−4.019	0.000[a]
NMSQ	10.42 ± 3.77	11.83 ± 4.86	−1.528	0.130
MRI features				
Lacunes (%)	1 (2.8)	9 (12.7)	1.718	0.190
PVWMH (IQR)	1.33 (0–3)	1.70 (0–3)	985.5	0.041[c]
DWMH (IQR)	0.81 (0–2)	1.07 (0–3)	1,025.5	0.046[c]
EPVS (IQR)	1.03 (0–3)	1.32 (0–4)	1,082	0.145
CMBs (%)	2 (5.6)	1 (1.4)	1.508	0.261
CSVD burden (IQR)	0.36 (0–2)	0.73 (0–3)	1,006.5	0.044[c]

ePD, early PD; aPD, advanced PD; LED, levodopa equivalent dose; MMSE, Mini-Mental State Examination, UPDRS, Unified Parkinson's Disease Rating Scale; H&Y stage, Hoehn and Yahr stage; HAMD, Hamilton Rating Scale for Depression; HAMA, Hamilton Rating Scale for Anxiety; PDQ-39, the Parkinson's disease questionnaire-39; NMSQ, non-motor symptoms questionnaire; IQR, interquartile range; [a]Independent t-test, P < 0.05. [b]Chi-square test, P < 0.05. [c]Mann-Whitney U test, P < 0.05.

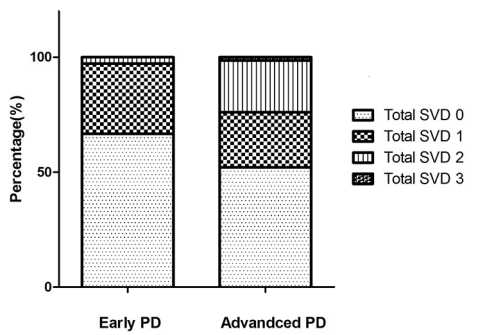

FIGURE 2 | The percentage of different total cerebral small vessel disease (CSVD) scores in the two Parkinson's disease (PD) subgroups. In early PD group, the total CSVD score 0–3 were present in 66.7%, 30.6%, 2.8%, 0% of the patients, respectively. In advanced PD group, the total CSVD score 0–3 were present in 52.1%, 23.9%, 22.5%, 1.4% of the patients, respectively. There were no patients with a score of 4 in our study.

addition, age was positively associated with PVWMH, DWMH, EPVS, and total CSVD scores. Diabetes mellitus was also related to increased DWMH, EPVS, and total CSVD burden (**Table 3**).

DISCUSSION

The present study suggested a significant difference in total CSVD score between the early and advanced PD groups. The H&Y stage was independently correlated with the total CSVD score, and as such a potential marker for monitoring PD progression.

In recent years, only three studies have investigated the association between CSVD and PD using the total CSVD score. Shibata et al. (2019) suggested a relationship between cognitive decline and increased CSVD score. While another two studies by indicated that comorbid CSVD may play a critical role in several PD domains, including motor deficits, cognition, depression, and anxiety (Chen et al., 2020, 2021). Although PD patients with more severe CSVD burden showed a higher H&Y stage, there were no significant differences in the H&Y stage between five subgroups according to the CSVD burden score. The association between CSVD burden and the H&Y stage had not been previously reported. In a cohort of 77 autopsy-confirmed PD patients, Schwartz et al. (2018) revealed the severity of SVD pathology characterized by globus pallidus interna pallor associated with the Hoehn and Yahr (H&Y) stage. Our study is the first to demonstrate a correlation between the total CSVD score and the H&Y stage in PD. The assessment of CSVD could be used as a clinically relevant neuroimaging marker in studies of disease progression in PD.

The pathologies of cerebrovascular diseases in PD have been investigated in several studies. The prevalence of cerebrovascular lesions in PD (44.0%) was higher than in controls (32.8%), including lacunes, amyloid angiopathy, white matter lesions, old and recent ischemic infarcts, and hemorrhages (Jellinger,

TABLE 3 | Correlation analysis between the CSVD markers clinical variables in PD.

	Clinical variables	OR	95% CI	X^2	*P*
Related to CSVD burden	H&Y stage	2.667	1.154–2.266	5.270	0.022*
	Age	1.305	1.186–1.435	30.098	0.000*
	DM	5.618	1.406–22.421	5.970	0.015*
Related to PVWMH	H&Y stage	2.237	1.084–1.696	4.753	0.029*
	Age	1.202	1.130–1.279	34.205	0.000*
Related to DWMH	Age	1.121	1.052–1.194	12.483	0.000*
	DM	4.609	1.098–19.317	4.363	0.037*
Related to EPVS	Age	1.158	1.090–1.232	21.943	0.000*
	DM	4.716	1.283–17.322	5.456	0.020*

**Multivariate ordered logistic regression analysis; DM, diabetes mellitus.*

2003). However, another study reported opposite results (Schwartz et al., 2012). The vascular pathology of PD includes capillary fragmentation and damage to the capillary network in multiple brain regions, particularly in the substantia nigra, middle frontal cortex, and brain stem nuclei (Guan et al., 2013). Further, widespread cerebral blood flow reduction has also been observed in patients with PD (Fernandez-Seara et al., 2012). These findings suggest shared pathogenic pathways between cerebrovascular diseases and PD (Kummer et al., 2019). We speculate that comorbid CSVD may lead to more widespread disruption, which could exacerbate PD progression.

In addition to the total CSVD burden, WMH has been related to motor symptoms in PD, especially bradykinesia and axial symptoms (Bohnen et al., 2011; Lee et al., 2020; Jeong et al., 2021). Other studies also indicated that WMH correlated with motor subtype and gait in patients with PD (Bohnen et al., 2011; Al-Bachari et al., 2017; Toda et al., 2019; Wan et al., 2019). Furthermore, there is an association between WMH and several non-motor symptoms in PD, such as cognitive dysfunction, depression, anxiety, fatigue, and quality of life (Lee et al., 2018; Huang et al., 2020). In another longitudinal study, Pozorski et al. found that greater WMH accumulation correlated with increased UPDRS motor sub-scores and impaired cognitive performance over an 18-month period in PD patients (Pozorski et al., 2019). Their findings suggest that WMH may worsen motor and cognitive functions in patients with PD. Our results showed that PD patients had higher PVWMH scores than NCs, which was also independently associated with the H&Y stage. The present finding is in line with previous studies, suggesting that PVWMH may be a promising marker for diagnosing and monitoring PD disease progression. The mechanism underlying WMH was associated with vascular changes including arteriolar tortuosity, decreased vessel density, occlusive venous collagenosis, and reduced myelin density due to Wallerian degeneration secondary to neuron loss, and low-grade inflammation (Smith, 2010; Wersching et al., 2010; Bohnen and Albin, 2011). WMH could also disrupt connectivity in widespread neural systems and exacerbate some motor and cognitive deficits in PD (Bohnen and Albin, 2011). Hence, comorbid white matter disease may provide a new sight for PD.

Regarding other CSVD markers, previous work showed that lacunes in the basal ganglia independently correlated with impaired gait and posture dysfunction in patients with PD (Chen et al., 2020). Moreover, EPVS in the basal ganglia is related to the tremor score (Wan et al., 2019), and may be a predictor of cognitive impairment in PD (Shibata et al., 2019). Yamashiro et al. also revealed that deep or infratentorial CMBs were more frequent in PD; risk factors include hypertension, orthostatic hypotension, and a history of ischemic stroke (Yamashiro et al., 2015). Patients with the postural instability gait disorder (PIGD) subtype exhibited a higher prevalence of CMBs compared to NCs (Kim et al., 2018). PD patients with CMBs were older and had higher CSVD scores than those without (Kim et al., 2018). In a Chinese cohort study, a history of cerebral ischemic events and hypertension was independently associated with CMBs presence in PD (He et al., 2017). However, our results did not show any differences in lacunes, EPVS, and CMBs between PD patients and controls. No marked relationship between these CSVD markers and clinical variables was observed. We speculate that WMH may play a more critical role in PD than other CSVD markers. To prove this point, larger sample size studies in this field are needed in the future.

This study has several limitations: (1) The sample size was relatively small, therefore, future longitudinal studies are warranted. (2) We did not explore the relationship between UPDRS subscores and CSVD markers. (3) The total CSVD score is a semi-quantitative method, and future integrated studies using multimodal structural, functional, and metabolic neuroimaging techniques are needed to provide new insights into the interaction between CSVD and PD.

In conclusion, we found that total CSVD and WMH scores were independently associated with disease stage in PD. These scores may be promising markers for monitoring PD progression. Comorbid CSVD may be an aggravating factor for the progression of PD, and immediate clinical and public health implications. Therefore, screening for CSVD should be considered in PD. The management of vascular risk factors may be helpful in patients with PD.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

XM, WS, and HC contributed to the conception and design of the study. SL, CL, RW, and MC organized the database. XM performed the statistical analysis, and wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Absolute Cardiovascular Disease Risk Is Associated With the Incidence of Non-amnestic Cognitive Impairment in Japanese Older Adults

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Background: The estimated absolute cardiovascular disease (CVD) risk level is known to be a useful surrogate marker for future cognitive impairment; however, evidence regarding its predictive validity in terms of cognitive subtypes is limited. We aimed to examine subtype-dependent differences in the associations between absolute CVD risk and the incidence of cognitive impairment in a community-dwelling older Japanese cohort.

Methods and Results: This study comprised 1,641 cognitively intact older Japanese participants without CVDs at baseline. We estimated absolute CVD risk using WHO region-specific risk estimation charts and included age, sex, diabetes mellitus, smoking, systolic blood pressure, and total cholesterol at baseline, and the CVD risk level was stratified into the three following risk categories: low (<10%), moderate (10 to <20%), and high (≥20%). Objective cognitive screening was performed using a multicomponent neurocognitive test at baseline and follow-up, and the incidence of cognitive impairment over 48 ± 2 months was determined. The incidence of cognitive impairment in low-, moderate-, and high-CVD risk participants was 1.2, 3.0, and 5.4%, respectively, for amnestic subtypes and 5.8, 10.1, and 14.0%, respectively, for non-amnestic subtypes. After adjusting for potential confounding factors, the absolute CVD risk level was significantly associated with non-amnestic impairment but not with amnestic impairment.

Conclusions: The absolute CVD risk estimated using region-specific risk estimation charts in old age is useful to predict incidence of cognitive impairment. Strategies to screen populations at risk of cognitive impairment and to prevent progression to dementia should be cognitive subtype-specific.

Keywords: cardiovascular disease, risk score, cognitive impairment, non-amnestic subtype, community setting

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INTRODUCTION

Prevalence rates concerning Alzheimer's disease (AD) and dementia are increasing rapidly along with an aging global population. According to predictions, the total number of people with dementia is likely to reach 82 million in 2,030 and 152 million in 2,050 (World Health Organization (WHO), 2019). Therefore, modifiable risk factors associated with dementia need to be urgently identified. Furthermore, along with efforts to determine risk factors for dementia, there is increasing interest in studying predictors of cognitive decline as it is now widely accepted that dementia has a long preclinical phase (Kaffashian et al., 2013).

Cardiovascular disease (CVD), as a modifiable risk factor for cognitive impairment or dementia, has become an area of interest. Previous studies have shown that traditional CVD risk factors including obesity, diabetes mellitus, smoking, hypertension, and hyperlipidemia are individually associated with cognitive decline (Carmelli et al., 1998). Regarding potential mechanisms, exposure to CVD risk factors might accelerate cognitive decline due to cerebral hypoperfusion, hypoxia, emboli, or infarcts, which lead to vascular and degenerative brain lesions (Qiu and Fratiglioni, 2015; Cohen, 2016). The point here is that CVD risk factors are correlated with each other, making it difficult to isolate their individual effects on cognitive decline remains challenging (Song et al., 2020).

Recently, multivariable CVD risk assessments have been advocated to estimate absolute CVD risk levels and to guide treatment concerning potential risk factors. Over the past decades, several CVD risk estimation tools involving multivariable risk factors have been developed to establish accurate estimation models for an individual's absolute risk of a CVD event (Conroy et al., 2003; Hippisley-Cox et al., 2007; D'Agostino et al., 2008). Previous large-scale cohort studies have shown that some CVD risk estimation tools have been useful in predicting not only a CVD event but also cognitive decline (Samieri et al., 2018; Song et al., 2020) and dementia (both all-cause dementia and AD) (Viticchi et al., 2017; Fayosse et al., 2020). Therefore, absolute CVD risk, estimated using multivariable risk factors, may be a useful surrogate marker of cognitive decline.

However, most CVD risk estimation tools have been developed based on data from Western countries (Conroy et al., 2003; Hippisley-Cox et al., 2007; D'Agostino et al., 2008), and it is unclear whether these tools are applicable to Asian populations. Previous studies have reported interethnic heterogeneity in terms of CVD risks and CVD events between Asian and Western countries. For example, Asian people have been found to have a higher predisposition to insulin resistance at a lesser degree of obesity than European people (Yoon et al., 2006), and the prevalence of adult obesity in most Asian countries is relatively low compared with Western countries such as the United States (Yoon et al., 2006). Interethnic heterogeneity is considered to be affected by lifestyle, environmental factors, and genetic predisposition (Yoon et al., 2006); thus, validation of a risk estimation tool in an Asian cohort is necessary for accurate CVD risk estimation in an Asian population.

The cognitive domain has previously been divided into amnestic and non-amnestic subtypes, with amnestic impairment hypothesized as more likely to progress to dementia due to AD (Jicha et al., 2006) and non-amnestic impairment more likely to progress to vascular and other forms of non-AD dementia (Luchsinger et al., 2009). Therefore, the mechanisms underlying CVD risk and cognitive impairment appear to differ between amnestic and non-amnestic subtypes and the strategies to prevent progression to dementia should be subtype-specific. However, the difference in the association between absolute CVD risk and cognitive impairment in amnestic and non-amnestic subtypes remains unclear.

Therefore, we examined the prospective associations of absolute CVD risk, based on region-specific risk estimation charts, with the incidence of cognitive impairment in amnestic and non-amnestic subtypes among Japanese older adults without CVDs, in a 4-year longitudinal cohort study. We hypothesized that the association between absolute CVD risk and cognitive impairment would be more robust in the non-amnestic subtype than in the amnestic subtype, because CVDs could directly lead to vascular dementia.

MATERIALS AND METHODS

Study Settings and Participants

This prospective cohort study involved community-dwelling older Japanese adults who were enrolled from a sub-cohort of the National Center for Geriatrics and Gerontology-Study of Geriatric Syndromes (NCGG-SGS). The NCGG-SGS is a Japanese national cohort study, the primary aim of which is to establish a screening system for geriatric syndromes and to validate evidence-based interventions to prevent such syndromes. Our study inclusion criteria comprised older adults (age, ≥65 years) at the time of the baseline assessment (from August 2011 to February 2012) who resided in Obu City (population of approximately 88,000), Aichi prefecture, Japan. At the registration of the Obu study cohort, individuals aged 65 years or older, living in Obu City, not hospitalized, not in residential care, not certified by the national long-term care insurance system as having a functional disability, and not participating in another study (n = 14,313) were sent an invitation letter. Overall, 5,104 individuals aged ≥65 years completed our baseline assessment. At baseline, our exclusion criteria comprised those with: (i) a history of neuropsychiatric diseases including AD, Parkinson's disease, and depression (n = 175); (ii) a history of CVDs, including stroke and heart diseases (i.e., angina, myocardial infraction, and aortic aneurysm) (n = 1,018); (iii) functional disability, based on the long-term care insurance system (n = 66); (iv) dependence in basic activities of daily living (n = 10); (v) suspected dementia, based on a Mini-Mental State Examination score <21 (Excellence NIfHaC, 2011) at baseline (n = 112); (vi) cognitive impairment (refer to details concerning the definition, as discussed later) at baseline (n = 880), and; (vii) missing data in the above criteria or missing data concerning assessments for CVD risks (n = 60). After exclusion, we included 2,783 cognitively intact participants

at baseline. Of these, 1,704 participants (61.2%) completed a follow-up assessment (from August 2015 to February 2016) that was conducted 48 ± 2 months from baseline. At this follow-up, we excluded participants with missing data for cognitive assessment (n = 63). Finally, data concerning 1,641 participants were available for analysis; a flow chart depicting the exclusion process is shown in **Figure 1**.

All baseline assessments were undertaken as health check-ups by well-trained nurses and study assistants in community centers. All staff received training from the authors in terms of the protocols for administering the assessments prior to study commencement.

Ethical Approval

The study protocol was developed in accordance with the Helsinki Declaration and was approved by the Ethics Committee of the National Center for Geriatrics and Gerontology (NCGG). Prior to study participation, written informed consent was obtained from all participants.

Estimation of Absolute CVD Risk

We estimated 10-year CVD risk using the revised World Health Organization (WHO) CVD risk estimation charts (2019) at baseline (WHO CVD Risk Chart Working WHO CVD Risk Chart Working Group, 2019). These estimation charts indicate the absolute risk of a CVD event according to an individual's risk status, and a higher risk score indicates a greater risk-factor burden. The development group calibrated prediction models for 21 global regions, and region-specific prediction charts were available. The estimation charts provided two types of estimation models: a laboratory-based model including medical history and blood data, and a non-laboratory-based model that consisted of convenient variables for resource-limited settings. In this study, we used a laboratory-based risk estimation model which included age, sex, current history of diabetes mellitus, smoking status, systolic blood pressure, and total cholesterol for the high-income Asia-Pacific region, including Japan.

Regarding each component of the WHO risk estimation model, we assessed diabetes mellitus, smoking status, systolic

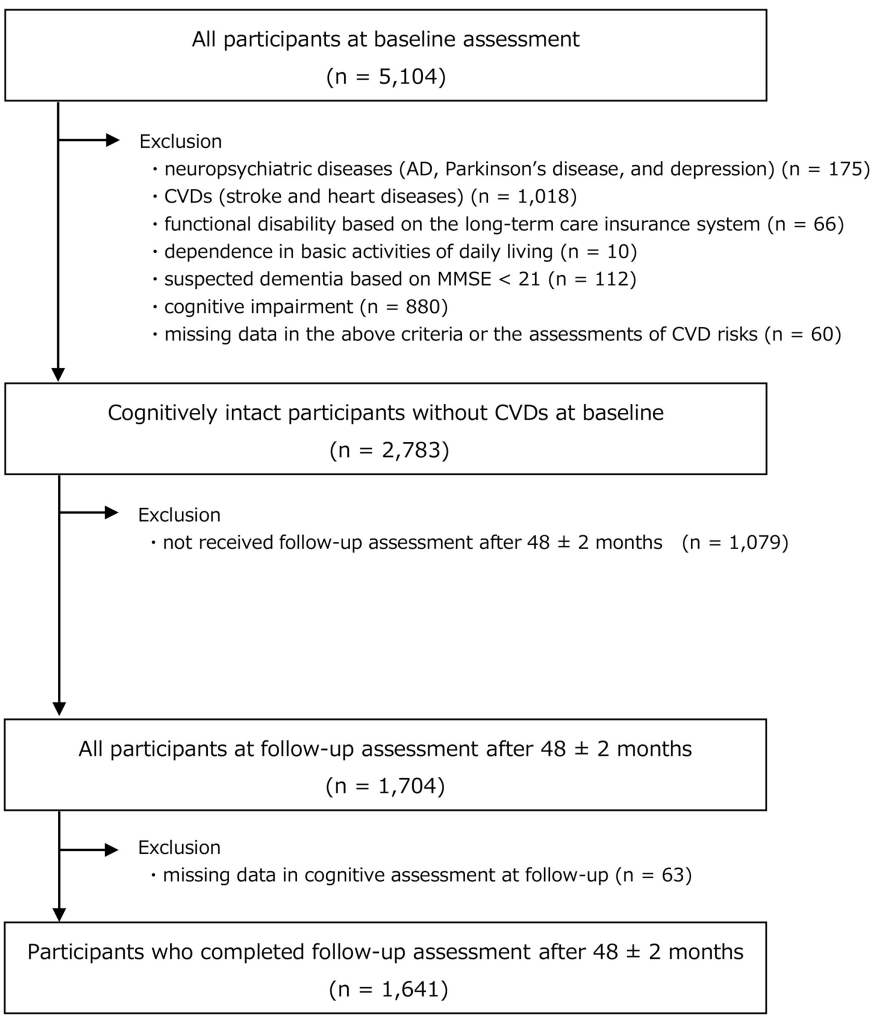

FIGURE 1 | Flowchart of participant recruitment and screening.

blood pressure, and total cholesterol levels, along with age and sex. A current history of diabetes mellitus was assessed through face-to-face interviews by nurses. Nurses measured systolic blood pressure using an automated sphygmomanometer, with participants in a seated position. Total serum cholesterol levels (in mmol/L) were measured by enzyme method at a laboratory (Good Life Design Co., Japan). Smoking status was assessed as the presence or absence of regular smoking (current vs. former/never) by the study assistants. Finally, we calculated absolute CVD risk (%) based on the above risk status using revised WHO CVD risk estimation charts and we stratified the CVD risk into three categories, namely, low (<10%), moderate (10 ≤ 20%), and high (≥20%) risk (World Health Organization (WHO), 2007).

Assessment of Cognitive Functions and Operational Criteria for Cognitive Impairment

Cognitive assessment was conducted using the NCGG–Functional Assessment Tool (NCGG-FAT) (Makizako et al., 2013). The detailed protocol of NCGG-FAT was described in a previous study (Makizako et al., 2013). The NCGG-FAT includes the following cognitive tests: (i) memory (word list memory-I [immediate recognition] and word list memory-II [delayed recall]); (ii) attention (a tablet-based version of the Trail Making Test [TMT]-part A); (iii) executive function (a tablet-based version of the TMT-part B); and (iv) processing speed (a tablet-based version of the Digit Symbol Substitution Test). Participants were given approximately 20 min to complete the test battery. This tool has previously been confirmed to have high test-retest reliability (intraclass correlation coefficients ranging from 0.79 to 0.94 in each test) and moderate-to-high validity (Pearson's correlation coefficients between the NCGG-FAT score and that of widely used clinical tests, including the subtest of the AD Assessment Scale-cognitive [delayed recall, modified], the written TMT-parts A and B, and the Digit Symbol-Coding subtest of the Wechsler Adult Intelligence-III ranging from 0.55 to 0.84) among community-dwelling older adults (Makizako et al., 2013). All tests had established standardized thresholds for defining objective cognitive impairments in the corresponding tests (a score of ≥1.5 standard deviations [SD] below the age- and education-specific means, based on our own algorithm sourced from a database including >10,000 community-dwelling older adults), which were derived from a population-based cohort (Shimada et al., 2013).

Cognitive impairment was defined as a participant score below the standardized thresholds (a score of ≥1.5 SDs below the age- and education-specific means) in one or more cognitive tests in the follow-up assessment. Additionally, cognitive impairment was classified as amnestic or non-amnestic impairment (Petersen, 2004). The former indicated individuals with a memory deficit (non-memory domain including attention, executive function, and processing speed remains intact) and the latter indicated individuals with a deficit in either attention, executive function, or processing speed (memory remains intact) in our study (Petersen, 2004).

Potential Confounding Factors

As covariates, education level, a medical history of pulmonary disease (i.e., pneumonia, tuberculosis, and chronic obstructive pulmonary disease), and the number of prescribed medications (total of overall drugs continuously prescribed by a doctor) were assessed through face-to-face interviews at baseline. We also included body mass index, alcohol consumption habits, slow gait speed, depressive symptoms, physical inactivity, living arrangements (living alone or cohabiting), employment status (the presence of paid work), and global cognitive function at baseline as covariates. Body mass index was calculated as bodyweight (kg) divided by the square of body height (m^2). Current alcohol consumption habits were assessed as the presence or absence of regular alcohol consumption (current vs former/never). Gait speed was measured in seconds using a stopwatch. Participants were asked to walk on a flat and straight surface at a comfortable walking speed. Two markers were used to indicate the start and end of a 2.4-m walk path, with a 2-m section to be traversed before passing the start marker so that participants were walking at a comfortable pace by the time they reached the timed path. Participants were asked to continue walking for an additional 2-m distance past the end of the path to ensure a consistent walking pace while on the timed path (Shimada et al., 2013; Doi et al., 2015). A gait speed <1.0 m/s was defined as a slow gait speed (Doi et al., 2015). Depressive symptoms were assessed using the 15-item Geriatric Depression Scale (GDS). The GDS was developed specifically for the screening of depression among elderly individuals and is used to quantify depressive symptoms. Participants could respond "yes" or "no" to 15 questions; thus, the total score ranged from 0 to 15. Participants who scored ≥6 on the GDS were considered to have depressive symptoms in this study (Ezzati et al., 2019). Physical inactivity was evaluated using the following questions: (i) "Do you engage in more than moderate levels of physical exercise or sports aimed at health?" and (ii) "Do you engage in low levels of physical exercise aimed at health?" Participants who responded "no" to both questions were defined as being inactive (Shimada et al., 2013). Global cognitive function was measured using the Mini-Mental State Examination (Folstein et al., 1983); and scores ranged from 0 to 30, with higher scores indicating better cognitive performance (Folstein et al., 1983).

Statistical Analysis

Baseline characteristics were compared between participants who completed the follow-up assessment and participants who were lost to follow-up using the Student's *t*-test for continuous variables and a χ^2 test for categorical variables. Furthermore, we compared baseline characteristics according to CVD risk levels (low-, moderate-, and high-risk levels) using a one-way analysis of variance for continuous variables and a χ^2 test for categorical variables. We then examined the association between baseline CVD risk levels and the incidence of cognitive impairment after 4 years. For this analysis, we used a χ^2 test and logistic regression analysis because we

dealt with non-time series data. In this analysis, we first examined CVD risk levels and the incidence of cognitive impairment (regardless of cognitive subtype) in all participants. Second, we examined CVD risk levels and the incidence of cognitive impairment divided according to cognitive subtype (amnestic or non-amnestic impairment). In the latter analysis, we compared participants who had remained cognitively intact over 4 years as a reference group and those who showed cognitive impairment only in the amnestic subtype or only in non-amnestic subtype to clarify the subtype-dependent difference in the association between CVD risk levels and the incidence of cognitive impairment in the true sense. Logistic regression analysis was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) at each CVD risk level at baseline and in relation to the incidence of cognitive impairment after 4 years, and univariate (crude) and multivariate (adjusted) regression models were developed. The multivariate regression model was adjusted for all potential confounding factors assessed in this study.

All analyses were performed using IBM SPSS Statistics 25 (IBM Japan, Tokyo, Japan) software. The level of statistical significance was set to $P < 0.05$.

TABLE 1 | Comparison of baseline characteristics between participants who completed follow-up assessment and participants who were lost to follow-up assessment.

		Completed follow-up *n* = 1,704	Lost to follow-up *n* = 1,079	*P*
Components of WHO risk estimation model				
Age	(Years)	70.8 ± 4.6	71.8 ± 5.6	<0.001
Female	(*n*, %)	939 (55.1)	543 (50.3)	0.014
Diabetes mellitus	(*n*, %)	153 (9.0)	131 (12.1)	0.007
Smoking status	(*n*, %)	150 (8.8)	126 (11.7)	0.013
Systolic blood pressure	(mmHg)	140.0 ± 20.2	144.6 ± 21.7	<0.001
Total cholesterol	(mmol/l)	5.5 ± 0.8	5.5 ± 0.9	0.431
Other characteristics				
Education level	(Years)	11.8 ± 2.5	11.6 ± 2.4	0.550
Pulmonary disease	(*n*, %)	198 (11.6)	113 (10.5)	0.349
Prescribed medication	(Number)	1.5 ± 1.6	1.7 ± 1.9	0.002
Body mass index	(kg/m^2)	22.8 ± 2.9	23.0 ± 3.3	0.055
Drinking habit	(*n*, %)	822 (48.2)	494 (45.8)	0.206
Slow gait speed	(*n*, %)	66 (3.9)	109 (10.1)	<0.001
Depressive symptoms	(*n*, %)	162 (9.5)	154 (14.3)	<0.001
Physical inactivity	(*n*, %)	415 (24.4)	341 (31.7)	<0.001
Living alone	(*n*, %)	160 (9.4)	106 (9.8)	0.704
Employment	(*n*, %)	528 (31.0)	340 (31.5)	0.771
MMSE	(Score)	27.0 ± 2.2	26.6 ± 2.3	<0.001

CVD, cardiovascular disease; MMSE, mini-mental state examination; WHO, World Health Organization.
Data are expressed as mean ± standard deviation or numbers (%)
P-values are based on the Student's t-test for continuous variables and χ^2 tests for categorical variables.

RESULTS

Participants' Baseline Characteristics

Of the 2,783 cognitively intact participants at baseline, 1,704 (61.2%) completed a follow-up assessment. Compared with participants who completed the follow-up assessment, those who were lost to follow-up assessment were significantly older ($P < 0.001$), had a significantly lower proportion of female subjects ($P = 0.014$), higher proportion of diabetes mellitus ($P = 0.007$) and current smokers ($P = 0.013$), had significantly higher systolic blood pressure ($P < 0.001$) and more prescribed medications ($P = 0.002$), had a significantly higher proportion of slow gait speed ($P < 0.001$), depressive symptoms ($P < 0.001$), and physical inactivity ($P < 0.001$), and showed a significantly lower MMSE score ($P < 0.001$, **Table 1**).

Of 1,641 individuals who were included in our longitudinal analysis, the group classification according to CVD risk levels was as follows: (i) low CVD risk ($n = 372$, 22.7%), (ii) moderate CVD risk ($n = 1{,}116$, 68.0%), and; (iii) high CVD risk ($n = 153$, 9.3%).

The differences in baseline characteristics between the three CVD risk levels are shown in **Table 2**. There were significant differences in all components of the WHO risk estimation model: age ($P < 0.001$), sex ($P < 0.001$), the prevalence of diabetes mellitus ($P < 0.001$), the proportion of current smokers ($P < 0.001$), systolic blood pressure ($P < 0.001$), and total cholesterol ($P = 0.028$). In addition, there were significant differences between CVD risk levels in the number of prescribed medications ($P < 0.001$), body mass index ($P < 0.001$), the proportion of participants who consumed alcohol ($P < 0.001$), the prevalence of a slow gait speed ($P = 0.003$), physical inactivity ($P = 0.001$), and the Mini-Mental State Examination score ($P < 0.001$).

Prospective Associations Between CVD Risk Levels and Cognitive Decline

The incidence of cognitive impairment according to baseline CVD risk levels is shown in **Figure 2**. Of 1,641 cognitively intact participants without CVDs at baseline, 213 (13.0%) participants had newly developed cognitive impairment in any domain after 4 years. The percentages for those with cognitive impairment in the low-, moderate-, and high-CVD risk categories were 8.1, 13.7, and 19.6%, respectively, and χ^2 test results showed that CVD risk levels at baseline were significantly associated with the incidence of cognitive impairment ($P = 0.001$). In the cognitive subtype analysis, of 1,641 cognitively intact participants without CVDs at baseline, 41 (2.4%) participants showed cognitive impairment only in the amnestic subtype and 149 (9.1%) participants showed cognitive impairment only in the non-amnestic subtype. The percentages for those with amnestic impairment in the low-, moderate-, and high-CVD risk categories were 1.2, 3.0, and 5.4%, respectively, and the percentages for those with non-amnestic impairment in the low-, moderate-, and high-CVD risk categories were 5.8, 10.1, and 14.0%, respectively. χ^2 test results indicated that CVD risk levels at baseline were significantly associated with the incidence of both amnestic and non-amnestic impairment ($P = 0.033$ and $P = 0.008$, respectively).

TABLE 2 | Baseline characteristics according to estimated CVD risk levels.

		Overall *n* = 1,641	CVD risk levels			*P*
			Low risk (<10%) *n* = 372	Moderate risk (10 ≤ 20%) *n* = 1,116	High risk (≥20%) *n* = 153	
Components of WHO risk estimation model						
Age	(Years)	70.9 ± 4.7	68.0 ± 3.0	71.6 ± 4.7	72.6 ± 5.0	<0.001
Female	(*n*, %)	900 (54.8)	363 (97.6)	515 (46.1)	22 (14.4)	<0.001
Diabetes mellitus	(*n*, %)	147 (9.0)	3 (0.8)	87 (7.8)	57 (37.3)	<0.001
Smoking status	(*n*, %)	146 (8.9)	1 (0.3)	81 (7.3)	64 (41.8)	<0.001
Systolic blood pressure	(mmHg)	140.1 ± 20.1	125.8 ± 15.3	141.7 ± 18.1	162.9 ± 18.5	<0.001
Total cholesterol	(mmol/l)	5.5 ± 0.8	5.6 ± 0.8	5.4 ± 0.8	5.6 ± 0.9	0.028
Other characteristics						
Education level	(Years)	11.8 ± 2.5	11.8 ± 2.1	11.7 ± 2.6	11.9 ± 2.5	0.734
Pulmonary disease	(*n*, %)	193 (11.8)	34 (9.1)	138 (12.4)	21 (13.7)	0.180
Prescribed medication	(Number)	1.5 ± 1.6	1.2 ± 1.4	1.6 ± 1.7	1.7 ± 1.8	<0.001
Body mass index	(kg/m^2)	22.7 ± 2.9	22.1 ± 2.8	22.9 ± 2.9	23.3 ± 3.0	<0.001
Drinking habit	(*n*, %)	789 (48.1)	132 (35.5)	563 (50.4)	94 (61.4)	<0.001
Slow gait speed	(*n*, %)	65 (4.0)	4 (1.1)	51 (4.6)	10 (6.5)	0.003
Depressive symptoms	(*n*, %)	158 (9.6)	33 (8.9)	111 (10.0)	14 (9.2)	0.808
Physical inactivity	(*n*, %)	396 (24.1)	69 (18.6)	277 (24.8)	50 (33.3)	0.001
Living alone	(*n*, %)	155 (9.4)	40 (10.8)	100 (9.0)	15 (9.8)	0.585
Employment	(*n*, %)	507 (30.9)	121 (32.5)	338 (30.3)	48 (31.4)	0.714
MMSE	(Score)	27.0 ± 2.2	27.5 ± 2.1	26.9 ± 2.2	26.6 ± 2.2	<0.001

CVD, cardiovascular disease; MMSE, mini-mental state examination; WHO, World Health Organization.
Data are expressed as mean ± standard deviation or numbers (%).
P-values are based on one-way analysis of variance for continuous variables and χ^2 tests for categorical variables.

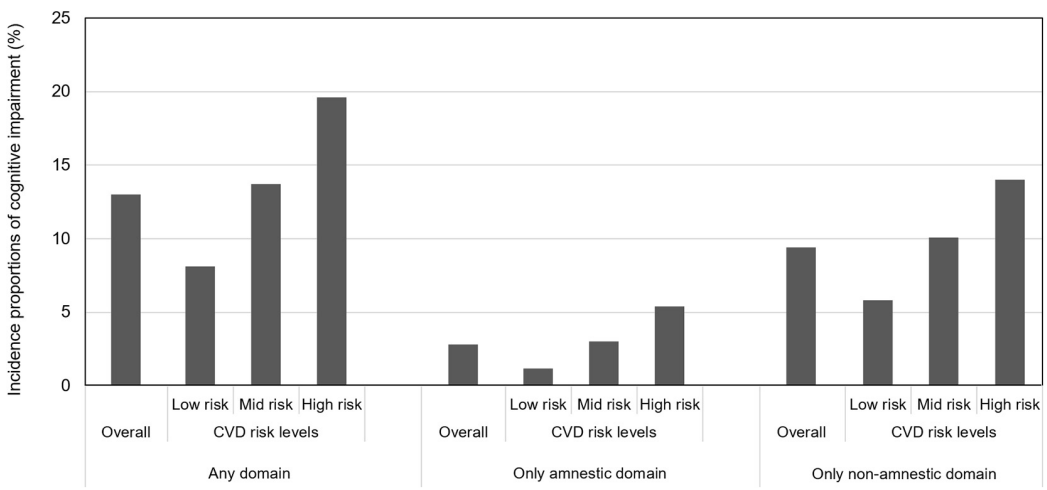

FIGURE 2 | Incidence proportions of cognitive impairment according to baseline CVD risk levels.

Logistic regression analysis showed that baseline CVD risk levels (reference: low risk) were significantly associated with the incidence of cognitive impairment (in any domain) after 4 years in both the crude model (moderate risk, OR 1.81; 95% CI 1.20–2.73; high risk, OR 2.78, 95% CI 1.61–4.80) and the adjusted model (moderate risk, OR 1.56, 95% CI 1.02–2.38; high risk, OR 2.21, 95% CI 1.24–3.95; **Table 3**). In the logistic regression analysis divided by cognitive subtype, the incidence of amnestic impairment was significantly associated with a high risk of CVD (OR 4.87, 95% CI 1.40–16.91) but not with a moderate risk of CVD (OR 2.66, 95% CI 0.93–7.62) in the crude model, and there was no significant relationship between CVD risk levels and the incidence of amnestic impairment in the adjusted model (moderate risk, OR 1.88, 95% CI 0.64–5.48; high risk, OR 3.21, 95% CI 0.89–11.57). However, incidence of non-amnestic impairment was significantly associated with moderate and high

risk in both the crude model (moderate risk, OR 1.83, 95% CI 1.13–2.96; high risk, OR 2.65, 95% CI 1.39–5.05) and in the adjusted model (moderate risk, OR 1.66, 95% CI 1.01–2.73; high risk, OR 2.40, 95% CI 1.22–4.73; **Table 4**).

DISCUSSION

Our longitudinal analysis indicated that absolute CVD risk, based on WHO risk estimation charts in old age, was significantly associated with the incidence of cognitive impairment among older adults without CVDs at baseline. Additionally, after adjusting for potential confounding factors, absolute CVD risk was found to be significantly associated with non-amnestic impairment but not with amnestic impairment. These results suggested that the association between absolute CVD risk and cognitive decline differed between amnestic and non-amnestic subtypes in older Japanese.

Previous studies have shown that CVD risk estimation based on multivariable risk assessment is a useful tool to predict not only a CVD event but also cognitive decline (Samieri et al., 2018; Song et al., 2020). Samieri et al. (2018) showed that baseline cardiovascular health levels, estimated according to the American Heart Association's Life's Simple seven metrics (smoking, body mass index, physical activity, diet, total cholesterol, fasting glucose, and blood pressure), predicted rates of cognitive impairment. Song et al. (2020). showed that the Framingham General Cardiovascular Risk Score (FGCRS), consisting of age, sex, smoking, blood pressure, medication for hypertension, total cholesterol, high-density lipoprotein cholesterol, and diabetes mellitus, predicted rates of cognitive impairment. Our results accorded with findings from these earlier studies. Importantly, components of a CVD risk estimation model are easily obtainable in clinical and research settings and may be useful for identifying individuals at the highest risk of future cognitive impairment and dementia. Moreover, to our knowledge, this study is the first to show the predictive validity of a region-specific CVD estimation model for the incidence of cognitive impairment in a non-Western country. In Japan, the average life span is approximately 81 years for men and 87 years for women (Ministry of Health, Labour and Welfare, 2019) and identifying effective interventions to expand healthy life expectancy remains an urgent issue. Further external validation of existing and international CVD risk estimation tools to predict cognitive impairment among older adults in non-Western countries is needed.

Participants in our study were followed up at 48 ± 2 months, whereas earlier studies involved relatively longer follow-up times [i.e., the maximum follow-up period was 16.6 years in Samieri et al.'s (2018) study and 21 years in Song et al.'s (2020) study]. Given more extended average life spans in many countries, early screening and intervention to mitigate the adverse effects of prolonged exposure to CVD risks in old age is essential, although control of CVD risk beginning early in life is optimal. Therefore, a relatively short-term CVD risk estimation in old age may be becoming increasingly important along with a longer-term estimation in middle age. Our results may have clinical significance because our study findings showed the potential for absolute CVD risk to predict relatively short-term cognitive decline in old age.

Most significantly, our study findings showed a possibility that associations between absolute CVD risk levels and cognitive impairment differ between amnestic and non-amnestic subtypes. While univariable analysis showed significant associations between CVD risk level and both amnestic and non-amnestic impairment, our multivariable analysis showed that the CVD risk level was significantly associated with non-amnestic impairment but not with amnestic impairment. Some previous longitudinal studies have examined the association between CVD risk level, calculated using multivariable risk factors, and cognitive impairment in amnestic and non-amnestic subtypes, and have reported contrasting results. Aljondi et al. (2020) reported that a high FGCRS-based CVD risk level was associated with executive function but not with episodic memory, semantic memory, and visuospatial ability. Kaffashian et al. (2013) showed that a high FGCRS-based CVD risk level was associated with faster cognitive decline in reasoning, phonemic fluency, semantic fluency, and vocabulary, but not with memory. Song et al. (2020) reported that a high FGCRS-based CVD risk level was associated with cognitive decline in episodic memory and working memory, as well as visuospatial ability and perceptual speed. However, regarding individual CVD risk factors, a subtype-dependent difference in the association between individual CVD risk factors and cognitive impairment has been observed. Reitz et al. found that hypertension predicted all-cause mild cognitive impairment (MCI) and non-amnestic MCI but not amnestic MCI (Reitz et al.,

TABLE 3 | Odds ratios and 95% confidence intervals for incidence of cognitive impairment according to CVD risk levels.

		Cognitive impairment			
		Crude model		Adjusted model	
		OR	95% CI	OR	95% CI
Cardiovascular disease risk					
Low risk (<10%)		ref		ref	
Moderate risk (10 ≤ 20%)		1.81	1.20–2.73	1.56	1.02–2.38
High risk (≥20%)		2.78	1.61–4.80	2.21	1.24–3.95
Potential confounding factors					
Education level	(Years)			0.94	0.88–1.00
Pulmonary disease	(*n*, %)			0.82	0.51–1.31
Prescribed medication	(Number)			1.10	1.01–1.20
Body mass index	(kg/m^2)			0.96	0.91–1.01
Alcohol consumption habit	(*n*, %)			1.21	0.89–1.65
Slow gait speed	(*n*, %)			0.77	0.35–1.68
Depressive symptoms	(*n*, %)			1.18	0.74–1.90
Physical inactivity	(*n*, %)			1.09	0.77–1.53
Living alone	(*n*, %)			1.36	0.85–2.19
Employment	(*n*, %)			0.73	0.52–1.03
MMSE	(Score)			0.84	0.79–0.90

CI, confidence interval; CVD, cardiovascular disease; MMSE, mini-mental state examination; OR, odds ratio.

The adjusted model was adjusted for all potential confounding factors assessed in the present study.

2007). Bae et al. (2017) reported that metabolic syndrome was associated with non-amnestic MCI but not with amnestic MCI. Our results concerning subtype-dependent differences in the association between CVD risk and cognitive impairment appear consistent with these previous findings.

The mechanisms involved in the subtype-dependent association between CVD risk and cognitive impairment are not fully understood; however, an earlier study suggested that the risk factors for vascular dementia characterized by non-amnestic impairment were almost identical to risk factors for CVD (O'Brien et al., 2003). Common risk factors, such as cortical or subcortical infarcts and small-vessel disease (O'Brien et al., 2003), might help explain the robust association found between CVD risk level and cognitive impairment in the non-amnestic subtype in contrast to the amnestic subtype in our study. However, AD and vascular cognitive impairment are known to share common pathology, including atherosclerosis and amyloid angiopathy, and clear discrimination is difficult. Additional studies including biomarkers related to dementia subtypes, such as amyloid status and brain magnetic resonance imaging, are required. As another explanation, one previous study reported that cognitive impairment in terms of episodic memory and working memory were linked with hippocampal volume; that is, typical markers of AD-related neurodegeneration and cognitive impairment in perceptual speed are linked with white matter hyperintensities that indicate microvascular lesions in cerebral white matter (Song et al., 2020). Furthermore, Wardlaw et al. (2013) noted the possibility that the effects of CVD risk factors on brain structure begin with white matter lesions of presumed vascular origin and then proceed to morphological neurodegenerative changes. Our results, according to cognitive subtype, might reflect temporal and regional differences in terms of adverse effects of CVD risk factors on brain function.

Absolute CVD risk estimated using region-specific CVD risk prediction charts in old age was found to be useful to predict the incidence of cognitive impairment, and the association between CVD risk levels and cognitive impairment was more significant in the non-amnestic subtype. Subtype-dependent differences in the association between absolute CVD risk and cognitive impairment in the present study may provide useful information for planning tailor-made strategies to prevent dementia.

Strengths and Limitations

A major strength of this study was that it was the first to examine the predictive validity of revised WHO CVD risk estimation charts in a non-Western country. Furthermore, we analyzed well-characterized cohort data and conducted multivariable analyses, adjusting for multiple confounding factors. Moreover, as we analyzed population-based data concerning older adults without CVDs at baseline, our findings can be generalized to community dwelling people in primary care settings.

However, our study had some limitations. First, approximately 39% of the participants dropped out during the follow-up period and their baseline characteristics were significantly different from those of participants who completed the follow-up assessment. This selection bias may have led to an underestimation of CVD risk and cognitive decline. Additionally, the number of new cases of cognitive impairment was limited particularly in subgroup analysis into amnestic and non-amnestic groups. This relatively small sample size might have affected our findings through decreased statistical power. Second, we did not undertake a

TABLE 4 | Odds ratios and 95% confidence intervals for incidence of cognitive impairment in each subtype according to CVD risk levels.

		Amnestic impairment				Non-amnestic impairment			
		Crude model		Adjusted model		Crude model		Adjusted model	
		OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Cardiovascular disease risk									
Low risk (<10%)		Ref		Ref		Ref		Ref	
Moderate risk (10 ≤ 20%)		2.66	0.93–7.62	1.88	0.64–5.48	1.83	1.13–2.96	1.66	1.01–2.73
High risk (≥20%)		4.87	1.40–16.91	3.21	0.89–11.57	2.65	1.39–5.05	2.40	1.22–4.73
Potential confounding factors									
Education level	(Years)			1.00	0.88–1.14			0.91	0.85–0.98
Pulmonary disease	(*n*, %)			1.88	0.86–4.10			0.55	0.29–1.02
Prescribed medication	(Number)			1.02	0.83–1.24			1.16	1.05–1.27
Body mass index	(kg/m^2)			1.05	0.94–1.17			0.93	0.87–0.99
Alcohol consumption habit	(*n*, %)			1.75	0.90–3.42			1.13	0.79–1.61
Slow gait speed	(*n*, %)			0.45	0.06–3.47			0.70	0.27–1.83
Depressive symptoms	(*n*, %)			2.03	0.85–4.86			1.12	0.64–1.95
Physical inactivity	(*n*, %)			0.97	0.47–2.03			1.06	0.71–1.58
Living alone	(*n*, %)			1.18	0.40–3.48			1.16	0.66–2.05
Employment	(*n*, %)			0.85	0.41–1.75			0.71	0.47–1.06
MMSE	(Score)			0.76	0.66–0.87			0.90	0.83–0.97

CI, confidence interval; CVD, cardiovascular disease; MMSE, mini-mental state examination; OR, odds ratio.
The adjusted model was adjusted for all potential confounding factors assessed in the present study.

comparison between the revised WHO CVD risk charts and other existing CVD risk estimation tools; therefore, we cannot confirm concurrent validity or discuss the relative merits of the WHO CVD risk charts. Third, we did not measure biomarkers related to the prognosis of MCI, such as apolipoprotein E genotype or amyloid status. Additional longitudinal studies to assess the relationships between CVD risk levels and cognitive decline in each domain using biomarkers reflecting the pathology of cognitive impairment are required. Finally, although we used the risk estimation model developed for "the high-income Asia-Pacific region" in accordance with region classification by WHO, our sample came from single country (only Japan). Therefore, there might be potential effects of socio-economic status on our findings and further examination is required to clarify whether our findings can be generalized to other countries.

CONCLUSION

In conclusion, absolute CVD risk estimated according to region-specific CVD risk estimation charts in old age was useful to predict the incidence of cognitive impairment among older Japanese adults. The absolute CVD risk level can be estimated using variables that are easily obtainable in clinical and research settings, and should be used for more effective early dementia risk screening. Additionally, the association between CVD risk level and cognitive impairment was more significant in the non-amnestic subtype, and strategies to screen populations at risk of cognitive impairment and to prevent progression to dementia should be cognitive subtype-specific.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because participants of this study did not agree for their data to be shared publicly. Requests to access the datasets should be directed to KM, kmakino@ncgg.go.jp.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the National Center for Geriatrics and Gerontology. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KM designed the study, analyzed and interpreted the data, and wrote and edited the manuscript. HS administered the project, acquired funding, and reviewed and edited the manuscript. SL, SB, and KH contributed to acquisition, analysis, and interpretation of data, and reviewed and edited the manuscript. IC, OK, and YS contributed to the discussion and reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Comorbidity Trajectories Associated With Alzheimer's Disease: A Matched Case-Control Study in a United States Claims Database

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Background: Trajectories of comorbidities among individuals at risk of Alzheimer's disease (AD) may differ from those aging without AD clinical syndrome. Therefore, characterizing the comorbidity burden and pattern associated with AD risk may facilitate earlier detection, enable timely intervention, and help slow the rate of cognitive and functional decline in AD. This case-control study was performed to compare the prevalence of comorbidities between AD cases and controls during the 5 years prior to diagnosis (or index date for controls); and to identify comorbidities with a differential time-dependent prevalence trajectory during the 5 years prior to AD diagnosis.

Methods: Incident AD cases and individually matched controls were identified in a United States claims database between January 1, 2000 and December 31, 2016. AD status and comorbidities were defined based on the presence of diagnosis codes in administrative claims records. Generalized estimating equations were used to assess evidence of changes over time and between AD and controls. A principal component analysis and hierarchical clustering was performed to identify groups of AD-related comorbidities with respect to prevalence changes over time (or trajectory), and differences between AD and controls.

Results: Data from 186,064 individuals in the IBM MarketScan Commercial Claims and Medicare Supplementary databases were analyzed (93,032 AD cases and 93,032 non-AD controls). In total, there were 177 comorbidities with a $\geq$ 5% prevalence. Five main clusters of comorbidities were identified. Clusters differed between AD cases and controls in the overall magnitude of association with AD, in their diverging time trajectories, and in comorbidity prevalence. Three clusters contained comorbidities that notably increased in frequency over time in AD cases but not in controls during the 5-year period before AD diagnosis. Comorbidities in these clusters were related to the early signs and/or symptoms of AD, psychiatric and mood disorders, cerebrovascular disease, history of hazard and injuries, and metabolic, cardiovascular, and respiratory complaints.

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Conclusion: We demonstrated a greater comorbidity burden among those who later developed AD vs. controls, and identified comorbidity clusters that could distinguish these two groups. Further investigation of comorbidity burden is warranted to facilitate early detection of individuals at risk of developing AD.

Keywords: Alzheimer's disease, comorbidity, principal component analysis, hierarchical cluster analysis, MarketScan, Medicare

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia, accounting for 60–70% of cases. The prevalence of AD increases with age, with a global prevalence of 5–8% in people 60 years and older [World Health Organization [WHO], 2021]. While AD has previously been considered to have discrete and clearly defined clinical stages, it is now more usually considered to be a seamless continuum from an asymptomatic phase through a long preclinical period, to a symptomatic phase in which cognitive and then functional impairment become increasingly evident (Dubois et al., 2016; Aisen et al., 2017; Jack et al., 2018). Furthermore, while the terms "mild cognitive impairment (MCI)" or "prodromal AD (pAD)" and "mild AD" have traditionally been used in clinical trials to describe the early stages of AD, these are often studied together and referred to as "early AD" patients (Siemers, 2021).

Evidence suggests that treatment earlier in the disease continuum is likely to achieve greater disease modification and slow the rate of cognitive and functional decline (Dubois et al., 2016; Aisen et al., 2017; Jack et al., 2018). However, AD is only usually diagnosed once clinical symptoms become apparent, which may be as long as 15 years after the first pathological changes occur, leading to delays in treatment and potentially lost clinical benefit (Dubois et al., 2016; Aisen et al., 2017). Even after symptoms of AD become clinically evident, there exists a large population living with dementia who remain undiagnosed (Lang et al., 2017; Amjad et al., 2018; Genovese et al., 2018; Grandal Leiros et al., 2018). It is thought that among older adults with probable dementia (including AD), most (58.7%) were either undiagnosed (39.5%) or unaware of the diagnosis (19.2%) (Amjad et al., 2018). A meta-analysis of 23 studies conducted between 1988 and 2015 in community and residential settings reported a 61.7% pooled rate of undetected dementia (Lang et al., 2017).

This underdiagnosis may be due in part to a low dementia diagnosis rate in primary care (Boise et al., 1999; Geldmacher and Kerwin, 2013; Jørgensen et al., 2015; Lang et al., 2017; Small, 2017). Currently in the United States (US), a diagnosis of dementia in primary care is largely reliant on the self-presentation of a patient on the basis of symptoms or caregiver concerns (Iliffe et al., 1991; McCormick et al., 1994; Bradford et al., 2009), such that many cases go undiagnosed until late in the disease (McCormick et al., 1994). For those patients who do present, referral to a specialist then requires the primary care physician to act on a clinical suspicion (Brayne et al., 2007), which is itself prone to being missed or delayed (Iliffe et al., 1991; Callahan et al., 1995; Bradford et al., 2009). The availability of specialist tools to help evaluate whether a patient needs to be referred for specialist care may save time and expedite any decision-making process, potentially increasing the rate of diagnosis of AD.

Certain chronic medical conditions, including type 2 diabetes (T2DM), hypertension, coronary artery disease, and depression, are established risk factors for cognitive decline (Artero et al., 2008; Vicini Chilovi et al., 2009; Li et al., 2012; Roberts and Knopman, 2013; Imtiaz et al., 2014; Johnson et al., 2015; Vassilaki et al., 2015; Fan et al., 2017). These conditions are also common in multimorbidity (defined as at least two comorbid conditions) in older adults, which may also be associated with biomarkers of the preclinical AD stages (Sperling et al., 2011; Jack et al., 2014; Sperling et al., 2014) and suspected non-amyloid pathophysiology (Jack et al., 2016; Vassilaki et al., 2019), even before clinically detectable cognitive decline becomes apparent. Not only is there an increase in the prevalence of comorbidities among patients at risk of AD, but multimorbidity, a distinctive hallmark of aging and potentially a clinical marker of accelerated aging (Fabbri et al., 2015), is also associated with increased risk of cognitive impairment (Palmer et al., 2007; Vassilaki et al., 2015; Santiago and Potashkin, 2021). There is also evidence suggesting that the trajectories of comorbidities among individuals at risk of AD differ from those who are simply undergoing the normal process of aging (Oveisgharan and Hachinski, 2010; Velayudhan et al., 2010; Xu et al., 2010). Therefore, an evaluation of comorbidities and their trajectories during the early stage of disease is highly relevant in characterizing the natural history of AD dementia. In this way, identifying distinctive patterns of comorbidities, including signs and symptoms of early AD, may enable more timely cognitive assessment and specialist referral for an evaluation of AD diagnosis.

A data-driven approach was used in this analysis to identify comorbidities that occur before AD diagnosis that are associated with the development of AD. Incident AD cases and matched non-AD controls from the general population were identified in a US claims database and used to investigate comorbid diagnoses that occurred during the 5 years prior to a first diagnosis of AD. A window of 5 years to capture patients with early AD was set on the basis that the median duration between the onset of dementia-related symptoms and assessment or diagnosis is typically up to 3 years, according to literature reports (Boise et al., 1999; Knopman et al., 2000; Wackerbarth and Johnson, 2002; Wilkinson et al., 2004; Fiske et al., 2005; Speechly et al., 2008; Carpentier et al., 2010; van Vliet et al., 2013; Zhao et al., 2016). The methodology used in this analysis is a new application of a standard method used to identify patterns inherent in data.

This analysis has two primary objectives: (1) to compare the prevalence of comorbidities between AD cases and non-AD controls during the 5 years prior to diagnosis; and (2) to identify

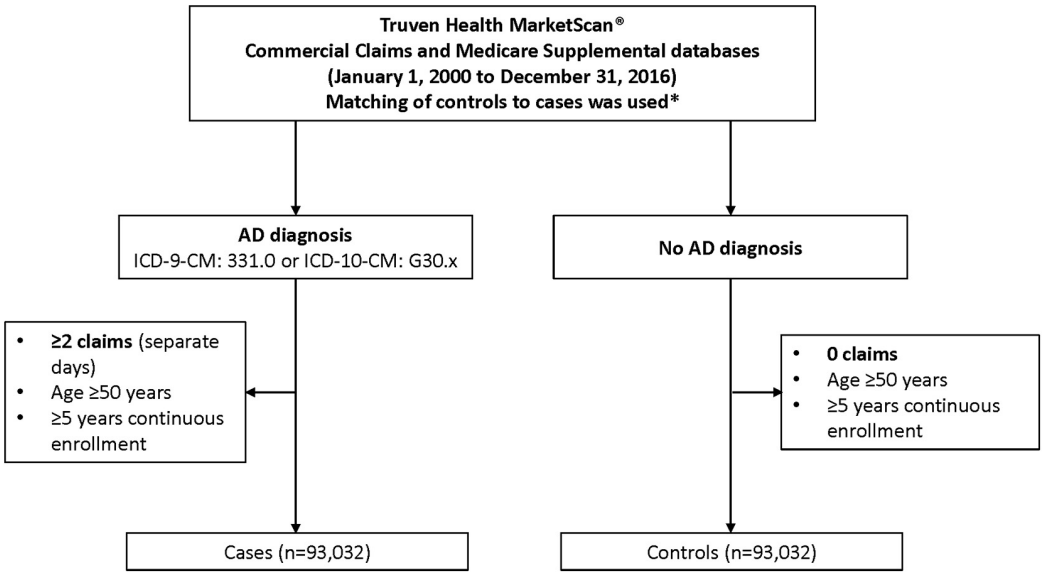

FIGURE 1 | Flowchart of case inclusion process. *Cases were required to have at least two claims for AD on separate days at age ≥ 50 years (ICD-9-CM: 331.0 or ICD-10-CM: G30.x) and a 5-year period of continuous enrollment prior to first AD diagnosis, while eligible controls for each case were required to have no claims for AD (ICD-9-CM: 331.0 or ICD-10-CM: G30.x) during the 5-year window prior to the respective cases index date. Matching was based on sex; year of birth; insurance plan type at index; relationship to insurance plan holder; (previous) employment industry; and US region. AD, Alzheimer's disease; ICD, International Classification of Diseases.

comorbidities with a time-dependent prevalence trajectory during the 5 years prior to AD diagnosis that is differential among cases, compared with controls.

MATERIALS AND METHODS

Study Design and Setting

This was a retrospective, observational, case-control study conducted in the US using data from the IBM MarketScan® Commercial Claims and Medicare Supplementary databases.

Study Population

The study population consisted of individuals with AD ("cases") and a matched group of individuals without AD ("controls") (**Figure 1**). Cases were required to have at least two claims for AD on separate days at age ≥ 50 years [International Classification of Diseases (ICD)-9-CM: 331.0 or ICD-10-CM: G30.x] and a 5-year period of continuous enrollment prior to first AD diagnosis, while eligible controls for each case were required to have no claims for AD (ICD-9-CM: 331.0 or ICD-10-CM: G30.x) during the 5-year window prior to the respective cases index date. All eligible cases from the database were included in the analysis. The index date for cases was the first AD diagnosis date. For controls, the index date was set to the same date as the individually matched case.

Matching

For each AD case, a control (1:1) was selected randomly and without replacement from the pool of eligible controls, as defined above. Matching was based on sex; year of birth (hence also age, given the same index date); insurance plan type at index [e.g., Health Maintenance Organization (HMO); Preferred Provider Organization (PPO); Point of Service (POS); comprehensive]; relationship to insurance plan holder (employee or spouse/other); employment industry; and US region (West, Northeast, Midwest, or South).

Data Source

Data for this analysis were extracted from the IBM MarketScan® Commercial Claims ("Commercial") database and the Medicare Supplementary ("Medicare") database. The commercial database contains active employees, early retirees, and dependents insured by employer−sponsored plans, while the Medicare database covers Medicare-eligible retirees (≥ 65 years) with employer-sponsored Medicare Supplementary plans. Both data sets were analyzed together in order to allow patients to be tracked from employment through into retirement.

Because the database is based on insurance claims, individuals are able to drop in and out of enrollment in the database. The "continuous enrollment period" was therefore defined on an individual-person level, based upon medical insurance coverage. Gaps in enrollment of up to 62 days (2 months) were allowed so long as this gap was contained by periods of documented enrollment before and afterward. Continuous enrollment periods had maximum boundaries of the study period (January 1, 2000 to December 31, 2016).

Comorbidity Definitions

The presence of individual comorbidities was evaluated in each of the 5-yearly intervals prior to AD diagnosis, based upon the occurrence of at least one diagnosis claim (code) in the relevant

time period. Diagnosis recorded in the database was based upon the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) up until 30 September 2015, and thereafter was based on ICD-10-CM criteria. For the purposes of this study, all ICD-10-CM codes were converted to ICD-9-CM prior to further grouping.

Comorbidities were grouped into approximately 1,200 categories, based upon the first three digits of each ICD-9-CM code. The decodes for these three-digit sub-chapters can be found in the "icd" r package (Wasey et al., 2021). Comorbidities with a total 5-year occurrence of ≥5% (across both AD cases and controls, combined) were kept.

Ethical Considerations

This was a retrospective, observational study of secondary use data. All personal information used was de-identified (with no possibility of linkage back to individual identified patients) and is compliant with the US Health Insurance Portability and Accountability Act (HIPAA).

Statistical Analysis

Descriptive Analyses

Demographic/personal characteristics of AD cases and non-AD controls from both cohorts were summarized using means and standard deviations (SDs) for continuous variables and frequencies and percentages for categorical variables. For both AD cases and non-AD controls, the proportion of patients with each comorbidity with ≥ 5% prevalence, over the 5 years prior to index date, were reported.

Associations Between Alzheimer's Disease and Comorbidities

Generalized estimating equations (GEE) were used to estimate the odds of each comorbidity as a function of time (yearly intervals prior to index), AD diagnosis status, and their interaction. Odds ratios (ORs) were estimated, and hypothesis tests were conducted on the following model:

$$\text{logit}(p_{ijk}) = \eta_i + \alpha_i AD_j + \beta_i Time_k + \gamma_i AD_j Time_k$$

where pijk = proportion of subjects who reported a claim for comorbidity i at year k prior index date (k = 1,. . ., 5), having AD diagnosis status (j = 1, 2). η is the logit's general mean, α is the log odds of AD vs. control, β is the change in the log odds by change in 1 year, and γ is the difference of log odds changes per year between AD cases and controls. Standard errors were computed using the sandwich robust variance estimator (Liang and Zeger, 1986), assuming an unstructured within-subject covariance matrix. Multiple testing correction (False Discovery Rate) was applied to account for multiple testing across comorbidities.

Multivariate Analysis: Principal Component Analysis and Hierarchical Cluster Analysis

In order to identify groups of comorbidities associated with AD that varied in terms of frequency changes over time and between AD and non-AD control groups, four metrics from the GEE models were considered; two denoting the magnitude of the differences between AD vs. controls (#1 and #2) and two related to the level of evidence of such differences (#3 and #4): (1) the difference in log odds of AD vs. non-AD controls (i.e., coefficient α centered at the mean follow-up time prior to AD diagnosis); (2) the $\log_{10}$ scaled p-value associated with the hypothesis test of the centered α; (3) the interaction term, γ, which denotes the difference of slopes between changes over time of AD patients vs. controls; (4) the $\log_{10}$ scaled p-value associated with the hypothesis test of γ. Metrics 1 and 2 assessed the overall difference in AD vs. controls comorbidities over the period prior to index date, whereas metrics 3 and 4 assessed the difference in the comorbidity trajectory over time between AD and controls, during the 5-year period prior to index date.

A data matrix of dimensions n (number of comorbidities) and p = 4 (the four metrics selected) was created and submitted to PCA, where a new set of orthogonal variables were obtained. The new data matrix was analyzed by hierarchical clustering with Ward's grouping algorithm along with Euclidean distances in order to identify AD comorbidities by their changes over time and between groups.

RESULTS

Demographic Characteristics

Data from 186,064 individuals in the IBM MarketScan® Commercial Claims and Medicare Supplementary databases were analyzed (93,032 AD cases and 93,032 non-AD controls) (**Table 1**). Overall, 59% of the population was female. The study population was predominantly older adults, with an average age of 82 years. Seventeen percent of participants were aged 90 years or older. The majority of the included population (57%) had comprehensive insurance, followed by PPO (32%). Most participants resided in the North-Central US region (45%), followed by the Southern states (29%).

Prevalence and Association of Comorbidities, Signs, and Symptoms During 5 Years Prior to Alzheimer's Disease Diagnosis

In total, 177 comorbidities were identified with a prevalence of ≥5% (**Supplementary Table 1**). Of these, the individual comorbidities [(ICD-9 code; prevalence (%)] with the highest 5-year pooled prevalence prior to index date across AD cases and in controls were: essential hypertension (401; 74.5%), general symptoms (780; 66.9%), symptoms involving the respiratory system (786; 65.6%), disorders of lipoid metabolism (272; 54.8%), and other and unspecified disorders of joint (719; 53.3%) (**Table 2**). However, the comorbidities with highest ORs in AD cases compared with controls in the period prior to index date were: persistent mental disorders due to conditions classified elsewhere (294), other non-organic psychoses (298), other cerebral degenerations (331), transient mental disorders due to conditions classified elsewhere (293), general symptoms (780), other conditions of the brain (348), episodic mood disorders (296), and depressive disorders not elsewhere classified (331), among others (**Supplementary Figure 1**).

TABLE 1 | Demographic characteristics.

Variable	Overall *N* = 186,064	Cases *N* = 93,032	Controls *N* = 93,032
Sex, % female	59.12	59.12	59.12
Age, years, mean (*SD*)	82.11 (8.1)	82.11 (8.1)	82.11 (8.1)
Age, years			
50–59, %	1.3	1.3	1.3
60–69, %	5.9	5.9	5.9
70–79, %	25.7	25.7	25.7
80–89, %	50.3	50.3	50.3
90+, %	16.8	16.8	16.8
Region, %			
North-Central	45.4	45.4	45.4
South	29.2	29.2	29.2
West	14.4	14.4	14.4
Northeast	10.8	10.8	10.8
Unknown	0.2	0.2	0.2
Plan holder %			
Current/previous employee	81.8	81.8	81.8
Spouse/child/other	18.2	18.2	18.2
Healthcare plan type %			
Comprehensive	56.9	56.9	56.9
PPO	32.5	32.5	32.5
HMO	6.0	6.0	6.0
POS	3.2	3.2	3.2
Other	0.8	0.8	0.8
Missing	0.8	0.8	0.8
Industry, n (%)			
Manufacturing, durable goods	42.7	42.7	42.7
Transportation, communications, utilities	18.1	18.1	18.1
Services	11.1	11.1	11.1
Other and missing	28.1	28.1	28.1

CDHP, Consumer Driven Health Plan; EPO, Exclusive Provider Organization; HDHP, High Deductible Health Plan; HMO, Health Maintenance Organization; POS, Point of Service; PPO, Preferred Provider Organization; SD, standard deviation.

Multivariate Analysis

Four principal components (PCs) were obtained from the PCA analysis conducted on the four metrics used to differentiate comorbidities. The first, second, third, and fourth PCs explained 70.3, 15.4, 11.0, and 3.3% of variance in the data, respectively. **Supplementary Figures 2A,B** display the distribution of the five clusters of comorbidities in biplots for the first and second PCs and in the first and third PCs, respectively. Five main clusters of comorbidities were found from the hierarchical cluster analysis conducted on the four PCs (**Figure 2** and **Supplementary Figure 3**). Clusters 1, 3, and 5 consisted of comorbidities with higher ORs and smaller *p*-values for the comparison of AD vs. controls mid-term prior to index date (**Figures 2C,D**). Although clusters 1, 2, and 3 contained comorbidities with higher ORs for the differential time trajectories between AD vs. controls, cluster 1 stood out as the collection of comorbidities with the largest differential time-dependent trajectories among AD vs. controls and smaller *p*-values for the interaction terms (**Figures 2A,B**).

TABLE 2 | Comorbidities with the highest pooled prevalence across cases and control.

Description	Overall *N* = 186,064 %	Cases *N* = 93,032 %	Controls *N* = 93,032 %
Essential hypertension	74.5	83.6	65.4
General symptoms*	66.9	86.3	47.5
Symptoms involving respiratory system and other chest symptoms	65.6	75.8	55.5
Disorders of lipoid metabolism	54.8	62.2	47.3
Other and unspecified disorders of joint	53.3	63.5	43.2
Other disorders of soft tissues	44.8	53.6	36.1
Cataract	44.1	48.8	39.4
Osteoarthrosis and allied disorders	43.0	50.5	35.5
Other and unspecified disorders of back	38.7	45.7	31.7
Cardiac dysrhythmias	38.5	44.8	32.2
Other symptoms involving abdomen and pelvis	37.8	45.0	30.6
Special screening for malignant neoplasms	37.2	40.5	33.9
Other disorders of urethra and urinary tract	36.4	45.4	27.4
Other forms of chronic ischemic heart disease	35.5	40.7	30.3
Symptoms involving digestive system	34.5	42.6	26.5
Encounter for other and unspecified procedures and aftercare	34.3	40.0	28.5
Other disorders of bone and cartilage	32.4	37.7	27.0
Other dermatoses	31.9	33.7	30.1
Symptoms involving skin and other integumentary tissue	31.7	38.3	25.1
Special investigations and examinations	31.3	34.9	27.8

Comorbidities are arranged in descending order of prevalence in the overall population.
**The frequency (%) of general symptoms were: Alteration of consciousness (22.5), hallucinations (2.1), syncope and collapse (30.3), convulsions (7.6), dizziness and giddiness (38.0), sleep disturbances (16.8), Fever and other physiologic disturbances of temperature regulation (14.9), malaise and fatigue (54.3), generalized hyperhidrosis (1.7), and other general symptoms (52.0).*
CI, confidence interval.

Description of the Clusters, Differential Trajectories of Comorbidities, Signs, and Symptoms During 5 Years Prior to Alzheimer's Disease Diagnosis Among Cases vs. Controls

The comorbidities included under each cluster are listed in **Supplementary Table 1**, trajectories are shown in **Supplementary Figure 4**.

Cluster 1 contained 18 comorbidities with a large difference in trajectories, increasing rapidly in the AD group prior to diagnosis, but not in the controls (**Supplementary Figure 4A**). Comorbidities were quite prevalent overall (generally >15% in AD and non-AD combined groups). This cluster included terms such as persistent mental disorder (294), non-organic

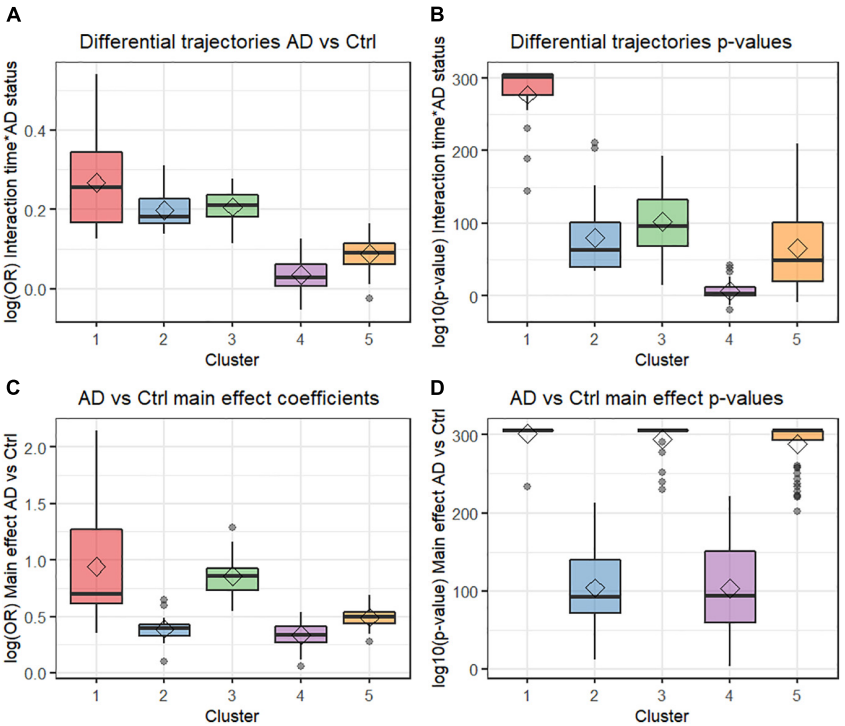

FIGURE 2 | Box plots from multivariate analysis. **(A)** Differential trajectories AD vs. control. **(B)** Differential trajectories *p*-values. **(C)** AD vs. control effect coefficients. **(D)** AD vs. control main effect *p*-values. AD, Alzheimer's disease; Ctr, Control; OR, Odds ratio.

psychoses (298), transient mental disorders (293), and other cerebral degenerations (331), but also included other wide-ranging comorbidities such as fluid and electrolyte imbalance (276), symptoms of respiratory system (786), heart failure (428), and dermatophystosis (110) (**Supplementary Figure 4A** and **Supplementary Table 1**).

Cluster 2 contained 19 comorbidities with marked differences in time trajectories between AD vs. non-AD (**Supplementary Figure 4B**), lower OR for the overall AD vs. non-AD comparison in the period prior to AD diagnosis (**Figure 3A**), and with lower overall prevalence (generally <15% in AD and non-AD combined, **Figure 3B**). This cluster included diseases of the kidneys, such as chronic (585) and acute (584), and other (593) kidney disease; and lungs, such as emphysema (492), pneumonia (486), and chronic bronchitis (491). Other comorbidities not related to lung or kidney were also included, such as bacterial infections (Wilkinson et al., 2004), septicemia (Speechly et al., 2008), vertebral fractures (805), and diseases of white blood cells (288) (**Supplementary Figure 4B** and **Supplementary Table 1**).

Cluster 3 contained 22 comorbidities, also with large trajectory differences between AD cases and controls (**Supplementary Figure 4C**). The difference with cluster 1 was that the statistical significance of the trajectory differences were not as strong and that comorbidities in this group had lower prevalence (**Figure 3B**). Comorbidities included psychiatric comorbidities such as depression (311) and anxiety (300) but also included terms related to care and rehabilitation procedures (V57), personal history of hazard to health (V15), nutrition, metabolism and development (783), fracture of femur (820), open wound of head (873), and contusions (of various body parts; 920 and 922–924). Additionally, various cerebrovascular disease comorbidities were included (434–438) (**Supplementary Table 1**).

Cluster 4 was the largest group with 65 comorbidities. This cluster included comorbidities with the most similar trajectories over time (**Figure 3A** and **Supplementary Figure 4D**), and a more similar prevalence between cases and controls. In addition, some comorbidities became less prevalent in both groups over time (e.g., benign neoplasm of skin; 216).

Cluster 5 comorbidities had both trajectory differences and overall AD vs. non-AD prevalence differences that were more subtle than shown for comorbidities in cluster 3 (**Figure 3A** and **Supplementary Figure 4E**), despite the relatively high prevalence of some symptoms such as symptoms of the digestive system (787), osteoarthritis (715), disorders of lipoid metabolism (272), and cataract (366). This cluster was also large (51 comorbidities), and contained a more heterogeneous group of comorbidities, both in terms of prevalence and the affected system and/or organ (**Figure 3B** and **Supplementary Table 1**).

DISCUSSION

This project used a novel application of longitudinal data modeling and multivariate analysis to identify clusters of comorbidities that occur in patients' records prior to diagnosis

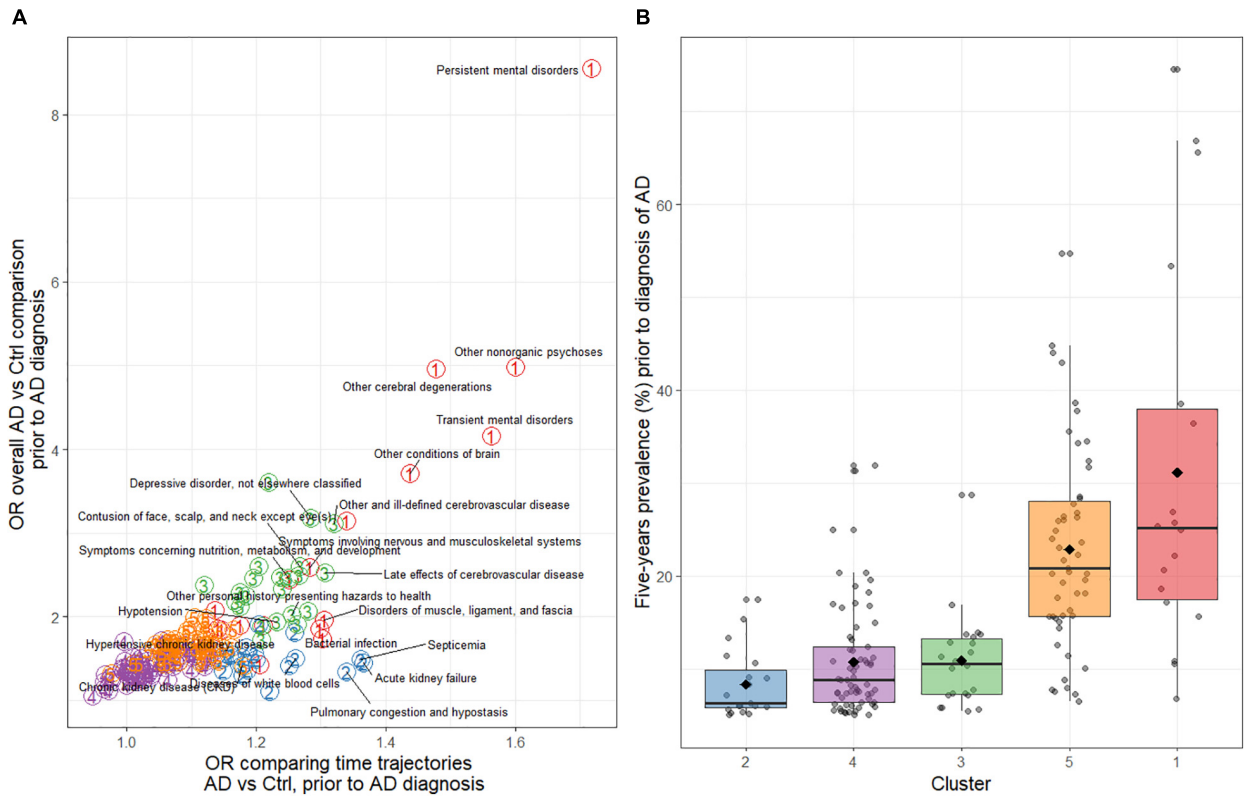

FIGURE 3 | (A) Odds ratios for the overall AD cases vs. non-AD controls comparison of comorbidities and for the time trajectories of comorbidities during the 5-year period before AD diagnosis. The colored circled numbers refer to the cluster that the comorbidity belongs to (i.e., red-colored 1 is cluster 1, etc.). **(B)** Distribution of the average comorbidity prevalence during the 5-year period before AD diagnosis by cluster. AD, Alzheimer's disease; Ctr, Control.

with AD. We identified clusters of AD comorbidities that may diverge in terms of frequency changes over time and between AD and non-AD control groups.

Cluster 1 offered the largest overall differences between AD cases and controls prior to diagnosis and, moreover, differential trajectories over time in AD cases compared with controls. The comorbidities in cluster 1 were fairly prevalent overall (>15% in AD and non-AD combined). Some comorbidities could be related to the early signs/symptoms of AD, such as persistent mental disorders and other cerebral degenerations (including MCI). However, also in cluster 1, with similar prevalence and trajectory differences prior to AD diagnosis, were serious comorbidities of other organs classes such as the respiratory and cardiovascular systems. These comorbidity classes have previously been shown to be associated with progression (Jutkowitz et al., 2017a,b; Koskas et al., 2017) and risk of AD and dementia (Bauer et al., 2014; Ruthirakuhan et al., 2019).

Similarly, other comorbidities in clusters 2, 3, and 5 showed differences between cases and controls, even if not directly related to AD. This shows that the comorbidity burden starts years prior to AD, during the early AD or MCI phase of progression or even earlier. For example, not only were relatively less frequent comorbidities of the kidneys and lungs (among others) more prevalent prior to AD diagnosis (cluster 2), but so were depression, anxiety, and comorbidities related to accidents or injuries (for example, history of personal hazards, open head wounds, and contusion to various body parts; cluster 3). These comorbidities have been associated with lower health-related quality of life in patients with AD (Barbe et al., 2018), as well as risk factors for cognitive impairment (Krell-Roesch et al., 2021). Falls are considered a marker of cognitive impairment, and an increased risk of falls has been reported among adults within the early, preclinical stage of AD (Stark et al., 2013). Together these and our findings reflect the need for additional measures to ensure patient safety around the home or in care facilities, respectively.

A potential limitation of this study is that comorbidities may be recorded more frequently in some patients' records, simply due to more encounters with the healthcare system during the work up of an AD diagnosis. However, cluster 4 was the largest group (65 comorbidities), and contains comorbidities without large differences between cases and controls. Small imbalances that do remain in cluster 4 are much lower in magnitude than for the differences between cases and controls in the other clusters, thus providing evidence of true elevated comorbidity burden in early AD, above any systematic differences due to reporting bias.

There is a real need to better characterize patients either at risk of developing AD or with AD early in the course of their disease,

to allow early intervention that could slow the rate of cognitive and functional decline (Dubois et al., 2016; Aisen et al., 2017; Jack et al., 2018). However, AD still tends to be diagnosed at a relatively advanced stage, meaning that the opportunity for early intervention is lost (Bature et al., 2018; Barnes et al., 2020).

AD usually has a slow progression (No authors listed, 2020), and although what defines AD as a unique neurodegenerative disease (among others conditions that could lead to dementia) are the β-amyloid plaques and the neurofibrillary tau deposits (Jack et al., 2018), there is still a lot of work to be done to delineate the AD pathogenesis causal pathways. Although, not all current findings are amenable to an easy interpretation, novel research (Wang et al., 2021) suggests that multiple pathological pathways could be involved in AD pathogenesis such as unresolved neuroinflammation, abnormal glucose metabolism, vascular alterations, mitochondria dysfunction; pathological processes present in many comorbidities (e.g., vascular conditions, diabetes, infections) in the present study.

Considerable research has been devoted over recent years to the use of biomarkers to diagnose AD early, and this approach has shown promising results (Frisoni et al., 2017; Blennow and Zetterberg, 2018; Giorgio et al., 2020). However, as most cases of AD are diagnosed in primary care, it is important to have a simple and convenient tool that is readily available to GPs to identify patients who may be at risk of progressing to AD dementia (Iliffe et al., 1991; McCormick et al., 1994; Bradford et al., 2009). It may be possible in a primary care setting to flag a patient's chart if a pattern of comorbidities is observed within a short period of time. This would then prompt healthcare professionals to inquire about memory concerns and possibly refer the patient to a specialist for cognitive testing and/or any imaging or fluid biomarkers available, including, but not limited to, magnetic resonance imaging (MRI), positron emission tomography (PET), and/or cerebrospinal fluid (CSF) Aβ and tau tests (Leocadi et al., 2020; Leuzy et al., 2021). The results of the current study add to and expand previous work toward the development of such a tool.

The study findings need to be viewed in light of the following limitations. There was an opportunity for misclassification of AD status among cases in this study because status was defined only by the presence of a diagnosis code for AD and not any biomarker or pathology data. Thus, the AD cases in this study are likely to have "Alzheimer's clinical syndrome," or what has been previously referred to as "clinically probably AD" (Jack et al., 2018). Another potential reason for misclassification of AD status is that the study cohort is skewed toward older age where seventeen percent are 90 years or older. It is possible that the older AD cases actually have other forms of dementia and/or neurodegenerative disorders with similar clinical presentation to AD and that are more common in older adults, such as limbic-predominant age-related TDP-43 encephalopathy (Nelson et al., 2019). Other forms of neurodegenerative disorders may be associated with a unique set of comorbidities, confounding interpretations. The statistical analysis also has a number of limitations, including the hierarchical clustering used to define groups. Although we used robust distance metrics on orthogonal data coordinates, alternative grouping-algorithms might have achieved different groupings. In addition, patients were grouped at the 3-digit ICD level as a short-hand for the more detailed patient histories collected. Although further refinement to lower-level codes may have yielded insights on more specific comorbidities, the scope of this study using an exploratory statistical approach was best suited for analyses with the higher-level groupings. Another potential limitation is that clusters were defined based on metrics related to overall differences in prevalence and trajectories; although our model assumed linear trajectories over time, and *p*-values are dependent on prevalence of comorbidities, not only the effect size. Finally, all comorbidities with prevalence $< 5\%$ across cases and controls were excluded, and no terms that captured associations other than linear shapes (or differences) were included based on the four metrics described in this study.

Strengths of the study include the large study size and follow up, taken from objectively and systematically collected data sources. Cases and controls were matched based on a number of factors including sex; year of birth; insurance plan type at index date; relationship to insurance plan holder; (previous) employment industry; and US region.

CONCLUSION

Although we demonstrated a greater comorbidity burden among those who later developed AD (vs. those who did not), it cannot be ruled out that the observed relationship between comorbidity burden and AD was due in part to residual confounding by underlying factors and/or mechanisms related to aging, given that multimorbidity is associated with accelerated aging (Fabbri et al., 2015). We also identified clusters of comorbidities that could distinguish AD cases and non-cases. Further investigation of comorbidity clusters is warranted to facilitate early detection of individuals at risk of developing AD.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Commercial claims databases are available with subscription payment. Requests to access these datasets should be directed to RH, richard.houghton@roche.com.

AUTHOR CONTRIBUTIONS

LB, RH, and GD-P developed the study concept, protocol, wrote the manuscript, and statistical analysis plan. LB, RH, AA, GD-P, and MV assisted in the interpretation of the findings and provided critical revision to the manuscript. GD-P and AA performed the statistical analyses. All authors read and approved the final 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/fnins.2021.749305/full#supplementary-material

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Vascular Risk Factors and Cognition in Multiple System Atrophy

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Objective: Vascular risk factors have been reported to be associated with cognitive impairment (CI) in the general population, but their role on CI in multiple system atrophy (MSA) is unclear. This study aimed to explore the relationship between vascular risk factors and CI in patients with MSA.

Methods: The clinical data and vascular risk factors were collected. The Montreal Cognitive Assessment tool was used to test the cognitive function of patients with MSA. Binary logistic regression was used to analyze the correlation between vascular risk factors and CI.

Results: A total of 658 patients with MSA with a mean disease duration of 2.55 ± 1.47 years were enrolled. In MSA patients, hypertension was recorded in 20.2%, diabetes mellitus in 10.3%, hyperlipidemia in 10.2%, smoking in 41.2%, drinking in 34.8%, and obesity in 9.6%. The prevalence of CI in patients with MSA, MSA with predominant parkinsonism (MSA-P), and MSA with predominant cerebellar ataxia (MSA-C) was 45.0, 45.1, and 44.9%, respectively. In the binary logistic regression model, patients with more than one vascular risk factors were significantly more likely to have CI in MSA (OR = 4.298, 95% CI 1.456–12.691, P = 0.008) and MSA-P (OR = 6.952, 95% CI 1.390–34.774, P = 0.018), after adjusting for age, sex, educational years, disease duration, and total Unified multiple system atrophy rating scale scores.

Conclusion: Multiple vascular risk factors had a cumulative impact on CI in MSA. Therefore, the comprehensive management of vascular risk factors in MSA should not be neglected.

Keywords: multiple system atrophy, cognition, vascular risk factor, non-motor symptom, neurodegenerative disorder

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Abbreviations: MSA, multiple system atrophy; CI, cognitive impairment; PD, Parkinson's disease; MSA-P, MSA with predominantly parkinsonian features; MSA-C, MSA with predominantly cerebellar ataxia; MRI, magnetic resonance imaging; SCA, spinocerebellar ataxia; UMSARS, unified multiple system atrophy rating scale; OH, orthostatic hypotension; BP, blood pressure; BMI, body-mass index.

INTRODUCTION

Multiple system atrophy (MSA) is a sporadic neurodegenerative disease clinically characterized by the combination of Parkinsonian, cerebellar, autonomic, or pyramidal signs and symptoms (Stefanova et al., 2009). The pathological hallmark of MSA is the presence of oligodendrocytic glial cytoplasmic inclusions consisting of α-synuclein. Patients with MSA only have a mean survival period of about 7–9 years after initial clinical presentation (Stefanova et al., 2009). However, the etiology of MSA is still unclear. Currently, symptomatic treatment is the only therapeutic option since disease-modifying therapy is not available.

Urinary failure, erectile dysfunction, orthostatic hypotension, sleep disorders, mood disorders, and cognitive dysfunction are common non-motor symptoms in MSA (Schrag et al., 2010; Cao et al., 2015b; Zhang et al., 2017). Cognitive dysfunction had been underestimated previously, but an increasing number of studies have reported that cognitive impairment (CI) can present as a single-domain deficit or as a wide spectrum of domains (Stankovic et al., 2014; Cao et al., 2015b; Lee et al., 2015). Specifically, frontal executive dysfunction is the most commonly affected domain, followed by the visuospatial, memory, and attention domains (Stankovic et al., 2014; Cao et al., 2015b). In addition, CI has been reported in autopsy-confirmed MSA patients (Wenning et al., 1997). A recent study reported that MSA patients with CI had a greater burden of neuronal cytoplasmic inclusions in the limbic regions (the dentate gyrus) (Koga et al., 2017).

Previous studies have found that vascular risk factors, such as smoking and alcohol drinking, were associated with CI at late life in the general population (Wu et al., 2018). Hypertension, hypercholesterolemia, and diabetes have also been reported to be associated with dementia in middle-aged people (Kivipelto et al., 2006). Vascular risk factors were associated with CI in patients with Parkinson's disease (PD) (Malek et al., 2016; Pilotto et al., 2016). Smoking was probably a protective factor in MSA (Vanacore et al., 2000, 2001; Vanacore, 2005), and increasing alcohol consumption may decrease the risk of MSA (Vidal et al., 2008). Levels of serum cholesterol have been reported to be insignificantly correlated with disease duration or severity, but low levels of total cholesterol and high-density lipoprotein may be associated with an increased risk of MSA (Lee et al., 2009). Our previous studies have shown that low levels of uric acid and severe motor symptoms were related to CI in patients with MSA (Cao et al., 2015a,b). However, the relationship between vascular risk factors and cognition has never been specifically studied in MSA. As such, this study aimed to provide a detailed prevalence of the vascular risk factors in MSA and evaluate the correlation between these vascular risk factors and CI in MSA.

MATERIALS AND METHODS

Patients

Consecutive patients with a clinical diagnosis of MSA and evaluated at the Department of Neurology, West China Hospital of Sichuan University between August 2013 and Jun 2021 were included in the current study. According to the second consensus criteria, the diagnosis of MSA was divided into three groups (Gilman et al., 2008). Definite MSA requires the neuropathologic demonstration of CNS α-synuclein–positive glial cytoplasmic inclusions with neurodegenerative changes in the striatonigral or olivopontocerebellar structures. Probable MSA requires a sporadic, progressive adult-onset disorder, including rigorously defined autonomic failure and parkinsonism or cerebellar ataxia that is poorly responsive to levodopa. Lastly, possible MSA requires a sporadic, progressive adult-onset disease, including parkinsonism or cerebellar ataxia, and at least one feature suggesting autonomic dysfunction plus one other feature that may be a clinical or a neuroimaging abnormality. Only patients diagnosed with probable MSA were included in the final analysis. Patients with predominantly parkinsonian features were designated as MSA-P, and patients predominantly presenting with cerebellar ataxia were designated as MSA-C. All patients included underwent magnetic resonance imaging (in our or other external hospitals) to exclude prominent cortical or subcortical infarcts, iron accumulation, or other atypical parkinsonian disorders. In order to exclude the common forms of spinocerebellar ataxia (SCA), patients were screened for SCA genes, including SCA1, 2, 3, 6, and 7.

The clinical data of age, sex, height, weight, educational years, and disease duration were collected by professional neurologists via face-to-face interviews. Disease onset referred to the initial presentation of any motor problems (whether parkinsonism or cerebellar) or autonomic features, except male erectile dysfunction (Gilman et al., 2008). Disease duration referred to the time from the disease onset date to the evaluation date. The Unified multiple system atrophy rating scale (UMSARS) was used to evaluate the disease severity (Wenning et al., 2004). Orthostatic hypotension (OH) was defined as a reduction in the systolic blood pressure (BP) by at least 30 mmHg and/or diastolic BP by at least 15 mmHg 3 min after standing up from a previous recumbent position for 10 min. A comprehensive and standardized cognitive battery (Montreal cognitive assessment) was applied to assess the global cognitive functions. The optimal cutoff scores for cognitive impairment screening were 19 for individuals with no more than 6 years of education, 22 for individuals with 7–12 years of education, and 24 for individuals with more than 12 years of education (Chen et al., 2016).

Vascular Risk Factors Evaluation

Vascular risk factors were evaluated during the clinical assessment. Hypertension was defined as a systolic BP $\geq$ 140 mm Hg, diastolic BP $\geq$ 90 mm Hg, self-reported use of antihypertensive medications, or lifetime diagnosis of hypertension. Nearly half of the patients completed the blood tests in our hospital. Diabetes mellitus was defined as fasting plasma glucose $\geq$ 126 mg/dL (7.0 mmol/L) for patients completed the blood tests in our hospital, reported use of hypoglycemic agents, or any self-reported history of diabetes. Hyperlipidemia was defined as total cholesterol $\geq$ 6.2 mmol/L or triglyceride $\geq$ 2.3 mmol/L for patients completed the blood tests in our hospital, use of lipid-lowering medications, or

lifetime diagnosis of hyperlipidemia. Personal history of smoking behavior was indicated by pack/years to quantify the packs smoked per day multiplied by years as a smoker, with the factor threshold set to 15 (Heinzel et al., 2014; Pilotto et al., 2016). Drinking was defined as an average alcoholic drink $\geq$ 50 mL at least once per week lasting more than half a year. The body mass index (BMI) was calculated as body weight (kg) divided by heights squared (m^2). Following the Chinese criteria for overweight/obesity, the patients were classified as normal (BMI 18.5–23.9 kg/m^2), overweight (24–27.99 kg/m^2), or obese ($\geq$ 28.0 kg/m^2).

This study was approved by the Ethics Committee of West China Hospital of Sichuan University. Informed consent was obtained from all participants.

Statistical Analysis

All continuous data are presented as the mean $\pm$ standard deviation, while all categorical variables are presented as numbers or percentages. The clinical characteristics and vascular risk factors prevalence of patients with and without CI were compared using the Student's *t*-test and the χ^2-test for continuous and dichotomous variables, respectively. A binary logistic regression model was used to explore the potential vascular risk factors related to CI in MSA. The presence or absence of CI was used as the dependent variable. All the vascular risk factors were considered covariables after adjusting for age, sex, subtypes, educational years, disease duration, and total UMSARS scores.

All the data analyses were performed using SPSS 22.0 (IBM, Chicago, IL). A p-value < 0.05 was considered statistically significant.

RESULTS

The demographic and clinical features of patients with MSA are presented in **Table 1**. Among the 658 patients included in the analysis, the following were observed: mean age of 60.13 $\pm$ 8.74 years, mean age at onset of 57.51 $\pm$ 8.60 years, and a mean disease duration of 2.55 $\pm$ 1.47 years. Furthermore, 57.1% were male (**Table 1**). In terms of the vascular risk factors, 20.2% of patients had hypertension, 10.3% had diabetes mellitus, 10.2% had hyperlipidemia, 41.2% were cigarette smokers, 34.8% were alcohol drinks, and 9.6% were obese. A total of 435 (66.1%) patients with MSA had at least one vascular risk factor, while 269 (40.95) patients had more than one.

The comparisons of the demographic and clinical features between patients with and without CI in MSA, MSA-P, and MSA-C are shown in **Table 2**. The prevalence of CI in patients with MSA, MSA-P, and MSA-C was 45.0, 45.1, and 44.9%, respectively. In the MSA, MSA-P, and MSA-C groups, the patients with CI were older; had late age at onset; higher UMSARS-I, UMSARS-II, UMSARS-IV, and total UMSARS scores than the patients without ($P < 0.05$). The proportion of hypertension, diabetes mellitus, hyperlipidemia, cigarette smoking, drinking, and obesity were not significantly different between the patients with and without CI ($P > 0.05$). Patients with CI had a greater number of vascular risk factors than those without, although this was not significantly different.

The correlations between vascular risk factors and CI in patients with MSA, MSA-P, and MSA-C in the binary logistic regression model are shown in **Table 3**. Patients with more than one vascular risk factor were significantly more likely to have CI in MSA (OR = 4.298, 95% CI 1.456–12.691, $P = 0.008$) and MSA-P (OR = 6.952, 95% CI 1.390–34.774, $P = 0.018$), after adjusting for age, sex, educational years, disease duration, and total UMSARS scores. However, there was no significant correlation between the number of vascular risk factors and CI in patients with MSA-C.

DISCUSSION

It has been reported that vascular risk factors such as hypertension, hypercholesterolemia, and obesity play important roles in the development of dementia in the general population (Kivipelto et al., 2006). However, their effect on the cognition of patients with MSA is still unknown. To the best of our knowledge, the present cross-sectional study was the first study to investigate the influence of vascular risk factors on cognition in a large cohort of patients with MSA who underwent a standardized global cognitive assessment, taking their demographic and clinical confounders into account.

In the current study, we found that CI was present in 45.0% of patients with MSA. Combining the subjective CI symptoms

TABLE 1 | Demographic and clinical features of the patients with MSA.

Variables	MSA
Total	658
Diagnosis (MSA-P, %)	304 (46.2%)
Sex (male, %)	376 (57.1%)
Age	60.13 $\pm$ 8.74
Age of onset	57.51 $\pm$ 8.60
Educational years	9.63 $\pm$ 3.74
BMI	23.63 $\pm$ 3.30
Disease duration	2.55 $\pm$ 1.47
MoCA score	22.06 $\pm$ 4.77
UMSARS-I	16.35 $\pm$ 6.48
UMSARS-II	18.11 $\pm$ 6.84
UMSARS-IV	2.12 $\pm$ 0.98
Total UMSARS scores	34.45 $\pm$ 12.47
OH (%)	232 (35.3%)
Hypertension	133 (20.2%)
Diabetes mellitus	68 (10.3%)
Hyperlipidemia	67 (10.2%)
Cigarette smoking	271 (41.2%)
Drinking	229 (34.8%)
Obesity	63 (9.6%)
Number of vascular risk factors	
0	223 (33.9%)
1	166 (25.2%)
$\geq$2	269 (40.9%)

MSA, multiple system atrophy; MSA-P, multiple system atrophy with predominately parkinsonism; CI, cognitive impairment; BMI, body mass index; MoCA, Montreal cognitive assessment; UMSARS, unified multiple system atrophy rating scale; OH, orthostatic hypotension.

TABLE 2 | The comparison of the demographic and clinical features between patients with and without CI in the MSA, MSA-P, and MSA-C groups.

	MSA			MSA-P			MSA-C		
Variables	**MSA without CI**	**MSA with CI**	***P*-value**	**MSA without CI**	**MSA with CI**	***P*-value**	**MSA without CI**	**MSA with CI**	***P*-value**
Total	362 (55.0%)	296 (45.0%)	–	167 (54.9%)	137 (45.1%)	–	195 (55.1%)	159 (44.9%)	–
Diagnosis (MSA-P, %)	167 (46.1%)	137 (46.3%)	0.969	–	–	–	–	–	–
Sex (male, %)	203 (56.1%)	173 (58.4%)	0.541	89 (53.3%)	77 (56.2%)	0.612	114 (58.5%)	96 (60.4%)	0.715
Age	58.67 ± 8.69	61.91 ± 8.47	<0.001*	60.09 ± 9.06	63.48 ± 8.69	0.001*	57.46 ± 8.19	60.56 ± 8.07	<0.001*
Age of onset	56.12 ± 8.62	59.20 ± 8.28	<0.001*	57.31 ± 9.11	60.62 ± 8.62	0.001*	55.11 ± 8.07	57.98 ± 7.80	0.001*
Educational years	9.93 ± 3.74	9.26 ± 3.72	0.024*	9.77 ± 3.76	9.23 ± 3.75	0.215	10.06 ± 3.72	9.29 ± 3.71	0.054
BMI	23.73 ± 3.24	23.51 ± 3.38	0.413	23.94 ± 3.47	23.35 ± 3.66	0.149	23.54 ± 3.02	23.66 ± 3.13	0.724
Disease duration	2.53 ± 1.43	2.57 ± 1.52	0.713	2.71 ± 1.53	2.80 ± 1.69	0.610	2.37 ± 1.33	2.37 ± 1.34	0.983
MoCA score	25.33 ± 2.48	18.05 ± 3.72	<0.001*	25.39 ± 2.48	18.20 ± 3.74	<0.001*	25.28 ± 2.48	17.91 ± 3.72	<0.001*
UMSARS-I	15.23 ± 5.86	17.71 ± 6.92	<0.001*	15.25 ± 6.40	17.97 ± 6.55	<0.001*	15.22 ± 5.38	17.48 ± 7.24	0.001*
UMSARS-II	16.58 ± 6.04	19.98 ± 7.29	<0.001*	17.55 ± 6.53	20.79 ± 7.32	<0.001*	15.75 ± 5.47	19.28 ± 7.22	<0.001*
UMSARS-IV	1.93 ± 0.84	2.36 ± 1.09	<0.001*	2.02 ± 0.92	2.34 ± 1.06	0.005*	1.85 ± 0.76	2.38 ± 1.11	<0.001*
Total UMSARS scores	31.81 ± 11.11	37.69 ± 13.27	<0.001*	32.79 ± 12.29	38.76 ± 12.85	<0.001*	30.97 ± 9.95	36.77 ± 13.59	<0.001*
OH (%)	127 (35.1%)	105 (35.5%)	0.917	40 (24.0%)	44 (32.1%)	0.113	87 (44.6%)	61 (38.4%)	0.236
Hypertension	65 (18.0%)	68 (23.0%)	0.111	34 (20.4%)	34 (24.8%)	0.353	31 (15.9%)	34 (21.4%)	0.185
Diabetes mellitus	38 (10.5%)	30 (10.1%)	0.879	19 (11.4%)	14 (10.2%)	0.747	19 (9.7%)	16 (10.1%)	0.920
Hyperlipidemia	42 (11.6%)	25 (8.4%)	0.183	11 (6.6%)	10 (7.3%)	0.807	31 (15.9%)	15 (9.4%)	0.072
Cigarette smoking	144 (39.8%)	127 (42.9%)	0.418	61 (36.5%)	52 (38.0%)	0.798	83 (42.6%)	75 (47.2%)	0.386
Drinking	122 (33.7%)	107 (36.1%)	0.512	58 (34.7%)	47 (34.3%)	0.938	64 (32.8%)	60 (37.7%)	0.335
Obesity	36 (9.9%)	27 (9.1%)	0.721	20 (12.0%)	13 (9.5%)	0.488	16 (8.2%)	14 (8.8%)	0.840
Number of vascular risk factors									
0	136 (37.6%)	87 (29.4%)	0.085	70 (41.9%)	44 (32.1%)	0.152	66 (33.8%)	43 (27.0%)	0.357
1	85 (23.5%)	81 (27.4%)		34 (20.4%)	38 (27.7%)		51 (26.2%)	43 (27.0%)	
≥2	141 (39.0%)	128 (43.2%)		63 (37.7%)	55 (40.1%)		78 (40.0%)	73 (45.9%)	

MSA, multiple system atrophy; MSA-P, multiple system atrophy with predominately parkinsonism; MSA-C, multiple system atrophy with predominately cerebellar ataxia; CI, cognitive impairment; BMI, body mass index; MoCA, Montreal cognitive assessment; UMSARS, unified multiple system atrophy rating scale; OH, orthostatic hypotension.
**Significant difference.*

TABLE 3 | The correlation between the vascular risk factors and CI in patients with MSA, MSA-P, and MSA-C in the binary logistic regression model.

Variables	MSA			MSA-P			MSA-C		
	OR	**95% CI**	***P*-value[a]**	**OR**	**95% CI**	***P*-value[b]**	**OR**	**95% CI**	***P*-value[b]**
Hypertension	0.778	0.453–1.336	0.363	0.701	0.316–1.555	0.383	0.834	0.393–1.772	0.638
Diabetes mellitus	0.587	0.315–1.092	0.093	0.410	0.152–1.104	0.078	0.700	0.305–1.607	0.401
Hyperlipidemia	0.466	0.244–1.107	0.055	0.883	0.308–2.529	0.816	0.353	0.149–1.106	0.059
Cigarette smoking	0.663	0.351–1.253	0.206	0.514	0.202–1.308	0.162	0.854	0.352–2.075	0.728
Drinking	0.582	0.312–1.085	0.089	0.391	0.154–1.101	0.054	0.662	0.275–1.594	0.358
Obesity	0.774	0.413–1.449	0.423	0.542	0.216–1.363	0.193	0.917	0.377–2.234	0.850
Number of vascular risk factors									
0	1 (reference)	1 (reference)	–	1 (reference)	1 (reference)	–	1 (reference)	1 (reference)	–
1	2.183	1.206–3.951	0.010*	2.835	1.182–6.799	0.020*	1.713	0.755–3.884	0.198
≥2	4.298	1.456–12.691	0.008*	6.952	1.390–34.774	0.018*	3.363	0.751–15.070	0.113

MSA, multiple system atrophy; MSA-P, multiple system atrophy with predominately parkinsonism; MSA-C, multiple system atrophy with predominately cerebellar ataxia; CI, cognitive impairment.
[a]Adjusted for age, sex, subtypes, educational years, disease duration, and total UMSARS scores.
[b]Adjusted for age, sex, educational years, disease duration, and total UMSARS scores.
**Significant difference.*

and different cognitive screening tests to assess CI in 102 MSA patients, Koga et al. (2017) showed that the prevalence CI was 32%. Fiorenzato et al. (2017) reported that the prevalence of CI was 30.6% in 72 MSA patients, based on the mini-mental state examination score < 27. The prevalences of CI in the above studies were slightly lower compared to our present finding, possibly due to their small sample sizes and difference in the cognitive assessment scales used. The mean disease duration of

our patients with CI was 2.57 years. Previous studies showed that the mean disease duration of MSA patients with CI ranged from 1.25 to 7 years (O'Sullivan et al., 2008; Stankovic et al., 2014; Auzou et al., 2015; Lee et al., 2015; Fiorenzato et al., 2017; Koga et al., 2017). The discrepancy could be due to populational bias or variations in the criteria used to access CI. It is noteworthy that CI can appear in an early stage of MSA.

Vascular risk factors are not uncommon in MSA, given that hypertension was recorded in 20.2%, diabetes mellitus in 10.3%, hyperlipidemia in 10.2%, smoking in 41.2%, drinking in 34.8%, and obesity in 9.6%. The prevalences of hypertension and diabetes mellitus in the general Chinese population (age $\geq$ 60 years) were estimated to be 60.0 and 20.0%, respectively, which were higher compared to our study population (Xu et al., 2013; Wang et al., 2014). The prevalence of hyperlipidemia in adults aged between 35 and 75 years in the Chengdu area was 23.53%, which was higher than in our patients (10.2%) (Liao et al., 2013). Meanwhile, the prevalence of smoking in Chinese adults (aged between 60 and 69 years) was about 38.0%, lower than our patients (41.2%) (Ding et al., 2016). The prevalence of drinking in the general Chinese population (aged between 55 and 65 years) was lower compared to our study population (30.0 vs. 34.8%) (Li et al., 2018). Lastly, the prevalence of obesity in the general Chinese population (aged between 35 and 72) was 14.0%, higher than in our patients (9.6%) (Zheng et al., 2015).

We found that a single vascular risk factor (e.g., hypertension, diabetes mellitus, hyperlipidemia, etc.) was not associated with CI in patients with MSA, MSA-P, and MSA-C. However, the current study showed the cumulative impact of multiple vascular risk factors on CI in MSA. It has been reported that the presence of more than two vascular risk factors was significantly associated with CI in patients with PD (Malek et al., 2016), which can support our results since MSA and PD belong to α-synucleinopathy. However, the pathophysiological mechanisms of the vascular risk factors associated with CI in patients with MSA remain unclear. Therefore, further mechanism studies are needed to elucidate these.

Hypertension has been reported as an important risk factor for the development of CI and dementia (DeCarli, 2015) due to the possible mechanistic endothelial dysfunction or vascular dysregulation, oxidative stress, and inflammation (Gorelick, 2014). Previous studies also suggested that hyperlipidemia was associated with the risk of mild CI and dementia (Carlsson, 2010; Panza et al., 2011). Kivipelto et al. (2006) found that obesity was one of the risk factors present at midlife, which can predict the future risk of dementia in the general population. Obesity is a risk factor for several metabolic diseases, such as insulin resistance and type 2 diabetes. Previous studies have revealed that insulin resistance may be important in the pathogenesis of Alzheimer's disease (Kuusisto et al., 1997; Watson and Craft, 2003). Furthermore, obesity may increase microglial activation, which has been observed in MSA through positron emission tomography molecular imaging (Niccolini and Politis, 2016). Furthermore, a recent study found that increased microglial activation and dendritic spine loss may be responsible for obesity-associated cognitive decline (Cope et al., 2018). Therefore, the mechanism of obesity involved in CI in MSA, which needs to be confirmed in further researches. Epidemiological studies have demonstrated that changes in lifestyle, including frequent physical exercise, can prevent and treat not only obesity/metabolic disorders but also improve cognitive function through epigenetic mechanisms (Barros et al., 2019). Therefore, physical exercise has been proposed as a non-pharmacological treatment of CI (Barros et al., 2019).

A long-term follow-up study (up to 27 years) found that frequent alcohol consumption was associated with an increased risk for dementia, compared to infrequent alcohol intake (Langballe et al., 2015). Researches have revealed that even moderate alcohol drinking in older people was associated with gray matter atrophy and reduced total brain volume and frontal and parietal gray matter densities (Mukamal et al., 2001; den Heijer et al., 2004; Paul et al., 2008; Sachdev et al., 2008). Similarly, another study analyzing a 30-year longitudinal data focused on the relationship between alcohol consumption and brain structure and function and found that even a moderate alcohol consumption was associated with hippocampal atrophy and cognitive decline (Topiwala et al., 2017). In the Chinese population, studies have shown that alcohol intake was associated with an increased risk of CI (Zhou et al., 2003; Wu et al., 2018). In addition, concomitant smoking and regular alcohol drinking at midlife had a much stronger impact than the individual factors on the risk of CI in late life in the general population (Wu et al., 2018). Therefore, we recommend reducing alcohol intake and smoking in MSA patients. The comprehensive management of multiple vascular risk factors may protect patients with MSA from CI.

The strength of our study was that it was the first study to focus on the prevalence of vascular risk factors and the relationship between vascular risk factors and cognition in a large sample of patients with MSA. However, we also acknowledge some limitations. First, we could not count the specific amount of alcohol consumption of each patient. Second, vascular risk factors were collected from interviews, which could lead to significant recall bias. Third, this was a cross-sectional study. Further prospective, longitudinal follow-up studies are required to confirm our results.

CONCLUSION

We found that vascular risk factors were common in patients with MSA. A single vascular risk factor may not show the impact on CI in MSA, however, the cumulative impact of multiple vascular risk factors on CI in MSA should be given proper attention. Patients with MSA may benefit from comprehensive management associated with vascular risk factors.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of West China Hospital of Sichuan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LZ: for the research project: conception, organization, execution; for the statistical analysis: design; for the manuscript: writing of the first draft. YH: for the statistical analysis: review and critique; patients enrollment. BC: for the statistical analysis: review and critique; patients enrollment. Q-QW, RO, JL, KL, TY, YX, and BZ: patients enrollment. HS: for the research project: conception; for the statistical analysis: review and critique; for the manuscript: review and critique. All authors contributed to the article and approved the submitted version.

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Diffusion Tensor Imaging Along the Perivascular Space Index in Different Stages of Parkinson's Disease

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Background: The aim of this study was to evaluate the glymphatic system activity in patients with Parkinson's disease (PD) using the diffusion tensor image analysis along the perivascular space (DTI-ALPS) methods.

Methods: In total, 71 patients with idiopathic PD and 36 age- and sex-matched normal controls (NCs) were involved. Patients with PD were divided into early (n = 35) and late (n = 36) subgroups, based on Hoehn and Yahr (HY) stages. We calculated the diffusivity along the perivascular spaces (ALPS), as well as projection fibers and association fibers separately, to acquire the ALPS index. Enlarged perivascular spaces (EPVS) and periventricular white matter hyperintensities were also rated. Differences in ALPS index between the PD group and NCs and between two PD subgroups and NCs were compared. In addition, a multivariate logistic regression analysis was conducted to investigate the association between ALPS index and clinical variables.

Results: Patients with PD revealed lower ALPS index than NCs (p = 0.010). The late PD group exhibited significantly lower ALPS index than NCs (p = 0.006). However, there were no marked differences noticed in ALPS index between NCs and early PD group and between the two PD subgroups. In the early PD group, there was a significantly positive correlation between ALPS index and Mini-Mental State Examination (MMSE) score (β = 0.021, p = 0.029) and a negative correlation between ALPS index and EPVS score (β = −0.050, p = 0.034), after controlling for multiple variables. In the late PD group, ALPS index was inversely associated with age (β = −0.012, p = 0.004).

Conclusion: Impairment of the glymphatic system is involved in PD. DTI-ALPS index could be a promising biomarker of glymphatic system in PD.

Keywords: Parkinson's disease, glymphatic system, magnetic resonance imaging, diffusion tensor imaging, enlarged perivascular spaces

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INTRODUCTION

Parkinson's disease (PD) is a common neurodegenerative disease, but the underlying mechanism is poorly understood. In addition to the loss of dopaminergic neurons, the aggregation of misfolded α-synuclein (α-syn) is also involved in the pathogenesis of PD (George et al., 2013; Poewe et al., 2017). The glymphatic system was first identified by Iliff et al. (2012), which was defined as a waste

excretion system in the brain. Cerebrospinal fluid (CSF) is exchanged with interstitial fluid along the perivascular space (PVS) to promote the elimination of soluble proteins, including misfolding proteins and metabolites (Iliff et al., 2012; Benveniste et al., 2019). Therefore, this system has been thought to play a critical role in the pathophysiology of PD.

Recently, Taoka et al. (2017) proposed a non-invasive method "diffusion tensor image analysis along the perivascular space" (DTI-ALPS) to assess the glymphatic function in human brain. They indicated that ALPS index positively correlated with the Mini-Mental State Examination (MMSE) score in Alzheimer's disease (AD; Taoka et al., 2017). Subsequently, this method has been used in patients with dementia (Steward et al., 2021), idiopathic normal pressure hydrocephalus (iNPH; Yokota et al., 2019; Bae et al., 2021), and type 2 diabetes mellitus (Yang et al., 2020). To our knowledge, there are only two studies exploring the glymphatic system activity in patients with PD, using DTI-ALPS method (Chen et al., 2021; McKnight et al., 2021). They highlighted that patients with PD with cognitive impairment showed lower ALPS index than normal controls (NCs). Moreover, patients with PD exhibited reduced ALPS index related to patients with essential tremor. However, the association between ALPS index and various clinical factors in different stages of PD has not yet been reported. Therefore, we aimed to investigate the glymphatic function in patients with PD, using this non-invasive method. We also examined the related factors of the ALPS index in different stages of PD.

In addition, several lines of evidence have emerged suggesting that PVS are associated with the clearance of interstitial fluid and waste from the brain. Enlarged perivascular spaces (EPVS) on MRI may reflect impairment of lymphatic drainage channels (Wardlaw et al., 2020). Lower grade of EPVS predicted lower CSF α-syn and t-tau in PD (Fang et al., 2020). However, it has not been established in humans as to whether MRI-visible EPVS are related to DTI ALPS index in PD. Hence, we also explored the relationship between EPVS and ALPS index in different stages of PD. This study may help us to unravel the glymphatic system activity in PD and find potential neuroimaging markers for diagnosing and monitoring the progression of PD.

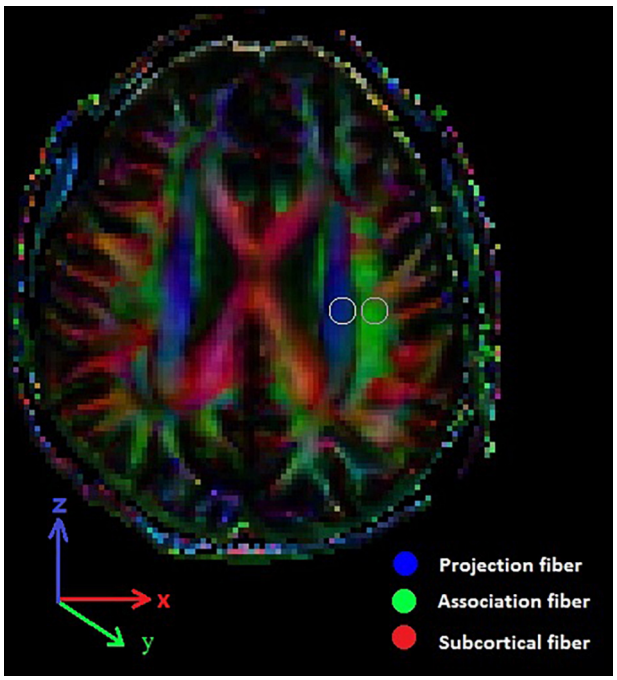

FIGURE 1 | DTI color map shows the direction of the projection fibers (blue; z-axis), association fibers (green; y-axis), and the subcortical fibers (red; x-axis). Two ROIs are placed to measure diffusivities of the projection and association fibers.

MATERIALS AND METHODS

Subjects and Clinical Assessments

Patients with idiopathic PD ($n = 71$, mean age: 64.68 ± 8.12 years) and age- and sex-matched NCs (n = 36, mean age: 62.00 ± 6.24 years) were involved in this study. All patients with PD were diagnosed based on the United Kingdom PD Society Brain Bank Clinical Diagnostic Criteria. All participants were right-handed Chinese natives. We excluded patients in whom PD was induced by cerebrovascular disease, medications, trauma, encephalitis, poisoning, and other neurodegenerative diseases. Neurological examinations were evaluated using MMSE score, Unified Parkinson's Disease Rating Scale (UPDRS) score, and Hoehn and Yahr (HY) stages. Patients with PD receiving dopaminergic medications were examined in the clinically defined "OFF" state. All neuropsychological scales were completed by one neurologist who was blinded to the clinical information. In addition, patients were divided into early PD group (HY 1–2, n = 35) and late PD group (HY 2.5–4, n = 36), based on HY stages. This study was approved by a local ethics committee, and written informed consent was obtained from each participant after a detailed description of the study was provided.

MR Image Acquisition

All MRI examinations were performed using a 3.0 T MRI scanner (Philips, Achieva TX). The head of the participant was immobilized with foam pillows inside the coil to diminish motion artifacts. Sequences consisted of high-resolution T1-weighted 3D (repetition time/echo time (TR/TE) = 7.4/3 ms, flip angle (FA) = 8, field of view (FOV) = 24 cm × 24 cm, and 1.2 mm slice thickness without slice gap), T2-weighted (T2WI; TR/TE = 2,500/100 ms; FOV = 24 cm × 24 cm, 5 mm slice thickness, and 1.5 mm slice gap), fluid-attenuated inversion recovery (FLAIR; TR/TE = 8,000/140 ms; TI = 2,400 ms; FOV = 24 cm × 24 cm, and 4 mm slice thickness without slice gap), susceptibility weighted imaging (SWI; TR/TE = 16/22 ms; FOV = 24 cm × 24 cm; and 2.8 mm slice thickness without slice gap), and diffusion tensor imaging (axially parallel to the anterior-posterior commissure (AC-PC), TR/TE = 5,472/93 ms; b = 1,000 s/mm^2; FOV = 24 cm × 24 cm; matrix = 128 × 128; MPG = 31 directions; 3 mm slice thickness without slice gap, 40 slices).

TABLE 1 | Demographic and clinical data of the subjects.

	NCs (n = 36)	PD (n = 71)	p	Early PD (n = 35)	Late PD (n = 36)	p
Age (years)	62.00 ± 6.24	64.68 ± 8.12	0.062	63.57 ± 8.93	65.75 ± 7.23	0.110
Sex (M/F)	18/18	31/40	0.534	17/18	14/22	0.590
MMSE	27.86 ± 2.20	28.03 ± 2.15	0.707	28.09 ± 2.47	27.97 ± 1.83	0.910
EDU (years)	10.44 ± 3.18	12.93 ± 3.30	0.000[a]	12.91 ± 3.48	12.94 ± 3.16	0.002[b]
ALPS index	1.53 ± 0.16	1.44 ± 0.16	0.010[a]	1.46 ± 0.15	1.42 ± 0.18	0.022[b]
Dur (years)	NA	8.38 ± 4.29	NA	6.86 ± 4.02	9.86 ± 4.06	0.002[b]
UPDRS-I	NA	3.04 ± 1.93	NA	2.80 ± 2.13	3.28 ± 1.72	0.300
UPDRS-II	NA	12.79 ± 4.93	NA	10.40 ± 4.31	15.11 ± 4.4	0.000[b]
UPDRS-III	NA	30.92 ± 11.58	NA	26.31 ± 9.80	35.39 ± 11.54	0.001[b]
UPDRS-IV	NA	2.68 ± 2.46	NA	2.14 ± 2.35	3.19 ± 2.49	0.072
UPDRS-V	NA	1.06 ± 0.95	NA	0.97 ± 1.04	1.14 ± 0.87	0.464
UPDRS-total	NA	50.48 ± 17.25	NA	42.63 ± 14.76	58.11 ± 16.19	0.000[b]
EPVS	NA	1 (0–4)	NA	1 (0–4)	1 (0–4)	0.663
PVWMH	NA	2 (0–3)	NA	1 (0–3)	2 (1–3)	0.130

M/F, male/female; NCs, normal controls; PD, Parkinson's disease; MMSE, Mini-Mental State Examination; EDU, education; ALPS, along the perivascular spaces; Dur, disease duration; UPDRS, Unified Parkinson's Disease Rating Scale; EPVS, enlarged perivascular spaces; PVWMH, periventricular white matter hyperintensities.
[a] *Two-sample t-test between NCs and PD.*
[b] *Two-sample t-test between early and late PD groups.*
$p < 0.05$.

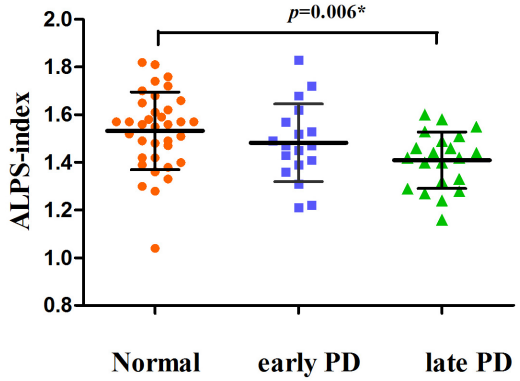

FIGURE 2 | Scatter plots showing the distribution of the ALPS-index values for normal controls, early and late PD subgroups. The p-value was obtained from the analysis of variance (ANOVA) *post hoc* tests.

MRI Analysis

The DTI data were calculated using DTI Studio software.[1] The software creates images of the diffusion tensor, including a color-coded fractional anisotropy (FA) map and a diffusivity map. Moreover, the diffusivity in the directions of x-axis, y-axis, and z-axis on each image can be calculated. We evaluated the diffusivity along the direction of the PVS compared with those of projection fibers and association fibers on a slice at the level of the lateral ventricle body (**Figure 1**). At this level, the direction of the PVS is perpendicular to the ventricle wall (mostly in the right-left direction/x-axis). This direction is also perpendicular to the direction of the projection fibers (mostly in the z-axis) as well as the association fibers (mostly in the y-axis) (**Figure 1**). Therefore, the diffusivity along the x-axis at regions with projection/association fibers will at least partly represent the diffusivity along the PVS. On a color-coded FA map, we placed a 5-mm-diameter spherical region of interest (ROI) in the area of the projection fibers (represented in blue in **Figure 1**) and the area of the association fibers (represented in green in **Figure 1**) in the left hemisphere. For each area, the diffusivity in the directions of the x-axis, y-axis, and z-axis was calculated.

We also calculated the ALPS index to assess the activity of the glymphatic system. According to a study by Taoka et al. (2017), this index is provided by the ratio of two diffusivity value sets, i.e., the ratio of the average values of the x-axis diffusivity in the area of the projection fibers (D_{xxproj}) and the x-axis diffusivity in the area of the association fibers ($D_{xxassoc}$) to the average value of the y-axis diffusivity in the area of the projection fibers (D_{yyproj}) and the z-axis diffusivity (D_{zzacoc}) of the association fibers area, shown as follows:

ALPS index = mean (D_{xxproj}, $D_{xxassoc}$)/mean (D_{yyproj}, $D_{zzassoc}$). The ALPS index represents the existence of the PVS. A higher ratio represents the more water diffusivity along the PVS.

In addition, PVSs were defined as punctate hyperintensities on T2WI at the same level of the lateral ventricle body, usually<3 mm in diameter, based on a previous work (Doubal et al., 2010). Periventricular white matter hyperintensities (PVWMH) were also investigated using the Fazekas scale from 0 to 3 (Fazekas et al., 1987). All MRI lesions were assessed by two trained neurologists who were blinded to the clinical information of participants.

Statistical Analysis

Statistical Package for the Social Sciences (SPSS) 25.0 software was used to perform the statistical analysis of clinical and demographic variables. Two-sample t-test and chi-square test were conducted to examine the clinical differences of continuous variables and categorical variables, respectively, between PD and

[1] https://www.mristudio.org

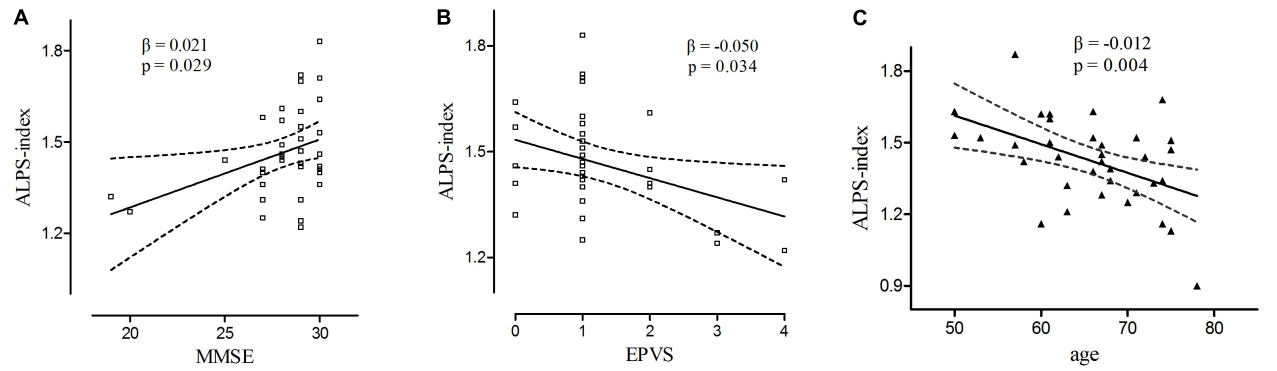

FIGURE 3 | (A) Relationships between ALPS-index and MMSE score in early PD group. **(B)** Relationships between ALPS-index and EPVS score in early PD group. **(C)** Relationships between ALPS-index and age in late PD group. Trend lines describe multivariate linear regression with 95% confidence intervals (dashed line).

NCs, and between ePD and lPD groups. In addition, analysis of variance (ANOVA) and *post hoc* tests were used to compare the differences between NCs and two PD subgroups. Multivariate linear regression analysis was carried out between the ALPS index and clinical variables in the early and late PD groups, respectively, including age, sex ratio (the ratio of males to females in a population), duration, MMSE score, EPVS score, UPDRS part III and total score, and HY stages. $p < 0.05$ was considered statistically significant.

RESULTS

We involved 71 patients with PD and 36 NCs in this study. There were no significant differences in age and sex ratio between the PD and NC groups. Demographic and clinical data of the subjects are presented in **Table 1**. Patients with PD revealed reduced ALPS index than NCs ($p = 0.010$). The late PD group exhibited significantly lower ALPS index than NCs ($p = 0.006$). However, there were no marked differences in ALPS index between the NCs and the early PD group, and between the two PD subgroups (**Figure 2**). These three groups did not differ in age, sex ratio, and MMSE score ($p > 0.05$). While two PD subgroups showed higher education than NCs. Compared with the early PD group, the late PD group revealed longer disease duration, higher UPDRS part II, part III, total score, and higher PDQ-39 score (**Table 1**). No significant difference in ALPS index and EPVS score between the two PD groups was observed.

In multivariate linear regression analysis, there was a significant positive correlation between ALPS index and MMSE score ($\beta = 0.021$, $p = 0.029$) (**Figure 3A**), and a negative correlation between ALPS index and EPVS score ($\beta = -0.050$, $p = 0.034$) (**Figure 3B**) in the early PD group. However, ALPS index did not associate with age, sex ratio, duration, UPDRS-III, UPDRS total score, HY stages, and PVWMH score in this group. In the late PD group, ALPS index negatively related with age ($\beta = -0.012$, $p = 0.004$) (**Figure 3C**). However, there was no significant relationship between ALPS index and other clinical status in this group ($p > 0.05$).

After controlling for sex ratio, disease duration, MMSE score, HY stages, UPDRS part III and total score, and PVWMH score, EPVS score was positively related to age in all PD participants ($\beta = 0.056$, $p = 0.000$), and in the early ($\beta = 0.059$, $p = 0.000$) and late PD ($\beta = 0.053$, $p = 0.023$) subgroups, respectively.

DISCUSSION

This study suggested that patients with PD exhibited lower ALPS index than NCs, especially in the late stage. ALPS index positively correlated with cognitive function in the early stage of PD and was inversely related to age in the advanced stage. Furthermore, we first demonstrated a negative association between the ALPS index and EPVS score in the early PD group. Our findings provided evidence supporting the involvement of glymphatic system in the pathogenesis of PD.

The glymphatic-lymphatic system is a recently discovered brain-wide paravascular pathway for the CSF and interstitial fluid exchange, which promotes efficient clearance of soluble proteins and metabolites from the brain (Iliff et al., 2013). Reduced clearance of protein waste from the brain has been observed in several neurological disorders (Rasmussen et al., 2018). However, the studies supporting the glymphatic system dysfunction in PD are relatively limited.

Lewy pathology has been hypothesized to a hallmark of PD, which consists of abnormal aggregates of α-syn protein. A previous work has highlighted the involvement of α-syn in the pathogenesis of PD (George et al., 2013). There was an association between α-syn species concentrations in the CSF and blood and motor progression in patients with PD (Parnetti et al., 2019). Ding et al. (2021) also confirmed the impairment of meningeal lymphatic system in patients with PD compared with atypical parkinsonian disorders. Their findings revealed that meningeal lymphatic drainage dysfunction may aggravate α-syn pathology and exacerbate PD progression (Ding et al., 2021). Ineffective elimination of α-syn, which is closely related to the dysfunction of glymphatic system, may be a contributory factor to the development of PD. Most of the studies investigating the role of the glymphatic system were conducted in animal models. Finding the effective glymphatic system biomarkers in human is still challenging. Recently, Taoka et al. (2021) proposed a new non-invasive method

DTI-ALPS to help assess the glymphatic system activity in human. They also suggested that ALPS index was robust under unified imaging method but was influenced by the imaging plane, the number of motion-proving gradient axes, and TE in the imaging sequence (Taoka et al., 2021). Until present, there are only two studies using DTI-ALPS method to evaluate glymphatic system activity in patients with PD (Chen et al., 2021; McKnight et al., 2021). Chen et al. (2021) indicated that cognitively impaired patients with PD showed lower ALPS index than NCs. Moreover, glymphatic system dysfunction was associated with increased oxidative stress status in PD (Chen et al., 2021). McKnight et al. (2021) recently demonstrated that in comparison with essential tremor (ET) patients, ALPS index was reduced in patients with PD. However, there was no significant relationship between ALPS index and mild cognitive impairment status after controlling for age, sex, diagnosis status (PD or ET), and Fazekas score (McKnight et al., 2021). Our findings provided evidence supporting the notion that dysfunction of the glymphatic system has been implicated in the pathogenesis of PD, especially in the late stage. However, there was no significant difference in ALPS index between the early and late PD groups. Future studies are needed to determine the utility of DTI-ALPS index in monitoring the progression of PD.

Furthermore, Taoka et al. proposed a new concept "central nervous system (CNS) interstitial fluidopathy" that represents diseases with impaired fluid dynamics of the CNS interstitial space (Taoka and Naganawa, 2021). These diseases include sleep disorders, AD, iNPH, traumatic brain injury, stroke, glaucoma, and other disorders. Our results support that PD has characteristics as a CNS interstitial fluidopathy. This term could also promote our understanding of the mechanisms and develop potential novel therapies in future.

A significant correlation between ALPS index and cognitive function has been observed in a previous study (Steward et al., 2021). Irwin et al. (2012) emphasized the importance of cortical Lewy bodies as pathological substrates of cognitive impairment and dementia in PD. Our result was consistent with previous studies. We first revealed that ALPS index was positively linked with MMSE score in the early stage of PD. While in the late stage, the correlation between ALPS index and age was noteworthy. Our findings provided insight into the role of glymphatic system on the cognitive function in PD. We also speculated that in the advanced stage of PD, increasing age may play a more vital role in the glymphatic system activity than other clinical factors.

In addition, our study first demonstrated a negative correlation between the ALPS index and EPVS score. EPVS on MRI has been considered as a marker of PVS dysfunction, which may reflect the impairment of normal brain fluid and waste clearance and microvascular dysfunction (Wardlaw et al., 2020). There is also a link between PVS in diffusion and structural scans. The influence of the PVS on the DTI-derived measures, including an increased mean diffusivity and decreased FA, has been demonstrated (Sepehrband et al., 2019). Previous work has indicated that the global and regional PVS volume fractions were increased in PD related to non-PD, especially in familial PD (FPD). A significant PVS volume fraction difference between idiopathic PD and FPD was also observed in the cuneus and lateral occipital regions (Donahue et al., 2021). Moreover, EPVS correlated with clinical symptoms in PD, such as tremor and cognitive decline (Park et al., 2019; Wan et al., 2019). However, a clinicopathological study found a lower prevalence of EPVS in PD group related to NCs (Schwartz et al., 2012). The interaction between EPVS and PD is still controversial. Recently, Li et al. (2020) revealed that patients with PD with EPVS in the substantia nigra (SN) showed greater expression of tau protein in CSF and a trend toward reduced DAT binding than those without SN-EPVS. Their results suggested an association between EPVS and SN degeneration in PD (Li et al., 2020). Another study also showed that patients with PD had a higher PVS burden in BG and midbrain than NCs, and the PVS number in BG significantly correlated with disease severity and L-dopa equivalent dosage (Shen et al., 2021). We speculated that a reduced glymphatic activity results in the accumulation of the misfolding proteins such as α-syn, which is implicated in the development of PVS dilation on MRI. Impaired clearance of large molecules may consequently stack in the PVSs, resulting in compensatory dilation.

There were some limitations in this study. (1) The sample size was relatively small. Larger sample size studies in this field are needed in future. (2) The DTI-ALPS method could calculate only the diffusivity along the direction of the PVS on one slice of the brain. We did not measure the diffusivity outside this area. (3) Head motion correction was not conducted in our study. But the head of the subject was immobilized with foam pillows inside the coil to diminish motion artifacts. Studies evaluating the optimal method are needed in future. (4) We could not use this method on an individual level. Further studies are warranted to develop new methods capable of differentiating patients on an individual level.

CONCLUSION

Our findings revealed that patients with PD showed lower ALPS index than NCs, especially in the late stage of the disease. We demonstrated the dysfunction of glymphatic system in PD, which was correlated with cognitive impairment, EPVS on MRI, and increasing age. Furthermore, DTI-ALPS is a sensitive method to assess glymphatic system activity. Further studies are needed to determine the utility of DTI-ALPS index in diagnosing and monitoring the progression of PD.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the original contributions presented in the study are

included in the article/supplementary material, further inquiries can be directed to the corresponding author. Requests to access the datasets should be directed to WS, suwenbjyy@163.com.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

XM, HC, and WS contributed to the conception and design of the study. XM, SL, CL, RW, and MC organized the database and performed the statistical analysis. XM wrote the first draft of the manuscript. All authors approved the final version of the manuscript.

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ORIGINAL RESEARCH
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The Impact of Multimorbidity Burden, Frailty Risk Scoring, and 3-Directional Morphological Indices vs. Testing for CSF Responsiveness in Normal Pressure Hydrocephalus

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Objective: Multimorbidity burden across disease cohorts and variations in clinico-radiographic presentations within normal pressure hydrocephalus (NPH) confound its diagnosis, and the assessment of its amenability to interventions. We hypothesized that novel imaging techniques such as 3-directional linear morphological indices could help in distinguishing between hydrocephalus vs. non-hydrocephalus and correlate with responsiveness to external lumbar drainage (CSF responsiveness) within NPH subtypes.

Methodology: Twenty-one participants with NPH were recruited and age-matched to 21 patients with Alzheimer's Disease (AD) and 21 healthy controls (HC) selected from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database. Patients with NPH underwent testing *via* the NPH programme with external lumbar drainage (ELD); pre- and post-ELD MRI scans were obtained. The modified Frailty Index (mFI-11) was used to stratify the NPH cohort, including Classic and Complex subtypes, by their comorbidity and frailty risks. The quantitative imaging network tool 3D Slicer was used to derive traditional 2-dimensional (2d) linear measures; Evans Index (EI), Bicaudate Index (BCI) and Callosal Angle (CA), along with novel 3-directional (3d) linear measures; z-Evans Index and Brain per Ventricle Ratio (BVR). 3-Dimensional (3D) ventricular volumetry was performed as an independent correlate of ventriculomegaly to CSF responsiveness.

Results: Mean age for study participants was 71.14 ± 6.3 years (18, 85.7% males). The majority (15/21, 71.4%) of participants with NPH comprised the Complex subtype (overlay from vascular risk burden and AD); 12/21 (57.1%) were Non-Responders to ELD. Frailty alone was insufficient in distinguishing between NPH subtypes. By contrast, 3d linear measures distinguished NPH from both AD and HC cohorts, but also correlated to CSF responsiveness. The z-Evans Index was the most sensitive volumetric measure of CSF responsiveness (p = 0.012). Changes in 3d morphological indices across timepoints distinguished between Responders vs. Non-Responders to lumbar testing. There was a significant reduction of indices, only in Non-Responders

and across multiple measures (z-Evans Index; $p = 0.001$, BVR at PC; $p = 0.024$). This was due to a significant decrease in ventricular measurement ($p = 0.005$) that correlated to independent 3D volumetry ($p = 0.008$).

Conclusion. In the context of multimorbidity burden, frailty risks and overlay from neurodegenerative disease, 3d morphological indices demonstrated utility in distinguishing hydrocephalus vs. non-hydrocephalus and degree of CSF responsiveness. Further work may support the characterization of patients with Complex NPH who would best benefit from the risks of interventions.

Keywords: normal pressure hydrocephalus, external lumbar drainage, ventricular morphology, ventricular volume, ADNI database

INTRODUCTION

The diagnosis of normal pressure hydrocephalus (NPH), first termed by Hakim and Adams (1965), requires supportive evidence from clinical history, physical examination and brain imaging (Schirinzi et al., 2015). It is characterized by the clinical triad of gait disturbance, mental deterioration and urinary incontinence, along with the enlargement of the cerebral ventricles (Relkin et al., 2005). Although the precise global incidence and prevalence of NPH are not known, NPH has been found to mainly implicate the geriatric population (Tanaka et al., 2009; Jaraj et al., 2014). Patients with NPH are thus also known to present with burden of concurrent comorbidities. Although attempts have been made to provide supplementary guidance for the needs of this challenging population (Malm et al., 2013), this cohort has previously been excluded from standard practice guidelines (Bergsneider et al., 2005; Klinge et al., 2005; Marmarou et al., 2005b,c; Relkin et al., 2005). We have previously described a particular subtype of NPH, with a heavy burden of concurrent comorbidities, termed "Complex NPH" as per Lock et al. (2019). Here, we have expanded on our definition for consistency, and further refined it *via* this work, as a subtype of NPH patients matching the following criteria:–(i) clinical symptoms and signs consistent with probable/possible NPH according to international/Japanese guidelines, (ii) with strong neuroradiological features supportive of the NPH diagnosis (such as DESH or other imaging biomarkers), (iii) but presenting with overlay from multiple comorbidities co-existing (such as significant cardiovascular risk burden or neurodegenerative disorders), and (iv) who are difficult to test using standard supplementary measures (due to poor cognitive/gait/balance/functional ability) or high risk for testing/surgical interventions (due to cardiac disease, antiplatelet or anticoagulation therapy, or spinal operations). In this cohort, invasive gold-standard testing may be difficult or inconclusive; the needs of such patients have elevated the importance of developing more precise risk stratification scoring and neuroimaging tools to characterize responsiveness to CSF drainage.

Given early and accurate diagnosis, symptoms of Classic NPH (gait disturbance, dementia, incontinence) can be reversed through ventricular shunting (Shprecher et al., 2008). There is also evidence that, in patients with Complex NPH, there is still a remediable component of responsiveness to external lumbar drainage (CSF responsiveness) that is amenable to interventions. External lumbar drainage (ELD), involving the drainage of cerebrospinal fluid (CSF) through a lumbar spinal catheter over several days, is a gold-standard supplementary test for shunt responsiveness (Ishikawa et al., 2008; Gallina et al., 2018). Several guidelines for the management of NPH have reported high sensitivities (50–100%) and high positive predictive values (80–100%) for the prognostic value of ELD (Walchenbach et al., 2002; Marmarou et al., 2005a; Ishikawa et al., 2008; Chotai et al., 2014). However, the perioperative morbidity of CSF shunting procedures are also significant (38% pooled rate of shunt complication including death, infection, seizures, shunt malfunction, subdural hemorrhage or effusion) (Hebb and Cusimano, 2001). Furthermore, improvements in cognitive deterioration have been found to be limited to only 30–50% of shunted patients (Black, 1980; Vanneste, 2000). Thus, there is a need for non-invasive supplementary screening tools to aid in the diagnostic and prognostic selection for shunt-responsive NPH patients.

By current clinical standards, 2-dimensional (2d) morphological indices such as the Evans Index (EI), Bicaudate Index (BCI) and Callosal Angle (CA), are used as diagnostic markers in differentiating NPH cohorts to AD and healthy control (HC) cohorts (Marmarou et al., 2005a; Ishikawa et al., 2008). However, recent volumetric studies demonstrating z-axial, as opposed to x-axial, ventricular expansion have suggested 2d morphological indices may be insufficient to fully describe the patterns of ventricular enlargement vs. brain atrophy across NPH and AD cohorts (Ambarki et al., 2010; Toma et al., 2011). Traditional linear morphological indices have also been found to be inadequate in characterizing intra-NPH cohorts such as NPH and secondary NPH due to variations in fluid distribution patterns within NPH cohorts (Yamada et al., 2017).

Other studies involving the use of morphological indices in NPH cohorts have since supported the potential utility of novel 3-directional (3d) linear indices (z-Evans index and brain per ventricle ratio, BVR) toward the differential diagnosis of NPH and AD cohorts (Yamada et al., 2016, 2017). The attractiveness of such methods are that 3d linear indices not only describe the directional expansion of brain ventricles, but are able to also characterize differences in fluid distribution patterns across differing CSF compartments amongst these disease cohorts.

In this study, we examined the use of a 3d morphological methodology to distinguish between cohorts with hydrocephalus vs. non-hydrocephalus and evaluated its performance in Asian patients with NPH, across both Classic and Complex subtypes (Lock et al., 2019). Here we present our findings on the relevance of multimorbidity burden and frailty risks and their associations between 3d linear indices, changes in ventricular size and responsiveness to CSF responsiveness *via* ELD.

MATERIALS AND METHODS

Data Source

Twenty-four patients with probable NPH (mean age 71 ± 6.3 years) who underwent the extended CSF drainage protocol *via* the NPH programme at the National Neuroscience Institute, Singapore, were recruited prospectively. All patients met criteria for probable or possible NPH according to published guidelines (Relkin et al., 2005), presenting with ventriculomegaly and at least one of three features of the NPH clinical triad. Additional details of the protocol have been previously published (Lock et al., 2019). Participants had one pre-intervention baseline MR scan and one post-intervention MR scan after CSF lumbar drainage (≥300 ml of CSF drained over 3 days, or otherwise determined by the treating consultant). Participants either had a lumbar drain insertion, or serial taps from an Ommaya reservoir. Three participants were excluded from analysis—one was unable to undergo MR scanning, another participant did not complete CSF drainage and was discontinued from the study due to a subarachnoid hemorrhage, and the third participant did not proceed with CSF drainage due to an incidental finding during pre-intervention clinical investigation. The study was approved by the National Healthcare Group Domain Specific Review Board (NHG DSRB; Ref 2014/00838) and the SingHealth Centralised Institutional Review Board (CIRB; Ref 2016/2627). Informed consent was obtained from all participants or their legal representatives, if applicable.

Data for comparator groups of twenty-one age-matched AD patients (9 males, mean age 73 ± 8.6 years) and healthy controls (HC; 7 males, mean age 73 ± 3.4 years) were obtained from baseline scans of patients enrolled to the ADNI 1 phase of the Alzheimer's Disease Neuroimaging Initiative (ADNI) study[1]. AD patients had mild AD, meeting NINCDS-ADRDA criteria for probable AD and a Clinical Dementia Rating of 0.5 or 1.0 (Alzheimer's Disease NeuroImaging1(ADNI1)Clinical Protocol, 2005; Petersen et al., 2010).

Image Acquisition and Pre-processing

MR imaging data for NPH participants were acquired with a 3-T MR Philips scanner (Ingenia, Philips Medical Systems, Best, the Netherlands), including 3D T1, T2, FLAIR, and DTI sequences. Three-dimensional axial T1-weighted imaging with sensitivity encoding (SENSE) was acquired (*TR* = 7.3ms, *TE* = 3.3 ms, flip angle = 8°, FOV = 256 × 256 mm, voxel size = 1.0 × 1.0 × 1.0 mm). Eight patients were downgraded to the 1.5-T scanner at equivalent specifications due to institutional MR safety protocol. AD and HC participants from ADNI were scanned in a 3-T MRI scanner (GE Healthcare, Philips Medical Systems, or Siemens Healthcare, depending on the ADNI scanning site). MRI scanning protocols for each scanner model are available online[2].

[1]adni.loni.usc.edu/study-design/

Modified Frailty Index-11

Frailty was quantified using the *modified Frailty Index-11* (mFI-11) and its components found in **Table 1**. The mFI-11 is a validated shortened version of the 70-point Canadian Study of Health and Aging Frailty Index (CSHA FI) (Fang et al., 2017). The mFI-11 is one of the more commonly utilized tool to assess frailty in various surgical subspecialities as it examines easily identifiable clinical information that can be extracted from available clinical notes, or obtained at bedside, with statistically simple and reproducible calculations (Pazniokas et al., 2020). Patients were given a binary score for each comorbidity assessed, then stratified by the extent of their frailty based on their

[2]adni.loni.usc.edu/methods/documents/mri-protocols/

TABLE 1 | Clinical characteristics of normal pressure hydrocephalus (NPH) cohort.

Characteristic	Number of subjects (*n = 21*)
Gender	
Male	18 (85.7)
Female	3 (14.3)
Mean age, years (SD)	71.14 (± 6.3)
Category of normal pressure hydrocephalus (NPH)	
Classic	6 (28.6)
Complex	15 (71.4)
External lumbar drainage (ELD) responsiveness	
Responder	9 (42.9)
Non-responder	12 (57.1)
Modified Frailty Index-11 (mFI-11)	
Hypertension	17 (81.0)
Impaired sensorium	13 (61.9)
Diabetes mellitus	7 (33.3)
Activities of daily living dependent	6 (28.6)
Myocardial infarction	3 (14.3)
Percutaneous coronary intervention/angina	3 (14.3)
Chronic/acute respiratory disease	2 (9.5)
Peripheral vascular disease	1 (4.8)
Coronary heart failure	0
Cerebrovascular accident/transient ischemic attack	0
mFI-11 score: 0–2	9 (42.9)
mFI-11 score: ≥3	12 (57.1)

Complex NPH; term as per Lock et al. (2019) and further refined in this work, a subtype of NPH patients matching the following criteria:- (i) clinical symptoms and signs consistent with probable/possible NPH according to international and Japanese guidelines, (ii) with strong neuroradiological features supportive of the NPH diagnosis (such as DESH or other imaging biomarkers), (iii) but presenting with overlay from multiple comorbidities co-existing (such as significant cardiovascular risk burden or neurodegenerative disorders) and (iv) who are difficult to test using standard supplementary measures (due to poor cognitive/gait/balance/functional ability) or high risk for testing/surgical interventions (due to cardiac disease, antiplatelet, or anticoagulation therapy or spinal operations).

cumulative scores (mFI-11 score: 0–2 and ≥3; with the highest mFI-11 score in our cohort being 6).

Morphological Features

We used the open-source quantitative imaging network tool, 3D Slicer 4.9, to derive traditional 2-dimensional (2d) linear and 3-directional (3d) linear measurements, as well as 3-Dimentional (3D) quantitative ventricular volumes[3] (Fedorov et al., 2012). This tool was selected as it functions akin to a radiology workstation that allows for versatile visualizations, but also provides advanced modular functionalities such as semi-automated segmentations, volumetry, and 3D quantitative measurements. This combined functionality allowed us to streamline optimization steps usually performed at the scanner workstation with subsequent morphological measurements, within a single continuous workflow.

Firstly, we reproduced the methodology of deriving measurements of morphological indices inaccordance with the work by Yamada et al. (2017), which includes traditional 2d linear measurements- Evans Index (EI), Callosal Angle (CA), Bicaudate Index (BCI) and 3d linear measurements- z-Evans, Brain-Ventricle Ratio (BVR). Intraclass Correlation Coefficients (ICCs) showed good intra-rater agreement for traditional 2d linear measures (EI, ICC = 0.980; CA, ICC = 0.953) and 3d linear measures (z-Evans, ICC = 0.967; BVR at AC, ICC = 0.972, BVR at PC, ICC = 0.989). The EI (**Figure 1A**) was defined as the ratio of the maximal width of the frontal horns of the lateral ventricles to the maximal width of the internal diameter of the cranium. The BCI (**Figure 1B**) was defined as the ratio of the maximum intercaudate distance to the width of the brain along the same line on the axial plane (Chen et al., 2017). The callosal angle (**Figure 1C**) was defined as the angle of the roof of the bilateral ventricles on the coronal plane at the level of the posterior commissure (PC). The z-Evans index (**Figure 1D**) was defined as the ratio of the maximum z-axial length of the frontal horns of the lateral ventricles to the maximum cranial z-axial length on the coronal plane, perpendicular to the anterior commissure-posterior commissure (ACPC) line. The brain-ventricle ratios (BVRs) at the AC and PC (**Figures 1E,F**) were measured as the maximal brain width above the lateral ventricles divided by the maximum height of the lateral ventricles, on the coronal plane with reference to the AC and PC levels, respectively. 3-Dimensional (3D) volumetric measures of ventricles were also derived in this study to validate results from their linear counterparts.

A flow chart of the methodology used on 3D Slicer is illustrated in **Figure 2**. Using the *ACPC Transform* and the *Resample Scalar/Vector/DWI Volume* modules, planes of the T1-weighted DICOM scans were re-aligned parallel to the ACPC line for accurate replication alignment at a radiology workstation. *Ruler Module* was used to extract measurements for the various morphological indices. *Segment Editor* was used for the semi-automated segmentation of the ventricles. *Segment Statistics* was used to derive ventricular volumes by semi-automatic counting of voxels in segments.

[3]www.slicer.org

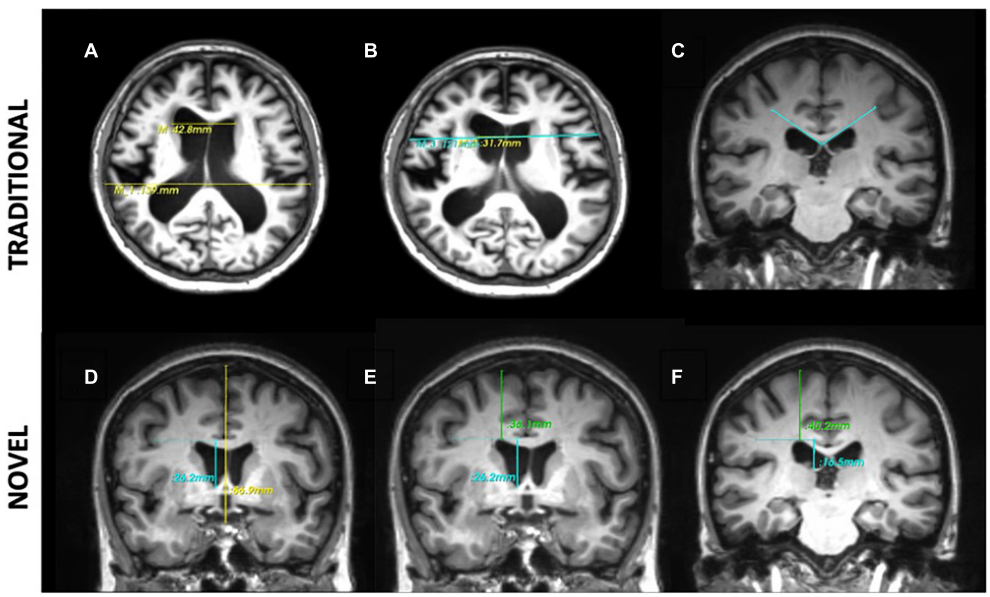

FIGURE 1 | Morphological indices. Traditional 2-dimensional (2d) linear measures: **(A)** Evans Index is the ratio of the maximal width of the frontal horns of the lateral ventricles (top line) to the maximal width of the internal diameter of the cranium (bottom line). **(B)** Bicaudate Index is the ratio of the maximum intercaudate distance (yellow line) to the width of the brain along the same line on the axial plane (blue line). **(C)** The callosal angle is measured as the angle of the corpus callosum through the posterior commissure perpendicular to the ACPC plane. Novel 3-directional (3d) linear measurements: **(D)** the z-Evans index is the ratio of the maximum z-axial length of the frontal horns of the lateral ventricles (blue line) to the maximum cranial z-axial length on the coronal plane (yellow line), perpendicular to the ACPC plane, on the anterior commissure. **(E,F)** The Brain-Ventricle-Ratios (BVRs) measured at the level of the anterior and posterior commissure, respectively, are measured as the maximal brain thickness above the lateral ventricles (blue line) divided by the maximum length of the lateral ventricles (green line), on the coronal plane.

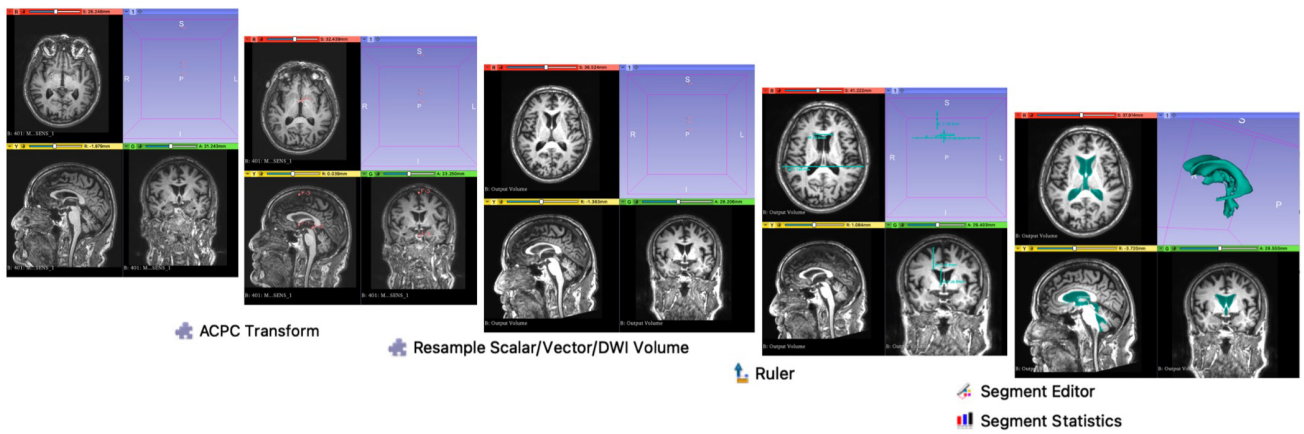

FIGURE 2 | Methodology on 3D Slicer. Using the *ACPC Transform* and the *Resample Scalar/Vector/DWI Volume* modules, planes of the T1-weighted DICOM scans were re-aligned parallel to the ACPC line for accurate replication of the alignment at a typical radiology workstation, as per Yamada et al. (2017). *Ruler Module* was used to extract measurements for the various morphological indices. *Segment Editor* was used for the semi-automated segmentation of the ventricles. *Segment Statistics* was used to derive ventricular volumes by automatic counting of voxels in segments.

Validation of our workflow on 3D Slicer was done by comparing inter-cohort morphological trends of our NPH, AD and HC cohorts to that of Yamada et al. (2016), which was conducted on a Siemens workstation (Yamada et al., 2017; **Supplementary Figure 1**). The lower BVR measurements between AD cohorts could be accounted for by the differences in the AD screening criteria set by ADNI Petersen et al. (2010) and Yamada et al. (2017) resulting in a more severe AD cohort in our study.

Statistical Analysis

Categorical data were described as numbers and percentages, continuous variables were reported as mean and standard deviation (SD). Categorical variables were compared and analyzed with Fisher's Exact test. Inter- and intra-group comparisons for morphological measures were tested with independent-samples Mann-Whitney *U* test and Wilcoxon signed rank test. All statistical tests were two-tailed and statistical significance was assumed at $p < 0.05$. The statistical analyses were performed using R statistical software version 3.3.3. (R Core Team, 2021)[4].

RESULTS

Clinical Characteristics

Twenty-one patients (mean age 71 ± 6.3 years; 18 males, 3 females) with NPH were recruited. Of these 21, six were found to be solely consistent with criteria for NPH diagnosis as per international guidelines and classified as Classic NPH; fifteen patients met both the international criteria for their clinico-radiological presentation and our described definition for Complex NPH. Subjects were classified as responders to ELD if their levels of improvement met the minimal clinically important difference (MCID) in at least one domain of NPH symptomatology sufficient to support consideration for shunt insertion. We defined the MCID using the following thresholds: an increase of ≥10% in any measure of inpatient gait, balance, or cognitive testing, matched with a ≥20% functional improvement reported by the patient or caregiver at home following discharge. Using this criteria, there were 9 Responders and 12 Non-Responders to CSF drainage *via* ELD within our NPH cohort. A summary of the demographics of our cohort is found in **Table 1**.

Frailty

Following frailty stratification by the mFI-11, 9/21 (42.9%) and 12/21 (57.1%) had a mFI-11 score of 0–2, and ≥3, respectively. The cohort was stratified relatively evenly with respect to ELD response ($p = 1.00$), and a majority of the frailer patients (mFI-11 ≥ 3) were classified as Complex NPH (Classic NPH 3/6, 50% vs. Complex NPH 9/15, 60%; $p = 1.00$). The distribution of our NPH subtypes (Responders vs. Non-Responders and Classic vs. Complex groups) stratified by frailty can be found in **Table 2**.

Morphological Indices: Inter-Cohort Comparisons

Both 2d and 3d linear measurements were able to distinguish between disease cohorts (NPH vs. AD), and between NPH and healthy controls ($p < 0.001$), as seen in **Table 3**. NPH patients also had significantly larger ventricles (characterized by higher EI and BCI values) and significantly tight high-convexities (characterized by lower CA and BVR values) in their morphology.

Morphological Indices: Intra-Cohort Comparisons

Compared to our patients with Complex NPH, our patients with the classic NPH subtype demonstrated a trend toward relatively larger ventricles (higher EI, BCI, and z-Evans index values)

[4]www.r-project.org/foundation

TABLE 2 | Stratification of frailty risks of the normal pressure hydrocephalus (NPH) cohort.

mFI-11 group	Responder (*n* = 9)	Non-responder (*n* = 12)	*p*-value	Classic (*n* = 6)	Complex (*n* = 15)	*p*-value
0–2	4 (44.4)	5 (41.7)	1	3 (50)	6 (40)	1
≥3	5 (55.6)	7 (58.3)		3 (50)	9 (60)	

mFI-11; *Modified Frailty Index-11.*
Complex NPH; *term as per Lock et al. (2019) and further refined in this work, a subtype of NPH patients matching the following criteria:- (i) clinical symptoms and signs consistent with probable/possible NPH according to international and Japanese guidelines, (ii) with strong neuroradiological features supportive of the NPH diagnosis (such as DESH or other imaging biomarkers), (iii) but presenting with overlay from multiple comorbidities co-existing (such as significant cardiovascular risk burden or neurodegenerative disorders) and (iv) who are difficult to test using standard supplementary measures (due to poor cognitive/gait/balance/functional ability) or high risk for testing/surgical interventions (due to cardiac disease, antiplatelet, or anticoagulation therapy or spinal operations).*

TABLE 3 | Inter-cohort comparisons of normal pressure hydrocephalus (NPH) vs. non-NPH *via* baseline morphological indices.

Baseline measures				*p*-values		
	NPH	AD	HC	NPH–AD	NPH–HC	AD–HC
Traditional 2d linear measures						
EI	0.380 ± 0.053	0.286 ± 0.033	0.277 ± 0.028	<0.001*	<0.001*	0.315
BCI	0.284 ± 0.027	0.186 ± 0.032	0.175 ± 0.025	<0.001*	<0.001*	0.216
Callosal angle (degrees)	57.1 ± 20.8	109.0 ± 15.1	106.4 ± 11.5	<0.001*	<0.001*	0.528
Novel 3-directional linear measures						
z-Evans index	0.448 ± 0.056	0.294 ± 0.037	0.273 ± 0.043	<0.001*	<0.001*	0.096
BVR at AC	0.678 ± 0.176	1.436 ± 0.257	1.589 ± 0.332	<0.001*	<0.001*	0.104
BVR at PC	0.835 ± 0.316	2.611 ± 0.964	3.281 ± 1.220	<0.001*	< 0.001*	0.055

**Significant at $\alpha < 0.05$.*
EI, Evans index.
BCI, Bicaudate index.
BVR, Brain-ventricle ratio.
AC, Anterior commissure.
PC, Posterior commissure.
NPH, Normal pressure hydrocephalus.
AD, Alzheimer's disease.
HC, Healthy control.
NPH cohorts consisted of classic and complex NPH patients. Differences in traditional 2d linear measures (EI, BCI, Callosal Angle) and 3-directional linear measures (z-Evans Index, BVR at AC and PC level), between NPH and AD, and between NPH and HC, were significant. No significant differences observed between AD and HC cohorts.

and tighter high-convexities (higher CA and lower BVR values) (**Table 4**). However, neither traditional 2d nor novel 3d linear measures distinguished between the two subtypes.

As there were no significant differences in morphological indices found between Classic vs. Complex NPH pre-testing, for subsequent comparisons between cohorts performed pre- and post-ELD, we considered these subtypes as one NPH cohort. Differences in morphological indices revealed a significant decrease in the z-Evans index values post-ELD ($p = 0.012$), and non-significant decreases in the EI and BCI values (**Table 5**).

When we classified patients by their testing timepoints, morphological indices alone were insufficient to distinguish between Responders vs. Non-Responders to CSF drainage, at either pre-or post-ELD (**Table 6**). However, when we classified patients by their responsiveness to ELD, changes in 3d morphological indices across timepoints were able to distinguish between Responders vs. Non-Responders to lumbar testing. The effect of ELD resulted in a significant reduction of 3d morphological indices; this only occurred in Non-Responders and was consistent across multiple independently derived measures (**Table 7**). These effects include a decrease in z-Evans Index ($p = 0.001$) and an increase in BVR at the level of the PC ($p = 0.024$), due to a decrease in the ventricular component of the BVR ($p = 0.005$). 3D volumetric analysis also supported the significant decrease in ventricular volumes, only in Non-Responders post-drainage ($p = 0.008$).

DISCUSSION

In this study, we examined the impact of multimorbidity burden, frailty risks and the efficacy of novel 3d linear measures in the Asian context of hydrocephalus vs. non-hydrocephalus and Classic vs. Complex NPH subtypes. Comorbidities, such as hypertension (81%), diabetes mellitus (33.3%) and myocardial infarction/percutaneous coronary intervention/angina (3%), were commonly found in our subjects. A higher comorbidity profile is known to decrease the chances of favorable outcomes in NPH patients who

TABLE 4 | Intra-cohort comparisons of Classic vs. Complex normal pressure hydrocephalus (NPH) *via* baseline morphological indices.

Pre-ELD measurements	Classic NPH (*n* = 6)	Complex NPH (*n* = 15)	*p*-value
Traditional 2d linear measures			
EI	0.392 ± 0.0577	0.373 ± 0.0534	0.494
BCI	0.287 ± 0.0261	0.281 ± 0.0279	0.779
Callosal Angle (degrees)	67.1 ± 23.9	54.6 ± 18.6	0.248
Novel 3-directional linear measures			
z-Evans index	0.436 ± 0.0588	0.449 ± 0.0565	0.602
BVR at AC	0.712 ± 0.158	0.674 ± 0.188	0.547
BVR at PC	0.922 ± 0.356	0.814 ± 0.312	0.494

ELD, External lumbar drain.
EI, Evans index.
BCI, Bicaudate index.
BVR, Brain-ventricle ratio.
AC, Anterior commissure.
PC, Posterior commissure.
Comparison of pre-ELD morphological indices between NPH subtypes (Classic vs. Complex). No significant differences were observed between Classic and Complex NPH cohorts.

undergo ventricular shunting (Meier and Lemcke, 2008). After ELD, we found that there was a higher proportion of Non-Responders as compared to Responders in our population. Given that ELD is known to accurately predict responsiveness to long-term ventricular shunting (Walchenbach et al., 2002), this suggests that a majority of our study population would not benefit from definitive ventricular shunting procedures (Chotai et al., 2014; Gallina et al., 2018). These data are consistent with expectations of reversibility of the NPH component in patients presenting with multiple comorbidities. Conversely, our study also provided evidence for the fallacies of screening NPH patient cohorts using comorbidities and frailty risks alone. Neither of these risks, nor traditional 2d linear radiological measures, were sufficient to predict CSF responsiveness within our cohort of Classic and Complex NPH subtypes.

To investigate the frailty risks of our population, we stratified our patients by means of a well-validated frailty score, in order to quantify biological, rather than purely chronological, age (Farhat et al., 2012; Pazniokas et al., 2020). Using mFI-11, we categorized our patient population into two discrete groups and assessed if there was any correlation with responsiveness to ELD. We found that a higher frailty score was neither correlated with CSF responsiveness nor the increased likelihood of being termed to have Complex NPH according to our criteria. Whilst there have been no definitive studies studying the link between frailty and shunt responsiveness in NPH patients, a recent study found that frailty was not associated with early post-operative patient-related complications post-ventricular shunting (Hadjiathanasiou et al., 2020). However, we should note that the study did not include patients with high frailty scores in their cohort, similar to our study in which the highest mFI-11 score was 6, out of a maximum of 11. Inclusion of patients with higher frailty scores may have led to different findings and should be the focus of future studies. Frailty has also been reported to be correlated with CNS elastance coefficient, suggesting that frailty is associated with pathological brain aging and the development of neurodegenerative diseases (Vallet et al., 2020). We would then expect that even if frailty was unable to predict CSF responsiveness, it would be significantly correlated to our Complex NPH group in which the proportion of neurodegenerative diseases are higher. However, our results suggest that frailty risks, which are also influenced by the presence of comorbidities, are likely to occur as an irreversible entity separate to the presentation of patients having what we term to be the subtype of Complex NPH. Instead, in Complex NPH, we believe the distinction to be that the probable/possible reversible NPH component still co-exists, except it does so in the presence of challenging overlay from irreversible risks of vascular risk burden/neurodegenerative disorders (that also contribute to the frailty score). Population differences (Asian vs. Caucasian) and our relatively small sample size may account for some of these discrepancies and global studies focused on patients with Complex NPH would be required to draw meaningful conclusions.

The association between changes in ventricular morphology and volumes with respect to ELD-responsiveness has been reported. Both Anderson et al. (2002), and more recently, Neikter et al. (2020), have shown that NPH responders demonstrate a significant decrease in ventricular volumes post-shunting. Yamada et al. (2019) also described increases in BVRs and reductions in ventricular volumes being correlated with excellent outcomes in NPH patients. In patients with idiopathic NPH, it has been shown that bilateral ventricle expansions occur in the z-axial direction, as opposed to the x-axial direction (Neikter et al., 2020). Thus 3d linear measurements, such as the z-Evans Index and BVRs, may be a better approximation of actual changes in brain-ventricular volumes as compared to traditional 2d indices (Neikter et al., 2020).

Interestingly, when we stratified our data according to responsiveness to ELD, this change in ventricular morphology was only significant amongst the Non-Responders. Changes in BVR values matched with a decrease in ventricular volumes, as demonstrated by Yamada et al. (2019). However, whilst these findings appear contradictory, we believe the changes to be reflective of the composition of our NPH cohort, with its large proportion of the Complex NPH subtype. In Complex NPH, the concurrent load of vascular risk burden and/or neurodegenerative diseases may contribute to brain changes more consistent with irreversible injury, such as atrophy and loss of tissue microstructure or elasticity. Conversely, such changes may promote the ease with which fluid moves between CSF compartments during test challenges such as ELD. As 3d linear measures are sensitive to changes in both ventricular volume and apparent overlying brain thickness due to distortion, large passive fluid movements would also be described by these morphological indices. Indeed, our negative results in Responders are also consistent with findings by Meier and Mutze (2004), and Lenfeldt et al. (2012), who

TABLE 5 | Comparison of normal pressure hydrocephalus (NPH) cohorts pre- and post-lumbar testing using morphological measures, regardless of responsiveness to external lumbar drainage (ELD).

Measurements	Pre-ELD	Post-ELD	*p*-value
Traditional 2d linear measures			
EI	0.380 ± 0.053	0.377 ± 0.051	0.437
BCI	0.284 ± 0.027	0.282 ± 0.031	0.662
Callosal angle (degrees)	57.1 ± 20.8	58.1 ± 20.2	0.344
Novel 3-directional linear measures			
z-Evans index	0.448 ± 0.056	0.440 ± 0.055	0.012*
BVR at AC	0.678 ± 0.176	0.672 ± 0.196	0.792
BVR at PC	0.835 ± 0.316	0.860 ± 0.284	0.233

**Significant at $\alpha < 0.05$.*
ELD, External lumbar drain.
EI, Evans index.
BCI, Bicaudate index.
BVR, Brain-ventricle ratio.
AC, Anterior commissure.
PC, Posterior commissure.
Comparison of pre- and post-ELD morphological indices within NPH cohort. As there were no significant differences in morphological indices found between Classic vs. Complex NPH pre-testing, for subsequent comparisons between cohorts performed pre- and post-ELD, we considered these subtypes as one NPH cohort. Differences in traditional 2d linear measures (EI, BCI, Callosal Angle) pre- and post-ELD were not significant. However, the differences between the z-Evans index, a 3-Directional linear measure, pre- and post-ELD was significant within the NPH cohort.

TABLE 6 | Classification of normal pressure hydrocephalus (NPH) cohorts by timepoints pre- vs. post-lumbar testing: morphological indices within groups compared by their responsiveness to external lumbar drainage (ELD).

	Pre-ELD			Post-ELD		
Measurements	**Responders (*n* = 9)**	**Non-responders (*n* = 12)**	**p-value**	**Responders (*n* = 9)**	**Non-responders (*n* = 12)**	***p*-value**
Traditional 2d linear measures						
EI	0.402 ± 0.054	0.363 ± 0.048	0.100	0.399 ± 0.045	0.360 ± 0.050	0.079
BCI	0.291 ± 0.026	0.278 ± 0.027	0.298	0.294 ± 0.028	0.274 ± 0.031	0.127
Callosal angle (degrees)	61.6 ± 21.0	53.8 ± 21.0	0.413	61.3 ± 19.7	55.8 ± 21.1	0.548
Novel 3-directional linear measures						
z-Evans index	0.461 ± 0.061	0.438 ± 0.052	0.359	0.460 ± 0.059	0.424 ± 0.049	0.152
BVR at AC	0.613 ± 0.172	0.726 ± 0.170	0.151	0.616 ± 0.159	0.714 ± 0.216	0.263
BVR at PC	0.789 ± 0.361	0.869 ± 0.290	0.581	0.773 ± 0.295	0.926 ± 0.269	0.230

ELD, External lumbar drain.
EI, Evans index.
BCI, Bicaudate index.
BVR, Brain-ventricle ratio.
AC, Anterior commissure.
PC, Posterior commissure.
Comparison of morphological indices between NPH response groups at pre- and post-ELD timepoints. We first classified patients by their drainage timepoints. Within the cohort, morphological indices were insufficient in distinguishing between Responders to Non-Responders of ELD at either timepoints.

demonstrated clinical improvement in NPH patients post-ELD without significant alterations in ventricular size and volume. As a minimum, our data have shown that significant reduction in intracranial CSF compartments was achieved *via* ELD in the Non-responder group to confirm that their lack of responsiveness was not confounded by insufficient CSF drainage during testing. However, taken together with findings from literature, our study also suggests that, using changes in 3d linear measures, it may also be possible to describe the spectrum of brain-ventricular changes across both Classic and Complex NPH subtypes. Significant changes in BVR and ventricular volumes may be present at both extremes of reversibility and irreversibility of brain injury patterns in NPH, reflecting differing underlying pathophysiological processes, from brain compression/distortion to alterations in its poroelastic properties.

There is evidence from other modalities of imaging to support the impact of changes in brain microstructure on

TABLE 7 | Classification of normal pressure hydrocephalus (NPH) cohorts by Responsiveness to CSF drainage: Morphological indices within groups compared at baseline vs. post-lumbar testing.

	Responders (*n* = 9)			Non-responders (*n* = 12)		
Measurements	**Pre-ELD**	**Post-ELD**	***p*-value**	**Pre-ELD**	**Post-ELD**	***p*-value**
Traditional 2d linear measures						
EI	0.402 ± 0.054	0.399 ± 0.045	0.789	0.363 ± 0.048	0.360 ± 0.050	0.091
BCI	0.291 ± 0.026	0.294 ± 0.028	0.357	0.278 ± 0.027	0.274 ± 0.031	0.215
Callosal angle (degrees)	61.6 ± 21.0	61.3 ± 19.7	0.866	53.8 ± 21.0	55.8 ± 21.1	0.201
Novel 3-directional linear measures						
z-Evans index	0.461 ± 0.061	0.460 ± 0.059	0.811	0.438 ± 0.052	0.424 ± 0.049	0.001*
BVR at AC	0.613 ± 0.172	0.616 ± 0.159	0.833	0.726 ± 0.170	0.714 ± 0.216	0.757
BVR at PC	0.789 ± 0.361	0.773 ± 0.295	0.643	0.869 ± 0.290	0.926 ± 0.269	0.024*
BVR at PC						
Brain	27.36 ± 5.583	27.12 ± 5.392	0.589	29.58 ± 4.774	30.23 ± 4.335	0.234
Ventricle	38.60 ± 0.490	38.22 ± 9.639	0.502	35.68 ± 5.772	33.89 ± 5.260	0.005*
3-Dimensional Volumetric Analysis						
Ventricular volume (cm^3)	160.56 ± 89.9	120.28 ± 39.0	0.388	156.05 ± 77.5	114.16 ± 38.0	0.008*

**Significant at $\alpha < 0.05$.*
ELD, External lumbar drain.
EI, Evans index.
BCI, Bicaudate index.
BVR, Brain-ventricle ratio.
AC, Anterior commissure.
PC, Posterior commissure.
Comparison of morphological indices between drainage timepoints within NPH response groups. We further classified patients by their responsiveness to ELD. Within these subgroups, changes in 3-directional morphological indices (z-Evans index and BVR at PC) across timepoints were able to distinguish between Responders vs. Non-Responders to lumbar testing. The effect of ELD resultsed in a significant reduction of morphological indices specifically in Non-Responders. This finding was consistent across multiple indipendetly derived measures (Ventricle component of BVR at PC, and 3-Dimentional volumentric analysis).

CSF movement between intracranial fluid compartments. Brain compression and stiffness in nonlinear elastic regions, as seen on Magnetic Resonance Elastography (MRE) have been hypothesized to lead to non-compliance, which may result in increased CSF drainage from the lateral ventricles, with no change in clinical response (Fattahi et al., 2016). Bugalho and Alves (2007) described irreversibility of NPH symptoms in patients with higher loads of white matter lesions on MRI analysis, suggesting that such parenchymal white matter lesions, thought to be a hallmark of brain microvascular disease, may result in poor response to ventricular shunting. Differential regions of stiffness on MRE imaging, in the temporal lobe for instance, may also portend failure in response to ventricular shunting (Perry et al., 2017). Our study cohort comprises predominantly Complex NPH patients, in which higher levels of brain atrophy and neurodegeneration would be expected as compared to cohorts of purely Classic NPH. Here, differences in ventricular morphology could be explained by loss of microstructural integrity, resulting in both the larger increase in BVR at PC in Non-Responders and the reduction in ventricular volumes. As the BVR measure describes a ratio, a significant drop of ventricular volume (the denominator) in our cohort would drive the larger increase in BVR and be reflected in the z-Evans Index and 3D volumetric analysis as a significant reduction in ventricular size and volume. Conversely, the same changes would still be consistent with that reported by Yamada et al. (2019) in his purer cohort of Classic NPH. In this context, a significant rise of the brain measurement (the numerator), due to improvement in compression, would also drive a larger increase in BVR and be reflected in the z-Evans Index and 3D volumetric analysis as a significant reduction in ventricular size and volume, due to successful CSF drainage *via* ELD. However, the differences between our two cohorts across the spectrum of NPH would be that, for the same significant volume of changes in ventricular morphology, loss of microstructural integrity would likely be less reversible than pathophysiological processes such as brain compression, stretching or tissue distortion.

In further unpacking such considerations, Diffusion Tensor Imaging (DTI), a methodology of modeling changes in white matter microarchitectural patterns by the use of water diffusion properties, may be a helpful adjunct. We have previously described the utility of DTI to describe differing concurrent changes in white matter injuries in response to NPH and interventions, across Classic (Keong et al., 2017) and Complex subtypes (Lock et al., 2019). Indeed, in our DTI analysis of the effect of ELD on Complex NPH patients, we described that a global reduction of DTI metrics across

all measures was consistent with passive fluid movement across compartments, suggestive of axonal disruption and brain atrophy. In addition, DTI profiles of white matter injury patterns in Responders still also demonstrated evidence of axonal disruption. However, despite the multiple changes occurring concurrently, we found that the DTI tissue signature of predominant stretch/compression, i.e., the white matter injury pattern most amenable to surgical intervention, was still consistent across Classic and Complex NPH cohorts (Lock et al., 2019). Such white matter changes are compatible with hypotheses suggested by the studies above involving 3d linear measures. Further work is needed to explore the coupling of brain-ventricular concepts in hydrocephalus and neurodegenerative cohorts *via* the concurrent use of 3d, as well as 3D, structural morphological indices and DTI methodology.

Limitations

Limitations of this study includes the relatively small sample size of our cohort, restricting the statistical power of the study. Nonetheless, testing of morphological indices on this small disease cohort yielded significant findings, consistent with other published work, which are encouraging for future studies to be conducted on larger NPH cohorts with multimorbidity burden and overlay from neurodegenerative disorders. Secondly, our methods of ventricular segmentation were semi-automated *via* 3D Slicer with manual exclusion of falsely included CSF-intensity voxels following anatomic identification by the software. This could have led to human error and inter-operator variability. However, this approach is consistent with similar methods performed *via* the radiology workstation. We minimized these considerations by having a standard operating protocol, refined by pilot testing and incorporating all subsequent post-processing steps within a continuous workflow for consistency, using available modules on 3D Slicer. Lastly, our patient population comprised a large proportion of patients with Complex NPH, and whilst this is reflective of the clinical practice in the Asian population, the impact of other concomitant neurodegenerative diseases may have influenced our results, as compared to the purer cohorts of Classic NPH previously published on the use of morphological indices.

CONCLUSION

Our study has shown that novel 3-directional (3d) linear indices can be applied to cohorts of Classic and Complex NPH, and to distinguish them from AD and HC cohorts. We also demonstrated that, contrary to traditional 2d linear measurements, these measures can also provide significant correlations to CSF responsiveness and 3D ventricular volumetry. In the context of multimorbidity burden and overlay from neurodegenerative disease, 3d morphological indices may provide a non-invasive tool to aid in the characterization of NPH cohorts at the clinical-research interface.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because de-identified data is not allowed to be made available to the public according to the study's IRB. Requests to access the datasets should be directed to NK, nk330@cantab.net.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the SingHealth Centralised Institutional Review Board (CIRB; Ref 2016/2627). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NK was primarily involved in the study design, protocol development, protocol implementation and analysis of the data at study site, as well as patient recruitment. SS, CL, SK, and JK were involved in patient recruitment and data collection. AT was involved in the frailty scoring. YL was involved in the 3D volumetric analysis. SS and AK were involved in the manuscript preparation with NK. CL and SS aided with the data analysis and statistical prowess. All authors have read and approved the final 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/fnins.2021.751145/full#supplementary-material

Supplementary Figure 1 | Validation of 3D Slicer workflow. NPH, Normal Pressure Hydrocephalus; AD, Alzheimer's Disease; HC, Healthy Control.

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Pathological Human Tau Induces Alterations in the Brain Insulin Signaling Cascade

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The process of neurodegeneration in Alzheimer's disease has been associated with a disruption of insulin signaling cascade in neurons, and to insulin resistance. T2DM correlates with Alzheimer's disease, but mechanisms of interaction are unknown. We have developed a mouse model of tau induced neurodegeneration expressing pseudo-phosphorylated tau [Pathological Human Tau (PH-Tau)] in neurons. This model (PH-Tau-Tg) recapitulated cognitive decline and neurodegeneration observed in AD. In this study we examined if expression of PH-Tau could affect neuronal excitability and insulin receptor signaling. Neuronal excitability was investigated using intracerebral recordings of extracellular field potentials from prefrontal cortex after insulin and kainic acid (KA) injection. Analysis of baseline recordings indicated an increased excitability of PH-Tau-Tg as evidenced by higher spectrum densities (PSDs) of high frequencies brain waves. Injection of insulin (1IU, s.c) led to a decrease of fast ripples PSDs, more pronounced in PH-Tau-Tg mice than controls. Subsequent injection of kainic acid (KA, 5 mg/kg, s.c) led to significant increase in firing rate, amplitude of extracellular field potentials and PSDs of high frequency brain waves in control mice only. To further investigate the role of insulin in PH-Tau-Tg mice, we subjected mice to a glucose tolerance test. We found that PH-Tau-Tg mice were significantly hyperglycemic prior to glucose injection. Interestingly, the PH-Tau-Tg mice showed a moderate increase at 30 min due to the higher baseline, indicating a low sensitivity of insulin receptor in these mice. This is consistent with increased levels of activated insulin receptors in the brain and the inhibitory effect of insulin on ictal activity post KA injection in PH-Tau-Tg mice. We suggest that these mice have reduced insulin sensitivity (hyperglycemia) and as a compensatory mechanism there is overactivation/expression of insulin receptor in the brain rendering neuronal circuits resistant to seizure induction after injection of insulin. These data indicate that insulin signal transduction pathway is altered in PH-Tau-Tg mice, and that injection of exogenous insulin reduces hypersynchronous bursting activity of field potentials recorded from cortical neuronal circuits. We propose that the appearance of abnormal tau might potentiate the toxic environment by interfering with the insulin signaling cascade in the brain of patients with Alzheimer's disease.

Keywords: Alzheimer's disease, hyperphosphorylated tau, tau transgenic mouse model, seizures, insulin resistance, brain waves, glucose tolerance test

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HIGHLIGHTS

- Transgenic mice expressing pathological human tau (PH-Tau) in the neurons exhibit hyperexcitability of neuronal circuits.
- PH-Tau-tg mice also exhibit features of type 2 diabetes, and are hyperglycemic.
- Insulin signal transduction pathway is altered in PH-Tau-Tg mice and have elevated expression of pS312 insulin receptors in the brain.
- Injection of exogenous insulin reduces hypersynchronous bursting activity from cortical neuronal circuits PH-Tau-Tg mice.
- Abnormal tau might potentiate the toxic environment by interfering with the insulin signaling cascade in the brain.

INTRODUCTION

Tauopathies are a group of neurodegenerative diseases characterized by the accumulation of hyperphosphorylated tau in the presence or absence of other lesions. These include Alzheimer's disease (AD), Fronto-Temporal Dementia, Chronic Traumatic Encephalopathy, Pick's Disease, tangle-only dementia to list a few (Lee et al., 2001; Iqbal et al., 2009; McKee et al., 2009; Murray et al., 2015; Alonso et al., 2018). Alzheimer's disease (AD) is a devastating neurodegenerative disorder affecting roughly 30 million people worldwide. AD is characterized by a cascade of pathological events, including the formation of amyloid plaques (made up of aggregated forms of Aβ), neurofibrillary tangles (composed of aggregated, hyperphosphorylated tau), synapse loss, brain hypometabolism, neuroimflammation, and brain atrophy that is accompanied by severe and progressive cognitive decline. Amyloid plaques, the other hallmark of AD are generated when Aβ peptide aggregate and accumulate in the extracellular space. The buildup of hyperphosphorylated and aggregated tau protein leads to the development of intracellular neurofibrillary tangles. Many factors, genetic and non-genetic, have been identified in the etiology of AD. Amongst them, type 2 diabetes (T2D), a disease of aging, has been shown to increase the risk for AD risk (Sims-Robinson et al., 2010).

Insulin receptors are widely expressed in the brain. Their regional specificity and the complexity of insulin signal transduction makes the effects of insulin on the brain pleiotropic (Unger et al., 1991). However, glucose utilization in the brain is insulin independent.

Insulin is primarily a metabolic hormone functioning on muscle, fat and liver *via* activation of insulin receptor (IR). Once insulin is secreted, it crosses the blood-brain barrier by a transporter-mediated saturable mechanism (Banks et al., 1997). Several studies have implicated IR activation in the regulation of excitatory and inhibitory neurotransmission. The expression of IR in the brain is activity-dependent (Plum et al., 2005). IR regulate the expression of potassium ion channel Kv1.3 in the olfactory bulb after intranasal insulin injection to mice (Marks et al., 2009). This led to increase to enhancement of memory in these mice (Marks et al., 2009), suggesting a cognitive enhancing role for insulin through activation of IR and increased expression of Kv1.3 channels. Additionally, insulin prevents cells death of hippocampal neurons deprived of glucose *in vitro* (Mielke et al., 2006). Insulin signaling in the brain has been shown to be important for both metabolic homeostasis and higher brain functions such as cognition. Insulin reduces brain excitability by lowering the threshold for extrasynaptic $GABA_A$ receptors activation, increasing therefore the GABA-mediated tonic inhibitory conductance (Jin et al., 2011). Impaired insulin signaling increases risk of Alzheimer's disease (Ronnemaa et al., 2008), cognitive disabilities in diabetes mellitus (Seaquist, 2010) whereas intranasal administration of insulin improves hippocampal-dependent memory function (Benedict et al., 2007).

Several studies provide significant insights and experimental evidence on the mechanistic link between AD and T2D and show the reciprocal actions between these two diseases, suggesting a shared common cellular and molecular mechanisms (Han and Li, 2010). In AD, it has been reported brain insulin resistance as an early sign of cognitive impairment and increase levels of Ser phosphorylated IRS-1 has been shown to precede cognitive decline in AD (Talbot et al., 2012). Insulin signaling alterations have been reported in the different mouse models of AD, like the triple transgenic mouse model or the APP/PS1 transgenic model or the one induced by the intracerebroventricular (icv) injection of amyloid-β oligomers (reviewed in Lyra e Silva et al., 2019). Changes in brain insulin resistance had been attributed to Abeta amyloid accumulation. But if the presence of hyperphosphorylated tau is related to insulin resistance is less explored. Activation of the insulin pathway has been related to increase in tau hyperphosphorylation, and it is currently viewed that tau hyperphosphorylation might be a consequence of an altered insulin signaling transduction pathway. Some studies are reporting metabolic alterations in the tau transgenic mice, like in a knocked in tau model that is hyperglycemic at 8 months of age and higher levels of phosphorylated tau were detected (Hull et al., 2020). Increase brain response to insulin was reported in a transgenic mouse model expressing a double FTDP-17 tau mutant (Leboucher et al., 2019). Furthermore, metabolic changes such as lower levels of leptin and insulin and resistance to high-fat diet were reported. Recently, the same group reported increased seizures susceptibility and excitability in the same tau transgenic model (Gomez-Murciaa et al., 2020). These are very intriguing observations, however, it should be considered that the level of transgenic tau expression in these animals is four to five times higher than tau endogenous levels at 3 months old animals and five to six-fold higher at 12 months of age (Schindowski et al., 2006).

We have demonstrated that a PH-Tau form (PH-Tau, R406Wtau pseudo-phosphorylated at Ser 199, Thr212, Thr231, and Ser262) mimics AD abnormal tau (Alonso et al., 2010), impairs learning and memory in Drosophila (Beharry et al., 2013) and in a transgenic mouse model in which it induces neuronal death and astrocytes activation (Di et al., 2016). In our transgenic mouse model PH Tau is expressed only in the neurons, this is a bigenic mouse model where the expression is controlled by the expression of a trans-activator, tTA, controlled by the CaCamII

promotor, inducing the expression in neurons. In this model we can prevent the expression of PH Tau with doxycycline, but for this report, all the mice were raised with food without the antibiotic. Under these conditions, PH Tau is expressed in all developmental stages but the levels of expression do not exceed 14% of the endogenous tau expression with no decrease on the native tau expression. Mice expressing PH Tau showed cognitive impairment, as well as neuronal death in the hippocampus and tau aggregation at 12 months of age. In the present report we set up to investigate the insulin signaling pathway in our mouse model of neurodegeneration in adult mice but at an earlier age than when we observed the structural changes in the brain. Therefore we chose 8 months of age to test the insulin signaling pathway, and we show that low level of expression of PH-Tau is enough to induce brain insulin resistance.

MATERIALS AND METHODS

Animals

A total of 24 males mice were used in this study. The control mice were 6-month-old C57BJ6 × 129 males. The PH-Tau-tg mice 8-months old mice were generated as described (**Supplementary Material**, Di et al., 2016). All mice were housed in groups of three-to-four in a pathogen-free room maintained on a 12 h light/dark cycle and given food and water. All procedures were approved by the Institutional Animal Care and Use Committee of the College of Staten Island/CUNY and were in conformity with National Institutes of Health Guidelines.

Drug Administration

Kainic acid and Insulin were dissolved in isotonic saline and injected at 5 mg, kg^{-1} and 1IU, kg^{-1}, respectively. Animals received the indicated drugs through a stationary butterfly needle inserted subcutaneously before the start of the recordings and attached to a 20 cm catheter to avoid any electrostatic artifacts during the injections. After each injection, the catheter was flushed with a small volume of saline so the whole dose of the drug could be delivered.

Intracerebral Recordings of Local Field Potentials

Mice were anesthetized with ketamine/xylazine mix (90/10 mg, kg^{-1} i.p.), scalps were shaved, then fixed on a stereotaxic apparatus. Right side craniotomies were made at AP 2.5 mm from bregma, L 0.5 mm (medial prefrontal cortex). Extracellular recordings were obtained with tungsten electrodes with impedances of 1–2 MΩ. Electrodes were placed in infragranular layers (0.5 lateral and 1.0–1.2 mm deep in prefrontal cortex). Local field potential (LFP) from the pre-frontal cortex were recorded. LabChart-8 (ADInstruments, Colorado Springs, CO, United States) was used for LFP recording this includes both frequency domain and time domain features that have been extracted. The low-frequency oscillations (LFO): delta 0.4–4 Hz, theta 5–7 Hz, alpha 7–12 Hz, beta 13–25 Hz, gamma 26–80 Hz. High frequency oscillations (HFO): slow ripples 125–250 Hz and fast ripples 250–500 Hz. All recordings were passed through a preamplifier connected to the electrode and amplified using model 1700 differential AC Amplifier (ADInstruments) and digitized at 10 kHz.

Immunohistochemistry

Cryosections were made and placed onto gelatin-subbed slides. Non-specific binding sites were blocked using 4% bovine serum albumin (BSA), 2% normal goat serum (NGS), and 0.05% Triton X-100 in 0.01 M phosphate-buffered saline (pH 7.2). Following the blocking step, the slides were rinsed in an antibody dilution cocktail (ABD) consisting of 2% BSA and 1% NGS in 0.01 M PBS. Primary antibodies (Life Sciences) employed were directed against the phosphorylated (activated) insulin receptor phosphor IR substrate 1 (p-Ser312-IRS1; Rabbit polyclonal) and Glucose transporter (Glut4; Mouse monoclonal) diluted 1:500 in ABD. The primary antibodies were incubated overnight at 4°C and then unbound antibodies rinsed with ABD. Secondary antibodies were all raised in goat and directed against appropriate primary antibody type. The anti-mouse IgG was conjugated to Cy5 and anti-rabbit was conjugated to Cy3 (1:500; Invitrogen/Molecular probes). Sections were rinsed in PBS and coverslipped with VectaShield mounting medium with DAPI (Vector Labs, Burlingame, CA, United States). Coverslips were sealed using clear nail polish (Electron Microscopy Sciences, Ft. Washington, PA, United States). Slides were stored in an opaque slide box at 4°C temperature until imaging. Images were obtained by confocal microscopy (Leica SP2 AOBS). Z stack images were acquired using a Plan-Apochromat 63X/1.4 oil objective. Stacks were collected at a 0.5 μm slice interval, stepping through the entire section. Frame size was set to 1024 × 1024 pixels. All image acquisition parameters including gain and offset were identical for all comparisons. Z-stacks were opened in Imaris in their native format. And automatically reconstructed into a multi-channel 3D model during input into Imaris, requiring no further image pre-processing. Background subtraction was used to separate immunoreactivity from the background signal. The auto-threshold value was utilized during background subtraction, without user adjustments. The size and shape of the generated surface were a direct map of the intensity distribution of p-Ser312-IRS1 and Glut4 immunolabeling and DAPI labeling within the sections as detected by Imaris. To quantify immunoreactivity, the Spots creation tool was used. Estimated XY diameter for Spot detection was 0.5 μm. To determine relative changes in protein expression, all quantification parameters initially set up for control z stacks were applied to the z stacks obtained from PH-Tau-Tg brain images. Changes in expression were also confirmed statistically using the Imaris ×64 software (Bitplane). Statistical significance was set at ($p < 0.05$).

Intraperitoneal Glucose Tolerance Test

Mice from both groups were fasted overnight (12 h) and then injected intraperitoneally with 0.02 ml/g of body weight D-glucose (7.5% stock solution in saline). Blood samples were taken by tail venesection at 0 min (just before glucose injection) and at 30-, 60-, and 120-min intervals after the glucose load.

Glucose was measured with Ascensia Breeze portable glucose meter (Bayer, Leverkusen, Germany). Mice were given only water during the test.

Statistical Analysis

The mean power spectral density was compared by generalized linear mixed model with log normal distribution. The fixed effects were modeled as treatment, treatment*genotype for each frequency band. The model was adjusted for inter electrode variability nested on animals using variance components from random effects. Tukey–Kramer was used as multiple comparisons test. Electrophysiological results are shown as the mean of each parameter, significance values were determined by one-way repeated measures ANOVA and a *post hoc* Dunnett test with $p < 0.05$. Peak amplitude of the response was calculated using LabChart software (ADInstruments, Colorado Springs, CO, United States) and data was analyzed using SPSS 8.0 software.

RESULTS

Insulin Reduced the Amplitude and Frequency of KA-Induced Epileptiform Discharge in PH-Tau-Tg Mice

In this study, we examined electrophysiologically the effects of insulin on neuronal excitability and seizure susceptibility in control and PH-Tau-Tg mice using the neurotoxin KA. KA activate a subtype of glutamate receptors (KA receptors), which are densely present in hippocampal and cortical interneurons and other principal neurons (Monaghan and Cotman, 1982; Wisden and Seeburg, 1993). KA receptors are channels that regulate Na^+, K^+ and Ca^{2+} conductance, responsible for fast synaptic transmission through generation of excitatory postsynaptic currents (EPSCs; Carta et al., 2014; Cossart et al., 2002; Ruiz et al., 2005). Activation of these receptors by KA, which trigger a cascade of events resulting in seizures, is the basis for the temporal lobe epilepsy model (El Idrissi et al., 2003). At a dose of 5 $mg.kg^{-1}$, KA first produced seizures with no motor expression and recorded as regular EEG spiking (**Figure 1**). Occasionally, these seizures ended with rapid tail shakes and could be followed by a recurrent seizure with high-frequency spikes and no behavioral concomitants. Here, we examined the effects of insulin receptor (IR) activation on neuronal excitability by quantifying the amplitude and frequencies of epileptiform discharges following KA injection. We injected insulin and subsequently injected low doses (5 mg/kg) of KA, a depolarizing agent with preferential binding to limbic structures. Synaptic activity triggers membranes currents that pass through the extracellular space and is measured by electrodes placed outside the neurons as local field potentials (**Figures 1A,C** upper traces). In control mice, intracerebral recording revealed epileptiform discharges about 20 min post KA injection (**Figure 1A**). Spike histogram analysis of recorded field potentials show a significant increase [$t(4,712) = 19.28$, $p < 0.001$] in the firing rate post KA injection, varying from 20 to 70/s (**Figure 1B**). In PH-Tau-Tg mice, however, addition of insulin led to an increase in the amplitude and frequency of population spikes and subsequent addition of KA in the presence of insulin resulted in a significant increase ($p < 0.01$) in the latency to the onset of epileptiform discharge, a significant decrease ($p < 0.01$) in the frequency of ictal events and an overall decrease in the amplitude and frequency of population spikes (**Figure 1C**). Spike histogram analysis of recorded field potentials shows a significant reduction in the firing rate in the presence of KA in the PH-Tau-Tg mice compared to controls [$t(4,712) = 19.28$, $p < 0.001$] (**Figure 1D**).

Insulin Suppresses KA-Induced Epileptiform Discharge and High Frequency Oscillations of Brain Waves in PH-Tau-Tg Mice

To further investigate how PH-Tau affects the function of neuronal circuits and their firing properties, we examined neuronal excitability by measuring cortical field potentials from mice treated with KA in the presence of insulin. Quantitative analysis of the power spectral density (PSD) obtained from the recorded field potential traces demonstrate a significant increase in neuronal firing from controls compared to PH-Tau-Tg mice $F(1) = 185.59$, $p < 0.001$ (**Figure 2C**). Segment analysis reveals that most of the increase in firing frequencies occurred in the presence of KA (**Figures 1A,C**), Therefore, we focused our quantitative analysis post KA injection and starting at 40 min into the recording. Peak increase in PSD amplitude could be seen around 100–600 Hz (**Figure 2C**). This range of high frequency oscillations (HFO) encompasses slow ripples 125–250 Hz and fast ripples 250–500 Hz as shown in **Figures 2C,D**. Therefore, field potential traces were filtered between 125 and 500 Hz and amplified. Ripple oscillations are HFOs ranging from 125 to 500 Hz, and they have been regarded as a potential marker of epileptogenicity (Bragin et al., 1999, 2000, 2004; Traub et al., 2002; Grenier et al., 2003; Dzhala and Staley, 2004; Jacobs et al., 2008; Zijlmans et al., 2009; Brázdil et al., 2010; Haegelen et al., 2013; Wang et al., 2013; Miao et al., 2014). Segment analysis revealed a significant increase in the PSD of slow ripples [$t(328) = 6.79$, $p < 0.001$] and fast ripples [$t(656) = 11.88$, $p < 0.001$] (**Figures 2C,D**) with the addition of KA, consistent with its epileptogenic effect. These increases were significantly suppressed by insulin in the PH-Tau-Tg mice $F(1) = 185.59$, $p < 0.001$ (**Figure 2D**). Segment analysis reveals that most of the increase in PSD of HFO was in the presence of KA. Fourier transform (FFT) analysis of raw data showed a significant increase in peak activity of both HFOs consistent with increased neuronal excitability (**Figures 2C,D**). PSD analysis of population spikes show a significant decrease of peak amplitude from PH-Tau-Tg recordings in the presence of insulin compared to controls $F(1) = 185.59$, $p < 0.001$ (**Figures 2C,D**). Insulin significantly suppressed KA-induced increase in peak amplitude of PSDs and in both the slow [$F(1) = 81.48$, $p < 0.001$] and fast [$F(1) = 9.95$, $p < 0.001$] ripple oscillations suggesting a role in elevating seizure threshold in the PH-Tau-Tg brains. These data clearly indicate that field potentials recorded from cortical neuronal circuits

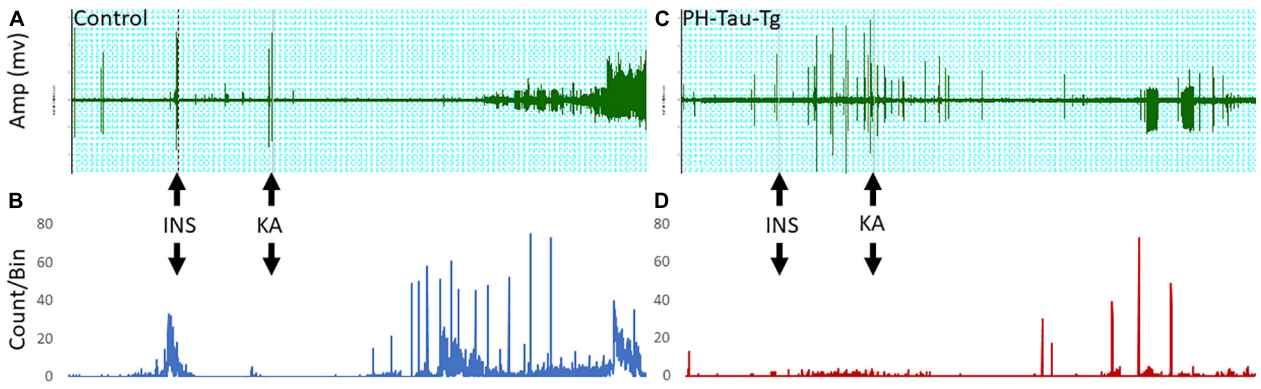

FIGURE 1 | Representative intracerebral recording from the prefrontal cortex of a control mouse **(A)** and PH-Tau-Tg mouse **(C)**. Recordings were performed for 15 min (baseline) followed by an i.p. injection of insulin (1IU) at 10 min later and KA (5 mg/kg) at t25. Onset of high frequency discharge and ictal activity occurred about 20 to 30 min post KA injection. In controls, once seizures occurred there was no recovery and the amplitude of the population spikes almost doubled and remained so for the duration of the recordings. In PH-Tau-Tg mice the latency was the same as controls, however, the amplitude of the population spikes was significantly lower and ictal activity was intermittent and interrupted with interictal events **(C)**. Spike histograms analysis of firing rates with a detection threshold of 0.1 mv and a Bin size of 1 s showed a significant increase after KA injection in controls **(B)** and drastic decrease post KA-injection in PH-Tau-Tg mice **(C)** (n = 3 of each group) Shown here are representative intracerebral recordings from individual mice.

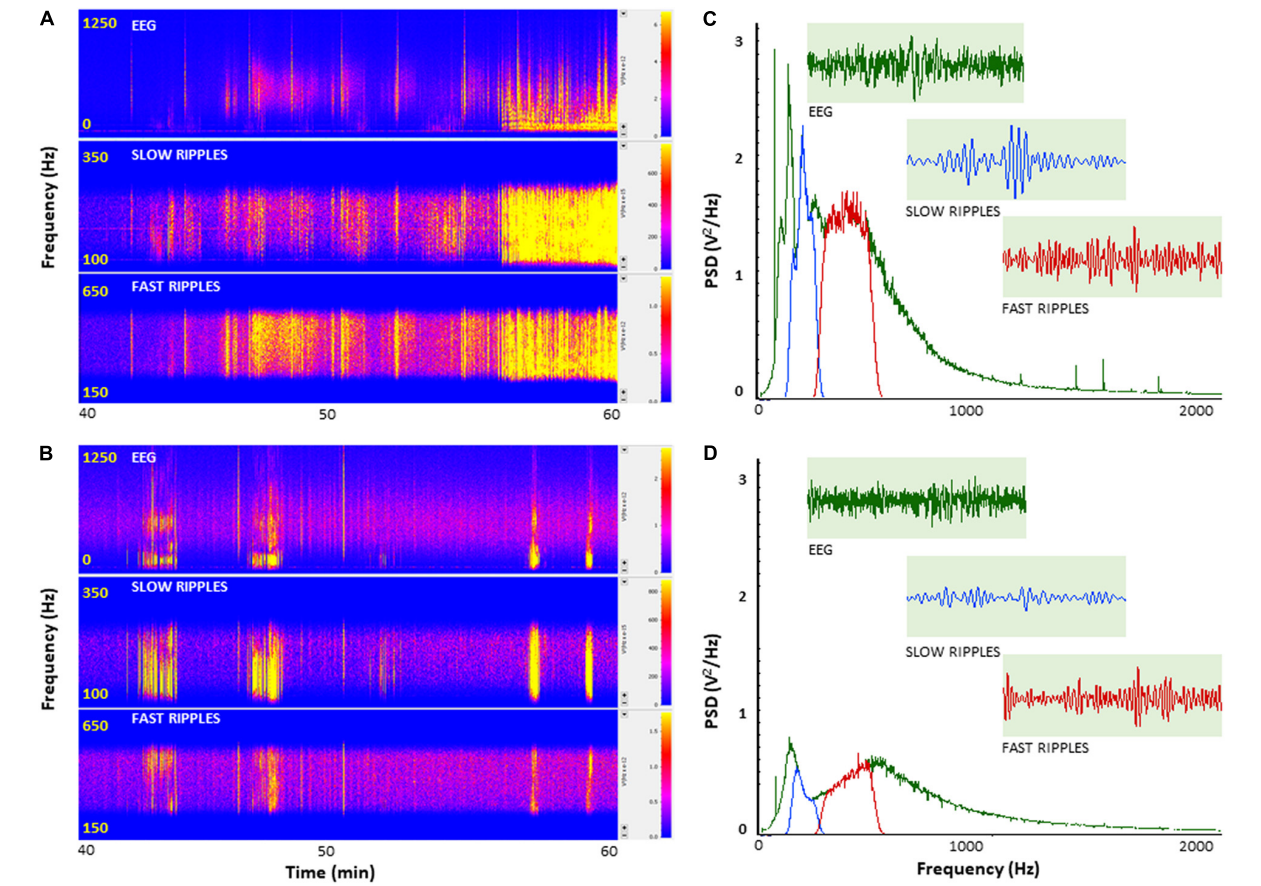

FIGURE 2 | Insulin selectively depresses cortical ripples in PH-Tau-Tg mice brains. PSDs analysis of field potential recordings from prefrontal cortex after KA injection. FFT of population spikes and filtered high frequency oscillations (HFO): slow ripples 125–250 Hz and fast ripples 250–500 Hz are shown in **(C,D)** with representative traces (inserts). The power density of field potentials and filtered frequencies were converted into heat maps **(A,B)**, Controls and PH-Tau-Tg mice, respectively). **(A,B)** Are representative heat maps of intracerebral recording, slow ripples and fast ripples from a control mouse **(A)** and PH-Tau-Tg mouse **(B)**. **(C,D)** Are FFT of PSDs obtained from pooled data of controls (n = 3) and PH-Tg mice (n = 3) respectively.

in control mice reflect hypersynchronous bursting activity consistent with hyperexcitability. Insulin significantly suppressed hypersynchronous firing in the brain of PH-tau-tg mice.

Insulin Suppresses Cortical Ripples in PH-Tau-Tg Mice

To further investigate the effects of insulin on neuronal excitability and seizure susceptibility, we analyzed the rate and amplitude of population spikes post KA injection, specifically during ictal activity (last 20 min of the recordings). Firing rate analysis with a detection threshold of 0.1 mv showed a significant increase [$t(4,712) = 19.28$, $p < 0.001$] in the firing frequency after KA injection in controls (as high as 80 population spikes/second, upper trace, **Figure 3**). On the other hand, recordings from PH-Tau-Tg mice had a significantly lower rate (upper right trace, **Figure 3**). Spike histogram analysis of filtered frequencies showed a significant reduction in the firing rate of both slow ripples [$t(328) = 6.79$, $p < 0.001$] (125–250 Hz) and fast ripples [$t(656) = 11.88$, $p < 0.001$] (250–500 Hz) post KA injection in the PH-Tau-Tg mice injected with insulin (**Figure 3**).

Insulin Reduces Seizures Severity and Propensity in PH-Tau-Tg Mice

Quantitative analysis of the power spectral density of the filtered frequencies obtained from the recorded field potential demonstrate a peak frequency distribution in neuronal firing after addition of KA around 100–500 Hz. Power spectrum calculates the area under the signal plot using the discrete Fourier Transform, the power spectrum density assigns units of power to each unit of frequency (**Figure 4A**). This frequency range correspond to HFOs encompassing slow ripples and fast ripples (slow ripples 125–250 Hz and fast ripples 250–500 Hz). Slow and fast ripple waves are commonly used as a biomarker of epileptogenic brain. **Figure 4** shows PSDs obtained from segment analysis of field potentials, slow ripples and fast ripples. As shown in **Figure 4**, addition of KA led to a selective increase in the power spectrum of HFOs corresponding to the ripples (slow and fast) with a significant difference in peak amplitude between controls and PH-Tau-Tg mice [$t(8,190) = 10.80$, $p < 0.001$]. Consistently, PDSs analysis of filtered HFOs (slow ripples 125–250 Hz and fast ripples 250–500 Hz) showed a significant increase in the amplitude of their power spectrum densities (**Figures 4B,C**, respectively) with the addition of KA [$F(1) = 185.59$, $p < 0.001$]. Injection of insulin prior to KA injection led to a significant decrease in PSD peak amplitude of both field potentials and HFOs [Slow ripples $t(328) = 6.79$, $p < 0.001$; fast ripples $t(656) = 11.88$, $p < 0.001$]. These data clearly indicate that insulin reduces hypersynchronous bursting activity and hyperexcitability of neuronal circuits of the PH-Tau-Tg mice. We suggest that the higher threshold for KA-induced seizures in PH-Tau-Tg-injected mice is due to an increase in GABA

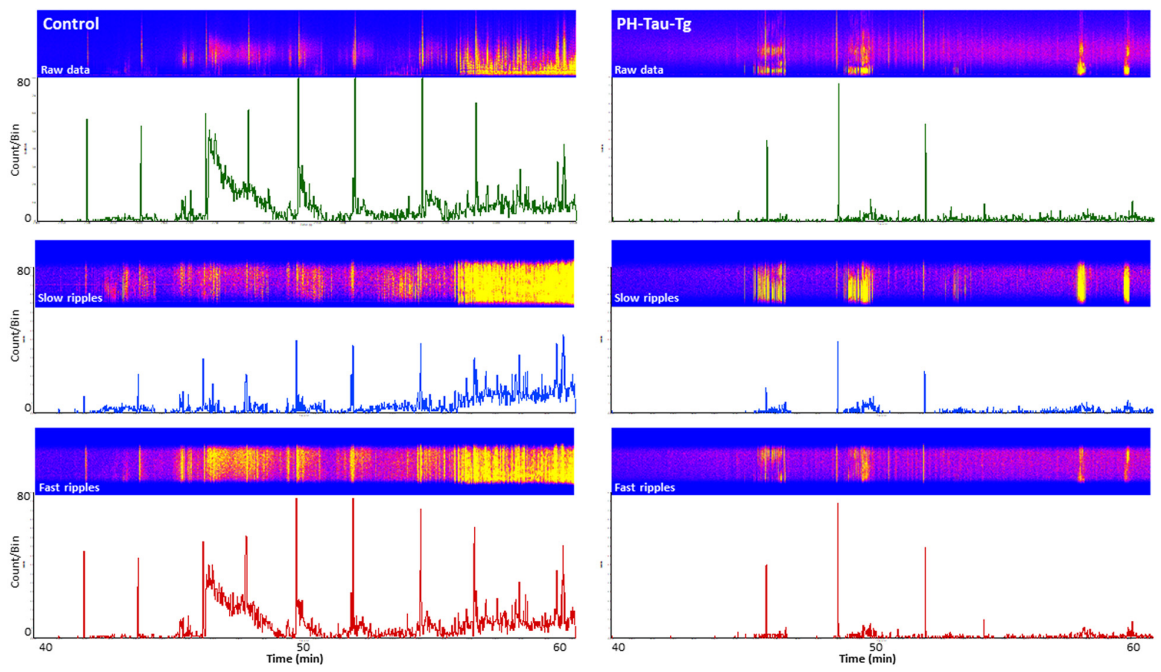

FIGURE 3 | Selective enhancement of cortical ripples after KA injections. Firing rate analysis with a detection threshold of 100 μv shows a significant increase after KA injection in controls **(left)** and a drastic decrease in PH-Tau-Tg mice **(right)**. Spike histograms of filtered frequencies show a significant increase in both slow ripples [$F(1) = 81.48$, $p < 0.001$] (125–250 Hz) and fast ripples $F(1) = 9.95$, $p < 0.001$ (250–500 Hz) post KA injection in controls in presence of insulin (left panel). Insulin injection led to a significant suppression of both HFOs post KA injection. Detection threshold for slow ripples and fast ripples were 0.02 and 0.04 mv, respectively. Bin size was set to 1 s. The power density of field potentials and filtered frequencies were converted into heat maps and shown on top of the respective spike histogram (left, Controls and right, PH-Tau-Tg mice). Heat maps are representatives of a control mouse and PH-Tau-Tg mouse. Spike histograms are obtained from pooled data of controls ($n = 3$) and PH-Tg mice ($n = 3$) respectively.

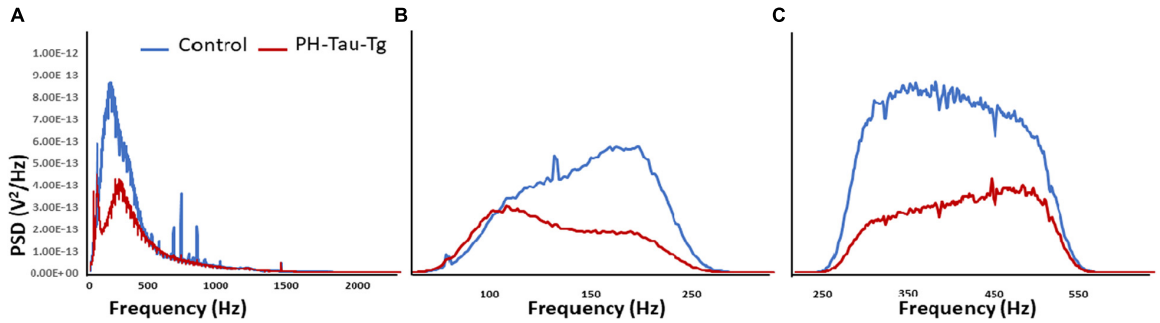

FIGURE 4 | Insulin reduces KA-induced increased in PSDs in PH-Tau-Tg mice. Field potential recordings from prefrontal cortex were filtered at the indicated frequencies and amplified and the respective power spectrum densities are shown above. **(A)** Represents the power spectrum density of the recording of field potentials obtained from controls (n = 3) and PH-Tau-Tg mice (n = 3) in response to addition of KA. **(B,C)** Represent the power spectrum density of slow ripples (125–250 Hz) and fast ripples (250–500 Hz) obtained from recordings after addition of KA. Insulin significantly reduced the amplitude PSDs for HFOs in the PH-Tau-Tg mice. Data represent means of pooled recordings from controls (n = 3) and PH-Tg mice (n = 3) respectively.

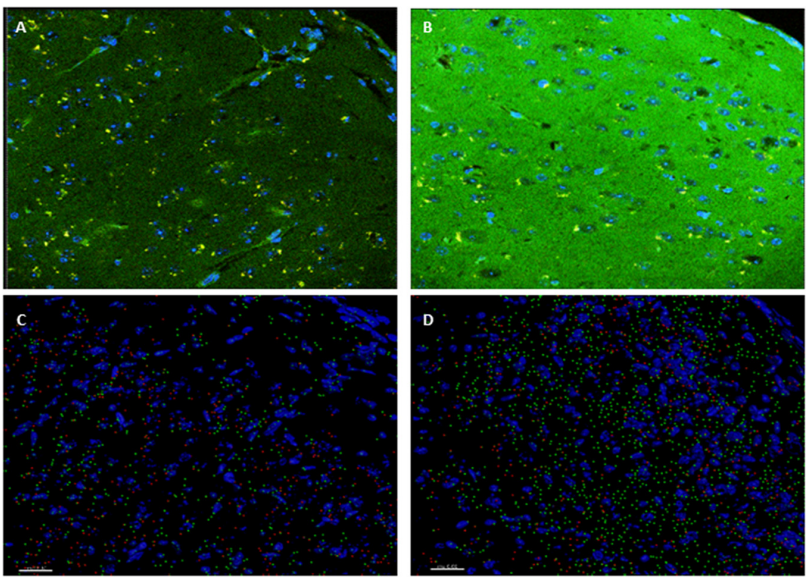

FIGURE 5 | PH-Tau-Tg mice have elevated levels of expression of activated insulin receptor and glucose transporter. **(A)** Control and **(B)** PH-Tau-Tg are representative images of a 30 μm cryosection probed with anti-p(Ser)-IRS1 (green) and anti-Glut 4 (red) showing the pattern and intensity of immunoreactivity in the cortex. DAPI (blue) was included in the mounting medium and used for nuclear localization. **(C)** Control and **(D)** PH-Tau- are Images that depict Imaris reconstructions of the z-stacks obtained with a confocal microscope of the same regions shown in **(A,B)**. Cortex from PH-Tau-Tg mice shows a significant ($p < 0.05$) increase in immunoreactivity for p(Ser)-IRS1 and Glut 4. Scale bar = 20 μm.

receptor function in the brain which increases the inhibitory drive rendering neural circuits seizure-resistant and might be mediated by direct modulation the $GABA_A$ receptors *in vivo*. This is supported by the finding that insulin reduces brain excitability by lowering the threshold for extrasynaptic $GABA_A$ receptors activation, increasing therefore the GABA-mediated tonic inhibitory conductance (Jin et al., 2011).

Altered Insulin Signaling in the PH-Tau-Tg Brains

Insulin signaling in the brain has been shown to be important for both metabolic homeostasis and higher brain functions such as cognition. Impaired insulin signaling increases risk of AD and cognitive disabilities in diabetes mellitus. To further investigate the involvement of insulin in the modulation of neuronal excitability, we examined the expression patter of the insulin receptor in the cortex. Interestingly, higher level of phosphorylated insulin receptor (p-Ser-IRS1) has been shown to be a consistent change in insulin signaling and a marker of insulin resistance in AD brains (Steen et al., 2005; Moloney et al., 2010; Bomfim et al., 2012; Talbot et al., 2012; Yarchoan et al., 2014). p(Ser)-IRS1 is a substrate for p-JNK, which also has also been found in elevated levels in AD brains (Bomfim et al., 2012; Talbot et al., 2012), suggesting some level of insulin resistance in AD. Consistent with this, we found a significant increase p(Ser)-IRS1

in PH-Tau-Tg mice compared to controls ($p < 0.05$) (**Figure 5**). Concomitant with the increase in p(Ser)-IRS1 in PH-Tau-Tg cortices, we also found a moderate increase in the expression levels of Glut 4. The expression levels of Gluts 4 has been shown to be dependent on the activation levels of IR (**Figure 5**).

PH-Tau-Tg Mice Are Hyperglycemic

To further investigate the role of insulin in PH-Tau-Tg mice, we subjected the mice to a glucose tolerance test. Mice were fasted for 12 h and injected with a glucose solution (7.5 mg/kg, s.c) then plasma glucose levels were monitored for 30, 60 and 120 min post injection. We found that PH-Tau-Tg mice were significantly hyperglycemic prior to glucose injection (**Figure 6**; baseline; $p < 0.05$). As expected, peak plasma glucose was observed at 30 min post glucose injection and at 120 min mice became normoglycemic. At 60 and 120 min PH-Tau-Tg mice glucose plasma levels were slightly but not significantly higher than controls, indicating a low sensitivity of insulin receptor in these mice. This is consistent with increased levels of activated insulin receptors in the brain and the inhibitory effect of insulin on ictal activity and epileptiform discharge post KA injection in PH-Tau-Tg mice.

DISCUSSION

In this study, we used a PH-Tau-Tg mouse to investigate the alteration in insulin signal transduction pathway and the resulting functional significance on neuronal excitability and seizure susceptibility. Insulin has been shown to play a pleiotropic role in the brain. Insulin receptors (IR) and have been identified in several regions of the brain that mediate important physiological effects, such as neuronal development, glucose uptake regulation, feeding behavior, and body weight, as well as cognitive processes, including attention, executive functioning, learning, and memory (Derakhshan and Toth, 2013). IRs are widely distributed throughout the brain (Fernandez and Torres-Alemán, 2012) and their expression is largely localized to neurons (Unger et al., 1989), although IR mRNA is present in glia and endothelial cells (Zhang et al., 2014). Insulin is a neuromodulators on mammalian CNS by affecting the function of certain neurotransmitter receptors therefore affecting electrophysiological properties of neurons and neuronal circuits. Insulin may play an important role in the control of GABA receptor density in the post-synaptic domain (Wang et al., 2003). Insulin also affects intracellular ion concentrations by modulating the activity of certain ion channels. In hypothalamic neurons, insulin activates ATP-dependent K^+ channels leading to membrane hyperpolarization, mechanistically similar to those in the β cells of the islets (Plum et al., 2005). In addition, insulin has stimulatory effects on Na^+/K^+ ATPase, producing an acute rise in intracellular Ca^{2+} concentration that triggers the release of inhibitory neuropeptides under high frequency firing (Jonas et al., 1997). There is also experimental evidence that insulin affects learning and memory through GABA receptors by stimulating the translocation of these receptors to the plasma membrane. This effect is abolished by the action of a PI3K inhibitor. Insulin also increases the functional GABA receptor expression on the post-synaptic and dendritic membranes of the CNS neurons (Ghasemi et al., 2013). Insulin also modulates glutamatergic neurotransmission at the synapses. This hormone induces long term depression by decreasing the amount of AMPA receptors in the post-synaptic membrane. This process is mediated by activation of insulin receptor and downstream activation of PI3-kinase (Huang et al., 2004). Furthermore, insulin has been shown to induce the phosphorylation of the GluR2 subunit in the AMPA receptors of hippocampal neurons leading to their internalization and a decrease in EPSPs amplitude and neuronal excitability (Ahmadian et al., 2004). Therefore, it is well established that insulin affects many aspects of neuronal development, metabolic homeostasis, and higher brain functions such as cognition. Disturbances of these processes through Impaired insulin signaling can lead to many pathological conditions including increased risk of AD and cognitive disabilities in T2D.

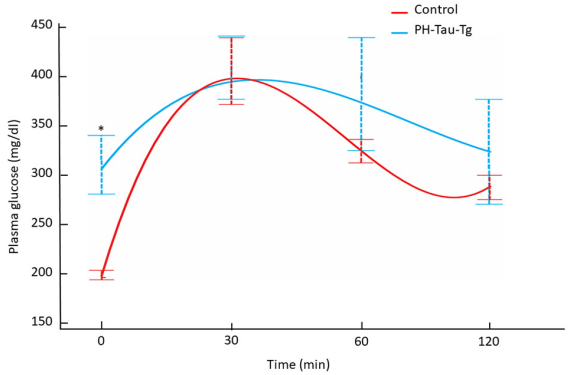

FIGURE 6 | Intraperitoneal glucose tolerance test on overnight fasted control mice ($n = 7$) and PH-Tau-Tg mice ($n = 9$). Values are expressed as means ± SEM obtained from three experiments. *$p < 0.05$ when compared with control group.

To further investigate the role of insulin in the regulation of neuronal excitability we pre-injected mice with insulin and measured the electrophysiological responses to KA. At a dose of 5 $mg.kg^{-1}$, kainate first produced seizures with no motor expression and recorded as regular EEG spiking, varying from 60 to 80/s (**Figure 1**). These filed potentials are a result of synaptic activity that triggers membrane currents that pass through the extracellular space and are recorded by electrodes placed outside the neurons. Field potential traces were filtered between 125 and 500 Hz and amplified. We focused our analysis on High frequency oscillations (HFO): slow ripples 125–250 Hz and fast ripples 250–500 Hz as shown in **Figures 2–4**. Ripple oscillations are HFOs ranging from 125 to 500 Hz, and have been regarded as a potential biomarker of epileptogenicity (Bragin et al., 1999, 2000, 2004; Traub et al., 2002; Grenier et al., 2003; Dzhala and Staley, 2004; Jacobs et al., 2008; Zijlmans et al., 2009; Brázdil et al., 2010; Haegelen et al., 2013; Wang et al., 2013; Miao et al., 2014). This activity was initially detected in human and animal epileptic tissue and in animals that develop spontaneous seizures (Bragin et al., 1999, 2000, 2004). In the brain, high-frequency

oscillations reflect coherent discharges of neurons in response to kainic acid activation of kainate receptors and are generated as a result of sequential and recurrent propagation of action potentials throughout the principal cell population. Using this paradigm, we found that injection of kainic acid (KA, 5 mg/kg, s.c) 15 min after insulin injection (1IU, s.c) led to significant increase in the firing rate, amplitude of extracellular field potentials and PSDs of high frequency brain waves in control mice only (slow and fast ripples: 125–250 and 250–500 Hz, respectively). Pre-injection of insulin prevented the KA-induced increase in ripples activity in the PH-Tau-Tg mice. Increased rates and PSDs of slow and fast ripple serve as a biomarker of epileptogenicity. By all measures used, we found that insulin significantly reduced neuronal excitability and seizure susceptibility in PH-Tau-Tg mice compared to controls (**Figures 1–4**). Indicating a strong effect of neuronal excitability. To further investigate the alterations in insulin signal transduction pathway in PH-Tau-Tg mice, we used an intraperitoneal glucose tolerance test. We found that PH-Tau-Tg mice have fasting hyperglycemia compared to controls (**Figure 6**). Hyperglycemia, hyperinsulinemia, and insulin resistance are hallmarks of T2D and has been shown to increase the risk for AD (Sims-Robinson et al., 2010). In this study, we found a significant increase p(Ser)-IRS1 in PH-Tau-Tg mice compared to controls (**Figure 5**), indicating alteration in IR signal transduction and some levels insulin resistance. Concomitant with the increase in p(Ser)-IRS1 in PH-Tau-Tg cortices, we also found a moderate increase in the expression levels of Glut 4 indicating alterations of glucose transport and utilization in PH-Tau-Tg mice. The expression levels of Gluts 4 have been shown to be dependent on the activation levels of IR. Interestingly, higher level of phosphorylated insulin receptor (p-Ser-IRS1) has been shown to be a consistent change in insulin signaling and a marker of insulin resistance in AD brains (Steen et al., 2005; Moloney et al., 2010; Bomfim et al., 2012; Talbot et al., 2012; Yarchoan et al., 2014). p(Ser)-IRS1 is a substrate for p-JNK, which also has also been found in elevated levels in AD brains (Bomfim et al., 2012; Talbot et al., 2012), suggesting some level of insulin resistance in AD. It has been reported that tau can regulate insulin signaling (Marciniak et al., 2017), and the lack of tau could be the cause of the insulin resistance observed in our system. Despite we cannot completely rule out this possibility, it seems unlikely since our mouse model has the same level of normal tau as the non-transgenic animals and the amount of PH-Tau expression is not more than 14% of that of the endogenous tau. Whether the insulin resistance observed in our mouse model is due to a lack of tau, this will reinforce the idea that PH-Tau has a gain of toxic function by binding to normal tau as we have previously described (Alonso et al., 1996). These findings further validate our mouse model for the study of the correlative relationship between AD and T2D. Consistent with this, a longitudinal study found that fasting hyperinsulinemia, even without T2D, doubled the risk of developing AD (Luchsinger et al., 2004). A cross-sectional study found that in AD patients without an APOE4 allele, hyperinsulinemia was also associated with an increased risk of AD (Kuusisto et al., 1997) and higher insulin was associated with amyloid deposition even before symptom onset (Willette et al., 2015) as shown by amyloid imaging on positron emission tomography scans. Furthermore, analyzes of the relationship between insulin resistance and AD biomarkers during the asymptomatic, preclinical stage in at-risk populations revealed that insulin resistance was associated with higher CSF tau, p-tau (Willette et al., 2015) and Aβ42 (Hoscheidt et al., 2016). Taken together, these studies suggest that alterations in insulin signal transduction pathway could play a causative role in AD. From the literature, an insulin imbalance could trigger tau hyperphosphorylation and then tau-induced neurodegeneration. The results presented here depict a different scenario, where the appearance of pathological tau in a neuron is enough to induce insulin resistance in the brain, that in turn could increase the levels of abnormal tau. It becomes evident that is vital to learn about the function of tau beyond a microtubule associated protein to attempt to design new therapeutics approaches to fight Alzheimer's disease.

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.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the College of Staten Island/CUNY.

AUTHOR CONTRIBUTIONS

AA generated and kept the transgenic animals. AA and AE obtained the electrophysiological recordings, immunocytochemistry and glucose levels and prepared the manuscript. AE analyzed the electrophysiological recordings. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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The Association of Pre-existing Diagnoses of Alzheimer's Disease and Parkinson's Disease and Coronavirus Disease 2019 Infection, Severity and Mortality: Results From the Korean National Health Insurance Database

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Objectives: Despite the numerous studies on coronavirus disease 2019 (COVID-19), data regarding the impact of pre-existing diagnoses of Alzheimer's disease (AD) and Parkinson's disease (PD) on the susceptibility to and outcome of COVID-19 are limited. We aimed to determine whether patients with AD/PD had a higher likelihood of contracting COVID-19 and experiencing worse outcomes.

Methods: Data from patients with confirmed diagnoses of COVID-19 (n = 8,070) from January to June 2020 and control participants (n = 121,050) who were randomly selected to match the patients on the basis of age and sex were extracted from the Korean National Health Insurance Database. Pre-existing diagnoses of AD and PD were identified based on medical claim codes. The associations of pre-existing AD or PD with contracting COVID-19, developing severe COVID-19 and dying due to COVID-19 were examined using a logistic regression model. The participants' age, sex, income, comorbidity score, and history of hypertension/diabetes were assessed as covariates.

Results: COVID-19 cases were more likely to have a pre-existing AD diagnosis (adjusted odds ratio [aOR] = 2.11, 95% confidence interval [CI] = 1.79–2.50, P-value < 0.001) than controls. COVID-19 cases were more likely to have a pre-existing PD diagnosis than controls, although this estimate did not quite reach statistical significance (aOR = 1.41, 95% CI = 1.00–2.00, P-value = 0.054). Pre-existing AD was related to severe disease and mortality from COVID-19 (aOR = 2.21, 95% CI = 1.64–2.98; aOR = 2.21, 95% CI = 1.00–2.00). Pre-existing PD was not associated with mortality (aOR = 1.54, 95% CI = 0.75–3.16) but was associated with severe disease (aOR = 2.89, 95% CI = 1.56–5.35).

Conclusion: We found that COVID-19 infection was significantly associated with a pre-existing diagnosis of AD but not with a pre-existing diagnosis of PD. Patients with pre-existing AD had higher odds of developing severe COVID-19 and dying. Pre-existing PD was only associated with a higher odds of developing severe COVID-19.

Keywords: Alzheimer's disease, Coronavirus disease 2019 (COVID-19), dementia, neurodegeneration, neurodegenerative disease, Parkinson's disease

INTRODUCTION

Coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has affected 224 countries, with more than 151,000,000 confirmed cases globally (World Health Oroganizations [WHO], 2021). This disease is primarily a respiratory disease that ranges in severity from mild to fatal, but it also impacts the functioning of the cardiovascular, renal, and nervous systems (Yuki et al., 2020). Although all individuals are susceptible to infection by SARS-CoV-2, older individuals and those with chronic diseases, specifically hypertension, coronary artery disease, obesity, and diabetes, are more susceptible to infection by SARS-CoV-2, the development of severe clinical symptoms of COVID-19 and mortality due to COVID-19 (Alzheimer's and Dementia, 2020). Furthermore, there is growing concern that the chronic diseases that make individuals more vulnerable to contracting COVID-19 and developing adverse outcomes could include neurodegenerative diseases. One recently published meta-analysis highlighted an imperative role of mental and neurological disorders in the context of COVID-19 and suggested instructions for recognizing and protecting these vulnerable individuals in the pandemic (Liu et al., 2021).

Neurodegenerative diseases are a heterogeneous group of diseases that are characterized by the progressive loss of neuronal cells of the central or peripheral nervous systems, including vascular dementia, Alzheimer's disease (AD), Parkinson's disease (PD), frontotemporal dementia (FTD), and various tauopathies (Yu et al., 2021). AD is the most common neurodegenerative disorder and the most frequent cause of dementia and is characterized by a progressive deterioration in cognition, particularly in memory function. PD is the most common movement disorder and represents the second most common degenerative disease of the central nervous system. The number of people suffering from these neurodegenerative disorders increases with age, and these disorders are often accompanied by various comorbidities. Therefore, it is plausible that patients affected by these neurodegenerative disorders would more likely be susceptible to SARS-CoV-2 infection. In addition, it is necessary to investigate whether people living with AD or PD are at greater risk of a severe clinical course and unfavorable outcomes of COVID-19. However, documented reports of the impacts of neurodegenerative disease on COVID-19 remain scarce. A recent cohort study used the UK Biobank to examine the associations of several risk factors, including all-cause dementia, AD and PD in particular, with COVID-19 positivity, severity (hospitalization), and death (Tahira et al., 2021). Another cohort study comprising 363 patients with AD and 259 patients with PD selected from a sample of 3,732 individuals concluded that inpatients with AD have a higher risk of 28-day mortality from COVID-19 (Fathi et al., 2021). In addition, an observational case series study investigating the frequency and mortality of COVID-19 among patients with a previous diagnosis of AD and FTD suggested that living in care homes was the most relevant factor for a higher risk of COVID-19 infection and death, with AD patients having a greater risk than those with FTD (Matias-Guiu et al., 2020).

We thus aimed to examine the associations between pre-existing AD and PD and COVID-19 infection, severity, and mortality using the Korean National Health Insurance Database.

MATERIALS AND METHODS

Ethics

Approval for this study was obtained from the ethics committee of Hallym University (2020-07-022). The need to obtain written informed consent was waived by the Institutional Review Board.

Study Population and Participant Selection

We used data extracted from the Korea National Health Insurance Database for Coronavirus disease 2019 (NHID-COVID DB). The NHID-COVID DB provided the data of all individuals who underwent testing for SARS-CoV-2 infection with real-time reverse-transcriptase-polymerase chain reaction (RT-PCR) assays of nasal or pharyngeal swabs in accordance with the World Health Organization (WHO) guidelines. The data covers the entire country without any exception based on medical claim codes between 2015 and 2020, including demographics, treatment outcomes, the isolation period, and the confirmation date of COVID-19 infection by PCR. In addition, all Korean citizens are registered with a lifelong 13-digit resident registration number and are required to register in the National Health Insurance Service (NHIS). Since the 13-digit resident registration number is used in all hospitals and clinics in Korea, all medical records of the entire population can be tracked and overlapping duplication can be prevented.

Data from patients with confirmed cases of COVID-19 were collected from 1 January 2020 to 4 June 2020. All included patients terminated treatment or died before 4 June 2020 (n = 8,070). The control participants were proportionally sampled in a 15:1 ratio with the COVID-19 patients from the Korean National Health Insurance Database after stratification by age and sex (n = 121,050). Then, the COVID-19 patients and control

participants were matched at a ratio of 1:4 based on age, sex, and income. Control participants without income records were excluded (n = 2,136). To mitigate potential selection bias, control participants were randomly chosen using clustered sampling. The index date was determined as the date of the confirmation of the diagnosis of COVID-19. The index date for each control participant was randomly assigned from 1 January 2020 to 4 June 2020. Ultimately, 8,070 COVID-19 patients were matched with 32,280 control participants (**Figure 1**).

Exposure (Alzheimer's Disease/Parkinson's Disease)

Participants were considered to have AD if they were diagnosed with AD [International Classification of Diseases and Related Health Problems (ICD-10) code: G30] or dementia in AD (F00). Patients with PD were those who had been diagnosed with PD (ICD-10 code: G20). To ensure the accuracy of the diagnosis, only participants who had received treatment ≥2 times were included, as in our previous work (Choi et al., 2019; Kim et al., 2019).

Primary Outcome (Coronavirus Disease 2019 Infection)

A confirmed diagnosis of COVID-19 was based on a positive assay for SARS-CoV-2.

Secondary Outcomes (Coronavirus Disease 2019 Severity and Mortality)

The severity of COVID-19 was divided into mild (n = 7,501) and severe (n = 569). Severe COVID-19 was defined based on admission to the intensive care unit (ICU), the use of invasive ventilation or extracorporeal membrane oxygenation (ECMO), and mortality. Additionally, COVID-19 patients were classified as having died (n = 237) or survived (n = 7,833).

Covariates

There were nine age groups at 10-year intervals: 0–9, 10–19, 20–29…, and 80+ years old. Income groups were classified as low, middle, and high. Individual comorbid conditions in this study were assessed according to the Charlson Comorbidity Index (CCI), which assesses 17 comorbidities and is presented as a continuous score from 0 to 29 (Quan et al., 2011), excluding dementia and diabetes.

Statistical Analysis

Differences in demographic data and general characteristics between the COVID-19 group and the control group and between the mild COVID-19 group and severe COVID-19 group were examined using the chi-square test or Fisher's exact test, as appropriate.

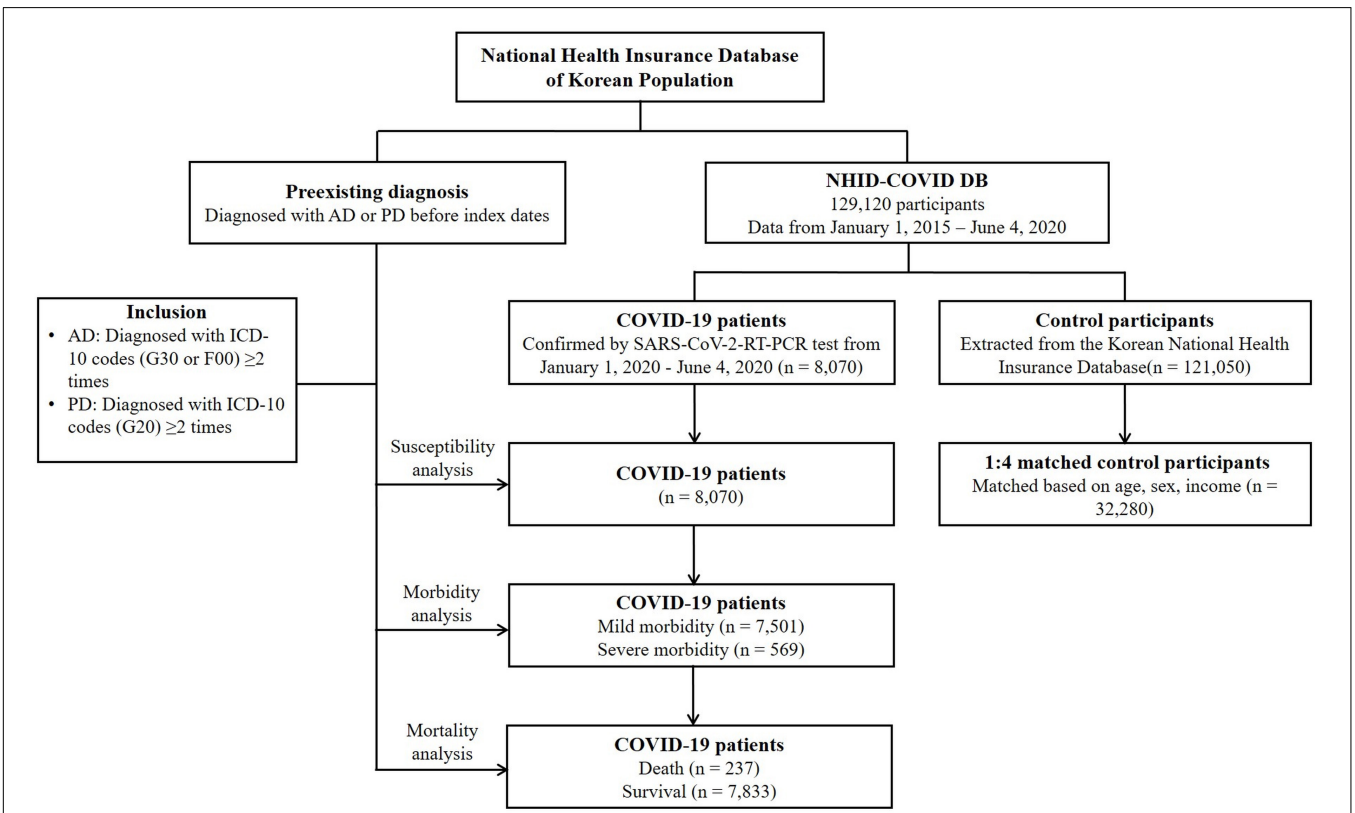

FIGURE 1 | A flowchart of the screening process used in this study. From a total of 129,120 participants, 8,070 COVID-19 patients were matched for age, sex, and income with 32,280 control participants. AD, Alzheimer's disease; ICD-10, International Classification of Diseases, Tenth Revision; NHID-COVID DB, National Health Insurance Database Coronavirus disease 2019; PD, Parkinson's disease; RT-PCR, reverse-transcriptase-polymerase chain reaction.

To estimate the odds of COVID-19 infection, odds ratios (ORs) with 95% confidence intervals (CIs) in patients with pre-existing AD or PD were calculated using crude (simple model) and adjusted conditional logistic regression models (adjusted for CCI score, hypertension, and diabetes).

To estimate the risks of severe COVID-19 and mortality in patients with pre-existing AD or PD, unmatched analyses were performed using unconditional logistic regression.

Stratified analyses were performed with subgroups of patients with different ages (<50 years old and ≥50 years old), sexes, incomes (low, middle, and high), CCI scores (0 points, 1 point, and ≥2 points), histories of hypertension, and histories of diabetes to test whether the effect of pre-existing AD or PD was consistent across different groups.

We used SAS version 9.4 (SAS Institute Inc., Cary, NC, United States) for the statistical analysis. All statistical tests were two-tailed, and probability values less than 0.05 were regarded as significant.

RESULTS

The distributions of age, sex, and income were the same between the COVID-19 and control groups owing to the matching process (P = 1.000). The COVID-19 group had higher proportions of individuals with a CCI score of 2 or higher, diabetes, AD, and PD than the control group (each $P < 0.001$). In the COVID-19 group, compared to patients with mild disease, the group of patients with severe disease was older and had a higher proportion of men. In addition, patients with severe COVID-19 were more likely to have a high income, a CCI score of 2 or higher, hypertension, diabetes, AD, and PD (each $P < 0.001$). The demographic and clinical characteristics of these cohorts are summarized in **Table 1**.

The crude and adjusted ORs of the association between pre-existing AD or PD and COVID-19 are shown in **Table 2**. COVID-19 cases were more likely to have a pre-existing diagnosis of AD than those of controls (adjusted OR in model 2 = 2.11, 95% CI = 1.79–2.50, $P < 0.001$). COVID-19 cases were more likely to have a pre-existing diagnosis of PD than those of controls, although the estimates did not reach statistical significance (adjusted OR in model 2 = 1.41, 95% CI = 1.00–2.00, $P = 0.054$).

The crude and adjusted ORs of the associations of pre-existing AD or PD with severe COVID-19 and COVID-19-related mortality are shown in **Tables 3**, **4**. Patients with a pre-existing diagnosis of AD had significantly higher odds of severe COVID-19 than patients without a pre-existing diagnosis of AD (adjusted OR in model 2 = 2.21, 95% CI = 1.64–2.98, $P < 0.001$).

TABLE 1 | General characteristics of the participants.

Characteristics	Total participants			COVID-19 patients		
	COVID-19 (n, %)	Control (n, %)	*P*-value	Severe morbidity (n, %)	Mild morbidity (n, %)	*P*-value
Total number	8,070 (100.0)	32,280 (100.0)		569 (100.0)	7,501 (100.0)	
Age (years)			1.000			<0.001*
0–9	81 (1.0)	324 (1.0)		6 (1.1)	75 (1.0)	
10–19	276 (3.4)	1,104 (3.4)		6 (1.1)	270 (3.6)	
20–29	2,057 (25.5)	8,228 (25.5)		31 (5.5)	2,026 (27.0)	
30–39	832 (10.3)	3,328 (10.3)		25 (4.4)	807 (10.8)	
40–49	1,036 (12.8)	4,144 (12.8)		30 (5.3)	1,006 (13.4)	
50–59	1,567 (19.4)	6,268 (19.4)		71 (12.5)	1,496 (19.9)	
60–69	1,199 (14.9)	4,796 (14.9)		116 (20.4)	1,083 (14.4)	
70–79	617 (7.7)	2,468 (7.7)		118 (20.7)	499 (6.7)	
80+	405 (5.0)	1,620 (5.0)		166 (29.2)	239 (3.2)	
Sex			1.000			<0.001*
Men	3,236 (40.1)	12,944 (40.1)		306 (53.8)	2,930 (39.1)	
Women	4,834 (59.9)	19,336 (59.9)		263 (46.2)	4,571 (60.9)	
Income			1.000			<0.001*
1 (low)	3,105 (38.5)	12,420 (38.5)		196 (34.5)	2,909 (38.8)	
2	2,347 (29.1)	9,388 (29.1)		161 (28.3)	2,186 (29.1)	
3 (high)	2,618 (32.4)	10,472 (32.4)		212 (37.3)	2,406 (32.1)	
CCI score†			<0.001*			<0.001*
0	6,725 (83.3)	29,637 (91.8)		333 (58.5)	6,392 (85.2)	
1	869 (10.8)	1,514 (4.7)		110 (19.3)	759 (10.1)	
≥2	476 (5.9)	1,129 (3.5)		126 (22.1)	350 (4.7)	
Hypertension	1,657 (20.5)	6,535 (20.2)	0.331	275 (48.3)	1,382 (18.4)	<0.001*
Diabetes	969 (12.0)	3,406 (10.6)	<0.001*	186 (32.7)	783 (10.4)	<0.001*
Alzheimer's disease	309 (3.8)	647 (2.0)	<0.001*	117 (20.6)	192 (2.6)	<0.001*
Parkinson's disease	52 (0.6)	109 (0.3)	<0.001*	275 (48.3)	1,382 (18.4)	<0.001*

CCI, Charlson comorbidity index; COVID-19, Coronavirus Disease 2019.

**Chi-squared or Fisher's exact test. Significance at p < 0.05.*

†*CCI score was assessed excluding dementia and diabetes.*

TABLE 2 | Crude and adjusted odds ratios of the association between pre-existing Alzheimer's disease or Parkinson's disease and COVID-19 in the total study participants.

Characteristics	COVID-19 (exposure/total, %)	Control (exposure/total, %)	Odds ratios (95% confidence interval) for COVID-19					
			Crude†	*P*-value	Model 1†‡	*P*-value	Model 2†§	*P*-value
Alzheimer's disease								
AD	309/8,070 (3.8%)	647/32,280 (2.0%)	2.41 (2.05–2.83)	<0.001*	2.17 (1.84–2.55)	<0.001*	2.11 (1.79–2.50)	<0.001*
Non-AD	7,761/8,070 (96.2%)	31,633/32,280 (98.0%)	1		1		1	
Parkinson's disease								
PD	52/8,070 (0.6%)	109/32,280 (0.3%)	1.94 (1.39–2.72)	<0.001*	1.78 (1.27–2.50)	0.001*	1.41 (1.00–2.00)	0.054
Non-PD	8,018/8,070 (99.4%)	32,171/32,280 (99.7%)	1		1		1	

AD, Alzheimer's disease; COVID-19, Coronavirus Disease 2019; PD, Parkinson's disease.
**Conditional logistic regression model; significance at $p < 0.05$.*
†*Model stratified for age, sex and income.*
‡*Model 1 was adjusted for Charlson comorbidity index scores, hypertension and diabetes.*
§*Model 2 was adjusted for model 1 plus Alzheimer's disease and Parkinson's disease.*

TABLE 3 | Crude and adjusted odds ratios of Alzheimer's disease and Parkinson's disease for morbidity in COVID-19 participants.

Characteristics	Severe participants (exposure/total, %)	Mild participants (exposure/total, %)	Odds ratios (95% confidence interval) for morbidity					
			Crude	*P*-value	Model 1†	*P*-value	Model 2‡	*P*-value
Alzheimer's disease								
AD	117/569 (20.6%)	192/7,501 (2.6%)	9.85 (7.68–12.64)	<0.001*	2.49 (1.87–3.32)	<0.001*	2.21 (1.64–2.98)	<0.001*
Non-AD	452/569 (79.4%)	7,309/7,501 (97.4%)	1		1			
Parkinson's disease								
PD	27/569 (4.7%)	25/7,501 (0.3%)	14.90 (8.59–25.84)	<0.001*	4.20 (2.34–7.54)	<0.001*	2.89 (1.56–5.35)	0.001*
Non-PD	542/569 (95.3%)	7,476/7,501 (99.7%)	1		1			

AD, Alzheimer's disease; COVID-19, Coronavirus Disease 2019; PD, Parkinson's disease.
**Unconditional logistic regression model; significance at $p < 0.05$.*
†*Model 1 was adjusted for age, sex, income, Charlson comorbidity index scores, hypertension and diabetes.*
‡*Model 2 was adjusted for model 1 plus Alzheimer's disease and Parkinson's disease.*

TABLE 4 | Crude and adjusted odds ratios of Alzheimer's disease and Parkinson's disease for mortality in COVID-19 participants.

Characteristics	Deceased participants (exposure/total, %)	Surviving participants (exposure/total, %)	Odds ratios (95% confidence interval) for mortality					
			Crude	*P*-value	Model 1†	*P*-value	Model 2‡	*P*-value
Alzheimer's disease								
AD	86/237 (36.3%)	223/7,833 (2.8%)	19.44 (14.45–26.14)	<0.001*	2.18 (1.52–3.15)	<0.001*	2.07 (1.42–3.02)	<0.001*
Non-AD	151/237 (63.7%)	7,610/7,833 (97.2%)	1		1		1	
Parkinson's disease								
PD	15/237 (6.3%)	37/7,833 (0.5%)	14.25 (7.71–26.34)	<0.001*	2.17 (1.09–4.30)	0.027*	1.54 (0.75–3.16)	0.236
Non-PD	222/237 (93.7%)	7,796/7,833 (99.5%)	1		1		1	

AD, Alzheimer's disease; COVID-19, Coronavirus Disease 2019; PD, Parkinson's disease.
**Unconditional logistic regression model; significance at $p < 0.05$.*
†*Model 1 was adjusted for age, sex, income, Charlson comorbidity index scores, hypertension and diabetes.*
‡*Model 2 was adjusted for model 1 plus Alzheimer's disease and Parkinson's disease.*

Similarly, compared with patients who had never been diagnosed with PD, patients who had been diagnosed with PD had a 2.89 times higher odds of developing severe COVID-19 (adjusted OR in model 2 = 2.89, 95% CI = 1.56–5.35, $P = 0.001$). Compared with participants who had not been previously diagnosed with AD, the odds of patients who had been diagnosed with AD were 2.07 times higher for COVID-19-related mortality, with statistical significance (adjusted OR in model 2 = 2.07, 95% CI = 1.42–3.02, $P < 0.001$). Patients who had been diagnosed with PD had a 1.54 times higher odds of COVID-19-related mortality than controls, although the estimates did not reach statistical significance (adjusted OR in model 2 = 1.54, 95% CI = 0.75–3.16, $P = 0.236$).

There were no substantial differences in the results of the subgroup analyses, and most subgroup analyses showed that patients with pre-existing diagnoses of AD or PD had

higher odds of developing severe COVID-19 and experiencing COVID-19-related mortality (**Supplementary Tables 2, 3**). The subgroups of patients with PD who were over 50 years of age, men, and did not have diabetes had significantly higher odds of contracting COVID-19 (**Supplementary Table 1**).

DISCUSSION

Our results showed that COVID-19 infection was significantly associated with a pre-existing AD diagnosis but not with a pre-existing PD diagnosis. Our findings confirmed that patients with pre-existing AD had higher odds of severe COVID-19 and COVID-19-related mortality. Although the patients with pre-existing PD were found to have higher odds of both severe disease and mortality due to COVID-19, the difference in mortality was not statistically significant.

Patients with AD are significantly more susceptible to infection with SARS-CoV-2 and have higher case-fatality rate because of certain clinical characteristics of AD. The explanation is thought to be that AD and dementia patients may be prone to having a high viral load because most of them are unable to comply with the recommendations to reduce the transmission of COVID-19 issued by the public health authorities due to their overall cognitive decline (Brown et al., 2020). In addition, the majority of the patients with AD and PD tend to live in care facilities where the risk of infection is considered high. Individuals with dementia, including AD, are also more likely than those without dementia to have cardiovascular comorbidities that are risk factors not only for AD but also for symptomatic and severe COVID-19 and are also more likely to have diabetes and pneumonia (Bauer et al., 2014). Additionally, advanced age, which was observed in most AD patients, has been identified as the most influential risk factor for mortality due to COVID-19 (Livingston and Bucher, 2020). Overall, it is unclear whether AD confers direct vulnerability to infection with SARS-CoV-2 or whether these comorbid conditions in AD contribute to an increased risk of poor outcomes of COVID-19. However, in this study, we identified that the pre-existing diagnosis of AD was closely associated with the risk of contracting COVID-19 and developing severe disease after adjusting for age and various comorbidities. Consistent with our results, a number of studies showed that dementia or AD, especially in the advanced stages of the disease, was associated with a higher risk of contracting COVID-19 and developing severe disease (Atkins et al., 2020; Bianchetti et al., 2020; Covino et al., 2020; Williamson et al., 2020; Yu et al., 2021; Zhou et al., 2021). In particular, one cohort study comprising 12,863 UK Biobank community-dwelling individuals more than 65 years old tested for COVID-19 highlighted that all-cause dementia and AD are age-independent risk factors for disease severity and death in COVID-19.

Meanwhile, a growing number of reports have suggested that AD-related neuropathology aggravates COVID-19 complications. First, amyloid fibrils, the pathologic hallmarks of AD, induce the activation of type I interferon (IFN) cytokines, which are innately produced in response to viral infections (Roy et al., 2020). Specifically, IFN plays a significant role in the host response to viral infection, AD pathology and disease severity and therefore might be a potential therapeutic target in both AD and COVID-19 (Naughton et al., 2020). Second, genomic studies recently demonstrated that angiotensin-converting enzyme 2 (ACE2) is the protein to which SARS-CoV-2 binds to gain entry into cells (Lu et al., 2020), and another recent study revealed that the protein expression level of ACE2 was upregulated in the brains of AD patients (Ding et al., 2020). Recent genomic research strongly indicated that ACE2 gene expression is elevated in the brain tissue of AD patients, which may be an important risk factor for COVID-19 transmission in AD patients (Lim et al., 2020). These neuropathological features of AD indicate that AD itself may be a direct cause of the increased susceptibility to contracting COVID-19 and developing severe disease and the consequent adverse clinical outcomes.

Similar to AD, several features of PD, such as respiratory muscle rigidity and impairment of the cough reflex in conjunction with pre-existing dyspnea, may lead to increased severity of COVID-19, particularly with regard to respiratory complications (Van Wamelen et al., 2020). Furthermore, it is conceivable that indirect consequences of the pandemic, such as increased stress levels, self-isolation, and anxiety, as well as prolonged immobility due to lockdown may worsen the outcomes in PD patients with COVID-19 (Prasad et al., 2020). Despite these characteristics, there are currently contradictory results regarding whether PD alone increases susceptibility to contracting COVID-19 and experiencing unfavorable outcomes. A recent large cohort study in Italy reported that the risks of contracting (7.1 vs. 7.6%) and dying from (age-adjusted OR = 0.45, P = 0.20) COVID-19 in patients with mild to moderate PD did not differ from those in the general population (Fasano et al., 2020). A community-based case-control study also reported 12 COVID-19 cases among PD patients (8.5%), whose mean age and disease duration (65.5 and 6.3 years, respectively) were similar to those in the non-PD population (Cilia et al., 2020). Conversely, one study by Antonini et al. (2020), which had a small sample size, investigated 10 PD patients and found that older age (mean, 78.3 years) and longer disease duration (mean, 12.7 years) were associated with an increased susceptibility to contracting COVID-19, with a high case-fatality rate of 40%. Meanwhile, a multicenter survey in Tuscany, Italy, reported a higher prevalence of COVID-19 in the PD population (0.9%) than the national average (0.24–0.35%) (Del Prete et al., 2021). Similarly, the results from the UK Biobank study showed that PD patients had a higher risk of SARS-CoV-2 infection (OR = 1.74, 95% CI = 1.34–2.27), but not of mortality, due to COVID-19 (Yu et al., 2021).

There are several neurobiological factors connecting PD and COVID-19. First, the ACE2 receptor, which is the protein to which SARS-CoV-2 binds to gain entry into host cells, is highly expressed in dopaminergic neurons (Antonini et al., 2020). It is possible to assume the penetration of SARS-CoV-2 into the brain through the ACE2 receptors is reduced in PD patients

because of degenerative changes in the dopaminergic neurons. Second, alpha-synuclein, which is considered the pathological hallmark of PD, is known to have multiple immunomodulatory functions, such as protection against pro-inflammatory responses (Lesteberg and Beckham, 2019). In addition, innate neuron-specific inhibitors of viral infection in the central nervous system have been identified (Ait Wahmane et al., 2020). Finally, several medications used for the treatment of PD have been found to have antiviral properties. Amantadine derivatives, such as amantadine, bananin, and memantine, might exert antiviral effects by inhibiting viral uncoating within the host cell endosome, blocking the enzyme helicase, or inhibiting ion channel activity (Tipton and Wszolek, 2020). However, it is unclear whether these agents are effective antiviral treatments for COVID-19.

Contrary to the above proposals, our results indicate that a pre-existing diagnosis of PD increases the risk of both contracting COVID-19 and experiencing poor outcomes after adjustment for potential confounders; however, the associations of a pre-existing diagnosis of PD with having a diagnosis of COVID-19 infection and COVID-19-related mortality were not significant. Despite the neurobiological background, the reason that pre-existing PD is associated with severe COVID-19 is because the two diseases have common clinical symptoms. The initial manifestations of COVID-19, such as fatigue, anosmia, flushing, or limb pain, could be masked by the non-motor symptoms of PD; thus, the early detection of COVID-19 in PD patients is somewhat challenging (Hainque and Grabli, 2020). It is plausible that PD patients affected by COVID-19 are likely to experience a severe clinical course that can be attributed to a delayed diagnosis.

Similar to the results of all participants, most subgroups stratified by income level showed that patients with a pre-existing diagnosis of AD or PD had a higher likelihood of contracting COVID-19 and experiencing severe COVID-19 disease. Intriguingly, however, only a subgroup of middle-income PD patients were less likely to die due to COVID-19 (aOR = 0.29, 95% CI = 0.05–1.74, $P = 0.177$). It can be inferred that the number of participants in the subgroups was relatively small.

The strengths of this study that warrant mention include the use of a nationwide dataset, which captured all validated cases of AD and PD and laboratory-confirmed cases of COVID-19 in the entire country. In addition, we adjusted for various comorbidities to minimize confounding. Nevertheless, several limitations should be noted. First, we did not assess the region of residence or residence in a care facility. There might be differences in susceptibility, disease severity or mortality according to region of residence because participants living in rural areas have limited access to the healthcare system. In addition, a considerable number of patients with chronic neurodegenerative diseases live in care facilities in Korea, and such facilities have consistently had clusters of COVID-19 cases. Therefore, further studies adjusting for residence type of the participants, which could influence disease susceptibility, are required to elucidate the contribution of pre-existing AD or PD to contracting COVID-19. Second, because of the inherent limitations associated with the use of insurance claims data, there was no information available on other possible confounding factors, such as genetic risk factors for AD or PD, lifestyle factors, stress, and the use of medications (Kuo et al., 2020; Magusali et al., 2021). We could not rule out the effects of these unmeasured confounders. Moreover, information regarding the disease stage or clinical severity of the neurodegenerative diseases was not available in the claims database, although these factors could be closely correlated to contracting COVID-19 and developing severe disease. In fact, although our result was not statistically significant, PD diagnosis was associated with COVID-19 diagnosis in a cohort study using the UK Biobank database (Tahira et al., 2021). Therefore, additional studies in larger populations with more information are warranted to clarify the correlation of the stage of neurodegenerative disease with the severity of COVID-19. Third, AD patients might have been more likely to have COVID-19 infection because they were older than the control participants. Although we calculated the adjusted conditional logistic regression including age as a factor and conducted additional stratified analyses with subgroups of patients by age (<50 years and ≥ 50 years old), we cannot exclude this limitation. Fourth, the number of severe COVID-19 cases ($n = 27$) and deaths ($n = 15$) among the PD patients was relatively small and thus unable show statistical significance. Fifth, we determined if AD or PD could be a risk factor for contracting COVID-19 by identifying subjects by outcome status in this case-control design study, but a cohort design, which follows the occurrence of COVID-19 infection in the pre-existing AD/PD group and control group (without pre-existing AD/PD), can be the best way to minimize the selection bias. At this time, it is impossible to obtain the results of a follow-up study design in the present study because access to the Korean NHID-COVID DB is currently limited. Therefore, future comparative studies are needed to establish whether the relationship between AD or PD and COVID-19 susceptibility can be replicated. Finally, we cannot rule out the possibility that individuals who were not tested for SARS-CoV-2 infection, namely, control participants, may have contracted COVID-19. Although we cannot exclude the effect of undiagnosed confirmed cases in the control group, the rate was reported to be extremely low in three antibody titer test surveys in Korea (Apio et al., 2020).

CONCLUSION

We found that COVID-19 infection was associated with a pre-existing diagnosis of AD but not with a pre-existing diagnosis of PD. In addition, patients with AD had higher odds of developing severe COVID-19 and experiencing COVID-19-related mortality, and patients with PD had higher odds of developing severe COVID-19.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the data analyzed in this study is subject to the following licenses/restrictions: The current article used a national sample cohort and does not involve data that can be available. Requests

to access the datasets should be directed to https://nhiss.nhis.or.kr/bd/ay/bdaya001iv.do.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Hallym University (2020-07-022). The Ethics Committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

JK and IC participated in the interpretation of the data and drafted and revised the manuscript. YK, CM, and DY participated in the data collection and data interpretation. HC designed the study, participated in the data collection and data interpretation, and revised the manuscript. All authors approved the final version of the manuscript for publication.

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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.2022.821235/full#supplementary-material

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Development and Validation of a Multimorbidity Index Predicting Mortality Among Older Chinese Adults

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Objective: This study aimed to develop and validate a multimorbidity index using self-reported chronic conditions for predicting 5-year mortality risk.

Methods: We analyzed data from the Chinese Longitudinal Healthy Longevity Survey (CLHLS) and included 11,853 community-dwelling older adults aged 65–84 years. Restrictive association rule mining (ARM) was used to identify disease combinations associated with mortality based on 13 chronic conditions. Data were randomly split into the training (N = 8,298) and validation (N = 3,555) sets. Two multimorbidity indices with individual diseases only (MI) and disease combinations (MIDC) were developed using hazard ratios (*HR*s) for 5-year morality in the training set. We compared the predictive performance in the validation set between the models using condition count, MI, and MIDC by the concordance (C) statistic, the Integrated Discrimination Improvement (IDI), and the Net Reclassification Index (NRI).

Results: A total of 13 disease combinations were identified. Compared with condition count (C-statistic: 0.710), MIDC (C-statistic: 0.713) showed significantly better discriminative ability (C-statistic: p = 0.016; IDI: 0.005, $p < 0.001$; NRI: 0.038, p = 0.478). Compared with MI (C-statistic: 0.711), the C-statistic of the model using MIDC was significantly higher (p = 0.031), while the IDI was more than 0 but not statistically significant (IDI: 0.003, p = 0.090).

Conclusion: Although current multimorbidity status is commonly defined by individual chronic conditions, this study found that the multimorbidity index incorporating disease combinations showed supreme performance in predicting mortality among community-dwelling older adults. These findings suggest a need to consider significant disease combinations when measuring multimorbidity in medical research and clinical practice.

Keywords: multimorbidity, measurement, mortality, restrictive association rule mining, older adults

INTRODUCTION

Multimorbidity, commonly defined as the coexistence of multiple chronic diseases and/or conditions within one individual, is prevalent among older populations (Salive, 2013). Multimorbidity has been associated with functional limitations (Kadambi et al., 2020), poor quality of life (Kanesarajah et al., 2018), and mortality (Nunes et al., 2016). With a rapidly aging global population, multimorbidity poses a great economic burden on both individuals and health care systems (Larkin et al., 2021; Soley-Bori et al., 2021). Identifying older patients with multimorbidity at high risks of adverse health outcomes in the community may inform clinicians and public health policymakers to prioritize those groups of people and enable early, effective, and targeted interventions to prevent premature death and reduce health costs (Charlson et al., 2014). Although previous research has made considerable efforts to assess multimorbidity (Nicholson et al., 2019), an international consensus regarding the standard measurement of multimorbidity has yet to be reached (Johnston et al., 2019). Therefore, further explorations for tools to measure multimorbidity are needed for patient care, resource allocation, and the prevention of multimorbidity progression and complications (Wei et al., 2016).

Among all multimorbidity assessments, weighted-multimorbidity indices have been widely used (Stirland et al., 2020). The majority of weighted indices for older adults were developed from hospital patients based on in-patient medical records (Diederichs et al., 2011). Moreover, current multimorbidity indices for community-dwelling adults were mostly developed from young or middle-aged populations in western countries (Lee et al., 2006; Wister et al., 2015; Wei et al., 2016). Given disparities in the spectrum of diseases in different regions and populations worldwide, these indices might not be generalizable to older Chinese adults (GBD 2017 Causes of Death Collaborators, 2018; Zhou et al., 2019).

Although current multimorbidity indices are able to distinguish and depict the influences of chronic conditions on mortality, little research considers the interaction of those conditions in the indices. Previous studies showed that disease combinations had discordant effects on mortality, but most of them identified disease combinations through traditional methods (Caughey et al., 2010; Ferrer et al., 2017). Association rule mining (ARM) is a data-driven approach that has been applied previously to discover significant disease combinations (Held et al., 2016; Yao et al., 2020). Therefore, a multimorbidity index considering the effects of important disease combinations derived from ARM might better capture the whole impact of multiple chronic conditions on mortality.

Developing an explicit and validated measurement tool is significant to help assess the health risks based on diagnoses of chronic diseases and classify older adults into different risk groups for targeted clinical treatment and health management. Moreover, identifying important combinations of diseases may facilitate the evidence-based co-treatment and further investigation into underlying mechanisms (Brown and Thorsteinsson, 2020). Therefore, this study aimed to investigate disease combinations significantly associated with 5-year mortality among community-dwelling older Chinese adults aged 65–84 years from the Chinese Longitudinal Healthy Longevity Survey (CLHLS), as well as develop a multimorbidity index incorporating disease combinations to predict 5-year mortality.

MATERIALS AND METHODS

Participants

This study used data from six waves of the CLHLS 2000–2014. The CLHLS is a prospective longitudinal study with the aim to assess determinants of healthy longevity in China. The survey was first conducted in 1998 and subsequent surveys were carried out every 2 or 3 years. The surveys were conducted in half of the counties and cities randomly selected from 23 provinces in China. The participants were enrolled *via* multistage disproportionate sampling (Gu, 2008). More details about the study design were provided elsewhere (Zeng et al., 2017). Duke University Medical Health System's Institutional Review Board (IRB), the National Bureau of Statistics of China, and the Ethical Committee of Social Science Division of Peking University reviewed and approved ethics for CLHLS. Written informed consent was obtained from participants or their proxies.

Among 40,359 participants from the six waves, we only included participants aged 65–84 years at baseline (N = 14,148), who were frequently defined as "older adults" in previous research and accounted for approximately 94% of adults aged ≥65 years in China (United Nations [UN], 2019; Greer et al., 2021). Additionally, the median survival time of participants aged 65–69, 70–74, 75–79, and 80–84 years at baseline was 16.9, 12.0, 8.8, and 5.8 years, respectively, all of which were more than 5 years (**Supplementary Figure 1**). After excluding those who were lost to follow up after baseline survey (N = 2,295), a total of 11,853 participants, with the mean follow-up of 4.1 [standard deviation (SD): 1.4] years, were included for analyses. The flowchart of the participant selection and sample sizes of the dynamic cohort is shown in **Supplementary Figure 2** and **Supplementary Table 1**.

Chronic Conditions

In this study, 13 chronic diseases or conditions (abbreviated hereafter as chronic conditions) at baseline were included, covering most somatic diseases and mental disorders frequently used in measuring multimorbidity (Diederichs et al., 2011; The Academy of Medical Sciences, 2018; Stirland et al., 2020). In addition, seven chronic conditions were ascertained by asking participants whether a doctor told them that they had diabetes, cerebrovascular disease, heart disease, cancer, lung disease (bronchitis, emphysema, asthma, pneumonia, and tuberculosis), Parkinson's disease, and arthritis. Blood pressure was measured by a trained physician with the electronic sphygmomanometer (Omron HEM-7200 Monitor) and the mean of two repeated measures was calculated. Participants were considered hypertensive, if their systolic blood pressure

was ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg, or if they self-reported being diagnosed with hypertension by the physicians (Writing Group of 2018 Chinese Guidelines for the Management of Hypertension et al., 2019). Cognitive impairment was defined as having either self-reported dementia or impaired cognitive function. Cognitive function was measured using the Chinese version of the Mini-Mental State Examination (MMSE). The MMSE score ranged from 0 to 30, and impaired cognitive function was defined as an MMSE score ≤18 as previously validated (Zhang et al., 2010). Depressive symptoms were assessed by a five-item Likert scale with scores ranging from 0 to 20 and the acceptable internal consistency reliability (Cronbach's $\alpha = 0.66$) (Shen et al., 2019). This scale has been commonly used to identify depressive symptoms in several studies using CLHLS data (Yi and Vaupel, 2004; Zeng et al., 2013; Feng et al., 2015; Shen et al., 2019). Those with the depression score ≤7, which was the median of all participants, were considered to have depressive symptoms. Participants were identified as having vision impairment, if they were unable to distinguish the break in the circle or see the circle clearly or blind, and/or reported having cataracts and/or glaucoma (Cao et al., 2021). Participants were considered to have hearing impairment, if they cannot hear clearly what the interviewers said despite using a hearing aid or cannot hear anything (Zhang et al., 2020). Sensory impairment was defined as having hearing impairment and/or vision impairment. Bedridden status was defined from either self-reported bedsore or being permanently bedridden in the past 2 years (Mervis and Phillips, 2019). Tooth loss was defined as having no natural teeth and without dentures (Yuan et al., 2020). Definitions of chronic conditions are shown in **Supplementary Table 2**. Participants answered questions about cognitive function and depressive symptoms on their own, while proxy respondents would answer other questions, if participants were unable to complete the interview due to cognitive and linguistic impairments (Gu, 2008; Lv et al., 2019). All diseases and conditions were defined as the binary variables.

Mortality

All-cause mortality was ascertained through a face-to-face interview with a close family member for those interviewees who had died during the follow-ups (Zeng et al., 2008). All-cause mortality is a common choice for an adverse outcome to understand the progression and severity of an exposure (e.g., chronic diseases), with a minor bias but easy to measure (Ferguson et al., 2013; Weiss, 2014). Additionally, mortality is one of the most commonly used outcomes to develop the multimorbidity index, which could facilitate the comparison between our indices and established indices (Nicholson et al., 2019). Follow-up time was defined as the period from the date of the baseline visit to the date of death or the last follow-up. At the 5-year follow-up, survivors were censored, which is the standard cutoff for evaluating the effect of screening or treatment for older adults with chronic diseases, such as cancer (Miller et al., 2020). Participants who were lost to follow-up during the 5-year follow-up were censored at the time of the last survey. The proportions for participants who were lost to follow-up or died within the 5-year follow-up were 16.3 and 25.2%, respectively.

Statistical Analyses

Baseline characteristics were summarized using frequencies (percentages) for categorical variables and median [interquartile range (IQR)] for continuous variables. The chi-square tests for categorical variables and Mann–Whitney *U*-test for continuous variables were used to compare baseline characteristics between survivors and non-survivors at 5-year follow-up (**Supplementary Table 3**).

Association rule mining was performed to identify the pairs of chronic conditions associated with mortality. ARM allows the identification of novel and potentially relevant associations of diseases without stating *a priori* hypotheses (Prados-Torres et al., 2014; Held et al., 2016). For association rules like ({A}→{B}) with an "antecedent" {A} and a "consequent" {B}, "support" refers to the frequency of the particular combination of A and B; "confidence" refers to how frequently B occurs conditionally on A; "lift" refers to how much more frequently A and B occur together compared with how often would be expected under statistical independence (Yao et al., 2020). The parameters of ARM were set as follows: minimum support >1.5%, minimum confidence >10%, lift >1.0, the number of items in the antecedent was limited to 2, and the consequent was restricted to 5-year mortality. Disease combinations were ascertained as the antecedents of association rules matching all parameters. For example, a disease combination of hypertension and sensory impairment was defined as the coexistence of these two chronic conditions based upon the rule of ({Hypertension, Sensory impairment}→{5-year mortality}).

Participants aged 65–84 years at baseline were randomly divided into training (70% of analytic sample) and validation (30% of analytic sample) sets. Multimorbidity indices with individual diseases (MI) or disease combinations (MIDC) were developed by Cox proportional hazards models in the training set. Model 1 included age, sex, and chronic conditions as independent variables, and Model 2 further added disease combinations derived from the restrictive ARM. All independent variables were based on baseline information, without considering disease evolution over time. The outcome was 5-year mortality for both Model 1 and Model 2. Adjusted hazard ratios (*HR*s) estimated by Model 1 were used to assign the weights for conditions in MI, while HRs from Model 2 were used for conditions and disease combinations in MIDC. Consistent with previous studies, a condition or disease combination with an $HR = 1.00–1.19$, 1.20–1.49, and ≥1.50, was assigned a weight of 1, 2, and 3, respectively (Charlson et al., 1987; Mukherjee et al., 2011). MI and MIDC were calculated by summing the weighted scores of conditions and/or disease combinations. The conditions or disease combinations with $HR < 1$ were excluded for the final calculation of MI and MIDC. More details of the process to develop MI and MIDC are shown in **Supplementary Figure 3**.

The base model with age and sex, and three models added simple condition count, MI, and MIDC at baseline, respectively, were employed to predict 5-year mortality in the validation set. We compared the performance to predict 5-year mortality

between every two of the models above by the concordance (C) statistic, the Integrated Discrimination Improvement (IDI), and the continuous Net Reclassification Index (NRI). The C-statistic generally ranging from 0.5 to 1.0 is an overall measure to compare the discrimination power of risk prediction models. A C-statistic closer to one indicates better performance of the predictive models (Harrell et al., 1996; Schroder et al., 2011). The IDI is defined as the comparative improvement of a new model in sensitivity and specificity for events. The NRI assesses the increase of model-based probabilities for events and the decrease of the probabilities for non-events. A positive IDI or NRI indicates that the new model predicts better than the comparator model (Kerr et al., 2011; Uno et al., 2013).

All data management and analyses were performed by R software version 4.0.0 (R Foundation for Statistical Computing, Vienna, Austria). Two-tailed $p < 0.05$ was considered statistically significant.

RESULTS

Baseline characteristics of all participants are summarized in **Table 1**. Among 11,853 participants, the median age was 76.0 (IQR: 69.0, 81.0) years and 53.0% were men. Hypertension, depressive symptom, and sensory impairment were the most common conditions, with the prevalence rates of 55.6, 36.0, and 23.7%, respectively.

Table 2 presents the results of restrictive ARM. Among all the participants, a total of 13 disease combinations were identified. The disease combination with the highest lift was hypertension and cognitive impairment. Older patients with hypertension and depressive symptoms, who died during a 5-year follow-up accounted for 6.6% among all participants. The prevalence rates of the co-occurrence of hypertension and depressive symptoms were 20.4% among all participants and 32.6% among participants who died during a 5-year follow-up.

Figure 1 shows the *HR*s and weights of each condition or disease combination. Cognitive impairment, diabetes, cancer, and bedridden status were the conditions with a weight of 3, while hypertension and arthritis with $HR < 1$ were excluded for the calculation of both MI and MIDC. Each of the six disease combinations derived from restrictive ARM and with $HR > 1$ was assigned a weight of 1 or 2. The total ranges of MI and MIDC were 0–23 and 0–31, respectively. A higher score indicates a greater burden of multimorbidity. The distributions of these two indices in the validation set were shown in **Supplementary Figure 4**. The Cronbach's α coefficient of MIDC in the validation set was 0.65, indicating acceptable internal consistency reliability. For validity, Pearson's correlation coefficients (**Supplementary Table 4**) showed that the MIDC was significantly related to

TABLE 1 | The baseline characteristics of training and validation sets*.

Characteristics	Total (*N* = 11,853)	Training set (*N* = 8,298)	Validation set (*N* = 3,555)	*P*-value†
Age (years), median (IQR)	76.0 (69.0, 81.0)	76.0 (69.0, 81.0)	76.0 (69.0, 81.0)	0.434
Male	6,287 (53.0)	4,398 (53.0)	1,889 (53.1)	0.908
Hypertension	6,596 (55.6)	4,626 (55.7)	1,970 (55.4)	0.753
Diabetes	384 (3.2)	278 (3.4)	106 (3.0)	0.326
Heart disease	1,240 (10.5)	893 (10.8)	347 (9.8)	0.110
Cerebrovascular disease	747 (6.3)	517 (6.2)	230 (6.5)	0.653
Parkinson's disease	58 (0.5)	43 (0.5)	15 (0.4)	0.586
Arthritis	2,165 (18.3)	1,514 (18.2)	651 (18.3)	0.952
Tooth loss	712 (6.0)	501 (6.0)	211 (5.9)	0.863
Lung disease	1,585 (13.4)	1,084 (13.1)	501 (14.1)	0.139
Cancer	61 (0.5)	47 (0.6)	14 (0.4)	0.288
Sensory impairment	2,815 (23.7)	1,991 (24.0)	824 (23.2)	0.351
Cognitive impairment	568 (4.8)	392 (4.7)	176 (5.0)	0.629
Bedridden status	132 (1.1)	90 (1.1)	42 (1.2)	0.715
Depressive symptoms	4,265 (36.0)	3,011 (36.3)	1,254 (35.3)	0.303

IQR, interquartile range.
**Data are presented as n (%) unless otherwise indicated.*
†The value of p was calculated using chi-square tests for categorical variables and Mann–Whitney U-test for continuous variables.

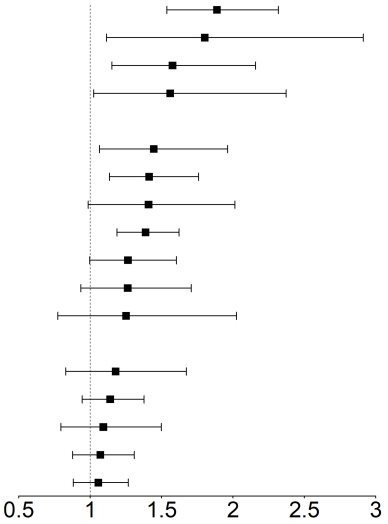
TABLE 2 | Results of association rule mining among participants aged 65–84 years (*N* = 11,853).

Rules		Support (%)*	Confidence (%)†	Lift	Prevalence (%)††
Antecedent	**Consequent**				
Hypertension, depressive symptoms	Five-year mortality	6.6	32.6	1.3	20.4
Hypertension, sensory impairment		5.1	36.0	1.4	14.2
Sensory impairment, depressive symptoms		4.6	39.2	1.6	11.6
Hypertension, lung disease		2.5	33.9	1.3	7.5
Lung disease, depressive symptoms		2.2	39.2	1.6	5.7
Arthritis, depressive symptoms		2.0	25.9	1.0	7.9
Cognitive impairment, depressive symptoms		2.0	58.0	2.3	3.4
Hypertension, heart disease		2.0	26.2	1.0	7.5
Sensory impairment, cognitive impairment		1.9	56.7	2.3	3.4
Hypertension, cerebrovascular disease		1.7	37.2	1.5	4.6
Hypertension, cognitive impairment		1.7	58.9	2.3	2.8
Hypertension, tooth loss		1.6	45.5	1.8	3.5
Lung disease, sensory impairment		1.6	40.6	1.6	3.9

**Support was the proportion of patients with the disease combination and died during a 5-year follow-up among all participants.*
†Confidence was the proportion of patients with the disease combination among participants who died during a 5-year follow-up.
††Prevalence was the proportion of patients with the disease combination among all participants.

Weight	Condition	HR (95%CI)
3	Diabetes	1.89 (1.54, 2.32)
	Cancer	1.80 (1.11, 2.91)
	Bedridden status	1.58 (1.15, 2.16)
	Cognitive impairment	1.56 (1.03, 2.37)
2	Hypertension, Tooth loss	1.45 (1.06, 1.96)
	Lung disease	1.41 (1.13, 1.76)
	Cognitive impairment, Depressive symptoms	1.41 (0.98, 2.01)
	Depressive symptoms	1.39 (1.19, 1.62)
	Heart disease	1.26 (1.00, 1.60)
	Cerebrovascular disease	1.26 (0.93, 1.71)
	Parkinson's disease	1.25 (0.77, 2.02)
1	Hypertension, Cerebrovascular disease	1.18 (0.83, 1.67)
	Sensory impairment	1.14 (0.94, 1.38)
	Hypertension, Cognitive impairment	1.09 (0.80, 1.50)
	Hypertension, Sensory impairment	1.07 (0.88, 1.31)
	Hypertension, Depressive symptoms	1.06 (0.88, 1.27)

A



Weight	Condition	HR (95%CI)
3	Cognitive impairment	1.91 (1.64, 2.23)
	Diabetes	1.84 (1.50, 2.26)
	Cancer	1.73 (1.07, 2.81)
	Bedridden status	1.52 (1.11, 2.07)
2	Cerebrovascular disease	1.42 (1.22, 1.66)
	Depressive symptoms	1.39 (1.27, 1.52)
	Lung disease	1.23 (1.10, 1.38)
	Tooth loss	1.21 (1.05, 1.41)
1	Parkinson's disease	1.20 (0.74, 1.94)
	Sensory impairment	1.08 (0.98, 1.20)
	Heart disease	1.05 (0.92, 1.21)

B

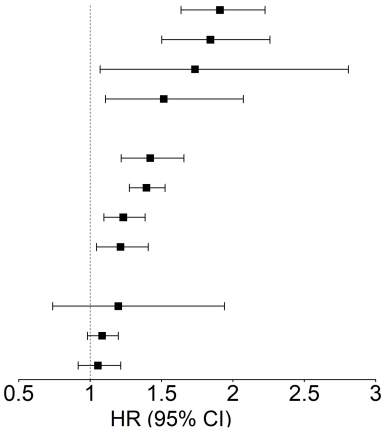


FIGURE 1 | Weights, *HR*s, and 95% CIs of MIDC **(A)** and MI **(B)** for 5-year mortality in the training set (*N* = 8,298). All models were adjusted for age and sex. A condition or disease combination with an *HR* = 1.00–1.19, 1.20–1.49, and ≥1.50, was assigned a weight of 1, 2, and 3, respectively. HR, hazard ratio; CI, confidence interval; MI, multimorbidity index with individual diseases; MIDC, multimorbidity index incorporating disease combinations.

condition count (r = 0.88, $p < 0.001$) and MI (r = 0.95, $p < 0.001$).

The results of predictive validity analyses are presented in **Table 3**. The C-statistics of condition count, MI, and MIDC were significantly higher for mortality prediction than that of the base model with only age and sex (all $p < 0.001$). Moreover, the reclassification measures of discrimination showed that all models, such as condition count, MI, and MIDC performed better in predicting 5-year mortality than the base model (IDI: more than 0, all $p < 0.001$; NRI: more than 0, all $p < 0.001$). Compared with condition count, MIDC showed significantly better discriminative ability (C-statistic: p = 0.016; IDI: 0.005, $p < 0.001$; NRI: 0.038, p = 0.478). Compared with MI, the C-statistic of the model using MIDC was significantly higher (p = 0.031), while the IDI was more than 0 but not statistically significant (IDI: 0.003, p = 0.090).

DISCUSSION

To the best of our knowledge, this is one of the first studies to develop and validate the MIDC to predict 5-year mortality in community-dwelling older Chinese adults aged 65–84 years. The MIDC showed greater predictive performance than simple condition count and MI in predicting mortality. This suggests that it is of great importance to consider the effect of specific disease combinations when assessing the impact of coexisting chronic conditions on mortality in older adults.

In our study, we found that 13 disease combinations and 5-year mortality were co-occurring more frequently than expected in older adults. Among these combinations, dyads of *hypertension and depressive symptoms*, as well as *hypertension and sensory impairment*, were most prevalent, which was in line with a large body of literature and could be explained by underlying pathophysiological mechanisms

TABLE 3 | C-statistics, IDIs, and NRIs for 5-year mortality in the validation set (N = 3,555).

Measures of multimorbidity	*C*-statistic	*P*-value	IDI	*P*-value	NRI	*P*-value
Base model*	0.701	Reference	–	Reference	–	Reference
Base model + *Condition count*	0.710	<0.001	0.017	<0.001	0.083	<0.001
Base model + *MI*	0.711	<0.001	0.020	<0.001	0.101	<0.001
Base model + *MIDC*	0.713	<0.001	0.022	<0.001	0.110	<0.001
Base model + *Condition count*	0.710	Reference	–	Reference	–	Reference
Base model + *MI*	0.711	0.231	0.002	0.259	0.017	0.478
Base model + *MIDC*	0.713	0.016	0.005	<0.001	0.038	0.478
Base model + *MI*	0.711	Reference	–	Reference	–	Reference
Base model + *MIDC*	0.713	0.031	0.003	0.090	−0.019	0.965

IDI, Integrated Discrimination Improvement; NRI, Net Reclassification Index; MI, multimorbidity index with individual diseases; MIDC, multimorbidity index incorporating disease combinations.
**The base model included age and sex as independent variables.*

(de Moraes Marchiori et al., 2006; Bhargava et al., 2012; Maatouk et al., 2016; Crews et al., 2017; Jin et al., 2019). It has been reported that the co-occurrence between hypertension and depressive symptoms could be because of neurobiological changes caused by vascular diseases and psychosocial stressors due to the diagnosis of hypertension (Maatouk et al., 2016; Jin et al., 2019). Microvascular damage in the retina and cochlea associated with elevated blood pressure, such as vascular stenosis and hemorrhage, has been proposed as a potential mechanism of hearing impairment and vision loss (de Moraes Marchiori et al., 2006; Bhargava et al., 2012). These findings highlighted that somatic diseases associated with comorbid mental health problems and age-related sensory changes in older people required more attention in primary care and clinical settings.

According to the cox regression results, higher mortality risks of the disease pairs, such as *hypertension and tooth loss*, and *cognitive impairment and depressive symptoms*, were observed. There has been little research comparing the effect of coexistence of hypertension and tooth loss on mortality to that of other disease pairs. However, a previous study reported that tooth loss increased the risk of hypertension due to insufficient intake of fiber and chronic systemic inflammation caused by periodontal disease (Da et al., 2019). Another cohort study of 7,674 Sweden adults also showed that tooth loss was significantly associated with all-cause mortality and cardiovascular diseases mortality (Holmlund et al., 2010). Furthermore, periodontitis has been shown to significantly increase the magnitude of endothelial dysfunction in patients with hypertension, which may accelerate the progression of carotid atherosclerosis and incidence of stroke, myocardial infarction, and cardiovascular diseases death (Higashi et al., 2008; Desvarieux et al., 2010). These results may imply that older people with both hypertension and tooth loss may be more likely to have cardiovascular diseases and further lead to premature mortality. Moreover, previous research has revealed that older patients with dementia and depression had higher mortality risks than those with most of the other chronic conditions (Schafer et al., 2018; Zheng et al., 2021). Late-life depression could interact with cognitive impairment by sharing common underlying pathogenetic mechanisms related to ischemic brain lesions (Linnemann and Lang, 2020). The co-occurrence of cognitive impairment and depression may indicate severe vascular dysfunction, leading to a high cardiovascular mortality risk (Georgakis et al., 2016). As a result, the combinations of these diseases may provide additional valuable information on predicting mortality in older patients with multimorbidity.

Our study compared the performance of condition count, MI, and MIDC in predicting mortality. As expected, based on C-statistics, we found that multimorbidity, measured by condition count, MI, and MIDC, showed significantly better discriminative ability in predicting mortality than age and sex. Although age has been found to be a strong predictor of mortality in several multimorbidity indices, multimorbidity should be additionally considered when assessing the mortality risks of older adults, as previously validated (Charlson et al., 1994; Lee et al., 2006; Nunes et al., 2016). Furthermore, in accordance with the study conducted in older adults aged ≥65 years in Canada, our finding showed that condition count, which was easy to use and understand in clinical settings, has an acceptable prediction performance for mortality among older adults (Quail et al., 2011).

In this study, MIDC performed better than condition count in predicting 5-year mortality. As numerous studies have noted that the association between different chronic conditions and mortality varies among older adults, considering the types and severity of diseases might capture the heterogeneity of their impacts on health (Wei et al., 2016; Stanley and Sarfati, 2017). In addition, we also found that the C-statistic of MIDC was significantly higher than that of MI. Prior research has indicated that specific combinations of diseases, especially cardiovascular and neuropsychiatric disease patterns, may have a synergistic effect on disability or mortality, which partially supports our findings of better performance of MIDC (Jackson et al., 2015; Zheng et al., 2021). Therefore, measuring multimorbidity through multimorbidity index including the effects of specific disease combinations provides a more nuanced risk classification of older patients with multimorbidity and a qualitative dimension that can be useful in clinical practice and research (Johnston et al., 2019; Stirland et al., 2020).

One of the strengths of this study is that we developed a multimorbidity index for community-dwelling older Chinese adults using the data from a multi-province longitudinal study. This approach can support that our index has good generalizability among this population. Moreover, our study included 13 chronic conditions covering most of the conditions prevalent among older Chinese adults and widely used in previous multimorbidity indices (Diederichs et al., 2011; Wang et al., 2020). In addition, compared to previous multimorbidity indices using only individual diseases, we conducted the restrictive ARM to obtain disease combinations important to predict mortality and considered the joint effects of multiple chronic diseases in our index. However, our study has several limitations. First, since our index focused on community-dwelling older adults, it is likely that our index will not be

applicable to a nursing home and other institutional populations. Second, most of the chronic conditions were assessed by self-reported questionnaires rather than clinical records, which might lead to recall bias, especially diabetes. However, previous studies also emphasized the importance of self-reported information on a population level since the accuracy of self-reported diseases may reflect the participants' health literacy (e.g., the awareness of diseases), which could improve the generalization of our index in the community (Smith et al., 2008; van den Akker et al., 2015). Third, the MMSE is not a sensitive measurement of cognitive impairment, and future studies need to assess cognitive function by better tools such as the Montreal Cognitive Assessment (MoCA) (Nasreddine et al., 2005; Arevalo-Rodriguez et al., 2015). Last, we did not have information on the severity, and treatment of each condition, and their influence on mortality needs to be considered in further research.

CONCLUSION

Multimorbidity index incorporating disease combinations, followed by multimorbidity index with individual diseases, improved the accuracy of 5-year mortality prediction. This may provide a tool for overall risk stratification, care management, and healthcare resource allocation among community-dwelling older Chinese adults. Moreover, our findings may also imply to researchers that considering significant disease combinations to capture synergistic effects is extremely valuable in predicting mortality among older adults with multimorbidity. Further studies are needed to investigate the association of the MIDC with other health outcomes and validate the MIDC in other populations. In addition, condition count may also be a good choice for measuring multimorbidity for its simplicity and the ease of data ascertainment.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: Peking University Open Research Data Platform, https://opendata.pku.edu.cn/dataset.xhtml?persistentId=doi:10.18170/DVN/WBO7LK.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Duke University Medical Health System's Institutional Review Board (IRB), the National Bureau of Statistics of China, and the Ethical Committee of Social Science Division of Peking University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL, HL, and BX designed the study. YL acquired the data, performed the statistical analyses, and drafted the manuscript. YL, ZH, HL, HX, HS, YC, YH, and BX revised the manuscript. All authors 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.2022.767240/full#supplementary-material

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Impact of Non-pharmacological Chronic Hypertension on a Transgenic Rat Model of Cerebral Amyloid Angiopathy

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Cerebral amyloid angiopathy (CAA), a common comorbidity of Alzheimer's disease (AD), is a cerebral small vessel disease (CSVD) characterized by deposition of fibrillar amyloid β (Aβ) in blood vessels of the brain and promotes neuroinflammation and vascular cognitive impairment and dementia (VCID). Hypertension, a prominent non-amyloidal CSVD, has been found to increase risk of dementia, but clinical data regarding its effects in CAA patients is controversial. To understand the effects of hypertension on CAA, we bred rTg-DI transgenic rats, a model of CAA, with spontaneously hypertensive, stroke prone (SHR-SP) rats producing bigenic rTg-DI/SHR-SP and non-transgenic SHR-SP littermates. At 7 months (M) of age, cohorts of both rTg-DI/SHR-SP and SHR-SP littermates exhibit elevated systolic blood pressures. However, transgene human amyloid β-protein (Aβ) precursor and Aβ peptide levels, as well as behavioral testing showed no changes between bigenic rTg-DI/SHR-SP and rTg-DI rats. Subsequent cohorts of rats were aged further to 10 M where bigenic rTg-DI/SHR-SP and SHR-SP littermates exhibit elevated systolic and diastolic blood pressures. Vascular amyloid load in hippocampus and thalamus was significantly decreased, whereas pial surface vessel amyloid increased, in bigenic rTg-DI/SHR-SP rats compared to rTg-DI rats suggesting a redistribution of vascular amyloid in bigenic animals. There was activation of both astrocytes and microglia in rTg-DI rats and bigenic rTg-DI/SHR-SP rats not observed in SHR-SP rats indicating that glial activation was likely in response to the presence of vascular amyloid. Thalamic microbleeds were present in both rTg-DI rats and bigenic rTg-DI/SHR-SP rats. Although the number of thalamic small vessel occlusions were not different between rTg-DI and bigenic rTg-DI/SHR-SP rats, a significant difference in occlusion size and distribution in the thalamus was found. Proteomic analysis of cortical tissue indicated that bigenic rTg-DI/SHR-SP rats largely adopt features of the rTg-DI rats with enhancement of certain changes. Our findings indicate that at 10

M of age non-pharmacological hypertension in rTg-DI rats causes a redistribution of vascular amyloid and significantly alters the size and distribution of thalamic occluded vessels. In addition, our findings indicate that bigenic rTg-DI/SHR-SP rats provide a non-pharmacological model to further study hypertension and CAA as co-morbidities for CSVD and VCID.

Keywords: cerebral amyloid angiopathy, hypertension, comorbidity, transgenic rat, cerebral microbleeds

INTRODUCTION

Cerebral small vessel diseases (CSVD) occur in many elderly patients, cause vascular cognitive impairment and dementia (VCID) and are contributors to ischemic and hemorrhagic strokes (Shi and Wardlaw, 2016; Mustapha et al., 2019; Pasi and Cordonnier, 2020). CSVD and VCID are growing concerns as the elderly population increases. Two general classes of CSVD are non-amyloidal CSVD and amyloidal CSVD. Non-amyloidal CSVD include lifestyle induced diseases such as hypertension (Cuadrado-Godia et al., 2018) and arteriolosclerosis (Li et al., 2018), but also hereditary diseases such as cerebral autosomal dominant arteriopathy with sub-cortical infarcts and leukoencephalopathy (CADASIL) (Mancuso et al., 2020). Cerebral amyloid angiopathy (CAA), an example of amyloidal CSVD, is a prevalent condition in the elderly and is a common comorbidity of Alzheimer's disease (AD) (Gilbert and Vinters, 1983; Thal et al., 2002; Viswanathan and Greenberg, 2011; Mehndiratta et al., 2012). CAA is characterized by deposition of fibrillar amyloid β-protein (Aβ) in blood vessels of the brain, including capillaries, arterioles and small arteries of the cortex and meninges (Auriel and Greenberg, 2012; Denver et al., 2019). Patients present with a decline in cognition as CAA contributes to development of VCID (Helman, 2018). CAA affects ≈80% of individuals over the age of 65 years (Boyle et al., 2015) and is present to some extent in nearly all AD patients (Ellis et al., 1996). Though the elderly are mainly affected by sporadic forms (Biffi and Greenberg, 2011), familial forms causing early onset of the disease also exist. Familial forms arise from mutations in the amyloid precursor protein (AβPP) gene that change the Aβ peptide sequence (Levy et al., 1990; Van Broeckhoven et al., 1990; Tagliavini et al., 1999; Grabowski et al., 2001; Biffi and Greenberg, 2011). These changes in the Aβ peptides appear to alter their biophysical properties and enhance fibrillogenesis (Van Nostrand et al., 2001; Melchor et al., 2008).

CAA exists in two types: 1 and 2. CAA type-2 is defined by amyloid deposits within the vessel wall and typically does not elicit a robust perivascular inflammatory response in the absence of amyloid infiltrating the surrounding brain parenchyma (Thal et al., 2002; Attems et al., 2011; Davis et al., 2018). In contrast, CAA type-1 amyloid is found along capillary vessel walls, allowing for interaction with the surrounding parenchymal tissue and induces a strong perivascular neuroinflammatory response (Thal et al., 2002; Attems et al., 2011).

Although CAA is recognized as a cause of intracerebral hemorrhagic strokes (ICH) in normotensive elderly patients (Auriel and Greenberg, 2012), hypertension (HTN), a non-amyloidal form of CSVD, is the most common cause of ICH (Broderick et al., 2020). Nearly 70% of individuals over the age of 65 in the United States are diagnosed with HTN (Mozaffarian et al., 2015), which is a common risk factor for dementia (Welsh et al., 2014). Despite their separate risks for ICH, it remains unclear how CAA and HTN potentially interact to impact ICH and VCID. For example, in a comparison of CAA patients and HTN patients that had ICH it was found that some differences between these two CSVDs exist, but it is still difficult to distinguish the two (Zhan et al., 2004).

Previously, we described the generation and characterization of a new transgenic rat model of CAA type-1, rTg-DI, that faithfully recapitulates many pathological features of the disease in humans (Davis et al., 2018). The rTg-DI model utilizes the expression of human AβPP in brain harboring two familial CAA mutations of Aβ, the Dutch (E22Q) and Iowa (D23N) mutations. rTg-DI rats develop early onset and progressive accumulation of CAA type-1 pathologies. Vascular amyloid first appears at ≈3 months with a subsequent emergence of behavioral deficits, perivascular neuroinflammation, microbleeds, small vessel occlusions and progressive loss of white matter (Zhu et al., 2020; Lee et al., 2021). These findings indicate that rTg-DI rats provide a useful preclinical platform to investigate the pathogenesis of CAA and microbleeds.

On the other hand, the spontaneously hypertensive stroke-prone (SHR-SP) rat is commonly used to investigate HTN and ICH. This particular model was derived by selective in-breeding of animals that present elevated blood pressure and HTN. SHR-SP rats also present with spontaneous strokes (Kimura et al., 2000). The HTN phenotype resulting from this breeding is due to multiple genetic factors that remain undefined; phenotyping rather than genotyping is therefore required. The SHR-SP rat provides a model that spontaneously develops HTN, is a more clinically relevant model and eliminates any undesired and potentially confounding side effects that could be introduced using pharmacological-induced HTN. In the present study, we bred rTg-DI rats with SHR-SP rats to generate a model of emerging CAA on a hypertensive background to investigate how the two distinct CSVDs interact to affect CAA progression and thrombotic vascular events.

MATERIALS AND METHODS

Animals

The goal of our study was to evaluate the impacts of chronic, non-pharmacological HTN on the development of CAA and related pathologies in experimental rats. To accomplish this, we used the CAA transgenic rat line (rTg-DI), which is a model

of early onset and robust cerebral microvascular amyloid as previously described (Davis et al., 2018). rTg-DI rats, generated on a Sprague-Dawley background, express low levels of human Swedish/Dutch/Iowa mutant AβPP under the control of the neuronal-specific Thy1.2 promoter and results in production of chimeric Dutch/Iowa CAA mutant Aβ peptides in the brain. Aβ accumulation and perivascular inflammation begin at ≈3 months of age and increase in a time dependent manner. Microbleeds and small vessel occlusions emerge at ≈6 months of age and become more numerous with advancing age.

For a model of chronic, non-pharmacological HTN, we used Spontaneously Hypertensive—Stroke Prone (SHR-SP) rats that were obtained from Charles River Laboratories (Kingston, NY). The SHR-SP model was derived from the spontaneously hypertensive (SHR) rat by inbreeding (Okamoto and Aoki, 1963). Phenotypes have been found to be autosomal dominant (Gratton et al., 1998) and can be identified by phenotyping rather than genotyping.

In the present study, rTg-DI rats and rTg-DI negative (WT) littermates served as CAA and controls. To obtain HTN CAA rats, SHR-SP rats were bred with heterozygous rTg-DI rats to produce bigenic rTg-DI/SHR-SP and rTg-DI transgene negative SHR-SP offspring. The rTg-DI/SHR-SP rats used in this study were backcrossed for 5 generational breedings with SHR-SP rats resulting in bigenic rats that possessed the human AβPP transgene and were > 95% on the hypertensive SHR-SP background. rTg-DI negative SHR-SP littermates served as HTN controls. Cohorts of each group of rats were aged to 7 or 10 M.

All rats were housed in a controlled room (22 ± 2°C and 40–60% humidity) on a standard 12 h light cycle. Rat chow and water were available *ad libitum*. All work with animals was approved by the University of Rhode Island Institutional Animal Care and Use Committee and in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals and was and in compliance with the ARRIVE guidelines.

Blood Pressure Measurements

Arterial blood pressures were measured using the CODA Non-invasive Blood Pressure System (Kent Scientific, Torrington, CT, United States). Rats were handled and acclimatized to the system for 15 min per day for 5 days before baseline measurements. Before starting, the infrared warming platform was warmed to a constant temperature between 33 and 38°C and maintained for all measurements. Rats were assigned appropriately sized restrainers with nose cones in which excess movement was limited and comfortable respiration was possible. The animals were encouraged to enter the restrainer with minimal guidance. While animals were in the restrainers and placed on the warming platform, tail occlusion cuffs were placed around the caudal region of the tails. The occlusion cuff size was determined based on tail thickness and ability to freely move up and down on the tail. The Volume Pressure Recording Sensor was placed on the tail about 2 mm distal to the occlusion cuff. This sensor was placed so that it could move freely along the tail and inflate enough to impede tail blood flow. Animal body temperatures were measured after 5 min of acclimatization in the restrainer and on the warming platform and ensured to read between 33 and 38°C. Tail temperatures were monitored until 32–35°C was maintained. Once body and tail temperatures were constant, systolic and diastolic blood pressures were measured automatically using the default settings for blood pressure monitoring on the blood pressure system. The averages of systolic and diastolic readings were calculated by the system.

Behavioral Testing

Open Field: To assess general exploration behavior, mobility and health the younger group of rats were subjected to Open Field testing at 7 M. After being habituated to the room for 30 min, animals were placed in the center of a 92 cm^2 field (Stoelting Co., Wood Dale, IL), inside a semi-opaque cylinder and held for 20 s to acclimate to the apparatus. After acclimation, animals were allowed to explore the open field for a total of 5 min. Total distance traveled for each animal was tracked using AnyMaze^{TM} tracking software (Stoelting Co., Wood Dale, IL) and the number of rearings were scored via manual keypress by the experimenter.

Rotarod: the rats were habituated to the test room for 30 min and then pre-trained by first being able to balance on the stationary rod for 10 s and then 60 s on a rod rotating at a speed of 5 rpm. For the testing protocol, the rod accelerated from 5 to 40 rpm in a period of up to 300 s or until the animal fell off the rod. The rats were returned to their home cages between trials for a total of three trials with 15 min ITIs. The mean latency to fall of the three trials is used to compare time spent on the rod.

Unreinforced Radial Arm Maze (URAM): This procedure followed an unrewarded version of the Radial Arm Maze (RAM) task where none of the arms were reinforced. At the start of each trial, rats were placed in the center circle of an 8-armed RAM apparatus (Stoelting Co., Wood Dale, IL). Arm entries were manually recorded by the experimenter and entry was defined as all four paws entered into the shaft of one arm. Trials terminated after 5 min or when the rat had successfully visited each arm of the apparatus once. In this configuration, this task reveals a slowed rate of arm entrances thought to reflect sensory-motor slowing in the rTg-DI rats as previously reported (Popescu et al., 2020).

Brain Collection and Tissue Preparation

Rats were injected with 1 mL/kg of ketamine and 0.5 mL/kg xylazine for deep anesthesia. The chest cavity was opened for intracardial perfusion with 1 M phosphate buffered saline (PBS) containing 0.05% heparin at 20 mL/min perfusion rate for 15 min. Forebrains were removed and cut in half along the mid-sagittal line. The right hemisphere was immediately embedded in Tissue-Plus OCT Compound (Fisher Healthcare, Houston, TX), frozen on dry ice, and stored in −80°C. These tissues were later sectioned on the sagittal plane at 20 μm thickness, mounted on Colorfrost/Plus slides (Thermo Fisher Scientific, Houston, TX) and stored at −80°C. In other cases, brain sections were collected and lysed with radioimmunoprecipitation assay (RIPA) buffer via 12 × 1 s bursts of sonication on ice followed by a 1 h incubation on ice. Samples were then normalized using BCA protein assay Kit (Thermo Fisher Scientific, Houston, TX).

The left hemisphere was immersion -fixed with 4% paraformaldehyde (PFA) for 24 h and then immersed in

30% sucrose in PBS for 48 h for cryoprotection. These tissues were then embedded in OCT Compound and frozen at −80°C. PFA fixed tissue was sagittal cut at 20 μm thickness using the Leica CM 3050S Cryostat (Leica Microsystems Inc., Buffalo Grove, IL), placed in a flotation PBS bath at −16°C, and then mounted on Colorfrost/Plus slides (Thermo Fisher Scientific, Houston, TX) coated using EMS Tissue Capture Pen (Electron Microscopy Sciences, Hatfield, PA) and stored at −80°C.

Immunoblot Quantitation of AβPP

The levels of AβPP in lysed forebrain tissue sections were determined by performing quantitative immunoblotting as described (Davis et al., 2004). Samples were probed with horseradish peroxidase (HRP) labeled-monoclonal antibody P2-1 (specific for human AβPP) (Suzukit et al., 1989) at a concentration of 1:1,000 overnight. HRP-catalyzed chemiluminescent signal was revealed using SuperSignalWest Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL, Thermo Fisher Scientific cat# 34096) and chemiluminescent signal was detected and quantified using an Odyssey Fc imager (LI-COR, Lincoln, NE).

Quantitation of Aβ Peptides

Total Aβ40 and Aβ42 levels were determined by ELISA of guanidine lysates of rat forebrain tissue. In the sandwich ELISAs, Aβ40 and Aβ42 were captured using their respective carboxyl-terminal specific antibodies mAb2G3 and mAb21F12 and biotinylated m3D6, specific for human Aβ, was used for detection (Johnson-Wood et al., 1997; DeMattos et al., 2002). All lysates were measured in triplicates and compared to linear standard curves of known concentrations of human Aβ using a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA).

Immunohistochemical Analysis

Antigen retrieval was performed by treating the tissue sections with proteinase K (0.2 mg/ml) for 10 min at 22°C. Tissue sections were then blocked in Superblock blocking buffer (cat. #37518, Thermo Fisher Scientific, Franklin, MA) containing 0.3% Triton X-100 at room temperature for 30 min and incubated with individual primary antibodies at the following dilutions overnight: rabbit polyclonal antibody to collagen IV to identify cerebral blood vessels (1:200, SD2365885, Invitrogen); goat polyclonal antibodies to glial fibrillary acidic protein (GFAP) to detect astrocytes (1:250, ab53554, Abcam) and ionized calcium-binding adapter molecule 1 (Iba-1) to detect microglia (1:250, NB100-1028, Novus). The primary antibodies were detected with Alexa Fluorescent 594- or 488-conjugated secondary antibodies (1:1,000). Staining for fibrillar amyloid was performed using thioflavin S (123H0598, Sigma-Aldrich). Prussian blue iron staining was performed to detect hemosiderin deposits reflecting signs of previous microbleeds (Gomori, 1936; Davis et al., 2018). Von Kossa calcium staining was used to detect small vessel calcified occlusion in the brain (Rungby et al., 1993; Davis et al., 2018).

Quantitative Measures of Cerebral Vascular Pathologies

The percent area amyloid coverage of cerebral microvessels, the number of microbleeds and the number of occluded/calcified vessels in the cortex, hippocampus and thalamus were determined in the rats using stereological principles as in previously described studies (Long et al., 1998; Davis et al., 2018). Diameters of vessel occlusions and thalamic area occupied by occlusions were measured. Diameters were measured using the "point to point function" and the free hand area function was used to delineate an area around the outermost occlusions in the analyzed section. Vessel occlusion diameters were categorized by size: < 30 μm (small) or > 30 μm (large). The number of occlusions in each size was reported as a percent of the total number of occlusions. The percent area CAA coverage was determined as the percent area of thioflavin stain overlapping with collagen IV stain. All images were collected with the Keyence BZ-X710 Microscope (RRID:SCR_017202) and analyzed with the Keyence BZ-X Analyzer Software Version 1.3.1.1 (Keyence Corp. Osaka, Japan). The number of microbleeds and occluded vessels were measured using with Image J Software Version 1.52a (ImageJ, RRID:SCR_003070, National Institute of Health, United States).

Protein Digest of Cortical Tissue

The cortical region from brain sections of WT, SHR-SP, rTg-DI and bigenic rTg-DI/SHR-SP rats was collected using laser capture microdissection (LCM). Tissue lysis and sample preparation for MS analysis was performed as previously described (Schrader et al., 2021). Briefly, tissue lysis was achieved via sonication in RIPA buffer. 25 μL dithiothreitol (100 mM) was added for protein denaturation with incubation and shaking (300 rpm) at 95°C for 15 min. Proteins were alkylated by incubation in the dark with 25 μL iodoacetamide (200 mM) 30 min at 20°C, and then precipitated and concentrated via chloroform-methanol-water (1:2:1) precipitation. Proteins were resuspended in sodium deoxycholate (DOC) (3% w/v in 50 mM ammonium bicarbonate) and digested with TPCK-treated trypsin (Sciex, Framingham, MA), in a barocycler (Pressure Biosciences Inc, Easton MA) as previously described (Schrader et al., 2021). DOC was precipitated by addition of formic acid and centrifugation as previously described, and the supernatant was collected for LC-MS/MS analysis.

Analysis by LC-QTOF/MS

A SCIEX 5600 TripleTOF mass spectrometer, operated in positive ion mode using a DuoSpray™ ion source (AB Sciex, Concord, Canada), coupled to an Acquity UPLC HClass system (Waters Corp., Milford, MA, United States) for chromatographic separation as was used for all proteomic experiments as previously described (Schrader et al., 2021). An Acquity VanGuard pre-column (2.1 × 5 mm, 300 Å, 1.7 μm) preceding an Acquity UPLC Peptide BEH C18 (2.1 × 150 mm, 300 Å, 1.7 μm) column were used for peptide separation according to the previously described method

(Schrader et al., 2021). TOF calibration was monitored by injection of trypsin-digested β-galactosidase every 4 samples. Analyst TF 1.7.1 software (AB Sciex, Concord, Canada) in data-independent acquisition (DIA) mode was used for data acquisition. All mass spec settings were exactly as previously described (Schrader et al., 2021).

Data Processing

Spectronaut (Biognosys, Schlieren, Switzerland), referencing our previously formed spectral library (Schrader et al., 2021) was used for all protein identification and quantification. Factory defaults were used for all Spectronaut settings, except "used Biognosys' iRT kit" and "PTM localization" were deselected. Spectronaut protein intensities were converted to molar concentrations (pmol/mg brain tissue) using the total protein approach (TPA) (Wiśniewski and Rakus, 2014). A baseline concentration (0.03 pmol/mg tissue) was imputed for concentrations of 0 in individual samples as previously described (Schrader et al., 2021). Threshold cutoffs of $\geq$ 50% increase or $\geq$ 30% decrease to identify differentially expressed proteins, and Student's *t*-test was used to determine statistical significance ($P \leq 0.05$) as previously described (Schrader et al., 2021).

Statistical Analysis

For blood pressure readings, ordinary one- way ANOVA, followed by an uncorrected Fisher's test LSD, with single pooled variance compared the means of each group. For behavioral data, it was noted that there was large and differing variance in the groups, therefore Welch's correction was used for ANOVA and individual *t*-test. Unpaired two tailed *t*-tests were completed for ELISAs of total Aβ and for CAA load. For number of occluded vessel and number of microbleeds, it was observed that the data was not normally distributed, therefore a non-parametric was indicated. For these data, non- parametric one-way ANOVA Kruskal Wallis test followed by Dunn's multiple comparisons test was completed. Unpaired, one tailed, parametric *t*-tests were used to compare percentages of vessel occlusions within the listed size ranges. A non-parametric, two tailed Mann-Whitney test was employed to compare percent area coverage of vessel occlusions in thalami. GraphPad Prism Version 9.1.2 was used for all statistical analyses.

RESULTS

Hypertension Phenotype Is Preserved in 7 Month Bigenic Rat Model of Cerebral Amyloid Angiopathy/Spontaneously Hypertensive, Stroke Prone Rats

Groups of rTg-DI, SHR-SP, bigenic rTg-DI/SHR-SP and WT rats ($n = 7$) were aged to 7 months (M). Systolic and diastolic blood pressure readings were acquired for each rat of the four strains as shown in **Figure 1**. Data analysis for systolic blood pressures was completed with one-way ANOVA having no matching or pairing with single pooled variance, comparing means of each group and an uncorrected Fisher's test LSD ($F = 5.418$, $p = 0.0041$, $R^2 = 0.3440$). Systolic blood pressure readings were significantly increased in SHR-SP and bigenic rTg-DI/SHR-SP rats compared to control groups of WT and rTg-DI rats. Systolic blood pressures of SHR-SP animals were significantly different from those of WT and rTg-DI animals ($P < 0.01$ and $P < 0.05$, respectively). Bigenic rTg-DI/SHR-SP systolic blood pressures were also increased compared to WT and rTg-DI ($P < 0.005$ and $P < 0.01$, respectively) as indicated by one-way ANOVA. Data analysis for diastolic blood pressures was also completed with one-way ANOVA having no matching or pairing with single pooled variance, comparing means of each group and an uncorrected Fisher's test LSD ($F = 1.454$, $p = 0.2456$, $R^2 = 0.1199$). Differences in diastolic blood pressures were only observed between rTg-DI/SHR-SP and WT animals ($P < 0.05$). These results indicate that systolic blood pressure is increased in SHR-SP animals and that this increase is maintained with addition of the rTg-DI transgene on the SHR-SP background.

Spontaneously Hypertensive, Stroke Prone Background Does Not Influence Human AβPP Transgene Expression

The levels of AβPP expression in rTg-DI and bigenic rTg-DI/SHR-SP rats were measured using quantitative immunoblotting (**Figure 2**). Unpaired *t*-test shows that there are no differences in human AβPP protein levels between rTg-DI and bigenic rTg-DI/SHR-SP animals. This indicates

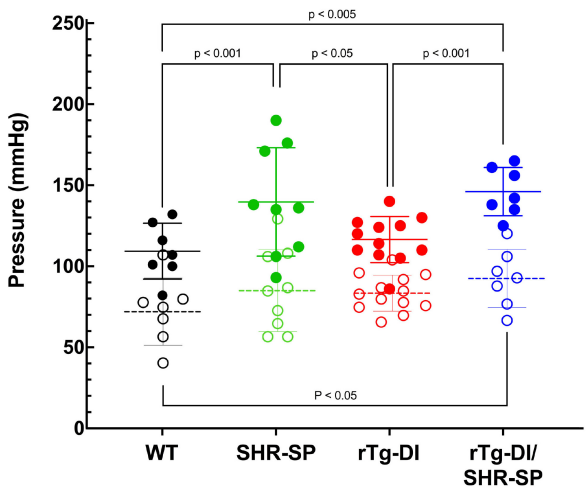

FIGURE 1 | Bigenic rTg-DI rats exhibit elevated blood systolic pressure at 7 M of age. Systolic (closed circles) and diastolic (open circles) blood pressure readings were measured in WT (black; $n = 7$), SHR-SP (green; $n = 9$), rTg-DI (red; $n = 12$), and bigenic rTg-DI/SHR-SP (blue; $n = 7$) rats at 7 M of age. Mean pressures are represented by solid or hashed horizontal lines ± SD. The systolic blood pressures of SHR-SP and rTg-DI/SHR-SP rats are significantly increased from those of WT and rTgDI rats. One-way ANOVA shows $P < 0.001$ between SHR-SP and WT, $P < 0.05$ between SHR-SP and rTg-DI, $P < 0.005$ between bigenic rTg-DI/SHR-SP and WT, and $P < 0.001$ between bigenic rTg-DI/SHR-SP and rTg-DI. These results indicate that increased systolic blood pressure is preserved with addition of the rTg-DI transgene on the SHR-SP background.

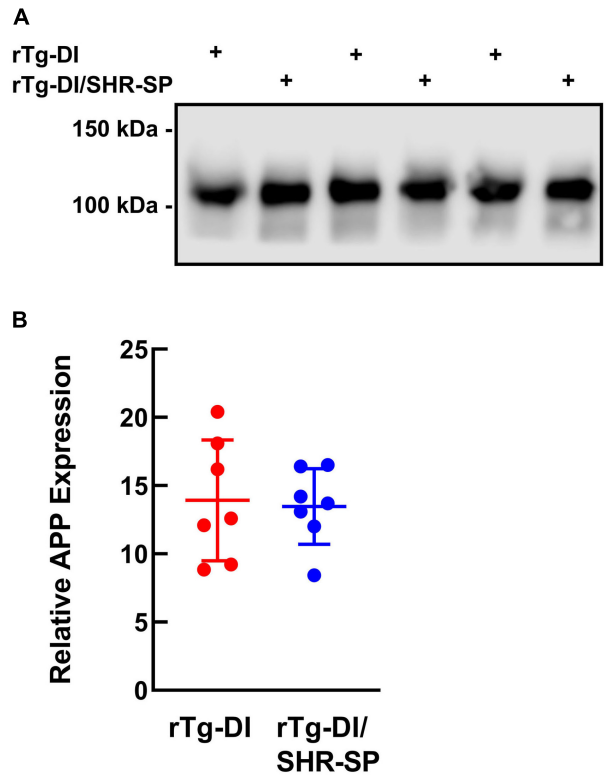

FIGURE 2 | Equivalent transgenic human AβPP expression in rTg-DI and bigenic rTg-DI/SHR-SP rats. **(A)** Representative immunoblots of human AβPP expression in total brain homogenates from 7M rTg-DI rats and bigenic rTg-DI/SHR-SP rats. **(B)** Quantitative immunoblotting was performed to measure human AβPP in brain homogenates of rTg-DI rats (red circles) and bigenic rTg-DI/SHR-SP rats (blue circles). The data presented are the means ± S.D. of $n = 7$ rats per each group. Unpaired t-test showed no differences in human AβPP expression in 7 M rTg-DI and bigenic rTg-DI/SHR-SP animals.

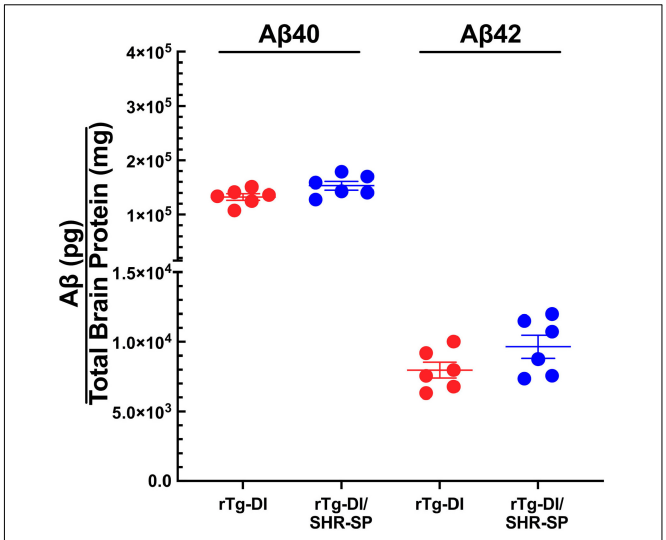

FIGURE 3 | Equivalent Aβ levels in 7 M rTg-DI and bigenic rTg-DI/SHR-SP rats. The levels of total Aβ40 and Aβ42 peptides in the forebrains of rTg-DI rats (red circles) and bigenic rTg-DI/SHR-SP rats (blue circles) were measured by ELISA as described under section "Materials and Methods." The data presented are the means ± S.D. of $n = 7$ rats per group. Unpaired t-test showed that there are no significant differences between Aβ40 and Aβ42 levels in rTg-DI and bigenic rTg-DI/SHR-SP animals.

equivalent expression of AβPP in both rTg-DI and bigenic rTg-DI/SHR-SP rats and preservation of the rTg-DI phenotype on the SHR-SP background.

Hypertension Phenotype Does Not Affect Brain Accumulation of Ab40 and Ab42 in 7 M Bigneic Rat Model of Cerebral Amyloid Angiopathy/Spontaneously Hypertensive, Stroke Prone Rats

We next measured the levels of total Aβ40 and total Aβ42 in rTg-DI and bigenic rTg-DI/SHR-SP rats using specific ELISA assays. Analysis of the data presented in **Figure 3** using unpaired, two tailed t-test showed that there were no differences between total Aβ40 and Aβ42 levels in rTg-DI and bigenic rTg-DI/SHR-SP animals. These findings indicate that the hypertensive SHR-SP background does not influence the accumulation of Aβ peptides in the rTg-DI model at 7 M of age.

Hypertension Phenotype Does Not Influence Behavioral Deficits in Bigenic Rat Model of Cerebral Amyloid Angiopathy/Spontaneously Hypertensive, Stroke Prone Rats at 7 M

rTg-DI rats were previously shown to exhibit a "sensory-motor slowing" phenotype with the onset of microvascular amyloid accumulation (Popescu et al., 2020). This phenotype was seen by comparing the 7 M WT vs. rTg-DI animal rearing measures in the open field (**Figure 4A**) and is emerging in the open field distance traveled (**Figure 4B**) and in the number of arm entries measure in the unreinforced radial arm maze (**Figure 4C**). No significant differences in distance traveled in open field or number of rears were seen between rTg-DI and bigenic rTg-DI/SHR-SP rats in any of the behavioral measures, indicating that most of the difference between the bigenic rats and SHR-SP rats is contributed by the rTg-DI genotype. All groups also performed similarly in rotarod task, indicating that differences in other assays were not due to motor impairment (data not shown). These findings indicate that non-pharmacological, chronic HTN does not alter the behavioral deficits observed in 7 M old rTg-DI rats.

Hypertension Phenotype Is Preserved in 10 M Bigenic Rat Model of Cerebral Amyloid Angiopathy/Spontaneously Hypertensive, Stroke Prone Rats

Since no profound changes were observed in 7 M bigenic rTg-DI/SHR-SP rats, a second cohort of rTg-DI, SHR-SP, and

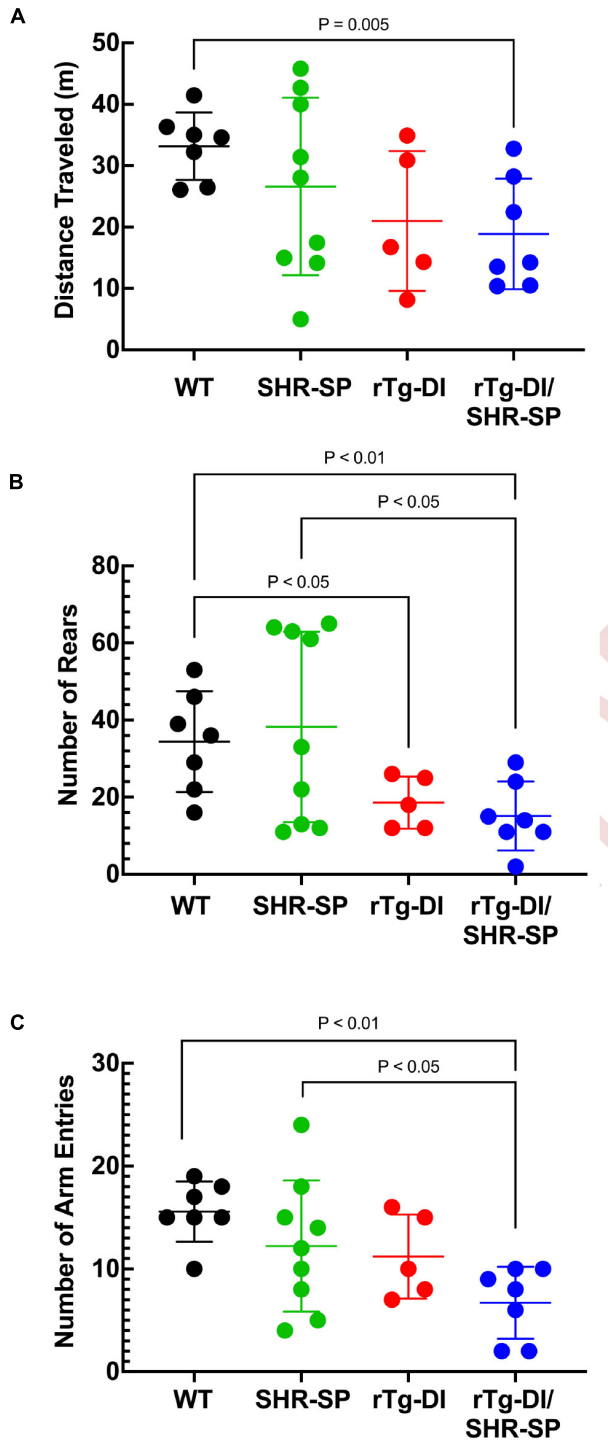

FIGURE 4 | Behavioral deficits of 7 M rTg-DI and bigenic rTg-DI/SHR-SP rats. Bigenic rTg-DI/SHR-SP rats were not significantly different than rTg-DI rats in the number of rears **(A)** or distance traveled **(B)** in the open field. Similarly, no significant differences were found between rTg-DI and bigenic rTg-DI/SHR-SP animals in the number of arm entries **(C)** in Unreinforced Radial Arm Maze (URAM). WT rats (black; $n = 7$), SHR-SP (green; $n = 9$), rTg-DI (red; $n = 5$), and bigenic rTg-DI/SHR-SP (blue; $n = 7$). Data presented are the mean ± SD. These data show that the hypertensive SHR-SP background does not significantly impact behavioral deficits of rTg-DI rats at 7 M of age.

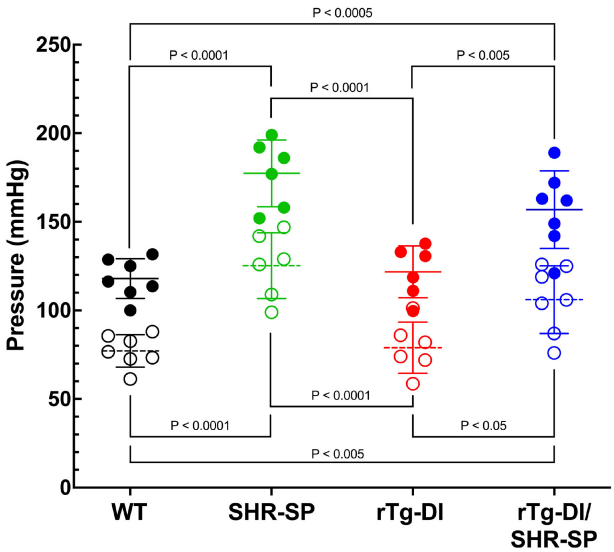

FIGURE 5 | Bigenic rTg-DI rats exhibit elevated systolic and diastolic blood pressure at 10 M of age. Systolic (closed circles) and diastolic (open circles) blood pressure readings were measured in WT (black; $n = 7$), SHR-SP (green; $n = 6$), rTg-DI (red; $n = 6$), and bigenic rTg-DI/SHR-SP (blue; $n = 7$) rats at 10 M. Mean pressures are represented by solid or hashed horizontal lines ± SD. The systolic blood pressures of SHR-SP and bigenic rTg-DI/SHR-SP rats are significantly increased from those of WT and rTg-DI rats. It is shown by one-way ANOVA that $P < 0.0001$ for SHR-SP compared to both WT and rTg-DI, $P < 0.0005$ for bigenic rTg-DI/SHR-SP compared to WT and $P < 0.005$ compared to rTg-DI. The same differences between groups are shown in diastolic pressures by one-way ANOVA. Comparisons of SHR-SP to WT and rTg-DI both are $P < 0.0001$. Diastolic pressures of bigenic rTg-DI/SHR-SP compared to those of WT and rTg-DI are $P < 0.005$ and $P < 0.05$, respectively. These results indicate that the increase in both systolic and diastolic blood pressures is maintained with addition of the rTg-DI transgene on the SHR-SP background.

bigenic rTg-DI/SHR-SP and WT rats were aged further to 10 M. Systolic and diastolic blood pressure readings were acquired for each rat of the four strains as shown in **Figure 5**. Data analysis for systolic blood pressures was completed with one-way ANOVA having no matching or pairing with single pooled variance, comparing means of each group and an uncorrected Fisher's test LSD ($F = 17.51$, $P < 0.0001$, $R^2 = 0.7048$). Systolic blood pressure readings were significantly increased in SHR-SP and bigenic rTg-DI/SHR-SP rats compared to control groups of WT and rTg-DI rats. Systolic blood pressures of SHR-SP animals were significantly different from those of WT and rTg-DI animals (both comparisons $P < 0.0001$). Bigenic rTg-DI/SHR-SP systolic blood pressures were similarly increased compared to WT and rTg-DI ($P < 0.0005$ and $P < 0.005$, respectively). Data analysis for diastolic blood pressures was completed in the same manner by one-way ANOVA having no matching or pairing with single pooled variance, comparing means of each group and an uncorrected Fisher's test LSD ($F = 13.53$, $P = < 0.0001$, $R^2 = 0.6485$). Similar to the systolic readings, diastolic blood pressure readings were significantly increased in SHR-SP and rTg-DI/SHR-SP rats compared to both control groups WT and rTg-DI. Diastolic pressures of SHR-SP animals were significantly

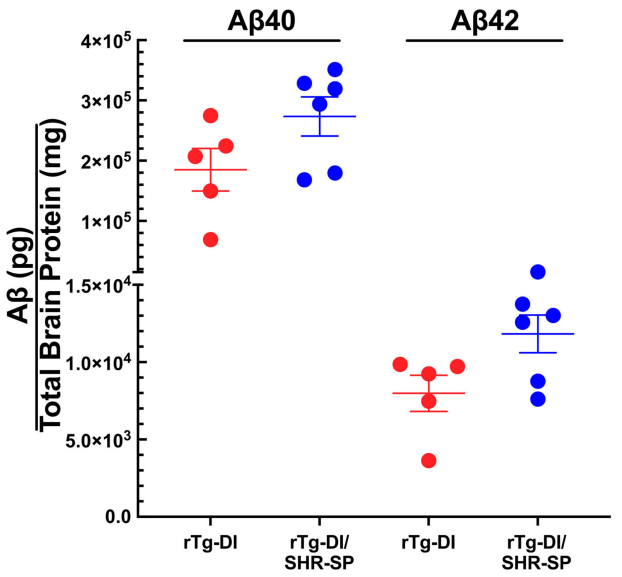

FIGURE 6 | Similar Aβ levels in 10 M rTg-DI and bigenic rTg-DI/SHR-SP rats. The levels of total Aβ40 and Aβ42 peptides in the forebrains of rTg-DI rats (red circles) and bigenic rTg-DI/SHR-SP rats (blue circles) were measured by ELISA as described under section "Materials and Methods." The data presented are the means ± S.D. of $n = 5$ for rTg-DI rats and $n = 6$ for bigenic rTg-DI/SHR-SP rats. Unpaired *t*-test showed that there are no significant differences between Aβ40 and Aβ42 levels in rTg-DI and rTg-DI/SHR-SP animals.

increased from those of WT and rTg-DI animals ($P < 0.0001$ for both comparisons). Bigenic rTg-DI/SHR-SP diastolic blood pressures were also increased compared to WT and rTg-DI by ($p < 0.0005$ and $p < 0.05$, respectively). No differences were observed between SHR-SP and bigenic animals in either blood pressure reading. These results indicate that systolic and diastolic blood pressures are both increased in 10 M SHR-SP animals and that this increase is maintained with addition of the rTg-DI transgene in the bigenic animals.

Hypertension Phenotype Does Not Influence the Brain Accumulation of Aβ40 and Ab42 in 10 M Bigenic Rat Model of Cerebral Amyloid Angiopathy/Spontaneously Hypertensive, Stroke Prone Rats

We next measured the levels of total Aβ40 and total Aβ42 in 10 M rTg-DI and bigenic rTg-DI/SHR-SP rats using previously described ELISA assays. Analysis of the data presented in **Figure 6** using unpaired, two tailed *t*-test shows that, similar to the 7 M old rats, there were no differences between total Aβ40 and Aβ42 levels in rTg-DI and bigenic rTg-DI/SHR-SP animals. These findings indicate that the hypertensive SHR-SP background does not influence accumulation of Aβ peptides in the rTg-DI rats as they age further to 10 M.

Hypertension Phenotype Alters the Distribution of Cerebral Amyloid Angiopathy in 10 M Bigenic Rat Model of Cerebral Amyloid Angiopathy/Spontaneously Hypertensive, Stroke Prone Rats

Although the SHR-SP background does not significantly alter the expression of human AβPP or the accumulation of Aβ peptides, we next evaluated if the hypertensive background influences the amount or distribution of vascular amyloid in 10 M rTg-DI rats. **Figure 7** shows representative images of thioflavin S-positive cerebral microvascular amyloid present in the cortex, hippocampus and thalamus, regions that were previously shown to accumulate robust levels of CAA type-1 (Davis et al., 2004; Zhu et al., 2020). In addition, representative images of surface pial vessels are also shown. The percent area of vessel coverage with amyloid in each area was measured for each rat. Unpaired, two tailed *t*-tests were completed for rTg-DI ($n = 6$) and bigenic rTg-DI/SHR-SP ($n = 7$) in each area of interest: cortex, hippocampus, thalamus, and surface pial vessels (**Figure 7I**). There were significant reductions ($P < 0.05$) in the amount of microvascular amyloid in the hippocampus and thalamus of rTg-DI/SHR-SP rats compared to rTg-DI rats. On the other hand, surface pial vessel coverage was significantly increased ($P < 0.05$) in bigenic rTg-DI/SHR-SP compared to rTg-DI. No thioflavin S-positive CAA was observed in any of the WT or SHR-SP rats (data not shown). These findings suggest that at 10 M of age the hypertensive SHR-SP background causes changes in the amount of vascular amyloid in the hippocampus, thalamus, and surface pial vessels.

Hypertension Phenotype Does Not Alter Thalamic Glial Activation in 10 M Bigenic Rat Model of Cerebral Amyloid Angiopathy/Spontaneously Hypertensive, Stroke Prone Rats

In rTg-DI rats, the presence of cerebral microvascular amyloid drives strong neuroinflammation indicated by a marked elevation of reactive perivascular glial cells (Davis et al., 2018; Zhu et al., 2020; Schrader et al., 2021). To determine if the SHR-SP hypertensive background influences this response in rTg-DI rats we performed immunolabeling studies for astrocytes and microglia. **Figure 8** shows that both rTg-DI rats and bigenic rTg-DI/SHR-SP rats exhibit a robust increase in thalamic astrocytes compared to WT rats and SHR-SP rats (**Figures 8A–D**). Similarly, both rTg-DI rats and bigenic rTg-DI/SHR-SP rats showed an increase in thalamic microglia compared to WT rats and SHR-SP rats (**Figures 8E–H**). It is also noteworthy that the microglia adopt an activated morphology in the rTg-DI rats and bigenic rTg-DI/SHR-SP rats whereas in the WT rats and SHR-SP rats the microglia exhibit a resting surveillance state with numerous extended processes. Together, these findings indicate that the robust neuroinflammation in response to microvascular

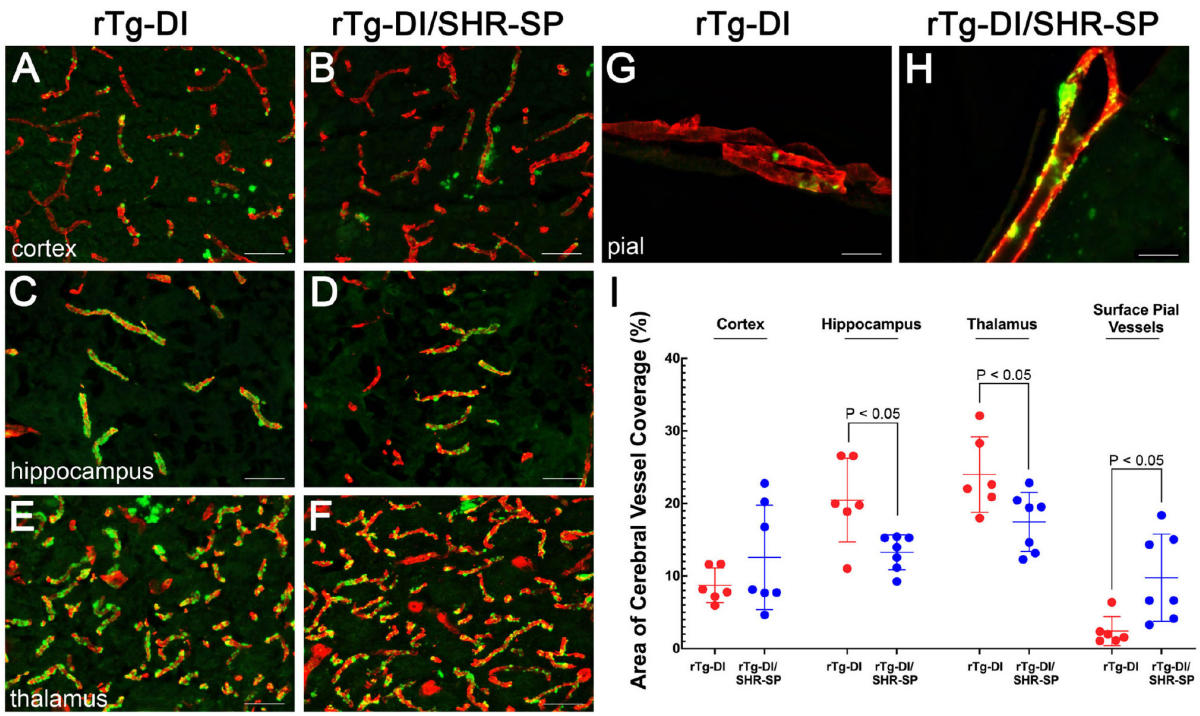

FIGURE 7 | CAA loads in 10 M rTg-DI rats and bigenic rTg-DI/SHR-SP rats. Representative images of brain sections from 10 M old rTg-DI rats **(A,C,E,G)** and bigenic rTg-DI/SHR-SP rats **(B,D,F,H)** were stained for fibrillar amyloid using thioflavin-S (green) and immunolabeled for collagen type IV to identify cerebral vessels (red). Scale bars = 50 μM. **(I)** Quantitation of thioflavin-S positive vascular amyloid load in different brain regions of rTg-DI rats (red circles) and bigenic rTg-DI/SHR-SP rats (blue circles). Data shown are mean ± S.D of n = 6–7 rats per group. Unpaired, two tailed t-tests show a significant decrease in CAA load in the hippocampus and thalamus of bigenic rTg-DI/SHR-SP rats compared to that of rTg-DI, both comparisons with p-values of $P < 0.05$. Contrarily, surface pial vessel coverage is significantly increased bigenic rTg-DI/SHR-SP rats compared to rTg-DI where $P < 0.05$. These data indicate a change in CAA load in when introducing the rTg-DI transgene onto the SHR-SP hypertensive background.

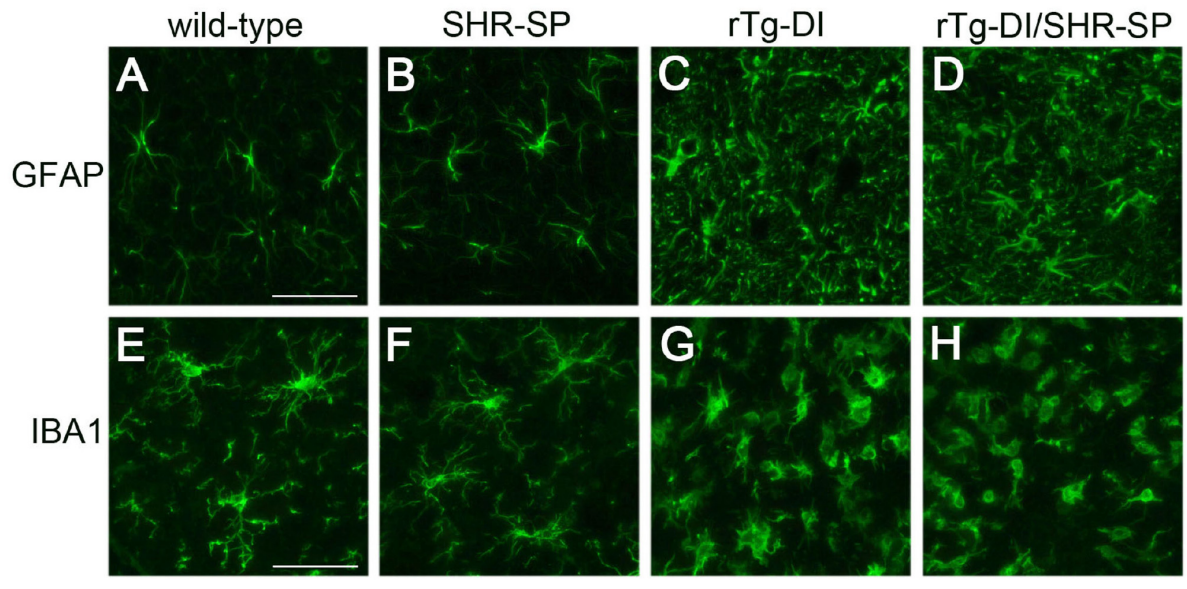

FIGURE 8 | Increased thalamic activated glial cells in 10 M rTg-DI rats and bigenic rTg-DI/SHR-SP rats. Representative images of the thalamus from rat brain sections immunolabeled for GFAP to identify astrocytes (upper panels) and IBA-1 to identify microglia (lower panels). WT rats **(A,E)**, SHR-SP rats **(B,F)**, rTg-DI rats **(C,G)**, and bigenic rTg-DI/SHR-SP rats **(D,H)**. Scale bars = 50 μm. Both rTg-DI and bigenic rTg-DI/SHR-SP rats exhibit increased numbers and activation of glial cells in the thalamus.

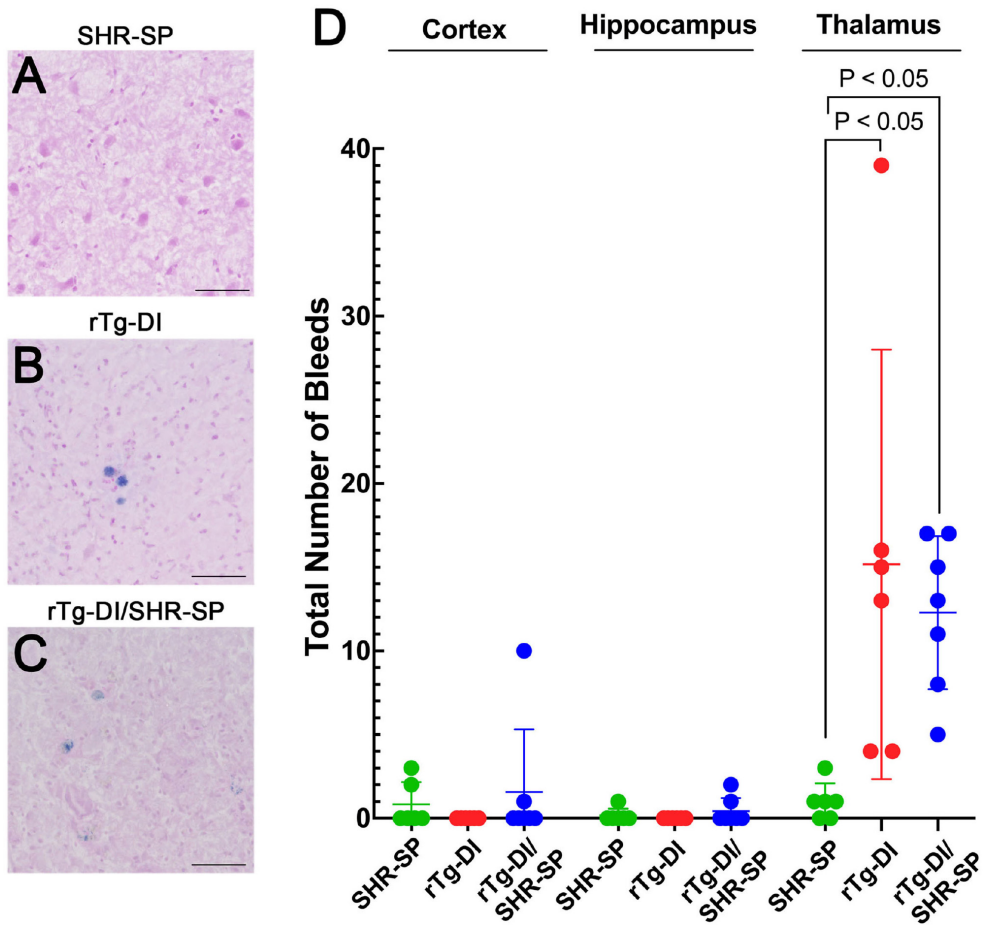

FIGURE 9 | Cerebral microbleeds in 10 M rats. Representative images of the thalamic region of 10 M old SHR-SP **(A)**, rTg-DI **(B)**, and bigenic rTg-DI/SHR-SP **(C)** stained with hemosiderin to identify cerebral microbleeds. Scale bars = 50 μm. **(D)** Number of microbleeds in different brain regions are shown in SHR-SP (n = 6) in green, rTg-DI (n = 6) in red, and rTg-DI/SHR-SP (n = 7) in blue. Data shown are means ± S.D. Significant differences are shown by one-way ANOVA between rTg-DI and SHR-SP ($P < 0.05$) and between bigenic rTg-DI/SHR-SP and SHR-SP ($P < 0.05$). These differences indicate a preservation of the microbleeding pattern observed in rTg-DI in bigenic rTg-DI/SHR-SP animals.

amyloid in rTg-DI rats is preserved in the bigenic rTg-DI/SHR-SP rats. Further, these findings show that glial activation is not appreciably observed with HTN alone in the SHR-SP rats.

Hypertension Phenotype Does Not Influence the Number of Microbleeds in the Brains of 10 M Bigenic Rat Model of Cerebral Amyloid Angiopathy/Spontaneously Hypertensive, Stroke Prone Rats

Thalamic microbleeds are a prominent pathological feature of rTg-DI rats that emerge at ≈6 M of age (Davis et al., 2018). Cerebral microbleeds also occur in different brain regions of aged SHR-SP rats (Schreiber et al., 2012, 2013). Therefore, we evaluated the presence of cerebral microbleeds in the cortex, hippocampus and thalamus of all rats by performing hemosiderin staining. **Figures 9A–C** shows representative images of microbleeds in the thalamic region of the rats. WT rats show no evidence of microbleeds in the thalamus or any other region (data not shown). Quantitation of microbleeds in the different brain regions of the rats was performed. Kruskal Wallis test of means comparison of each positive group followed by a Dunn's test was completed for the cortex, hippocampus and thalamus to compare the total number of bleeds in SHR-SP (n = 6), rTg-DI (n = 6), and rTg-DI/SHR-SP (n = 7) (**Figure 9D**) revealing several findings. First, in the cortex several SHR-SP and bigenic rTg-DI/SHR-SP rats showed some cerebral microbleeds whereas the rTg-DI rats did not show any. The same trend is observed for microbleeds in the hippocampus. Lastly, the thalamus is most affected with numerous microbleeds in rTg-DI and rTg-DI/SHR-SP while SHR-SP show lower levels similar to those observed in the cortex. The Kruskal-Wallis test showed significance with (P = 0.0004) and a Kruskal-Wallis statistic value of 11.80. Differences were found between number of bleeds in the thalamus of SHR-SP and rTg-DI ($P < 0.05$) and SHR-SP and rTg-DI/SHR-SP ($P < 0.05$). These results indicate the number of microbleeds in thalamus

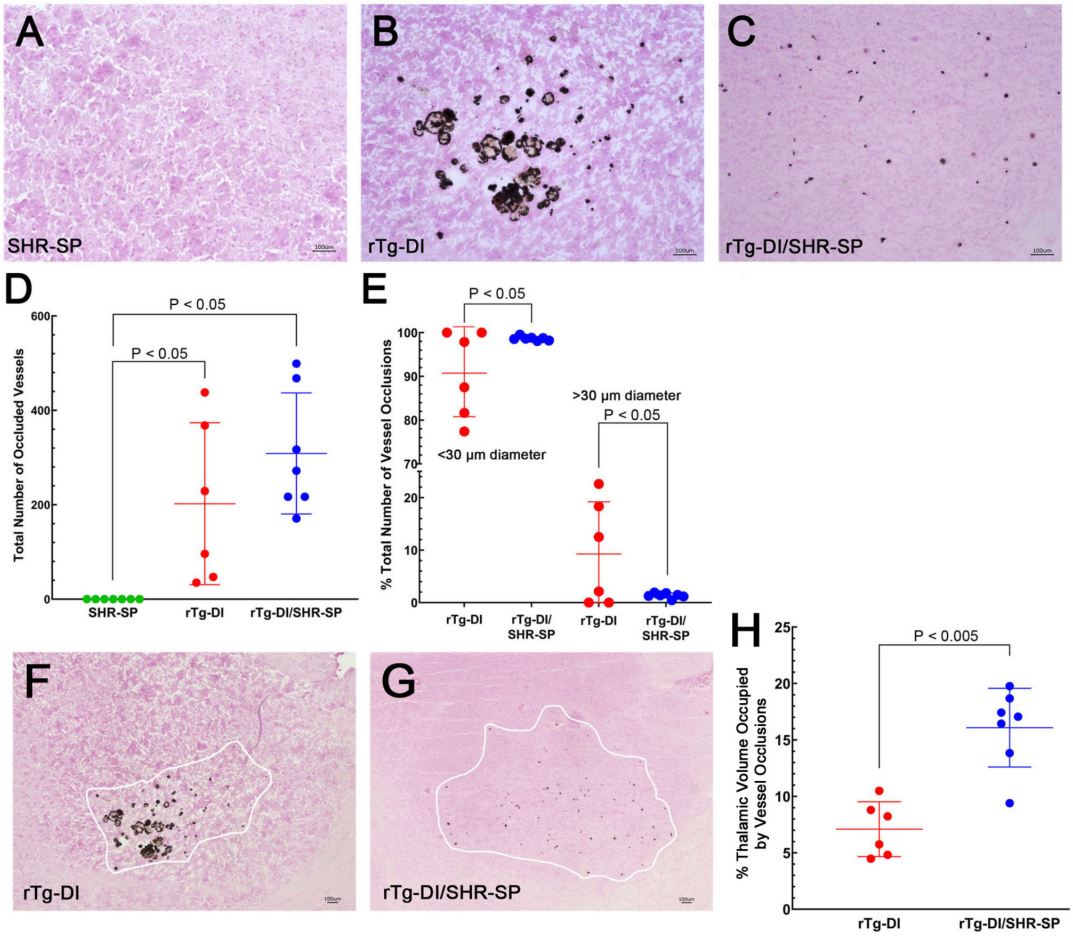

FIGURE 10 | Thalamic small vessel occlusions in 10 M rats. Representative images of 10 M brain sections of SHR-SP **(A)**, rTg-DI **(B)**, and bigenic rTg-DI/SHR-SP **(C)** rats showing thalamic calcified, occluded vessels. Scale bars = 100 μm. **(D)** Total number of thalamic occluded vessels in SHR-SP (n = 7) in green, rTg-DI (n = 6) in red and bigenic rTg-DI/SHR-SP (n = 7) in blue **(D)**. Data presented are the means ± S.D. Significant differences were found by one-way ANOVA between SHR-SP and rTg-DI ($P < 0.05$) and between SHR-SP and bigenic rTg-DI/SHR-SP ($P < 0.05$). **(E)** The percentage of total thalamic vessel occlusions < or > 30 μm in diameter in rTg-DI (n = 6; red) and bigenic rTg-DI/SHR-SP (n = 7; blue). Data presented are the means ± S.D. Unpaired t-test shows an increase in the percentage of thalamic vessel occlusion diameters < 30μm or > 30 μm in bigenic rTg-DI/SHR-SP compared to rTg-DI ($P < 0.05$). Lower magnification representative images of 10 M brain sections of rTg-DI **(F)** and bigenic rTg-DI/SHR-SP **(G)** rats showing the range of thalamic calcified, occluded vessels. White tracings depict the borders of small vessel occlusions in thalamus. Scale bars = 100 μm. **(H)** The percent thalamic volume presenting with small vessel occlusions in rTg-DI (n = 6) in red and bigenic rTg-DI/SHR-SP (n = 7) in blue. Data presented are the means ± S.D. Unpaired t-test shows a significant increase in thalamic volume with small vessel occlusions in bigenic rTg-DI/SHR-SP rats compared to rTg-DI rats ($P < 0.005$). Together, these data indicate that the number of thalamic vessel occlusions does not change with the introduction of the rTg-DI transgene onto the SHR-SP background, although there is a change in size and extent of spatial distribution of these vessel occlusions in the thalamus.

of rTg-DI and rTg-DI/SHR-SP are similar and higher when compared to SHR-SP rats. Thus, there is a preservation of rTg-DI thalamocentric pattern of microbleeds with addition to the hypertensive SHR-SP background.

Hypertension Phenotype Impacts Thalamic Small Vessel Occlusions in 10 M Bigenic Rat Model of Cerebral Amyloid Angiopathy/Spontaneously Hypertensive, Stroke Prone Rats

In the rTg-DI model, calcified small vessel occlusions emerge at ≈6 M and increase with further aging (Davis et al., 2018). We next determined if the hypertensive SHR-SP background impacts the presence and numbers of these small vessel occlusions. **Figure 10** shows representative images of calcified, occluded small vessels in the thalamic region of the different rats (**Figures 10A–C**). Occluded vessels were observed only in the thalamus of rTg-DI and bigenic rTg-DI/SHR-SP rats. Kruskal Wallis test of means was completed for the total number of occluded vessels in the thalamus of SHR-SP, rTg-DI, and bigenic rTg-DI/SHR-SP (**Figure 10D**). Significant differences were found between SHR-SP rats and rTg-DI and bigenic rTg-DI/SHR-SP ($P < 0.05$) since SHR-SP itself showed no vessel occlusions. Though the total number of occlusions did not differ between rTg-DI and bigenic rTg-DI/SHR-SP animals, a qualitative difference was

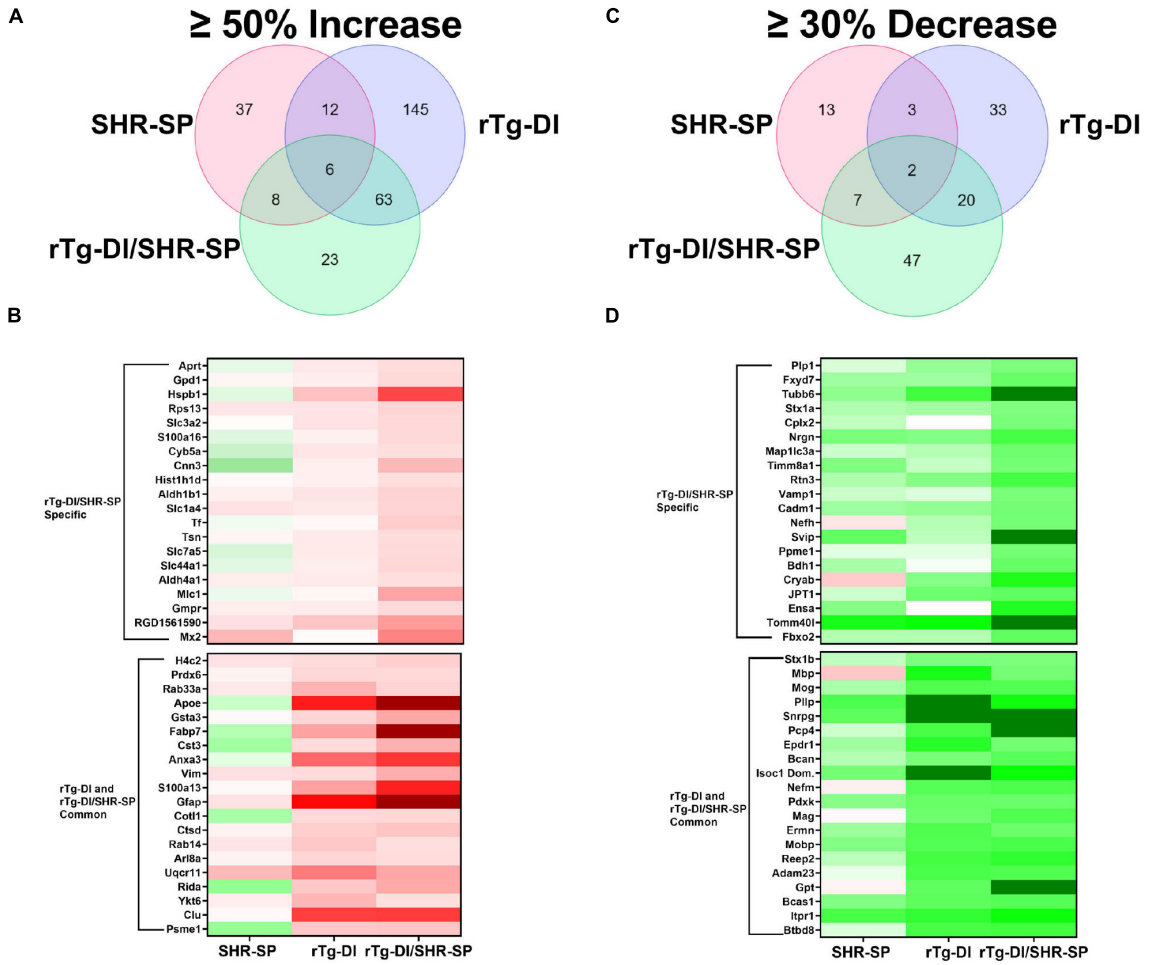

FIGURE 11 | Comparison of significantly enhanced and reduced proteins in the 10 M SHR-SP, rTg-DI and bigenic rTg-DI/SHR-SP cortex. **(A)** Venn diagram comparing significantly ($P < 0.05$) enhanced proteins by ≥ 50% of the WT concentration in the cortex of 10 M SHR-SP, rTg-DI and bigenic rTg-DI/SHR-SP rats ($n = 5, 6$). **(B)** Heat map depicting relative expression of most abundantly enhanced cortical proteins in the bigenic rTg-DI/SHR-SP only (top) or common to both rTg-DI and bigenic rTg-DI/SHR-SP, along with the corresponding expression in the SHR-SP cortex, with red indicating enhanced expression, green representing reduced expression, and color intensity representing the degree of expression change. **(C)** Venn diagram comparing significantly ($P < 0.05$) reduced proteins by ≥ 30% of the WT concentration in the cortex of 10 M SHR-SP, rTg-DI and bigenic rTg-DI/SHR-SP rats ($n = 5, 6$). **(D)** Heat map depicting relative expression of most reduced proteins arranged as in **(B)** with color shading as in **(B)**.

observed in the sizes and spatial distribution of the occlusions (**Figures 10B,C**). We next measured the diameters of occlusions and sorted them based on size. The number of small and large occlusions are shown as percentage of total number of vessel occlusions in the thalamus (**Figure 10E**). Unpaired *t*-test shows an increase in the percentage of small vessel occlusions in bigenic rTg-DI/SHR-SP thalamus compared to rTg-DI thalamus ($P < 0.05$). On the other hand, a significant increase was found in the percentage of larger occlusions in the thalamus of rTg-DI rats compared to bigenic rTg-DI/SHR-SP rats ($P < 0.05$). A difference was also observed in the percent of thalamic volume that presented small vessel occlusions between rTg-DI and bigenic rTg-DI/SHR-SP rats by two-tailed, unpaired parametric *t*-test ($P < 0.005$) (**Figures 10F–H**). These findings show that even though the total number of small vessel occlusions did not differ between rTg-DI rats and bigenic rTg-DI/SHR-SP rats the latter exhibited primarily small occlusions that were more broadly distributed in the thalamus.

Elevated Cortical Proteins in Spontaneously Hypertensive, Stroke Prone, Rat Model of Cerebral Amyloid Angiopathy and Bigenic Rat Model of Cerebral Amyloid Angiopathy/Spontaneously Hypertensive, Stroke Prone

To further understand the impact of the SHR-SP hypertensive phenotype on the pathology of the rTg-DI rats we conducted proteomic analysis of cortical tissue from WT, SHR-SP, rTg-DI, and bigenic rTg-DI/SHR-SP rats via Sequential

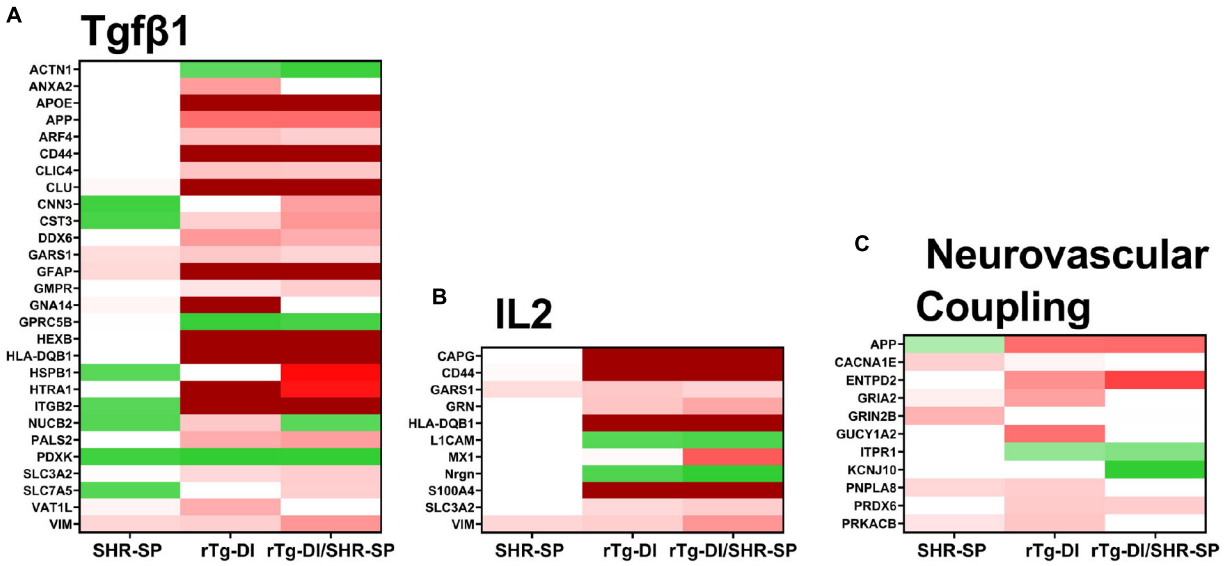

FIGURE 12 | IPA identified upstream regulators, causal networks, and disease functions. **(A)** Heat map depicting the differentially expressed proteins (≥ 50% increase or ≥ 30% decrease, $P < 0.05$) in the SHR-SP, rTg-DI, and bigenic rTg-DI/SHR-SP cortex associated with the upstream regulator Tgfβ1 **(A)**, Interleukin 2 causal network **(B)** and the pathway neurovascular coupling **(C)**. Red indicates increased, green indicates decreased, and white indicates not differentially expressed proteins, and color intensity correlates with degree of change.

Acquisition of all Theoretical Mass Spectra (SWATH-MS), a data independent acquisition (DIA) protein mass spectrometry approach, as previously described (Schrader et al., 2021) and in section "Materials and Methods." Protein identification and quantification from DIA data was performed using Spectronaut (Biognosys), referencing a previously compiled spectral library (Schrader et al., 2021). Protein intensities from Spectronaut were converted to molar concentrations using the "total protein approach" (TPA) (Wiśniewski and Rakus, 2014). Differentially expressed proteins were then determined by comparison of molar concentrations (pmol/mg total protein) with the corresponding WT concentrations. As previously reported, multiple testing corrected false discovery rates (FDR) are often too restrictive in small *n* proteomics studies (Pascovici et al., 2016; Hondius et al., 2018), and thus we used threshold cutoffs with uncorrected *P*-values to manage the FDR as before (Schrader et al., 2021). Significantly increased proteins were defined as ≥ 50% increase with $P \leq 0.05$. From this analysis 63, 226, and 100 proteins were found significantly elevated in the cortex of the SHR-SP, rTg-DI, and bigenic rTg-DI/SHR-SP rats, respectively (**Figure 11A**), and lists of these proteins can be found in **Supplementary Tables 1–3**. The rTg-DI and bigenic rTg-DI/SHR-SP rats displayed the greatest commonality, as a total of 69 elevated proteins were shared between the two, whereas only 6 proteins were common to all three models. The bigenic rTg-DI/SHR-SP and SHR-SP models shared only 14 elevated proteins (**Figure 11A**). Heat maps depicting the relative expression of the 20 most abundant elevated proteins in the bigenic rTg-DI/SHR-SP rats along with a heat map depicting the 20 most abundant elevated proteins common to the rTg-DI and bigenic rTg-DI/SHR-SP rats is shown in **Figure 11B**, including the corresponding expression in the SHR-SP cortex. Red shading indicates increased, green decreased, and white no change in expression, with color intensity relative to the degree of change. Of note among these proteins is Hspb1, which is strongly enhanced in bigenic rTg-DI/SHR-SP cortex, but not in that of the other models. We have previously reported upregulation of Hspb1 other brain regions of rTg-DI rats that display greater CAA burden and more severe CAA related pathology (Schrader et al., 2021). Furthermore, many of the significantly enhanced proteins shared by the rTg-DI and bigenic rTg-DI/SHR-SP rats display greater increases in the bigenic rTg-DI/SHR-SP cortex. For example, Apoe, Gsta3, Fabp7, Cst3, Anxa3, Gfap, and S100a13 were all previously reported as commonly elevated in the cortex, thalamus, and hippocampus of rTg-DI rats (Schrader et al., 2021). All of these proteins are commonly elevated in the rTg-DI and bigenic rTg-DI/SHR-SP cortex (**Figure 11B**), but to a greater extent in the bigenic rTg-DI/SHR-SP animals. Taken together, this could suggest an enhancing effect of the SHR-SP hypertensive phenotype on the rTg-DI model, as proteins that were elevated in the cortex of rTg-DI rats are elevated to a greater degree in the bigenic rTg-DI/SHR-SP rats. In addition, Anxa3, Vim, Clu, and Ctsd, all previously reported to be elevated in brain regions of 12 M rTg-DI rats, were commonly elevated in the 10 M rTg-DI and bigenic rTg-DI/SHR-SP cortex. Of particular note is Anxa3, which has been previously suggested as a marker of microglia activation (Junker et al., 2007; Smithson and Kawaja, 2010), and proven to be an indicator of microgliosis in rTg-DI rats (Schrader et al., 2021). The finding that many of these proteins are not elevated in the SHR-SP cortex further suggests that the presence of CAA is responsible for their consistent expression profile between rTg-DI and bigenic rTg-DI/SHR-SP rats.

Reduced Cortical Proteins in Spontaneously Hypertensive, Stroke Prone, Rat Model of Cerebral Amyloid Angiopathy and Bigenic Rat Model of Cerebral Amyloid Angiopathy/Spontaneously Hypertensive, Stroke Prone Rats

We also compared the significantly reduced (≥30% decrease, $P \leq 0.05$) proteins in the cortex of each model (**Figures 11C,D**). In this comparison 25, 58, and 76 proteins were decreased in the SHR-SP, rTg-DI, and bigenic rTg-DI/SHR-SP cortex, respectively, and lists of these proteins can be found in **Supplementary Tables 4–6**. Again, the rTg-DI and bigenic rTg-DI/SHR-SP rats shared the greatest commonality, as 22 reduced proteins were shared between the two, whereas only 9 were shared between the SHR-SP and bigenic rTg-DI/SHR-SP rats (**Figure 11C**). Notable among the shared proteins are myelin oligodendrocyte glycoprotein (Mog), myelin associated glycoprotein (Mag), myelin basic protein (Mbp), myelin oligodendrocyte basic protein (Mobp), and neurofilament medium polypeptide (Nefm). Mog, Mag, Mbp, and Mobp (Yoshikawa, 2001; Pronker et al., 2016; Weil et al., 2016; Peschl et al., 2017). All contribute to axonal myelination, and thus significant reduction in their expression could lead to demyelination and disruption of axonal integrity, while alteration in neurofilament expression is often indicative of neurodegeneration (Liu et al., 2011). These results are consistent with the diffuse white matter loss previously reported in brain regions of the rTg-DI rats, along with our previous findings of their differential expression (Lee et al., 2021; Schrader et al., 2021). Proteolipid protein 1 (Plp1) and neurofilament heavy polypeptide (Nefh), listed as specifically down regulated in the bigenic rTg-DI/SHR-SP cortex, did not meet our threshold cutoffs in the rTg-DI cortex, though their 25 and 18% respective decreases were statistically significant. Considering their implicated roles in myelination (Plp1) (Gould et al., 2018) and axonal integrity (both) (Liu et al., 2011; Gould et al., 2018), this is consistent with our other findings. In any case, like the upregulated proteins, the observed trends in the down regulated proteins suggest that the bigenic rTg-DI/SHR-SP rats adopt a proteome much more similar to the rTg-DI rats than the SHR-SP.

Pathway Analysis of Differentially Expressed Cortical Proteins in Spontaneously Hypertensive, Stroke Prone, Rat Model of Cerebral Amyloid Angiopathy and Bigenic Rat Model of Cerebral Amyloid Angiopathy/Spontaneously Hypertensive, Stroke Prone Rats

To provide functional context to the similarities and differences observed in the different rat model proteomes, we performed comparative pathway analysis using Ingenuity Pathway Analysis (IPA) (QIAGEN Inc.).[1] Only proteins meeting our imposed threshold cutoffs were included in the analysis. IPA predicts activation (z score > 2) or inhibition (z score < -2) states of upstream regulators, causal networks, or disease functions based on the directional differential expression of downstream or associated target proteins (Krämer et al., 2014). Comparative analysis predicted activation of the upstream regulator TGF-β1 in the rTg-DI and bigenic rTg-DI/SHR-SP cortex, but not in the SHR-SP. This is consistent with our previous reports of increased mRNA expression of TGF-β1 in the brain of rTg-DI rats and IPA predicted activation of TGF-β1 in brain regions of 12 M rTg-DI rats (Zhu et al., 2020; Schrader et al., 2021). A heat map comparing relative fold changes in each model of downstream proteins associated with TGF-β1 is displayed in **Figure 12A**, with red color indicating increased expression, green decreased, and color intensities relative to the degree of change. Proteins that did not meet the imposed effect threshold cutoffs are depicted in white as they were not considered in the IPA analysis. Many of the depicted proteins, such as Apoe, App, clusterin (Clu), Gfap, serine protease Htra1 (Htra1), vimentin (Vim), and integrin β-2 (IGB2) are not only common to rTg-DI and bigenic rTg-DI/SHR-SP cortex, but were also previously found to be elevated in other brain regions of 12 M rTg-DI rats (Schrader et al., 2021). Also noteworthy is Hspb1, which, as stated above, is specifically upregulated in the bigenic rTg-DI/SHR-SP cortex, but not in the rTg-DI nor the SHR-SP cortex.

IPA also indicated activation of Interleukin 2 (IL2) in the bigenic rTg-DI/SHR-SP cortex, but not in that of the SHR-SP. Only 2 of the 11 proteins were somewhat enhanced in the SHR-SP cortex. A heat map comparing relative fold changes in each model of downstream proteins associated with IL2 is depicted in **Figure 12B**, with color shading as above. IL2 has been reported to enhance astrocyte recruitment and activation of astrocytes and lead to decreased amyloid load in the mouse hippocampus (Alves et al., 2017). Additionally, IL2 has been shown to influence activation of macrophages by directly mediating the release of TGF-β1 (Nelson et al., 1994). On the other hand, IL2 also disrupts BBB integrity and can lead to vascular leak syndrome (Wylezinski and Hawiger, 2016), which could exacerbate microbleeds. Interestingly, many of the proteins indicated here for bigenic rTg-DI/SHR-SP rats are also differentially expressed in the rTg-DI cortex, though it did not meet the threshold (z score > 2) for predicted activation. Thus, these results, along with the activation of TGF-β1 mentioned above, further suggest that the bigenic rTg-DI/SHR-SP rats closely resemble that of the rTg-DI rats, where the SHR-SP hypertensive phenotype exacerbates changes in the cortex.

IPA also predicted activation of the Neurovascular Coupling pathway in the cortex of rTg-DI rats, but not the bigenic rTg-DI/SHR-SP nor the SHR-SP cortex (**Figure 12C**). Neural vascular coupling is the mechanism responsible for altering localized cerebral blood flow in response to enhanced neuronal activity (Hendrikx et al., 2019). Neurovascular coupling will regionally enhance cerebral blood flow to areas of enhanced neuronal activity to support neuronal function while aiding in waste

[1] massive.ucsd.edu/ProteoSAFe/static/massive.jsp

removal (Kaplan et al., 2020). Considering its role in removal of waste, neurovascular coupling may be important for amyloid clearance. Although some of these features are lost in the bigenic rTg-DI/SHR-SP cortex it still more closely resembles rTg-DI cortex rather than SHR-SP.

DISCUSSION

The impact of HTN on CAA and ICH remains controversial. Clinically, ICH in CAA patients occurs mostly in the cortex of elderly patients (Sahni and Weinberger, 2007; Mehndiratta et al., 2012). CAA patients have a lower mortality rate but higher incidence of recurrence than HTN patients (Mehndiratta et al., 2012). On the other hand, HTN generally affects younger patients and associated bleeds typically occur in deeper regions of the brain, particularly the basal ganglia, cerebellum and pons (Sutherland and Auer, 2006; Sahni and Weinberger, 2007). A clinical study of risk of ICH showed that patients diagnosed with both HTN and CAA had lower incidences of ICH (mixed ICH) compared to patients with only CAA (Pasi et al., 2018). On the other hand, mixed ICH patients seemed to have a higher incidence of ICH than only HTN ICH occurrences (Pasi et al., 2018) suggesting a possible protective effect of HTN in CAA. Another study reported similar results; CAA patients with HTN had better clinical outcomes after ICH events (Zhang et al., 2020). Treating with anti-hypertensive therapies was shown to reduce incidence of ICH and stroke in HTN patients (Qureshi et al., 2001) and was also found to decrease ICH incidence in CAA patients (Arima et al., 2010). Overall, clinical data suggests that HTN and CAA as comorbidities could provide a protective effect against characteristic ICH events of CAA but further investigation is required.

There have been previous experimental studies on the effects of acute and chronic HTN in AD and CAA in murine models. For example, it was reported that AD-like symptoms in Tg-SwDI mice worsened with pharmacologically induced chronic HTN (Kruyer et al., 2015). In this study, the endothelial nitric oxide synthase (eNOS) inhibitor, L-NAME (Wu et al., 2020) was used. In similar studies, HTN was pharmacologically induced by eNOS inhibition in conjunction with administration of angiotensin II in the Tg2576 mouse model of AD-like pathology (Passos et al., 2016; Nyúl-Tóth et al., 2020). In both cases, there was an increase in cerebral microbleeds. However, the inhibition of eNOS can be controversial as this has been shown to cause an increase in AβPP expression in mice (Austin et al., 2013) and pharmacological inhibitors can target other NOS isoforms (Alderton et al., 2001). In another study HTN was induced in mice by transverse aortic coarctation resulting in brain injury in the cortex and hippocampus and increased Aβ in brain (Carnevale et al., 2012). Although this is not a pharmacological intervention, transverse aortic coarctation is used to induce heart failure and could cause excessive cardiac effects with undesired consequences (deAlmeida et al., 2010).

In the present study, we investigate how chronic, non-pharmacological HTN interacts as a comorbidity of CAA, a prominent amyloidal CSVD and cause of stroke, using novel rat models of disease. There are several advantages to our approach to study these interactions. First, in contrast to the studies mentioned above that used murine models, here we use rat models of CAA and HTN. Rat models may be more suited for investigating human disease. In particular, the rTg-DI rat model of CAA faithfully recapitulates many of the pathological features of clinical CAA including perivascular neuroinflammation, cerebral microbleeds, small vessel occlusions, progressive white matter loss and progressive behavioral decline (Davis et al., 2018; Popescu et al., 2020; Zhu et al., 2020; Lee et al., 2021). Second, the use of rTg-DI rats focuses specifically on CAA pathology without parenchymal AD-like pathologies observed in most murine models. Lastly, due to the genetic origin of the phenotype, the use of a spontaneous, non-pharmacological rat model of HTN eliminates the potential confounds of any pharmacological or surgical side effects. In this regard, the SHR-SP model is appropriate due to its clinically relevant pathologies such as patterns of bleeding (Yamori et al., 1976). In addition, both the SHR-SP and rTg-DI models have consistent timelines of emerging pathology. SHR-SP rats are shown to develop and maintain hypertensive systolic blood pressure starting at ≈10 weeks of age (Okamoto and Aoki, 1963). This is harmonious with the rTg-DI rats that begin to accumulate CAA at ≈3 M of age (Davis et al., 2018; Zhu et al., 2020). The reliable timing of both models produces a cross that steadily exhibits clinically relevant pathologies of both CSVDs.

Crossbreeding of the SHR-SP rats and rTg-DI rats showed a preservation of both model phenotypes. Systolic blood pressures of SHR-SP rats and bigenic rTg-DI/SHR-SP rats were increased compared to WT rats and rTg-DI rats at 7 M. Despite the elevated blood pressures in bigenic rTg-DI/SHR-SP rats this had no appreciable impact on the level of transgene human AβPP expression or in the accumulation of Aβ peptides in the brain at 7 M (**Figures 2**, **3**). HTN at this age also did not affect cognitive decline characteristic of rTg-DI rats. Because 7 M bigenic rTg-DI/SHR-SP rats showed no changes from rTg-DI other than increased systolic blood pressure, we bred a second cohort of animals that were aged to 10 M to determine the effects of HTN in rTg-DI rats that exhibit more advanced pathologies.

In addition to already increased systolic pressure observed in 7 M animals, SHR-SP and bigenic rTg-DI/SHR-SP rats at 10 M also exhibited increased diastolic blood pressures compared to WT and rTg-DI rats. Though AβPP expression (not shown) and Aβ peptide accumulation were not different between 10 M rTg-DI and bigenic rTg-DI/SHR-SP animals the SHR-SP background was correlated with a significant redistribution of CAA load in the bigenic rTg-DI/SHR-SP rats. The thalamic and hippocampal vascular amyloid loads of the bigenic rTg-DI/SHR-SP rats were significantly decreased whereas the surface pial vessel amyloid load was more than doubled in the same rats indicating a significant shift in amyloid distribution to a different vascular bed. Though the microvascular CAA load in the cortex was not significantly different, there appears to be a modest increase in the rTg-DI/SHR-SP rats and that could indicate an emerging change. It was previously reported that CAA spontaneously develops in SHR-SP rats (Carnevale et al., 2012; Jandke et al., 2018; Denver et al., 2019). However, we were unable to detect

the accumulation of vascular Aβ or fibrillar amyloid in the 10 M SHR-SP rats used in this study. This redistribution of vascular amyloid load in bigenic rTg-DI/SHR-SP rats could result from different effects of HTN. For example, HTN is known to lower cerebral vasoreactivity (Hajjar et al., 2010). Also, cerebrospinal fluid (CSF) flow is driven by arterial pulsations and is reduced in HTN (Mestre et al., 2018). In this regard, we recently found that rTg-DI rats exhibit a hyperdynamic CSF flow coupled with reduced glymphatic clearance compared with WT rats (Chen et al., 2022). In future studies it will be interesting to investigate if the HTN phenotype impacts CSF flow and glymphatic clearance in bigenic rTg-DI/SHR-SP rats.

The rTg-DI rats typically present with microbleeds that are largely restricted to the thalamus and emerge at ≈6 M of age (Davis et al., 2018). SHR-SP animals were reported to exhibit microbleeds emerging at ≈3 M which increase in severity and number with age (Schreiber et al., 2012). These previous findings in the CAA and HTN rat models are consistent with clinical ICH where CAA bleeds affect more elderly individuals whereas HTN patients with bleeds are generally younger (Sahni and Weinberger, 2007; Mehndiratta et al., 2012). In our study, SHR-SP rats showed some microbleeds in all brain regions whereas in rTg-DI rats they were mostly observed in the thalamic region. The presence of HTN in bigenic rTg-DI/SHR-SP rats appears to somewhat enhance the number of microbleeds in the thalamus, although no significant difference was observed.

Similarly, small vessel thalamic occlusions also emerge at ≈6 M (Davis et al., 2018). SHR-SP rats showed no small vessel occlusions in the thalamus or in any other brain region. It should be noted that infarcts occurring in SHR-SP animals are associated with blood-brain barrier (BBB) compromise rather than vessel occlusions (Schreiber et al., 2013), supporting the lack of vessel occlusions found in the SHR-SP rats. Although there was no significant difference found between the number of small vessel occlusions in the thalamus of rTg-DI rats and bigenic rTg-DI/SHR-SP rats, a clear qualitative difference was observed in the size and area occupied by vessel occlusions (**Figures 10A–C**). Further quantitation of size and spatial distribution of occlusions confirmed that there is a change in the characteristics of vessel occlusions when rTg-DI rats are on the SHR-SP background. Bigenic rTg-DI/SHR-SP rats have smaller and more dispersed occlusions in the thalamus than rTg-DI rats. It has been reported in several studies that blood vessel lumens decrease in diameter due to contraction of the blood vessels, thickening of vessel walls, and overall change in function of vessels as a result of HTN (Schiffrin, 1992; Intengan and Schiffrin, 2000; Mulvany, 2002; Pires et al., 2013). Studies of SHR-SP rats have shown that remodeling of cerebral arterioles occurs in older (6–10 M) rats and is characterized by thickening of the blood vessel wall and decrease of lumen diameter (Baumbach et al., 1988; Baumbach and Heistad, 1989). This HTN decrease in vessel lumens could physically be preventing the characteristic larger occlusions observed in rTg-DI rats from forming in bigenic rats. It is also possible that the global remodeling of arterioles caused by HTN is impacting the area affected within the thalamus leading to the wider distribution of occluded vessels in bigenic rats, although this would need further study.

Proteomic analysis of the SHR-SP, rTg-DI and bigenic rTg-DI/SHR-SP cortex revealed much greater similarity between the rTg-DI and bigenic rTg-DI/SHR-SP models, as they shared 91 differentially expressed proteins compared to only 23 common differentially expressed proteins between the SHR-SP and bigenic rTg-DI/SHR-SP cortex (**Figures 11A,C**). Many of the commonly reduced proteins including Mbp, Mobp, Mog, Mag, and Nefm, are associated with myelination, axonal integrity, and neuronal degeneration. Thus, these results are consistent with our previous findings of diffuse white matter loss and down regulation of these proteins in similarly aged rTg-DI rat brains (Lee et al., 2021; Schrader et al., 2021). Notable among the commonly elevated proteins are Apoe, Anxa3, and Gfap. We have previously reported elevation of Apoe in brain regions of 12 M rTg-DI rats with strong co-localization of Apoe and vascular amyloid deposits (Schrader et al., 2021). Thus, elevated Apoe in the bigenic rTg-DI/SHR-SP cortex is not surprising due to the abundant vascular amyloid in these animals. It is not surprising that Gfap, a well-known astrocyte marker, is robustly elevated in both bigenic rTg-DI/SHR-SP and rTg-DI rats since these animals similarly present with increased astrocytes (**Figure 8**; Davis et al., 2018; Zhu et al., 2020; Schrader et al., 2021). Similarly, we previously reported Anxa3, a marker of activated microglia, is elevated in rTg-DI rats (Schrader et al., 2021). Thus, elevated levels of Anax3 in bigenic rTg-DI/SHR-SP rats is consistent with increased microglial activation observed in both rTg-DI rats and bigenic rTg-DI/SHR-SP rats (**Figure 8**). Taken together, these results suggest that bigenic rTg-DI/SHR-SP rats adopts a proteome more similar to the rTg-DI rats than the SHR-SP rats, and the SHR-SP hypertensive phenotype may enhance the differential expression of many of these shared proteins.

IPA analysis of the proteomes obtained from the three models predicted common and distinct activation of pathways and regulators related to inflammation, BBB integrity/permeability, and changes in cerebral blood flow. Activation of TGF-β1 was predicted in the cortex of rTg-DI and rTg-DI/SHR-SP rats (**Figure 12A**). We have previously shown increases in TGF-β1 mRNA expression and IPA indicated activation of TGF-β1 in 12 M rTg-DI rats, and other studies have linked upregulation of TGF-β1 mRNA in Dutch-type CAA to increased CAA severity (Moursel et al., 2018; Zhang and Yang, 2020; Zhu et al., 2020; Schrader et al., 2021). Interestingly, it has been reported that TGF-β1 deficiency in the neurovascular unit increases BBB permeability and that TGF-β1 released from astrocytes promotes BBB integrity (Garcia et al., 2004; Derada Troletti et al., 2016). Considering these reported roles of TGF-β1 in the neurovascular unit, it is interesting that the neurovascular coupling pathway was only predicted to be activated in the rTg-DI cortex (**Figure 12C**). Neurovascular coupling, mediated by vascular smooth muscle, astrocytes and neurons, is responsible for regional changes in cerebral blood flow in response to neuronal activity (Hendrikx et al., 2019; Kaplan et al., 2020). These changes in blood flow promote essential nutrient delivery and waste removal (Kaplan et al., 2020), and therefore may be important for Aβ clearance. Thus, the lack of activation of neurovascular coupling in the bigenic rTg-DI/SHR-SP cortex could suggest altered deposition

or clearance of vascular amyloid, and may lead to changes in CAA pathology, however, this requires further investigation. IPA predicted activation of IL2 only in the bigenic rTg-DI/SHR-SP rats, although 10 of the 11 implicated proteins were also differentially expressed in the rTg-DI rats. IL2 activity has been reported to promote clearance of Aβ through the activation and recruitment of astrocytes (Alves et al., 2017). On the other hand, IL2 may be detrimental to BBB integrity and can lead to vascular leak syndrome (Wylezinski and Hawiger, 2016). Thus, IL2 could have beneficial or damaging functions in CAA related pathology depending on its context. Nevertheless, the differential expression of proteins associated with IL2 activation further suggests that the bigenic rTg-DI/SHR-SP proteome is most closely related to the rTg-DI model, and may not only enhance these protein changes, but the activation of pathways as well.

Despite the new information gained there are several noted limitations of the present study. Our investigations focused primarily on 10 M old rats where CAA and associated vascular pathologies are developed but, still progressing. Potential chronic effects of HTN at this stage of disease may not be prominent but could be become much more robust as the animals continue to age and disease progresses. For example, a recent study similarly bred SHR-SP rats with a rat model of AD-like pathologies (Denver et al., 2019). Aging of these particular bigenic animals to 16–18 M showed a worsening of several AD-like pathologies in the brain including vascular changes, neuroinflammation and mitochondrial stress, but no effect on behavioral deficits (Denver et al., 2019). This underscores how further aging of our bigenic rTg-DI/SHR-SP rats may be necessary to observe more robust effects of HTN on CAA related pathologies. Second, the rTg-DI rat is a model of CAA type-1 that primarily affects small microvessels and capillaries. Perhaps HTN introduced by the SHR-SP crossing would be more impactful on a model of CAA type-2 that targets larger vessels in the brain. Finally, the rTg-DI model involves cerebral vascular deposition of chimeric Dutch/Iowa familial CAA mutant Aβ. HTN could have a more prominent impact on CAA accumulation and associated pathologies in a model that accumulates non-mutated Aβ. In any case, the changes caused by HTN that are observed in this study suggest that over time, the CAA pathologies could be further altered and perhaps have a significant impact on cognitive deficits. The hypertensive bigenic rTg-DI/SHR-SP rats generated in this study provide a preclinical platform to further investigate the consequences of chronic, non-pharmacological HTN on CAA and VCID.

DATA AVAILABILITY STATEMENT

Raw mass spectrometry data can be found in the MassIVE repository (massive.ucsd.edu/ProteoSAFe/static/massive.jsp), project ID#: MSV000088300, and password: SvdCortComp21.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Rhode Island Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

AS performed experiments, analyzed the data, and wrote the manuscript. JS performed the experiments, analyzed the data, and edited the manuscript. XZ, JM, MM, and FX performed the experiments and analyzed the data. AH performed the experiments. JR designed the experiments and analyzed the data. WV conceived the study, designed the experiments, edited the manuscript, and secured funding. 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/fnins.2022.811371/full#supplementary-material

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Comorbidity in patients with first-ever ischemic stroke: Disease patterns and their associations with cognitive and physical function

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The present study examined the prevalence and pattern of comorbidity among Chinese patients with first-ever acute ischemic stroke, and assessed the associations of specific comorbidity patterns with physical and cognitive functioning after stroke occurrence. A hospital-based cross-sectional study was conducted among 2,151 patients with first-ever ischemic stroke (age ≥40 years; 64.2% men) who were admitted to two university hospitals in Shandong, China between 2016 and 2017. Data on demographics, lifestyles, chronic health conditions, and use of medications were collected through in-person interviews, clinical examinations, and laboratory tests. Physical functioning was assessed by the Barthel index (BI) and the modified Rankin Scale (mRS) while cognitive functioning was assessed by the Montreal Cognitive Assessment test. The results showed that comorbidity was present in 90.9% of the stroke patients (women vs. men: 95.2 vs. 88.7%, $P < 0.001$). Exploratory factor analysis identified three patterns of comorbidity, i.e., patterns of degenerative-cardiopulmonary, heart-gastrointestinal-psychiatric, and metabolic-kidney diseases. The number of comorbidities was significantly associated with a higher likelihood of moderate-to-severe physical dependence [odds ratio (95% CI) = 1.15 (1.06–1.25) for BI and 1.12 (1.04–1.21) for mRS, all $P < 0.01$] and cognitive impairment

[odds ratio (95% CI) = 1.11 (1.02–1.20), P = 0.017], after adjusting for multiple covariates. Almost all the three comorbidity patterns were associated with increased likelihoods of physical dependence (range for odds ratios: 1.26–1.33) and cognitive impairment (range for odds ratios: 1.25–1.34). No significant association was found between degenerative-cardiopulmonary pattern and mRS. These findings suggest that comorbidity is associated with poor physical and cognitive functioning during the acute phase of ischemic stroke. Routine assessments of comorbidity and cognitive and physical function among patients with acute ischemic stroke should be considered in stroke research and clinical practice.

KEYWORDS

stroke, comorbidity, functional dependence, cognitive impairment, China

Introduction

Stroke is the second leading cause of disability and death worldwide. In China, ∼2.4 million new stroke cases and ∼1.1 million stroke-related deaths occurred in 2013 (Wu et al., 2019), and the numbers are expected to rise over the next few decades primarily due to population growth and aging. Survivors of stroke often present with poor physical function and impaired cognition, leading to significantly decreased quality of life (Davis et al., 2019; She et al., 2021). Approximately 70–80% of stroke survivors require rehabilitation and long-term care (Nichols-Larsen et al., 2005). Post-stroke physical and cognitive conditions have been associated with demographics (e.g., age and gender), stroke features (e.g., size, location, and type), walking capacity, and psychosocial factors (e.g., social support, depression, and balance self-efficacy) (Salbach et al., 2006; Yoon et al., 2017; Ilunga Tshiswaka et al., 2018). As post-stroke function deficits may increase the risk of readmission, mortality, and early death (Gaynor et al., 2018), exploring factors associated with poor functional status among survivors of patients with stroke is greatly warranted if we seek to achieve the overall goal of rehabilitation services.

Stroke survivors commonly have comorbid conditions, especially older stroke patients. Evidence suggests that a clinical stroke could occur in the absence of comorbid conditions in less than 6% of cases (Nelson et al., 2017). Multimorbidity, the co-occurrence of two or more chronic diseases in the same person, is sometimes used interchangeably with comorbidity, which is defined as the presence of at least one long-term condition alongside an index condition (Gallacher et al., 2019). The coexisting of multiple chronic conditions and the presence of comorbidity patterns may be due to the fact that these health conditions share common risk factors and pathophysiology (e.g., heart disease and stroke) or causal/precursor relationship (e.g., atrial fibrillation and stroke) (Schäfer et al., 2010; Gallacher et al., 2019). Previously, community-based studies indicated that the burden and patterns of multimorbidity were associated with impaired physical functioning, poorer quality of life, and more frequent use of health care services (Ryan et al., 2015; She et al., 2019). However, there is a dearth of studies that have focused specifically on comorbidity patterns in the stroke population. We only identified a few studies that investigated the associations between comorbidity (mainly operationalized as a count of numbers of chronic health conditions or comorbidity index) and poor physical function among stroke patients, which reported mixed findings (Schmidt et al., 2014; Kabboord et al., 2016; Gallacher et al., 2019).

Thus, in this hospital-based cross-sectional study, we aimed to investigate the burden and pattern of chronic disease comorbidity among Chinese patients with first-ever ischemic stroke; and further to explore the associations between the number and patterns of comorbidity and physical and cognitive function outcomes among stroke patients.

Materials and methods

Patients

The participants were derived from a randomized controlled multimodal behavioral intervention trial among patients with acute ischemic stroke or transient ischemic attacks (TIAs), who were hospitalized in two university hospitals in Jining, Shandong, China. The inclusion criteria were (Wang et al., 2011): (1) first-ever ischemic stroke or TIAs that was confirmed by brain computed tomography (CT) or magnetic resonance imaging (MRI) scans; (2) aged ≥40 years; (3) patients, family members or caregivers can provide consent. Patients with severe symptoms (e.g., unconsciousness or aphasia) were excluded. From January 2016 to February 2017, 2,187 eligible stroke

patients were recruited and completed the assessments within 2 weeks of admission to hospitals. We excluded 36 (1.7%) participants who were diagnosed with dementia, leaving 2,151 persons for the current analysis.

Ethical statement

The study protocols were approved by the Ethics Committee at the relevant institution (No. 2015B006). Written informed consent was obtained from all participants, or in the case of cognitively impaired persons, from informants. Research had been conducted in accordance with the ethical principles expressed in the Declaration of Helsinki. The trial was registered in the Chinese Clinical Trial Registry (ID: ChiCTR-IOR-16007741).

Data collection

Baseline data were collected within 2 weeks after hospitalization when patients' clinical conditions became stable, following the standardized questionnaire through face-to-face interviews, clinical examinations, psychological testing, and laboratory tests by trained staff at the two hospitals. We collected data on sociodemographics (e.g., age, sex, and education), lifestyles prior to hospitalization (i.e., smoking and alcohol drinking), use of medications in the 2 weeks before hospitalization, and cognitive and physical function following the hospitalization. Weight and height were measured in light clothes with no shoes. Body mass index was calculated as weight (kg) divided by height (meters) squared. Arterial blood pressure was measured in the sitting position on the right arm after at least a 5-min rest, using an electronic blood pressure monitor (Omron HEM-7127J, Omron Electronics Inc., Japan). Blood pressure was measured three times on one occasion, and the mean of the three readings was used in the analysis. Peripheral blood samples were obtained after an overnight fast. Fasting plasma glucose (FPG) and total cholesterol (TC) were measured using an automatic Biochemical Analyzer at the hospital laboratories.

Assessments of health conditions

Chronic diseases and comorbidity

We defined and categorized 16 chronic disease categories following the methods previously described (Calderón-Larrañaga et al., 2017). We defined hypertension as blood pressure ≥140/90 mmHg or use of antihypertensive drugs (Song et al., 2014; Wang et al., 2015), diabetes as FPG ≥7.0 mmol/l or use of oral antidiabetic agents or insulin injection (American Diabetes Association, 2014), obesity as a body mass index ≥28 kg/m^2 (Song et al., 2014), and dyslipidemia as total serum cholesterol >6.22 mmol/l or triglycerides ≥2.26 mmol/l or low density lipoprotein cholesterol ≥4.14 mmol/l or use of hypolipidemic drugs (Calderón-Larrañaga et al., 2017). Gastrointestinal diseases were ascertained as clinical diagnosis of gastric or duodenal ulcer or chronic gastritis or use of antacids. Respiratory diseases were ascertained by clinical diagnosis of chronic obstructive pulmonary disease (COPD) or asthma or use of antiasthmatic drugs. Kidney diseases were ascertained by clinical diagnosis of nephritis or kidney failure. Depression was defined as having a clinical diagnosis of major depression according to the structured clinical interview of the fifth edition of Diagnostic and Statistical Manual of Mental Disorders, significant depressive symptoms defined as the 15-item Geriatric Depression Scale (GDS-15) score ≥5 (Chan, 1996; Almeida and Almeida, 1999), or using antidepressants during the hospital admission. Cancer, coronary heart disease, migraine, fracture, heart failure, arrhythmia, cerebrovascular malformation, and arthritis were ascertained by integrating information from clinical examination, instrumental examination (e.g., electrocardiogram and B-mode ultrasonic examination), blood test, and discharge diagnosis. Chronic diseases with a prevalence of <0.5% were not included in the analysis in order to avoid spurious associations and obtain epidemiologically coherent patterns, such as brain injury (0.1%), epilepsy (0.2%), Parkinson's disease (0.2%), and thyroid dysfunction (0.4%). Multimorbidity was defined as the co-occurrence of two or more diseases in the same individual; therefore, the presence of one or more of the 16 comorbidities in patients with stroke indicated the presence of multimorbidity.

Physical function

Physical function was evaluated using the Barthel Index (BI) (McLennan et al., 2011) and the modified Rankin Scale (mRS) (Banks and Marotta, 2007). The BI included 10 basic self-care activities, i.e., bowel control, bladder control, personal hygiene, toilet transfer, bathtub transfer, feeding, dressing, wheelchair transfer to and from bed, walking, and ascending and descending stairs. The total score ranged from 0 to 100, with higher scores indicating higher degrees of independence. The cutoff score of ≤75 was used to denote the presence of moderate-to-severe physical dependence in stroke patients (Supervía et al., 2008). The mRS is a commonly used scale for measuring the degree of disability or dependence in the daily activities of people who have suffered from a stroke or other causes of neurological disability. The mRS comprises 6 grades of stroke severity ranging from 0 (no significant disability) to 5 (severe disability). Unfavorable outcome on the mRS was defined as the score ≥3 (moderate-to-severe disability) (Diener et al., 2008).

Cognitive function

Global cognitive function was assessed with the validated Changsha version of Montreal Cognitive Assessment (MoCA)

(Nasreddine et al., 2005). MoCA is a 30-point test, which measures language, memory, attention, abstraction, orientation, and executive functions. Based on a national population-based study among Chinese elderly people, cognitive impairment in patients with stroke was defined as MoCA score <14 for illiterate individuals, <20 for individuals with 1 to 6 years of education, and <25 for individuals with 7 or more years of education (Lu et al., 2011).

Covariates

Demographic features including age (years), sex, education (no formal schooling, primary school, and middle school or above), marital status (married vs. single or widowed or divorced), enrolled hospital, type of stroke (cerebral infarction, TIA, and lacunar infarction), and lifestyle factors (i.e., smoking, alcohol drinking, and whether or not having difficulty in falling asleep) were controlled as covariates.

Statistical analysis

Characteristics of study participants by sex were present and compared using *t*-test for continuous variables or chi-square test for categorical variables. Exploratory factor analysis was performed to identify comorbidity patterns based on a tetrachoric correlation matrix and using principal factor analysis (Mislevy, 1985). Eigenvalues greater than 1 and the scree plot were used to determine the number of retained factors (Prados-Torres et al., 2012). A chronic condition was considered to characterize a given pattern of comorbidity if its loading was ≥0.25 in that pattern. When the factor loading of a certain disease was ≥0.25 in more than one group, this disease was clustered into the group with a larger factor loading value (Wang et al., 2015). To facilitate the interpretation of the factors, an oblique rotation (Oblimin) was applied. For each comorbidity pattern, we used regression method (a least squares regression approach) to estimate the factor scores of participants with respect to their factor loading values and the factor scores for each comorbidity pattern were divided into tertiles (Distefano et al., 2008). From the lowest to the highest tertile, the participant's expression of the comorbidity pattern associated with the specific component increased.

Multiple logistic regression models were utilized to examine the associations between the number of comorbidities with physical and cognitive functional outcomes, respectively, controlling for sociodemographic variables, lifestyle factors, and type of stroke. A total of 526 patients (24.5%) had missing data on at least one item of studied variables, such as sociodemographic and lifestyle factors (16.0%), measurement of comorbidities (9.5%), the MoCA test (6.3%), BI (1.3%), and mRS (1.2%). Missing values were dealt with multiple imputations ($n = 20$), which shows advantages (e.g., reducing bias, increasing validity, and preserving statistical power) over most of the existing methods dealing with missing data (McCleary, 2002). Relative variance increased (RVI) as a measurement for evaluation of multiple imputation was reported, which is interpreted as the proportional increase in the sampling variance of the parameter of interest that is due to the missing data. The closer this number is to zero, the less effect missing data have on the variance of the estimate. The odds ratios (OR) and 95% confidence intervals (CIs) of cognitive or physical impairment associated with tertile of factor scores for each comorbidity pattern were also estimated, in which the first tertile was used as the reference category. SPSS 23.0 Statistics for Windows (IBM Corp., Released 2015, Armonk, NY, United States: IBM Corp.) and Stata Statistical Software: Release 12.0 (StataCorp 2011, College Station, TX, United States: StataCorp LP) were used for all statistical analyses. Two-tailed P-value < 0.05 was considered statistically significant.

Results

Characteristics of the study participants

The mean age of the 2,151 participants was 61.5 (SD, 9.8) years and 64.2% were men. Of all the stroke patients, 93.7% were diagnosed with cerebral infarction, 3.6% with TIAs, and 2.7% with lacunar infarction; 40.3% were current smokers; 36.3% had alcohol drinking habits in the past year, and 13.9% had difficulties in falling asleep. The average number of comorbidities per patient was 2.0 (SD 1.2). Only 9.0% did not present any other comorbidities, and 29.6, 30.1, and 31.3% had one, two, and three or more comorbidities, respectively. Multimorbidity affected 91.0% of all stroke patients. Compared with patients who had <2 comorbidities, stroke patients with two or more comorbidities were more likely to be female (72.1 vs. 55.4%, $P < 0.001$), have employee health insurance (24.7 vs. 18.6%, $P < 0.001$), have sleep problems (16.4 vs. 12.2%, $P = 0.011$) while less likely to be current smokers (35.6 vs. 48.5%, $P < 0.001$) and drink alcohol (33.3 vs. 42.8%, $P < 0.001$). No differences in age, marital status, and stroke subtypes were observed between patients with ≥2 and <2 comorbidities. Overall, the most common comorbidities in the stroke patients were hypertension (74.7%), diabetes (28.5%), dyslipidemia (23.3%), coronary heart disease (19.1%), and obesity (17.7%) (**Figure 1**).

Moderate-to-severe physical dependence defined by BI ≤75 or mRS ≥3 was found in 35.4 and 36.4% of the patients, respectively. Cognitive impairment was present in 57.5% of the patients. Female stroke patients were older, less educated, more likely to be widowed/divorced and have difficulties in falling asleep, less likely to smoke and drink alcohol, had more comorbidities, and were more likely to have multimorbidity and physical dependence (**Table 1**).

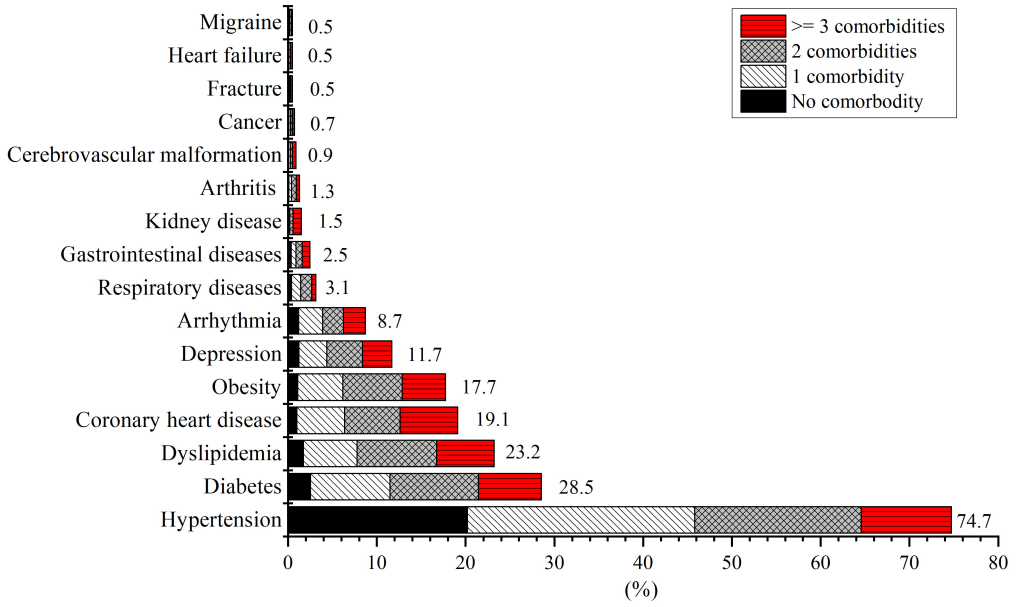

FIGURE 1
Prevalence (per 100 patient population) and co-occurrence of chronic diseases among patients with first-ever acute ischemic stroke (n = 2,151). Comorbidity refers to chronic diseases other than the specific condition and stroke.

Patterns of chronic comorbidity

We identified three patterns of chronic comorbidity among stroke patients, which could explain 63.7% of the total variance (Table 2). Pattern 1 was referred to as degenerative-cardiopulmonary disease pattern, which included degenerative disorders (arthritis, fracture, and cancer), heart failure, respiratory diseases, and other diseases (migraine and cerebrovascular malformation). Pattern 2 was called as heart-gastrointestinal-psychiatric disease pattern, which included heart diseases (coronary heart disease and arrhythmia), gastrointestinal diseases, and depression. Pattern 3 was referred to as metabolic-kidney disease pattern that included metabolic disorders (hypertension, obesity, diabetes, and dyslipidemia) and kidney disease.

Comorbidity, physical function, and cognitive function

The increasing number of comorbidities was associated with an increased likelihood of physical dependence [OR = 1.15 for BI and 1.12 for mRS; $P < 0.01$ for all] and cognitive impairment (OR = 1.11; $P = 0.017$) (Table 3). The presence of multimorbidity was positively associated with physical dependence assessed by BI (OR = 1.56; $P = 0.013$) and mRS (OR = 1.41; $P = 0.049$). Consistently, participants with two comorbidities and three or more comorbidities (OR = 1.34 and 1.56 for BI and OR = 1.28 and 1.51 for mRS, respectively; $P < 0.05$ for all) were more likely to have physical dependence than those who had no or only one comorbidity. Similarly, compared to participants who had no or only one comorbidity, those with three or more comorbidities were more likely to have cognitive impairment (OR = 1.36; $P = 0.011$) (Table 3).

Of the three comorbidity patterns, the upper tertile of degenerative-cardiopulmonary disease pattern (vs. lower tertile) was significantly associated with physical dependence assessed by BI [OR (95% CI) = 1.27 (1.01–1.61)] and cognitive impairment [OR (95% CI) = 1.34 (1.04–1.71)]. Compared to participants in the lower tertile of heart-gastrointestinal-psychiatric disease pattern, those in the upper tertile had a higher likelihood of physical dependence [OR (95% CI) = 1.24 (1.00–1.53) for BI and 1.26 (1.02–1.56) for mRS] and cognitive impairment [OR (95% CI) = 1.25 (1.01–1.56)]. Similarly, participants in the upper tertile of metabolic-kidney disease pattern were more likely to have physical dependence [OR (95% CI) = 1.31 (1.05–1.65) for BI and 1.33 (1.06–1.68) for mRS] and cognitive impairment [OR (95% CI) = 1.28 (1.01–1.62)] than those in the lower tertile. There was no significant association between the comorbidity pattern of degenerative-cardiopulmonary diseases and functional outcome assessed with mRS (Figure 2).

Discussion

In this hospital-based study, comorbidity was present in nine out of the ten patients with first-ever acute stroke.

TABLE 1 Characteristics of the study participants.

Characteristics	Total sample (n = 2,151)	Men (n = 1,380)	Women (n = 771)	P-value*
Sociodemographic and lifestyle characteristics				
Age (years), mean (SD)	61.5 (9.8)	60.4 (9.7)	63.3 (9.8)	<0.001
Education[b]				
No formal school	545 (26.4)	137 (10.4)	408 (54.7)	<0.001
Primary school	544 (26.3)	366 (27.7)	178 (23.9)	
Middle school or above	979 (47.3)	819 (62.0)	160 (21.5)	
Marital status[b]				
Married	2,068 (96.7)	1,340 (97.8)	728 (94.7)	<0.001
Widowed/Divorced/Single	71 (3.3)	30 (2.2)	41 (5.3)	
Health insurance[a, b]				
Urban residents basic medical insurance	1,172 (55.7)	688 (50.8)	484 (64.5)	<0.001
Employee health insurance	466 (22.1)	372 (27.5)	94 (12.5)	
NRCMS health insurance	390 (18.5)	245 (18.1)	145 (19.3)	
Others (e.g., Public expense)	77 (3.7)	50 (3.7)	27 (3.6)	
Current smoking[b]				
No	1,269 (59.7)	550 (40.5)	719 (94.0)	<0.001
Yes	855 (40.3)	809 (59.5)	46 (6.0)	
Alcohol drinking[b]				
No	1,228 (63.7)	489 (41.8)	739 (97.2)	<0.001
Yes	701 (36.3)	680 (58.2)	21 (2.8)	
Difficulties in falling asleep[b]				
No	1,819 (86.1)	1,213 (89.5)	606 (80.1)	<0.001
Yes	293 (13.9)	142 (10.5)	151 (19.9)	
Clinical characteristics				
Stroke type				
Cerebral infarction	2,015 (93.7)	1,301 (94.3)	714 (92.6)	0.302
TIAs	77 (3.6)	44 (3.2)	33 (4.3)	
Lacunar infarction	59 (2.7)	35 (2.5)	24 (3.1)	
Comorbidities[b]				
No. of chronic conditions, mean (SD)	2.0 (1.2)	1.8 (1.1)	2.3 (1.2)	<0.001
Multimorbidity (≥1 comorbidity)				
No	175 (9.0)	142 (11.3)	33 (4.8)	<0.001
Yes	1,771 (91.0)	1,112 (88.7)	659 (95.2)	
No. of comorbidities				
≤1	752 (38.6)	559 (44.6)	193 (27.9)	<0.001
2	585 (30.1)	376 (30.0)	209 (30.2)	
≥3	609 (31.3)	319 (25.4)	290 (41.9)	
Physical and cognitive function				
Physical dependence (Barthel Index ≤75)[b]				
No	1,373 (64.1)	907 (66.6)	466 (61.0)	0.009
Yes	752 (35.4)	454 (33.4)	298 (39.0)	
Physical disability (mRS ≥3)[b]				
No	1,350 (63.6)	897 (66.0)	453 (59.4)	0.002
Yes	773 (36.4)	463 (34.0)	310 (40.6)	
Cognitive impairment (MoCA)[b]				
No	857 (42.5)	553 (42.7)	304 (42.2)	0.803
Yes	1,158 (57.5)	741 (57.3)	417 (57.8)	

Data are n (%), unless otherwise specified. MoCA, Montreal Cognitive Assessment; mRS, modified Rankin Scale. [a]Urban resident basic medical insurance was a government-subsidized, household-level voluntary medical insurance for urban residents that were not covered by employee health insurance. Employee health insurance was for urban working and retired employees in public and private sectors, in which employers and employees contributed to the insurance system in proportion to the employee's salary. New Rural Co-operative Medical Scheme (NRCMS) was for rural residents provided by local and central governments covering various rates of expense in all level public healthcare facilities. [b]The number of patients with missing values was 83 for education, 12 for marital status, 46 for health insurance, 27 for current smoke, 222 for alcohol drinking, 39 for difficulties in falling asleep, 205 in comorbidities, 26 for BI, 29 for mRS, and 136 for MoCA. Missing values were dealt with multiple imputations (n = 20) in the regression analysis. *P value is for the test of difference between men and women.

The greater number of comorbidities was associated with an increased likelihood of physical dependence and cognitive impairment. Furthermore, three patterns of comorbidity were identified among stroke patients, which generally showed significant positive associations with physical dependence and cognitive impairment. To the best of our knowledge, this is

TABLE 2 Rotated loadings for each of the 16 chronic disease categories by three groups from factor analysis.

Chronic diseases	Degenerative-cardiopulmonary diseases	Heart- gastrointestinal-psychiatric diseases	Metabolic-kidney diseases
Fracture	**0.84**	−0.10	0.20
Arthritis	**0.58**	−0.15	−0.06
Heart failure	**0.57**	0.40	−0.32
Respiratory diseases	**0.46**	−0.09	−0.46
Migraine	**0.38**	0.35	−0.11
Cancer	**0.35**	0.23	−0.12
Cerebrovascular malformation	**0.29**	0.25	0.07
Coronary heart disease	−0.15	**0.76**	0.07
Gastrointestinal diseases	0.12	**0.35**	−0.02
Arrhythmia	−0.09	**0.32**	−0.24
Depression	0.04	**0.29**	0.06
Diabetes	0.15	−0.03	**0.73**
Dyslipidemia	−0.02	0.20	**0.43**
Kidney disease	0.33	0.34	**0.40**
Obesity	−0.07	−0.13	**0.26**
Hypertension	−0.22	0.22	**0.26**
Eigenvalue	2.65	1.42	1.20
Cumulative percentage	32.1%	49.2%	63.7%

Values in bold indicate the factor loadings of diseases that were assigned to the corresponding comorbidity pattern.

TABLE 3 Associations of physical and cognitive functions with number of chronic health conditions.

Chronic health conditions	Moderate-to-severe physical dependence				Cognitive impairment	
	Barthel Index ≤75		mRS ≥3		Low MoCA score[a]	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
No. of comorbidities	**1.15 (1.06, 1.25)**	**0.001**	**1.12 (1.04, 1.21)**	**0.005**	**1.11 (1.02, 1.20)**	**0.017**
Multimorbidity						
No	1.00 (reference)		1.00 (reference)		1.00 (reference)	
Yes	**1.56 (1.10, 2.23)**	**0.013**	**1.41 (1.00, 1.99)**	**0.049**	1.19 (0.84, 1.69)	0.336
No. of comorbidities						
≤1	1.00 (reference)		1.00 (reference)		1.00 (reference)	
2	**1.34 (1.05, 1.71)**	**0.019**	**1.28 (1.01, 1.61)**	**0.042**	1.15 (0.91, 1.45)	0.240
≥3	**1.56 (1.23, 1.98)**	**<0.001**	**1.51 (1.19, 1.94)**	**0.001**	**1.36 (1.07, 1.72)**	**0.011**

Odds ratio (95% confidence interval) was adjusted for age, sex, education level, marital status, type of health insurance, enrolled hospital, type of stroke, smoking status, alcohol drinking, and difficulties in falling asleep. Significant odds ratios (95% confidence interval) with $P < 0.05$ are presented in bold. OR, odds ratio; CI, confidence interval; mRS, modified Rankin Scale; McCA, Montreal Cognitive Assessment (Changsha version). [a]Cognitive impairment in patients with stroke was defined as MoCA score <14 for illiterate individuals, <20 for individuals with 1 to 6 years of education, and <25 for individuals with 7 or more years of education.

the first study in a large sample of Chinese stroke patients that investigates the burden and patterns of comorbidity as well as their associations with cognitive and physical functional outcomes, which provides a novel perspective to assess the health impacts of comorbidities on post-stroke functions.

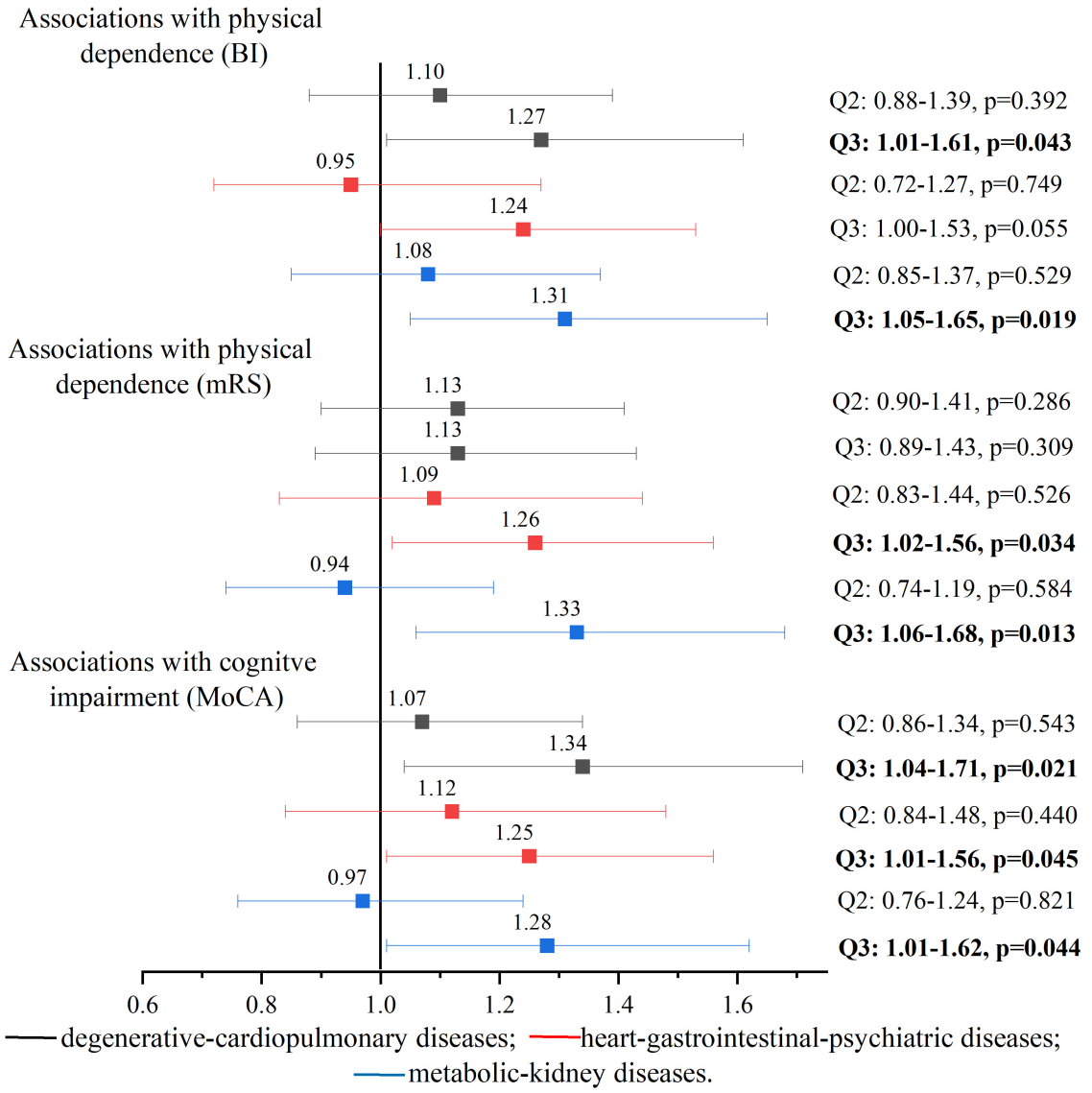

FIGURE 2
Associations between comorbidity patterns and functional outcomes among patients with first-ever ischemic stroke. Odds ratios and 95% confidence intervals were adjusted for age, sex, education, marital status, type of health insurance, enrolled hospital, type of stroke, smoking status, alcohol drinking, and difficulties in falling asleep. The lower tertile (Q1) was used as the reference group; Q2: the medium tertile; Q3: the upper tertile. Missing data were dealt with multiple imputation and the RVI for multiple logistic regression models ranged from 0.0298 to 0.0388.

The overall prevalence of comorbidity was generally comparable with that of the previous examination in stroke patients, which showed great variations across studies due to differences in methodology (e.g., sample characteristics and the number, definition, and measurement of morbidities) (Gallacher et al., 2014). Patients with two or more comorbidities were more likely to have sleep problems while less likely to be current smokers and drink alcohol in the past year than those having no or only one comorbidity. Stroke patients with more comorbidities may be more inclined to be recommended by their doctors to adopt a healthy lifestyle prior to the occurrence of clinical stroke. The vulnerability of sleep disorders in stroke patients with multiple comorbidities warrants attention and further investigation. Corroborating previous studies, female patients were more likely to have poor physical functional status following an acute stroke (Gargano and Reeves, 2007). In the present study, female patients were older, less educated, and had more comorbidities and higher prevalence of multimorbidity, which might contribute to worse physical functioning. This suggests that greater attention should be paid to female stroke survivors to properly manage comorbidities and physical dependence.

We further identified three patterns of comorbidity among the stroke patients (i.e., degenerative-cardiopulmonary, heart-gastrointestinal-psychiatric, and metabolic-kidney diseases) and assessed their relationships with functional outcomes. The first pattern was mainly characterized by degenerative diseases (e.g., joint diseases), heart failure, cancer, and respiratory diseases. Relatedly, a previous study of patients in the primary care setting in Spain identified a comorbidity pattern of psychogeriatric diseases, which covered geriatric diseases, heart failure, stroke, and neurocognitive diseases (Prados-Torres et al., 2012). Another observational study based on electronic health records also suggested that the pattern of degenerative diseases (e.g., arthropathy, cataract, osteoporosis, and hearing loss) was one of the most prevalent comorbidity patterns in Spanish patients with heart failure, possibly explained by the shared risk of aging and physical limitation (Gimeno-Miguel et al., 2019). Corroborating a prior study among the Chinese elderly population, cancer was clustered with degenerative diseases, reflecting the strong age dependence of these conditions (Gu et al., 2017). A community-based survey of older adults in China also identified the comorbidity pattern of cancer and pulmonary diseases and suggested that cancer tended to affect most frequently the respiratory tracts (Wang et al., 2017). While no prior studies have explored comorbidity patterns among stroke patients, future studies are warranted to confirm whether this comorbidity pattern remains across different populations and geographic regions.

The second pattern was represented by cardiovascular diseases, gastrointestinal diseases, and depression. The close associations between gastrointestinal diseases and affective disorders have been frequently reported in the general and clinical populations (Mayer et al., 2001). Acute life-threatening stressors and the central nervous system mechanisms play an important role in the development of gastrointestinal and psychiatric symptoms. It is worth noting that cardiovascular and gastrointestinal diseases as a separate pattern was not common in previous studies. However, the recently discovered contribution of gut-microbiota-derived molecules in the development of heart disease and its risk factors has significantly increased attention toward the connection between gut and heart diseases (Ahmadmehrabi and Tang, 2017). For instance, gastroesophageal reflux disease can lead to atrial fibrillation, in which multiple mechanisms may be involved in such as inflammation, autoimmunity, and exacerbated autonomic stimulation (Martins et al., 2015). Future research should focus on the underlying pathogenesis connecting these medical conditions, and the role of age and aging in the development of cardiovascular and gastrointestinal diseases.

Consistent with prior literature of studies among the community-dwelling elderly people (Wang et al., 2015), hypertension, diabetes, dyslipidemia, and obesity, the main components of metabolic syndrome, are established risk factors for cardiovascular diseases. This finding corroborates a prior systematic review that identified the major multimorbidity pattern of cardiovascular-metabolic diseases in the adult populations (Prados-Torres et al., 2014). Additionally, we found that kidney disease was clustered with metabolic factors and disorders. Extensive literature has indicated that metabolic syndrome and its individual components (e.g., obesity and diabetes) are associated with heightened risks of chronic kidney diseases (Kumar et al., 2013). For instance, it is known that chronic high blood pressure is a major cause of kidney disease, while chronic kidney disease is one of the most common causes of secondary hypertension (Freedman and Cohen, 2016). Patients with long-term hypertension (e.g., >5 years) were more likely to suffer from kidney disease with the small renal arteries of the glomerulus pathological changes (Mulè et al., 2008). The epidemiologic data also revealed that the prevalence of diabetic kidney disease increased with the growing epidemic of diabetes (Harjutsalo and Groop, 2014). Therefore, kidney disease should be recognized and prevented as an important complication of cardiovascular and metabolic diseases. Our study indicated that these metabolic diseases were among the most prevalent comorbidities among stroke patients. Metabolic syndromes are arguably prominent risk factors in the development of stroke and thus they are common comorbid diseases among patients with stroke.

Furthermore, we found that the number of comorbidities was associated with an increased likelihood of both physical dependence and cognitive impairment among stroke survivors, which is consistent with literature among the general older population (She et al., 2019). Notably, the associations between comorbidity and poor physical function were overall consistent when using the two different tools for assessment of post-stroke functional outcomes (i.e., BI and mRS), supporting the robustness of the findings. The results corroborate a previous study showing that comorbidity assessed using the Charlson comorbidity index was associated with physical dependence among stroke patients (Jiménez Caballero et al., 2013). Other studies also suggested that the presence of comorbidities such as heart disease and COPD were risk factors of cognitive impairment among stroke patients (Valkova and Peychinska, 2012).

Specifically, the three comorbidity patterns identified in our study showed generally significant associations with physical and cognitive functional outcomes. These results extended the findings from previous studies of comorbidity patterns and health outcomes among the general populations by disentangling the relationship between comorbidity patterns and functional status among stroke survivors. The heart-gastrointestinal-psychiatric disease and metabolic-kidney disease patterns showed relatively consistent associations with the three measurements of cognitive and physical functional outcomes. Similarly, prior studies indicated the associations between heart

diseases and metabolic syndromes with various health outcomes (e.g., morbidity, functional decline, muscular weakness, and cognitive function) among stroke patients (Akbal et al., 2012; Li et al., 2016; Specogna et al., 2017). In contrast, the degenerative-cardiopulmonary pattern was associated with an increased likelihood of moderate-to-severe physical dependence assessed by BI but not by mRS. This may indicate the discrepancy of BI and mRS in monitoring the impact of degenerative-cardiopulmonary diseases on physical function in stroke patients. Prospective follow-up studies will help further clarify the longitudinal associations between comorbidity patterns and post-stroke functional status as well as the concordance between functional outcome parameters in patients with stroke.

Our hospital-based study covered a broad range of chronic conditions in a large sample of stroke patients that were defined by integrating information from face-to-face interviews, clinical examinations, and instrumental and laboratory tests. However, our study also has limitations. First, the cross-sectional nature of the study design does not allow causal inference of the observed associations and mediations, and the findings might be subject to selective survival bias. Thus, caution is needed when interpreting the findings. Second, the study participants from the two local general hospitals might not be representative of the stroke patient population in China, which should be kept in mind when generalizing our findings to a broad patient population. Third, although factor analysis has been most frequently used in the literature to explore comorbidity patterns (Ng et al., 2018), the results may vary with the definition, type, number, and prevalence of included chronic diseases as well as participants' sociodemographic characteristics. For instance, some common heart diseases (i.e., heart failure, coronary heart disease, and arrhythmia) did not cluster into a single group, as indicated in our study. Therefore, the interpretation of findings should be incorporated with pathophysiological and clinical features of the chronic conditions. Fourth, the prevalence of arthritis might have been underestimated because the misdiagnosis and underdiagnosis of arthritis was fairly common in China, particularly in primary care settings (Tian et al., 2021). Fifth, functional outcomes were assessed within 2 weeks after hospitalization. The findings may be relevant to the functional status of stroke patients in the acute phase and may have implications for early rehabilitation intervention. Future studies that assess cognitive and physical functioning of patients during the period of a few months post-stroke are warranted to confirm the associations between comorbidity and functional outcomes. Finally, we were not able to explore the potential influences of stroke characteristics (e.g., severity, size, and location) on the associations between comorbidities and functional outcomes due to lack of detailed CT or MRI data.

Conclusion

Our study indicates that comorbidity is highly prevalent among Chinese stroke patients and that chronic conditions are clustered in certain patterns (e.g., patterns of degenerative-cardiopulmonary, heart- gastrointestinal-psychiatric, and metabolic-kidney diseases). Furthermore, the burden and patterns of comorbidity are associated with both poor physical and cognitive function in patients with acute ischemic stroke. These findings may have implications for the proper management of comorbidity among stroke patients in order to maintain and improve post-stroke physical and cognitive functioning outcomes but warrant further investigation in longitudinal studies. Assessments of comorbidity should therefore be routinely included in stroke research and clinical practice.

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 study protocols from the Ethics Committee at the Jining No. 1 People's Hospital, Shandong (No. 2015B006). The patients/participants provided their written informed consent to participate in this study.

Author contributions

RS, ZY, YH, ZZ, YD, JD, BB, and CQ: conception and design of the study. ZY, YH, ZZ, and BB: execution. RS: statistical analysis. RS and CQ: writing the first draft of the manuscript. DV, JL, and CQ: supervision. All authors revised the manuscript.

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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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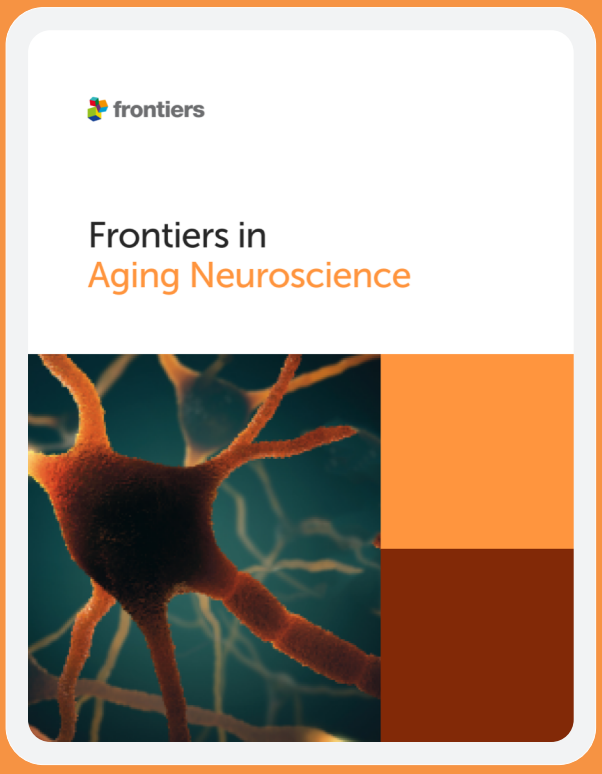

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