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Original Article

Cross-Tissue Proteomic Mendelian Randomization Identifies Therapeutic Targets for Vascular dementia

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ABSTRACT: Vascular dementia (VD) is the second most prevalent form of dementia, yet no disease-modifying treatments are available. Proteins in the brain, cerebrospinal fluid (CSF), and plasma with strong genetic associations represent promising therapeutic targets. However, a comprehensive, proteome-wide screening for VD-associated proteins has not been conducted. We employed a cross-tissue proteomic Mendelian randomization (MR) framework, integrating pQTL data from brain, CSF, and plasma with VD GWAS. We identified causal VD-associated proteins, assessed their consistency across VD subtypes, explored metabolite-mediated effects via metabolomic MR, determined cell-type specificity using single-nucleus RNA sequencing (snRNA-seq), and prioritized therapeutics through drug-target interaction analysis and molecular modeling. MR analysis identified APOE as the strongest VD-associated protein in both CSF and plasma, indicating systemic relevance. Several proteins, including the 14-3-3 family, AREG, SMOC1, and UBE2G2, were exclusively associated in CSF, suggesting CNS-specific roles. Metabolomic MR revealed key APOE-mediated metabolites linked to disease progression. snRNA-seq showed APOE upregulation in excitatory neurons of VD patients. Drug screening highlighted benserazide and puromycin as top candidates, validated by molecular simulations. This study systematically identifies genetically validated proteomic targets for VD, establishing APOE as a central molecular driver. By integrating proteomics, genomics, metabolomics, and drug discovery, we propose a comprehensive framework for targeted therapeutic development, supporting precision medicine in VD treatment.

Keywords: Vascular dementia, Genome-wide association study, Mendelian randomization, Protein

INTRODUCTION

Dementia is a leading cause of death worldwide, with VD being the second most prevalent subtype [1]. This neurodegenerative disorder is primarily characterized by impaired cerebral blood flow and vascular damage, accounting for at least 20% of dementia cases globally [2]. In 2019, an estimated 57.4 million people were living with dementia, with projections suggesting this number will rise to 152.8 million by 2050 [3]. Concurrently, the incidence of VD is increasing, posing a significant financial burden and straining healthcare systems. Despite progress in managing modifiable vascular risk factors to prevent VD, a substantial residual risk remains [4, 5], underscoring the urgent need for novel therapeutic targets to address the unmet medical needs in its prevention and treatment.

Although several previous studies have explored potential drug targets for VD—either through observational associations, transcriptomic profiling, or drug repurposing pipelines—these efforts remain fragmented and often lack clinical utility [6-10]. First, most studies fail to account for tissue or fluid specificity, limiting the ability to distinguish between central (brain,

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cerebrospinal fluid) and peripheral (plasma) processes. Second, many findings are based on correlation rather than causation, reducing their therapeutic relevance. Third, few studies incorporate large-scale genetic data or multi-omics integration, resulting in limited mechanistic insight and low translational success. These gaps highlight the pressing need for a robust, genetically informed strategy capable of resolving compartment-specific biological signals to identify viable therapeutic targets for VD.

Previous studies have identified several proteins linked to VD risk as potential therapeutic targets [11]. However, the high attrition rate in drug development underscores the need for ongoing investigation of proteins involved in the pathogenesis of VD prior to clinical trials. Notably, drug targets with strong genetic support are twice as likely to gain market approval [12]. Recent advancements in high-throughput protein quantification have facilitated genome-wide association studies (GWAS), enabling the simultaneous identification of genetic determinants for brain, cerebrospinal fluid, and blood proteins [13]. Thus, integrating genetic and proteomic data through MR analysis offers a powerful strategy to uncover novel and effective drug targets for VD.

MR is a statistical genetic framework designed to evaluate the causal relationship between exposure (e.g., protein levels) and outcome (VD), while accounting for potential confounders through sensitivity analyses [14]. This approach offers a key advantage over traditional observational studies by reducing bias from confounding factors. Another strength of MR is its ability to simulate randomized controlled trials by grouping subjects based on allele presence or absence, allowing for the assessment of allele effects without the extensive resources required for clinical trials [15]. Despite these advantages, no studies have yet explored drug targets using brain, cerebrospinal fluid, and plasma proteomes for VD interventions, and no potential therapies have been identified. In this study, we aim to investigate potential drug targets for VD, providing a theoretical foundation for the development of novel, effective treatments to address the significant unmet medical needs in this area. The schematic overview of the research workflow is illustrated as follows (Fig. 1).

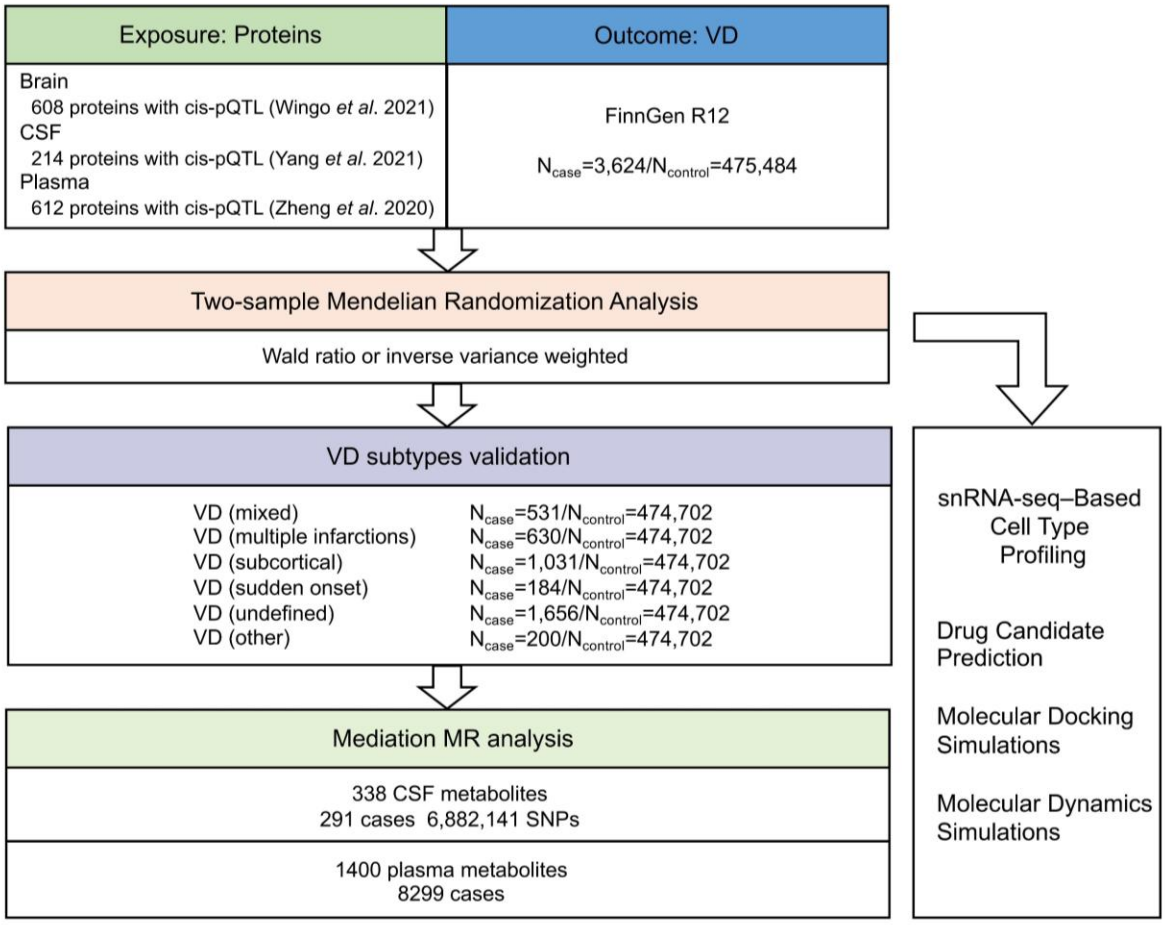

Figure 1. Graphical representation of the research design.

MATERIALS AND METHODS

pQTLs in Brain, Cerebrospinal Fluid, and Plasma

For the primary analysis, we utilized brain pQTL data from the study by Wingo et al [16]. The included pQTLs met the following criteria: (1) demonstrated genome-wide significant association ($P<5\times10^{-8}$); (2) showed independent association ($r^2<0.001$ in linkage disequilibrium within a 10,000 kb window); and (3) exhibited robust SNPs, as determined by an F-statistic ≥10. To ensure that the genetic variant was not directly associated with the outcome, we examined each SNP in the GRASP v2.0 database to determine whether it was significantly associated with the outcome ($P<5\times10^{-8}$) [17]. Given that our two-sample MR was performed using two independent datasets (i.e., no overlapping samples between exposure and outcome), it can be assumed that confounding factors were minimized. In total, 616 cis-pQTLs corresponding to 608 proteins were identified. CSF pQTL data were obtained from Yang et al [18]. and followed the same screening criteria as the brain pQTL dataset, resulting in 233 cis-SNPs for 214 proteins. Finally, plasma pQTL data were sourced from Zheng et al [19]. and integrated with five previously published GWAS, comprising 616 cis-SNPs for 612 proteins in the primary analysis (Supplementary Table 1).

Summary of GWAS Statistics

The summary statistics for our primary outcome, VD, were obtained from the FinnGen study, a nationwide meta-analysis of GWAS conducted across 13 cohorts and biobanks in Finland. This study includes 3,624 cases and 475,484 controls, making it the largest GWAS of VD to date [20]. VD cases were defined based on inpatient or outpatient diagnoses coded according to the International Classification of Diseases, 10th revision (ICD-10 [F01.x]). To further explore potential subtype-specific associations, we extracted GWAS summary statistics for six VD subtypes from the FinnGen R12 release. Subtypes were classified according to ICD-10 subcodes under F01, with the following sample sizes: VD (mixed): 531 cases / 474,702 controls; VD (multiple infarctions): 630 cases / 474,702 controls; VD (subcortical): 1,031 cases / 474,702 controls; VD (sudden onset): 184 cases / 474,702 controls; VD (undefined): 1,656 cases / 474,702 controls; VD (other): 200 cases / 474,702 controls. All subtype definitions and phenotype categorizations were directly based on the FinnGen clinical endpoint registry. To further assess the generalizability of our findings, we additionally incorporated a publicly available, independent GWAS dataset for VD (GCST90435838) [21], which includes 189 VD cases and 402,383 controls of British ancestry. Although the case number is limited, this dataset offers a valuable external reference point. This cross-cohort validation enhances the robustness of our conclusions and allows for an assessment of replicability in a distinct European population.

Mendelian Randomization Analysis of VD

We conducted MR using SNPs associated with brain, CSF, and plasma proteins as exposures and VD as the outcome, employing the "TwoSampleMR" package. When a single pQTL was available for a protein, the Wald ratio method was applied; otherwise, the inverse variance weighted (IVW) method was used [22]. Due to the limited number of instrumental variables (IVs), post-MR analyses (e.g., Cochran Q test for heterogeneity, MR-Egger intercept test for horizontal pleiotropy, MRPRESSO test for outlier detection, and I^2 GX test for "no measurement error") were not feasible [23]. For the primary analysis, we adjusted for multiple testing using the Bonferroni correction, setting a threshold of 0.05 divided by the number of proteins analyzed to prioritize results for further examination. The corrected significance thresholds were set at $P < 8.22 \times 10^{-5}$ for brain, $P < 2.34 \times 10^{-4}$ for CSF, and $P < 8.17 \times 10^{-5}$ for plasma.

Targeted MR Analysis of VD Subtypes

Given that risk proteins may vary or remain consistent across different etiologies of VD, we examined the potential causal role of risk proteins identified in major VD datasets, focusing on the following subtypes: mixed-type VD, VD due to multiple infarctions, subcortical VD, sudden-onset VD, and undefined or unspecified VD [20].

Mediation MR Analysis

To investigate the potential mediating role of CSF metabolites in the relationship between APOE and VD, we conducted a mediation MR analysis leveraging summary-level GWAS data. A total of 338 CSF metabolites were included as candidate mediators, derived from a dataset comprising 291 cases and 6,882,141 single nucleotide polymorphisms (SNPs) [24]. Publicly accessible genetic variation data can be found at EBI GWAS Catalog. The analysis was structured as a two-step approach [25]. In the first step, MR analysis (β_1) was applied to assess the causal effect of APOE protein levels (exposure) on CSF metabolites (outcome). In the second step, risk-associated metabolites identified in step one were analyzed as exposures to determine their causal association (β_2) with VD (outcome). Metabolites meeting the statistical threshold ($P < 0.05$) were classified as risk-

associated mediators of VD. To quantify mediation, the indirect effect of APOE on VD through each metabolite was estimated as the product of β_1 (effect of APOE on CSF metabolites) and β_2 (effect of CSF metabolites on VD) [25, 26]. Standard errors were derived using the delta method, ensuring robust variance propagation and statistical inference [27]. This analytical framework provides a comprehensive evaluation of metabolic pathways that may bridge APOE and VD pathology, offering insights into potential mechanistic targets. Furthermore, p-values in the mediation MR analysis were not adjusted for multiple testing. This decision reflects the exploratory nature of the analysis, which aimed to generate hypotheses rather than make definitive causal inferences. Given the two-step design and the limited number of genetically instrumented metabolites, applying multiple testing correction at both stages would have substantially reduced statistical power and increased the risk of false negatives. Therefore, metabolites meeting nominal significance thresholds ($P < 0.05$) in both steps were retained as candidate mediators. These findings should be interpreted with caution and will require future validation in independent cohorts.

In addition to CSF metabolites, we further performed a parallel mediation MR analysis using plasma metabolites to investigate peripheral metabolic pathways linking APOE to VD. Summary statistics for 1400 plasma metabolites were obtained from a large-scale GWAS of 8,299 European individuals [28], publicly available via the EBI GWAS Catalog. The same two-step MR framework was applied: first, we assessed the causal effects of APOE protein levels on each plasma metabolite (β_1); second, we evaluated the causal association between risk-associated plasma metabolites and VD (β_2). Indirect effects were estimated as the product of β_1 and β_2, with standard errors derived using the delta method. Metabolites passing a nominal significance threshold ($P < 0.05$) in both steps were retained as candidate mediators.

Cell Type-Specific Analysis

To investigate the cell type-specific expression of VD-associated genes in the brain, we analyzed a single-nucleus RNA sequencing (snRNA-seq) dataset derived from individuals with and without VD (GSE282111). The dataset included periventricular white matter samples from four age-matched healthy controls and four VD patients, enabling a high-resolution characterization of gene expression across distinct brain cell populations and providing insights into the cellular mechanisms underlying VD pathogenesis. For quality control, genes expressed in fewer than three cells were excluded, and cells expressing fewer than 200 genes were filtered out. Doublet removal was performed using Scrublet, and further quality control was applied based on mitochondrial and ribosomal gene content, removing cells with >2.20% mitochondrial gene counts and >1.13% ribosomal gene counts. Library size correction was applied, and the data matrix was normalized using the "scanpy.pp.normalize_total" function in Scanpy. The resulting logarithmically normalized data matrix was used for downstream analyses.Dimensionality reduction and unsupervised clustering followed the standard Scanpy workflow [29]. Highly variable genes (n = 4000) were selected using "scanpy.pp.highly_variable_genes", and total counts per cell, mitochondrial gene expression percentage, and ribosomal gene expression percentage were regressed using "scanpy.pp.regress_out". To standardize gene expression, features were scaled to unit variance using "scanpy.pp.scale" with a maximum value of 10. Principal component analysis (PCA) was performed to reduce dimensionality, and batch effects were corrected using "sc.external.pp.harmony_integrate" with n_pcs = 40. Further dimensionality reduction was applied using Uniform Manifold Approximation and Projection (UMAP) via "scanpy.tl.umap". Finally, cells were clustered using the Leiden algorithm to construct a neighborhood graph, enabling the identification of distinct cellular populations contributing to VD. To annotate cell types, we adopted a graph-based clustering approach and defined major populations based on canonical marker gene expression. Specifically, oligodendrocytes were identified by PLP1; microglia by SLC7A14-AS1 and DOCK8; oligodendrocyte precursor cells (OPCs) by FYB1; excitatory neurons by NRGN; inhibitory neurons by GAD2; astrocytes by MEGF11; ependymal cells by CD44; endothelial cells by COL1A2; and pericytes by COL6A3. To assess APOE expression across disease states, we further performed cell-type-specific differential expression analysis using the "FindMarkers" function in the Seurat package (v4.3) [30] after converting the AnnData object to a Seurat object. This enabled us to quantify APOE expression differences between VD and control groups within excitatory neurons. Violin plots with annotated p-values were used to visualize the findings.

Construction of Drug-Target Protein Interaction Networks and Functional Pathway Analysis

To assess the functional relevance of the identified targets, we constructed protein–protein interaction (PPI) networks using STRING [31] and performed enrichment analysis with Enrichr [32]. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were evaluated under stringent criteria (adjusted $P < 0.05$). The top 10 enriched pathways were prioritized and

visualized using ggplot2 in R to highlight key biological processes and signaling cascades.

Drug Candidate Prediction

To predict candidate pharmacological agents, we analyzed drug–target interactions using the Drug Signature Database (DSigDB) via the Enrichr platform [33]. Target genes identified in this study were queried against DSigDB, and drug candidates were prioritized based on adjusted p-values (< 0.05).

Molecular Docking Simulations

To further explore the molecular interactions and assess the binding affinity between drug candidates and core drug target proteins, molecular docking simulations were performed. The crystal structures of the drug targets were retrieved from the Protein Data Bank (/www.rcsb.org/) for subsequent docking analysis. The molecular structures of the potential drug candidates were obtained from the ZINC database (https://zinc.docking.org/). Docking simulations were systematically conducted for all drug candidate-core drug target protein combinations. Prior to docking, receptor preparation involved essential steps, including the addition of nonpolar hydrogens, charge merging, removal of lone electron pairs, and elimination of solvent molecules near the binding site. Semi-flexible docking was then performed using an automated docking program to generate diverse conformational orientations, facilitating the investigation of electrostatic and van der Waals interactions between ligand molecules and the binding site.

Molecular Dynamics Simulations

The steady-state binding conformation of the UBE2G2 protein and Puromycin was initially determined through molecular docking and subsequently imported into the Maestro workbench within the Schrödinger software suite (version 2022-1), using the Desmond engine, for further molecular dynamics (MD) simulations. The simulation workflow was executed sequentially using the following modules: First, protein preparation was performed to ensure structural integrity. The protonation states of ionizable residues were calculated under physiological conditions (pH = 7.4), followed by a structural inspection to retain the most representative single conformation from multiple possibilities. The polypeptide chains were capped, chemical bond orders were validated, and missing hydrogen atoms were added. Hydrogen bond assignments were further optimized to minimize steric clashes, and non-essential molecules, including crystallographic water and solvent molecules, were removed. Next, the system setup was configured. The SPC explicit water model was employed as the solvent environment, with Na^+ ions introduced to neutralize the system charge, while no additional salt components were included. The system was encapsulated within a rectangular periodic boundary box, maintaining a minimum distance of 10 Å between the protein and the box edges, followed by automated box size optimization. The OPLS_4 force field was applied to ensure accurate molecular representation and energetic calculations. Following system preparation, molecular dynamics simulations were conducted. The system underwent default relaxation protocols to stabilize prior to simulation. The total system size comprised approximately 25,000 atoms, and simulations were executed for 100 ns with a 2 fs time step. The trajectory data were saved every 1 ns (100 frames), and system energy profiles were recorded at 100 ps intervals (1,000 data points). The NPT ensemble (298.15 K, 1 atm) was employed, with temperature regulation via the Nose-Hoover chain thermostat and pressure control via the Martyna-Tobias-Klein barostat, while maintaining OPLS_4 as the force field throughout. Upon completion of the MD simulation, interaction analyses were performed using the Simulation Interaction Diagram module, which facilitated a comprehensive assessment of protein-ligand interactions, including hydrogen bonding, hydrophobic interactions, ionic interactions, and water-mediated contacts. This module also enabled the extraction of raw simulation data, visualizations, and analytical reports, providing an extensive evaluation of the stability and dynamic behavior of Puromycin within the UBE2G2 binding pocket.

RESULTS

Screening the proteome for VD

MR analysis was conducted to identify potential drug targets for VD in CSF and plasma proteins. While no significant associations were observed for brain proteins, multiple CSF proteins exhibited strong causal associations with VD. Notably, APOE was the only protein identified as a significant drug target in both CSF and plasma, with a robust association in CSF (β = 15.5236; 95% CI: 12.9015-18.1458, $P = 3.95 \times 10^{-31}$) and a more moderate effect in plasma (β = 0.3340; 95% CI: 0.1863- 0.4818, $P = 9.39 \times 10^{-6}$), underscoring its systemic relevance in VD pathogenesis (Fig. 2). In contrast, several additional proteins were identified in CSF, including AREG, SMOC1, YWHAZ, and the 14-3-3 protein family complex (YWHAB/YWHAE/YWHAG/YWHAH/YWHAQ/YWHAZ/SFN), as well as AIMP1, CKAP2, CRYZL1, PGK1, PSME1, SNRPF, and UBE2G2, all of which were exclusively detected in CSF.

These findings highlight APOE as the only shared protein target between CSF and plasma, suggesting its unique role in both central and systemic mechanisms of VD pathogenesis, while other identified CSF proteins may represent more localized brain-specific contributors to disease progression. Mendelian Randomization Analysis of All Proteins (Supplementary Table 2, Supplementary Table 3)

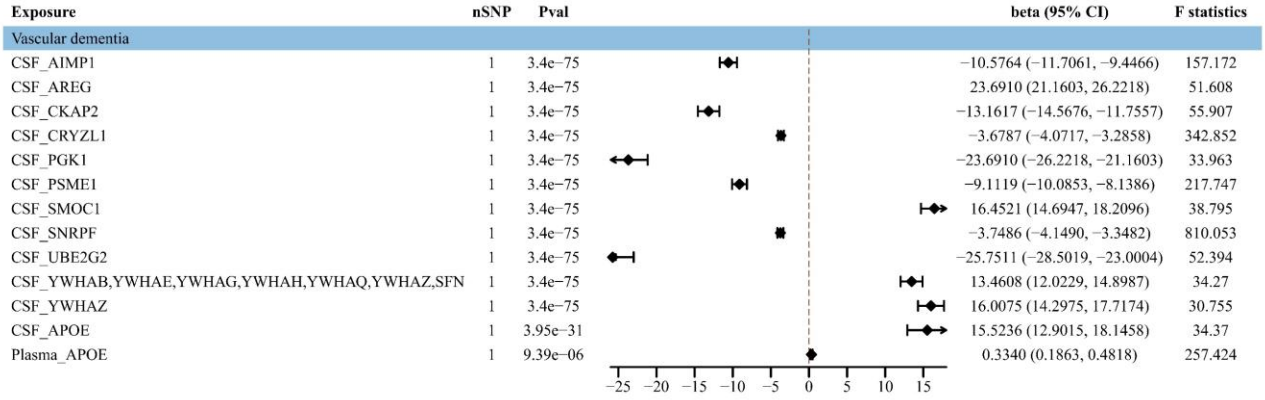

Figure 2. Cross-Tissue Mendelian Randomization Analysis Identifies Causally Associated CSF and Plasma Proteins in VD. Forest plot illustrating the effect estimates (β) and 95% confidence intervals (CIs) for CSF and plasma proteins associated with VD identified through two-sample MR. APOE was the only protein identified as a significant VD risk factor in both CSF and plasma, highlighting its systemic relevance in disease pathogenesis. Several additional proteins—including AIMP1, AREG, CKAP2, CRYZL1, PGK1, PSME1, SMOC1, SNRPF, UBE2G2, and members of the 14-3-3 protein family—exhibited exclusive CSF associations, suggesting their roles in central nervous system-specific pathology. Effect estimates were derived using IVW MR or the Wald ratio method, with statistical significance determined by Bonferroni-corrected thresholds. Higher F-statistics indicate stronger instrumental variable strength, ensuring the robustness of causal inference. Negative effect estimates indicate protective associations, while positive values indicate risk-enhancing effects.

To further corroborate the robustness of our findings, we conducted an independent two-sample Mendelian randomization analysis leveraging a publicly available VD GWAS dataset (GCST90435838). Despite the modest sample size, this external validation consistently recapitulated the directional causal associations between all CSF-identified candidate drug targets and VD. The concordance of these effects across independent cohorts reinforces the credibility of our primary results and suggests that the CSF-specific proteins uncovered herein may represent biologically conserved mediators implicated in the pathogenesis of VD. A detailed summary of replication outcomes is provided in Supplementary Table 4.

Key Therapeutic Target Proteins Across VD Subtypes

To identify potential therapeutic targets for VD, we examined whether risk-associated proteins exhibit consistent effects across different VD subtypes. Our analysis revealed strong and significant associations between CSF APOE levels and all VD subtypes, reinforcing APOE as a central driver in VD pathogenesis. Effect estimates were stable across subtypes, with the strongest associations observed in VD (subcortical) ($\beta = 16.52$, $p = 1.03 \times 10^{-11}$) and VD (undefined) ($\beta = 16.97$, $p = 2.85 \times 10^{-18}$), alongside significant associations in VD (multiple infarctions), VD (sudden onset), and VD (mixed) (Fig. 3, Supplementary Table 5). The consistently positive associations suggest that APOE-driven mechanisms may underlie diverse VD subtypes, potentially through shared cerebrovascular and neurodegenerative pathways. These findings establish APOE as a fundamental genetic determinant of VD and underscore its potential as a universal therapeutic target, paving the way for targeted interventions across all VD subtypes.

Investigation of CSF Metabolites as Potential Mediators in the APOE-Driven Pathogenesis of VD Subtypes

In the initial MR analysis, we identified 18 CSF metabolites with a putative causal association with APOE from a total of 338 metabolites ($p < 0.05$). Among these, elevated APOE levels were associated with increased expression of 14 metabolites, while decreased expression was observed for four metabolites (Supplementary Table 6). In the second MR step, we further identified 5 metabolites linked to distinct subtypes of VD, including three metabolites associated with VD, and one metabolite associated with each of the following subtypes: VD (multiple infarctions), VD (sudden onset), VD (mixed),

VD (undefined), and VD (other) (Supplementary Table 7).

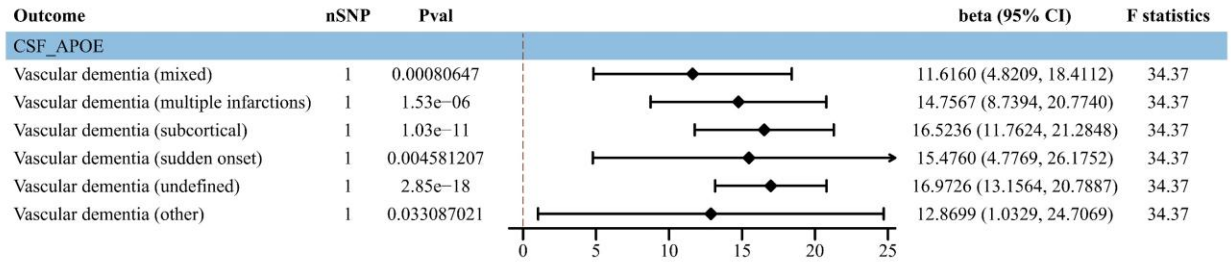

Figure 3. APOE as a Key Genetic Risk Factor Across VD Subtypes. Forest plot depicting the causal associations between CSF APOE levels and various VD subtypes, as determined through MR. APOE demonstrated significant positive associations across all major VD subtypes, including mixed VD, multiple infarct VD, subcortical VD, sudden-onset VD, undefined VD, and other VD classifications. Effect estimates (β) and 95% confidence intervals (CIs) were derived using the IVW MR method or the Wald ratio test. Consistent F-statistics across models indicate robust instrumental variable strength. These findings highlight APOE as a potential universal therapeutic target for VD intervention.

Subsequent two-step mediation analysis revealed that CSF_APOE may influence VD through distinct metabolic pathways. Notably, ethyl β-glucopyranoside levels demonstrated a potential mediating role in the relationship between CSF_APOE and VD (other), contributing 1.68 to the total effect of 12.87, with a direct effect of 11.19. This suggests that while ethyl β-glucopyranoside plays a role, additional mechanisms are likely to contribute to disease pathogenesis. Similarly, threonine levels were identified as a potential mediator in the association between CSF_APOE and VD (mixed), contributing 0.87 to the total effect of 11.62, with a direct effect of 10.75 (Supplementary Table 8). These findings implicate ethyl β-glucopyranoside and threonine in the metabolic cascade linking CSF_APOE to VD subtypes, underscoring their potential as mechanistic targets for further investigation.

Investigation of Plasma Metabolites as Potential Peripheral Mediators in the APOE–VD Axis

We next investigated the role of plasma metabolites as potential mediators linking peripheral APOE expression to VD. In the initial MR step, we identified 218 plasma metabolites with nominally significant causal associations with APOE levels from a total of 1400 metabolites ($P < 0.05$; Supplementary Table 9). Among these, APOE levels were positively associated with 103 metabolites, while negative associations were observed for 115 metabolites.

In the subsequent MR step, we evaluated the effects of these APOE-associated plasma metabolites on VD risk （Supplementary Table 10）. Our mediation analysis revealed that the plasma metabolite 1-stearoyl-2-docosahexaenoyl-GPC (18:0/22:6) significantly mediates the effect of circulating APOE levels on VD risk (Supplementary Table 11). Specifically, higher APOE levels were associated with increased levels of 1-stearoyl-2-docosahexaenoyl-gpc ($\beta_1 = 0.134$, SE = 0.056), which in turn was positively associated with VD risk ($\beta_2 = 0.151$, SE = 0.037). The estimated indirect effect (β_indirect = 0.020, SE = 0.010, P = 0.042) accounted for approximately 6.1% of the total APOE→VD effect, suggesting a modest but statistically significant mediating role. The mediation pathway showed consistent directionality, with both exposure–mediator and mediator–outcome effects aligned in the same direction. This implicates peripheral lipid signaling, particularly involving DHA-containing phosphatidylcholines, as a potential mechanistic link between APOE dysregulation and vascular cognitive decline.

Cell Type-Specific Expression of Risk Genes in the Human Brain

To further investigate the cell type-specific expression of the risk-associated gene APOE in the human brain, we analyzed snRNA-seq data from the GSE282111 dataset, which includes periventricular white matter samples from four healthy controls and four VD patients. Clustering analysis identified major brain cell populations, including astrocytes, endothelial cells, ependymal cells, excitatory neurons, inhibitory neurons, microglia, oligodendrocyte precursor cells (OPCs), oligodendrocytes, and pericytes, with their relative distributions between control and VD groups visualized in Supplemtary Figure 1. Supplemtary Figure 2 presents dot plots illustrating the expression patterns of canonical marker genes across each identified cell subset.

Consistent with its well-established role in lipid metabolism and neuroinflammation, APOE expression

was primarily enriched in oligodendrocytes and microglia (Fig. 4A, 4B). Notably, in VD patients, APOE expression was significantly upregulated in both excitatory neurons (Figure 4C, Supplemtary Fig. 3), suggesting an increased neuronal susceptibility to APOE-mediated risk in the context of VD. These findings highlight a potential APOE-driven neuronal dysfunction beyond its canonical role in glial cells, underscoring its broader impact on neuronal homeostasis and neurovascular pathology in VD.

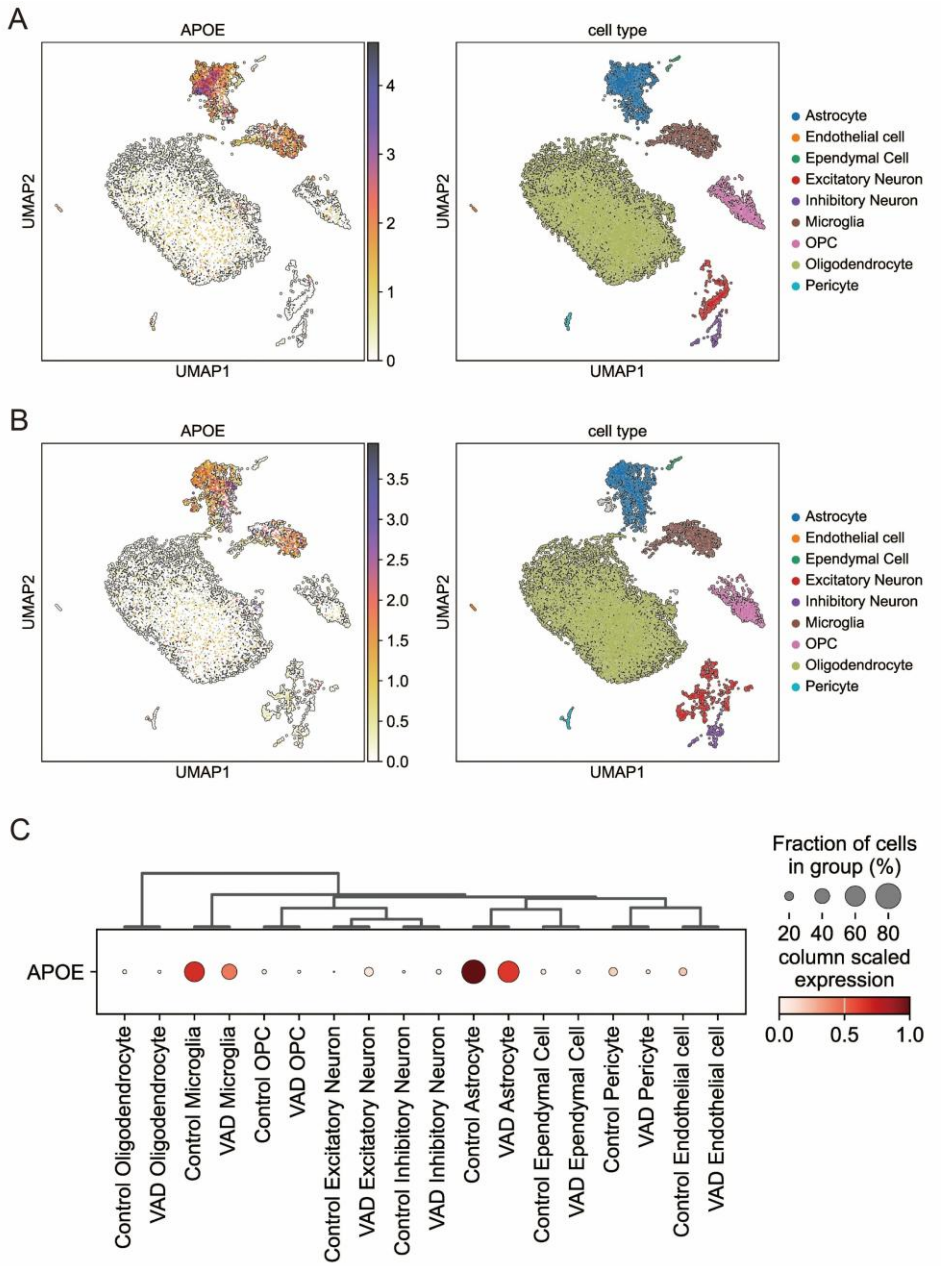

Figure 4. Cell Type-Specific Expression of APOE in the Human Brain in VD. (A-B) Uniform Manifold Approximation and Projection (UMAP) plots displaying APOE expression across major brain cell types in healthy controls (A) and VD patients (B). Cell populations include astrocytes, endothelial cells, ependymal cells, excitatory neurons, inhibitory neurons, microglia, oligodendrocyte precursor cells (OPCs), oligodendrocytes, and pericytes. **(C)** Dot plot illustrating the fraction of cells expressing APOE across different brain cell types in HC and VD groups.

Protein Interaction Network and Pathway Enrichment Analysis of Potential Therapeutic Target Proteins in Cerebrospinal Fluid

To elucidate the molecular interactions and functional pathways associated with potential therapeutic target proteins in CSF, we conducted a PPI network analysis using the STRING database, applying a medium-confidence interaction threshold (score ≥ 0.4). The resulting PPI network comprised 17 nodes and 29 edges, significantly exceeding the expected six edges (enrichment $P = 7.06 \times 10^{-12}$), indicating a highly interconnected protein network with biological relevance (Supplemtary Fig. 4A).

To further dissect the functional implications of these candidate therapeutic targets in the pathogenesis of VD, we performed GOBP enrichment analysis (Supplemtary Fig. 4B). The results revealed a significant overrepresentation of pathways related to mitochondrial outer membrane permeabilization and apoptotic signaling, implicating mitochondrial dysfunction in the neurodegenerative processes underlying VD.

Additionally, KEGG pathway enrichment analysis (Supplemtary Fig. 4C) highlighted several signaling cascades with potential mechanistic relevance to VD, including the PI3K-Akt signaling pathway, Hippo signaling pathway, and cholesterol metabolism, as well as pathways linked to proteasome function and cell cycle regulation. These findings underscore the diverse biological roles of the identified proteins, providing mechanistic insights into VD pathology while also highlighting novel targets for therapeutic intervention.

Drug Prediction for Potential Therapeutic Targets in Cerebrospinal Fluid

To identify candidate therapeutics targeting key gene interventions in cerebrospinal fluid, drug prediction analysis was conducted using the Drug Signature Database (DSigDB). A total of 17 target genes were assessed, and potential small-molecule compounds were ranked based on statistical significance. The top four candidate drugs with the highest therapeutic potential were prioritized according to the adjusted p-value (Supplementary Table 12).

To evaluate the pharmacological potential of the four candidate compounds, we conducted molecular docking analyses to assess their binding affinities to respective target proteins. Binding affinity was quantified using docking scores (kcal/mol), where lower values indicate stronger molecular interactions. In general, docking scores below −5 kcal/mol are considered indicative of robust binding interactions with potential biological relevance. Among the tested compounds, benserazide and puromycin emerged as the most promising candidates, exhibiting docking scores below this threshold, suggesting strong molecular interactions with their target proteins. Notably, puromycin, a well-characterized protein synthesis inhibitor, demonstrated the strongest binding affinities across multiple targets, with docking scores consistently below −5 kcal/mol. Its highest-affinity interactions were observed with YWHAE (PDB: 2BR9), PGK1 (PDB: 3C39), SFN (PDB: 1YZ5), UBE2G2 (PDB: 2CYX), YWHAZ (PDB: 3RDH), and YWHAH (PDB: 2C74). Similarly, benserazide exhibited strong binding interactions with UBE2G2 (PDB: 2CYX), YWHAE (PDB: 2BR9), and YWHAZ (PDB: 3RDH), further supporting its potential as a therapeutic candidate (Supplemtary Fig. 5).

Furthermore, molecular dynamics simulations were performed to validate the interaction between puromycin and UBE2G2, which exhibited the lowest binding energy in molecular docking studies. The simulation confirmed that puromycin remained stably bound within the UBE2G2 binding pocket throughout the 100-nanosecond trajectory (Supplemtary Fig. 6), with minimal conformational drift. These findings underscore puromycin's strong binding affinity and structural stability, supporting its potential as a therapeutic candidate for VD.

DISCUSSION

In this study, we leveraged multi-compartment proteomic profiling of brain tissue, CSF, and plasma to identify proteins causally implicated in VD. Our analysis uncovered 17 candidate proteins with potential therapeutic relevance, among which APOE was uniquely detectable in both CSF and peripheral circulation, while the remaining proteins were confined to CSF. Strikingly, across diverse subtypes of VD—including mixed VD, multi-infarct VD, subcortical VD, and sudden-onset VD—our findings consistently identified APOE dysregulation as a convergent molecular signature. Further mechanistic interrogation via mediated Mendelian randomization in CSF revealed that elevated APOE levels drive a reduction in CSF threonine concentrations, thereby exacerbating the risk of mixed VD. Additionally, single-cell analysis demonstrated that APOE is upregulated in both excitatory and inhibitory neuronal populations in patients with VD, underscoring its critical role in the neuronal damage underlying disease pathogenesis.

Our study underscores the critical value of integrating three distinct tissue sources to unravel the molecular mechanisms underlying VD, laying the foundation for the development of targeted therapeutic interventions. Notably, our analysis identifies APOE protein levels in plasma and CSF as key determinants in the pathogenesis of VD. Encoded by the APOE gene, APOE exists in three major allelic forms—ε2, ε3, and ε4—which translate into the isoforms apoE2, apoE3, and apoE4, respectively [34]. While APOE has been extensively studied in the context of lipid metabolism, cardiovascular disease, and Alzheimer's disease (AD)-related dementia, its precise role in VD remains incompletely understood. Emerging evidence suggests that apoE influences cerebrovascular integrity, neurovascular function, and blood-brain barrier (BBB) stability [35]. Intriguingly, previous studies indicate that apoE-mediated cerebrovascular damage disrupts BBB integrity, exacerbating neurovascular

dysfunction [36]. Moreover, the loss of the primary apoE receptor, low-density lipoprotein receptor-related protein 1 (LRP1), induces cerebrovascular dysfunction and cognitive impairment in a genotype-dependent manner. Specifically, apoE ε4 mice with LRP1 deletion exhibit impaired spatial memory, hyperactivation of perivascular glial cells, reduced cerebrovascular collagen IV, and compromised BBB integrity, whereas apoE ε3 mice with LRP1 deletion do not display these deficits [37]. These findings suggest that LRP1 in vascular wall cells plays a crucial role in maintaining cerebrovascular function in an apoE genotype-dependent fashion. Additionally, apoE ε4-mediated neurovascular impairment may be influenced by border-associated macrophages, particularly intracranial perivascular macrophages (PVMs), which modulate neuroinflammation and vascular homeostasis [38]. Lastly, an intriguing link has been established between APOE expression and blood pressure variability (BPV)—a known risk factor for cognitive decline and cerebrovascular disease burden [39]. Collectively, these findings highlight the multifaceted role of APOE in VD pathogenesis, emphasizing its potential as a therapeutic target for preserving cerebrovascular integrity and cognitive function.

We investigated the cell type distribution of APOE gene expression in the brains of vascular VD patients and observed a notable upregulation of APOE in neurons. This finding aligns with previous studies suggesting that neuronal APOE4 exerts detrimental effects on synaptic integrity and neuronal network function [40]. In mice with a murine ApoE-knockout (ApoE-KO) background, neuronal-specific expression of APOE4 has been shown to induce synaptic loss and cognitive deficits, manifesting as learning and memory impairments by 6–9 months of age [41, 42]. A potential mechanism underlying APOE4-driven synaptic loss involves the hyperphosphorylation of vasodilator-stimulated phosphoprotein (VASP) at serine 235 (S235). This modification disrupts VASP's interactions with key cytoskeletal components, including actin filaments and tubulin, thereby impairing synaptic architecture and neuronal stability [43]. In parallel, neuronal APOE4 compromises mitochondrial function and structural integrity, likely leading to energetic deficits that exacerbate neurodegeneration [44, 45]. Specifically, primary neurons from transgenic mice expressing neuronal APOE4 exhibit a significant reduction in mitochondrial respiratory complex levels compared to those expressing APOE3, thereby impairing oxidative phosphorylation and ATP production [46]. This mitochondrial dysfunction-driven energy failure may serve as a pivotal mechanism linking APOE4 expression to neuronal vulnerability and synaptic degeneration in VD.

Although APOE is a well-known genetic risk factor for various dementias, including VD, its role in VD—particularly mixed subtypes—remains less understood mechanistically. Recent meta-analyses confirm that the APOE ε4 allele is significantly associated with increased VD risk across populations [47], but these findings are largely associative in nature. To explore potential mechanisms, we applied mediation MR and identified CSF threonine as a potential intermediary linking APOE expression to VD-mixed. Our results showed that APOE reduces CSF threonine levels, suggesting a metabolic pathway through which APOE may contribute to disease development. This aligns with prior metabolomic studies implicating threonine dysregulation in cognitive decline [48]. Threonine plays key roles in mitochondrial function and neurotransmitter synthesis. Its depletion could impair neurovascular integrity, providing a plausible link between APOE genotype and VD pathogenesis. This hypothesis is supported by the 2023 AAIC update, which highlighted the context-specific effects of APOE, including its interaction with metabolic and vascular pathways [49].We also extended the mediation analysis to plasma metabolites, identifying peripheral metabolic changes consistent with APOE-related systemic effects. While exploratory, these findings suggest that APOE may act via metabolically mediated pathways rather than direct neurotoxicity, especially in the context of VD-mixed.

Our study offers novel insight by shifting the focus from whether APOE is involved in VD—a well-accepted fact—to how it might act via specific metabolic intermediates, helping refine future research directions and potential intervention targets. Collectively, these findings highlight the therapeutic potential of blood- and CSF-derived proteins as intervention targets in VD, underscoring the need for further clinical validation and mechanistic studies to fully elucidate the interplay between APOE, threonine metabolism, and VD pathogenesis.

Taken together, while APOE emerges as a central node in VD pathogenesis, our findings also highlight the relevance of other CSF-restricted proteins that may act through complementary neurovascular mechanisms. Notably, SMOC1 has been implicated in neurovascular communication and cerebrovascular development. Su et al. showed that neuronal SMOC1 secreted during cortical development engages endothelial TGFBR1 via the Smad2/3 signaling axis, orchestrating angiogenesis and maintaining neurovascular integrity [50]. Loss of SMOC1 disrupts this signaling, resulting in reduced vessel density and impaired neuron-to-endothelium communication. Supporting this, proteomic analyses in AD cohorts have shown elevated SMOC1 levels in both CSF and plasma, with strong correlations to vascular protein modules associated with cerebral amyloid angiopathy (CAA) and

tau pathology, underscoring its potential as a biomarker of cerebrovascular dysfunction [51]. Similarly, 14-3-3 family proteins (notably YWHAZ and YWHAG), which were also enriched in CSF, are known to regulate tau phosphorylation, synaptic plasticity, and neuroinflammatory cascades. These proteins have been found elevated in patients with acute stroke and neurodegenerative diseases, likely reflecting astrocytic activation, neuronal stress, and BBB disruption [52]. Collectively, these findings support a broader role for CSF-derived proteins in VD through mechanisms involving vascular inflammation and disruption of neurovascular unit (NVU) homeostasis [53].

This study offers several key strengths. First, the systematic interrogation of protein markers across plasma, brain, and CSF provides a comprehensive framework for identifying novel therapeutic targets in VD. By integrating multi-omic analyses, we identified candidate pathogenic proteins implicated in disease progression and explored existing pharmacological agents that interact with these targets. Notably, our findings highlight two compounds—benserazide, and puromycin—that may modulate disease-relevant pathways, offering potential avenues for therapeutic intervention. Although puromycin showed strong binding affinity and stability in our docking and MD simulations, it is a broad-spectrum protein synthesis inhibitor that causes premature chain termination during translation [54]. Its cytotoxicity has been widely documented, and puromycin has therefore been primarily used as a laboratory tool or as an antineoplastic antibiotic, rather than as a therapeutic agent in chronic neurodegenerative diseases [55, 56]. These pharmacological characteristics limit its direct clinical applicability in VD, but its identification in our computational screen provides proof-of-concept for target engagement. More importantly, puromycin may serve as a structural scaffold for the development of analogues or derivatives with improved selectivity and reduced toxicity, an approach that has been successfully applied in drug discovery for other broad-spectrum inhibitors [57].

In contrast, benserazide is an approved drug with established safety in humans, widely used as a peripheral dopa decarboxylase inhibitor in combination with levodopa for Parkinson's disease [58]. Its clinical use demonstrates the feasibility of drug repurposing in neurological disorders. Furthermore, there is no direct evidence supporting a role for benserazide in modulating vascular or neurodegenerative pathology relevant to VD. Thus, while its identification highlights a potential avenue for repurposing, the translational value of benserazide in the context of VD should be regarded as preliminary.

Together, these considerations underscore the importance of balancing computational predictions with pharmacological reality. Computational drug prioritization provides a valuable starting point but must be interpreted alongside established knowledge of compound specificity, off-target effects, and safety profiles. Future studies should therefore focus on experimental validation of these interactions, screening for safer structural analogues, and evaluating whether compounds with similar scaffolds may offer neuroprotective benefits without the inherent limitations of puromycin or benserazide.

Furthermore, proteins identified in CSF and plasma may play a direct role in the pathogenesis of VD, providing mechanistic insights into disease progression while also serving as potential biomarkers for diagnosis and prognosis. Importantly, the key pathogenic proteins identified in this study exhibit broad relevance across multiple subtypes of VD, underscoring their potential as universal therapeutic targets. Future treatment strategies are likely to focus on modulating these risk-associated proteins, opening promising new directions for targeted intervention in VD.

The interpretation and generalizability of our findings should be considered in the context of several limitations. First, the precision of protein quantification may be influenced by genetic variation and qualitative protein modifications, including amino acid substitutions, post-translational modifications, and alternative splicing events. Such molecular heterogeneity could impact the accuracy of our causal inferences. Second, the majority of participants in our analysis were European ancestry, reflecting data availability constraints. Given the genetic and environmental diversity across populations, future GWAS incorporating non-European cohorts will be essential to enable cross-ancestry MR analyses and enhance the broader applicability of our findings. Additionally, our ability to conduct heterogeneity and pleiotropy assessments was limited by the fact that all prioritized proteins were linked to a single cis-acting SNP without trans-pQTL associations. This contrasts with conventional MR approaches in GWAS, which typically leverage multiple SNP instrumental variables, necessitating rigorous heterogeneity and pleiotropy testing. Future MR studies investigating drug targets may benefit from deeper proteome sequencing and expanded pQTL datasets to improve statistical robustness and facilitate these critical assessments. Finally, while our findings suggest interactions between VD-associated proteins and existing clinical drug targets, these results should be interpreted as hypothesis-generating rather than definitive. Further experimental and clinical studies are required to validate the causal roles of these proteins in VD pathophysiology and to assess their therapeutic potential.

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Disclosure Statement

The authors have no conflicts of interest to declare.

Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2025.0538.

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