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Original Article

The role of serum vitamins in mediating the effect of neurodegenerative diseases on subcortical brain volume

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ABSTRACT

Background: Neurodegenerative diseases (NDs) lead to a progressive loss of neuronal cells and link to atrophy of subcortical brain structures, but the causal intermediates are not known. To test whether major NDs (Alzheimer's disease (AD), Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis) causally affects subcortical atrophy, and whether serum vitamin level play a mediating role in this process.

Methods: Using large-scale genome-wide association study (GWAS) summary data, we performed two-sample Mendelian randomization (MR) to assess the causal effect of NDs on the volume of seven subcortical structures, and then adopted two-step multivariable MR approach to quantify the proportion of the effect of NDs on the volume of subcortical regions mediated by serum vitamin level. Finally, we utilized animal experiments to validate results and explored the potential molecular mechanisms.

Results: Genetically predicted AD was associated with atrophy of the nucleus accumbens (NAc) (β = -0.09; $p = 5.13 \times 10^{-5}$), amygdala (β = -0.07; $p = 8.44 \times 10^{-4}$), and hippocampus (β = -0.07; p = 0.001), as well as with low serum vitamin D level (β = -0.02; $p = 6.84 \times 10^{-6}$). Specifically, decreased serum vitamin D level mediated 3.99 % (95 % CI: -0.006 to -5.82×10^{-5}) and 3.97 % (95 % CI: -0.007 to -2.94×10^{-4}) of the total effect of AD on hippocampal and NAc atrophy, respectively. Animal experiments further confirmed significant delays in hippocampal and NAc atrophy, a significant reduction of β-amyloid deposits and an increase of vitamin D receptor expression in hippocampus in AD mice with high-dose vitamin D diet.

Conclusions: These findings provide important insights into the effect sizes of vitamin D-mediated roles in AD and atrophy of subcortical structures. Interventions to increase serum vitamin D levels at a population level might attenuate damage to hippocampus in patients with AD.

1. Introduction

Neurodegenerative disorders (NDs) are a group of diseases characterized by progressive dysfunction and loss of neurons affecting distinct parts of central nervous system [1], which pose a notable global health challenge with a growing incidence. The most common NDs include Alzheimer's disease (AD) [2], Parkinson's disease (PD) [3], multiple sclerosis (MS) [4], and amyotrophic lateral sclerosis (ALS) [5]. Subcortical brain regions form circuits with cortical areas to coordinate movement, memory, and learning. Many observational neuroimaging studies have demonstrated varying degrees of atrophy in different subcortical regions by using magnetic resonance imaging (MRI) technology in patients with NDs [6–9]. However, the observed alterations from these studies cannot reflect a causal relationship. It is not clear whether subcortical structural atrophy is the etiology of NDs or the pathological changes during the course of the disease.

Although the exact pathogenesis of NDs remains largely indistinct, several factors have been proposed to contribute to neuroanatomical changes, including genetic, environmental, and nutritional predisposition. Among numerous lifestyle and environmental factors, vitamins play an indispensable role in the proper functioning of our brain. A shortage or lack of regulation of vitamins can adversely impact neuronal

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metabolism. Some literature reports vitamin deficiencies, either singular or multiple, in patients with the major NDs [10–13]. Among these, studies on vitamin D deficiency or insufficiency are relatively plentiful. Vitamin D deficiency is a global issue, affecting approximately 7 % of the population [14]. Additionally, vitamin D deficiency impacts its neurosteroid effects, contributing to neurodegeneration through the onset of neuroinflammation, oxidative stress, disruption of calcium homeostasis, impairment of neurotrophic factor synthesis, and neurotransmitter imbalances [15]. However, most studies are based on cross-sectional design, from which the causality cannot be inferred. A recent systematic review raised the possibility that the consistent finding of reduced serum vitamin levels in patients with NDs may simply reflect reverse causation, as the onset of diseases may lead to dietary changes and/or limited outdoor activity, which in turn result in low vitamins concentrations [16].

Mendelian randomization (MR) is a promising genetic approach that employs single nucleotide polymorphisms (SNPs) acquired by genome-wide association studies (GWAS) as genetic instrumental variables (IVs) for the exposure [17]. Given that SNPs are randomly assigned and fixed at conception, and they do not change in response to traits or diseases, thereby significantly reducing methodological problems related to confounding biases and reverse causation inherent in observational studies. Three key assumptions must be met when conducting MR analysis: (I) the IVs must have a strong association with the exposure; (II) the IVs should be independent of any potential confounders affecting exposure-outcome relationship; and (III) the IVs should influence the outcome exclusively through the exposure [18]. Recent studies using the MR approach have explored causal relationships between NDs and brain imaging traits, primarily focusing on AD and brain structures, thereby improving our understanding of the causal impact of AD on brain traits [19–22]. However, the AD-related genetic variants used were obtained through meta-analysis of GWAS data from clinically diagnosed AD and AD-by-proxy (based on parental diagnoses) participants, which may somewhat attenuate the true genetic effects of AD. To minimize such bias, we used GWAS data from clinically diagnosed AD patients exclusively for our MR analysis. Moreover, these MR studies are limited to simple causal analyses between two variables and do not incorporate mediating factors to further explore the underlying mechanisms. They lack validation through in vivo animal experiments. Therefore, we aimed to elucidate the causal relationship between four NDs and changes in subcortical structures, as well as to determine whether and to what extent serum vitamin level can explain these causal relationships. The flowchart of MR design is shown in Fig. 1. In addition, based on the MR results, we further validated whether subcortical structural atrophy in AD model mice was alleviated by a high-dose vitamin D diet and explored the potential molecular mechanisms.

2. Materials and methods

2.1. Study design of MR analyses

Briefly, we conducted a two-sample, two-step multivariable MR (MVMR) analysis to investigate whether four types of vitamins (C, D, and E (α-tocopherol and γ-tocopherol)) mediated the causal relationships between four NDs (AD, PD, MS and ALS) risk and seven subcortical structures (amygdala, caudate nucleus, hippocampus, nucleus accumbens (NAc), pallidum, putamen, and thalamus). It is worth mentioning that GWAS summary data for other common vitamins (such as vitamin A, B6, B9, and B12) are not acquired due to their public unavailability. Additionally, the largest GWAS summary data from various institutions and organizations were retrieved, ensuring the sample independence of each exposure-outcome pair. This step is crucial because sample overlap can increase the risk of type I error and introduce bias in conventional MR methods [23]. To ensure ethnic consistency in the GWAS data for exposure, outcome, and mediator, we used data exclusively from European populations to minimize bias associated with population stratification [24,25]. All data used in our study are publicly available GWAS summary statistics, and thus no additional ethical approval is required.

2.2. Data sources

The GWAS summary statistics for genetic prediction of risk of NDs included those from the International Genomics of Alzheimer's Project (IGAP) [26], the International Parkinson's Disease Genomics Consortium (IPDGC), the International Multiple Sclerosis Genetics Consortium (IMSGC) [27] and the Project MinE [28]. Subcortical structures GWAS data were obtained from the Enhancing Neuro Imaging Genetics through Meta-Analysis (ENIGMA) consortium, which aims to investigate the impact of common genetic variants on the volume of the seven aforementioned subcortical regions [29]. After excluding non-European ancestry and publicly unavailable data, we integrated the following four types of serum vitamin-related GWAS summary-level data: Vitamin C [30], 25-hydroxyvitamin D (25(OH)D) [31], and Vitamin E (α-tocopherol and γ-tocopherol) [32]. All details of the chosen GWAS data are available in Supplementary Table 1–3.

2.3. Instrumental variable selection

All the SNPs strongly associated with the exposure at genome-wide significance level ($p < 5 \times 10^{-8}$) were selected as potential IVs. To ensure the independence of each SNP, linkage disequilibrium (LD) $r^2 < 0.001$ and a window of 10 mb were used as parameters for clumping. We subsequently harmonized the data sources to ensure that the genetic variants associated with the exposure matched the effect estimates of the same alleles. In addition, SNP-phenotype correlations were identified using the PhenoScanner database (http://www.phenoscanner.medschl.cam.ac.uk/) [33]. SNPs associated with common potential confounding factors at $p < 5 \times 10^{-8}$ such as socioeconomic status, drinking behavior, smoking behavior, and educational attainment [34–38] were removed. SNPs associated with sun behavior (time spent outdoors in summer and winter time and sun protection) [39], serum calcium and phosphorus levels which were reported to impact serum 25(OH)D level, were also excluded before MR analyses. Finally, to determine whether the MR estimate was influenced or skewed by pleiotropic SNPs, the RadialMR method was applied for outlier detection (with $p < 0.05$ indicating the presence of pleiotropic SNPs). This method also addressed horizontal pleiotropy by removing these outliers [40]. The remaining SNPs were then used to conduct MR analyses.

F-statistic was calculated to assess the strength of each IV to minimize weak instrumental bias, $F = R^2 \times (n-2)/(1-R^2)$, where n is the sample size of exposure GWAS [41]. R^2 equals the proportion of total variation in the exposed phenotype. The formula for R^2 is $R^2 = \beta^2 \times 2 \times MAF \times (1-MAF)$, where β represents the effect estimate of the IV in the exposure [42]. We considered the IV to be weak when the F value was less than 10 [23].

2.4. MR and sensitivity analyses

In univariate MR analyses, we estimated the potential causal relationships between four major NDs and the volumes of seven subcortical regions. The primary MR approach was inverse-variance weighted (IVW) method because it has the greatest empirical power and interprets the heterogeneity in the causal estimates derived from single variants [43]. We additionally used two-step MVMR approach to determine the role of circulating vitamins in mediating the causal associations between NDs and subcortical volume. The total effect of an exposure on an outcome can be divided into direct effects (those not mediated through a mediator) and indirect effects (those mediated through a mediator). The method of the product of coefficients was used to calculate the mediating effect of circulating vitamin levels. In our study, the total effect of each type of NDs on subcortical volume was divided into (i) the direct

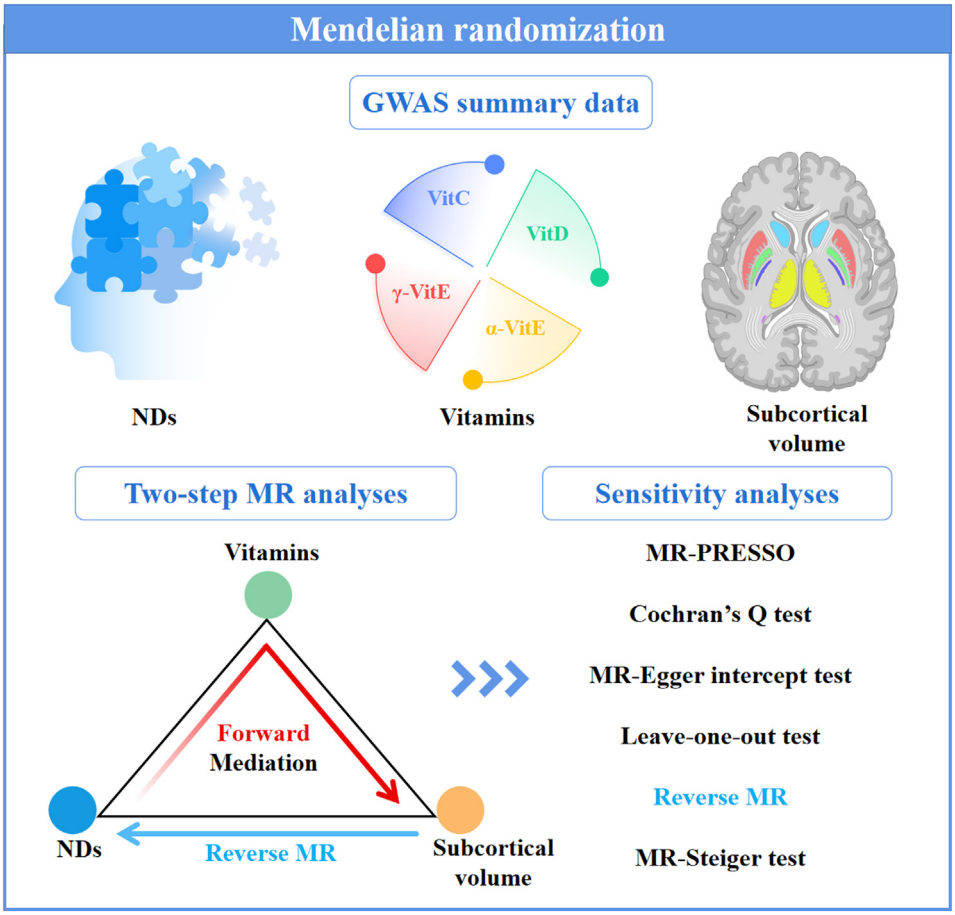

Fig. 1. Flowchart of the Mendelian randomization design. (1) Identify the GWAS summary data for the exposure (NDs), mediator (vitamins), and outcome (subcortical volumes); (2) Use the two-sample MR and two-step multivariable MR (MVMR) approaches to determine the causal relationships of NDs on vitamins and subcortical volumes. Additionally, apply the product-of-coefficients method to assess the mediation effect of vitamins in the causal relationship between NDs and subcortical volumes; (3) Conduct sensitivity analyses, including tests for heterogeneity, pleiotropy, and directional influence. Abbreviations: GWAS, Genome-Wide Association Studies; MR-PRESSO, Mendelian randomization-pleiotropy residual sum and outlier; NDs, neurodegenerative diseases.

effect of each type of NDs on subcortical volume after adjusting for each mediator (circulating vitamin level), and (ii) the indirect effect of each type of NDs through each mediator individually. The ratio of indirect effect to total effect was used to estimate the proportion of total effect of each exposure on each outcome that was mediated by a specific type of serum vitamin level.

The purpose of sensitivity analyses is to enhance the reliability of our causal estimates obtained by the IVW method. Firstly, three complementary approaches relying on different MR assumptions (weighted median, weighted mode and MR-Egger) were conducted to address heterogeneity and the pleiotropy effect of IVs in different ways. Specifically, the weighted median method generates a robust estimate of causality, even when up to 50 % of the IVs are invalid [44]. The weighted mode method estimates the causal effect for the subset containing the most SNPs by grouping them based on the similarity of their causal effects. This method produces an unbiased causal estimate even when most instruments are invalid [45]. Meanwhile, MR-Egger provides a consistent estimate of the causal effect under a less stringent assumption, even when all IVs exhibit pleiotropic effects [46]. Secondly, Mendelian Randomization Pleiotropy Residual Sum and Outlier (MR-PRESSO) analysis aims to detect potentially horizontal pleiotropic outliers and recalculate the causal effect after removing the outliers. Thirdly, the MR-Egger intercept test can be used to assess the potential bias due to directional pleiotropy [47]. The p value greater than 0.05 indicates the absence of significant horizontal pleiotropy bias in the results. Cochran's Q statistics were also calculated to quantify the level of heterogeneity with the p value smaller than 0.05, suggesting significant heterogeneity among IV. Moreover, we performed the MR-Steiger test and reverse MR analysis (i.e. subcortical volume as the exposure and each ND as the outcome) to explore whether the exposure was directionally causal for the outcome [48]. In the reverse MR analysis, the threshold for selecting exposure-related IVs was set at $p < 1 \times 10^{-5}$ due to the limited availability of significant SNPs. Finally, we conducted a leave-one-out analysis to re-estimate the causal effect after removing each SNP individually, in order to test whether a specific SNP was the main driver of the observed effect [17].

The statistical significance level for Bonferroni correction for multiple testing was $p < 0.0018$ (0.05/28, 4 exposures × 7 outcomes). All MR analyses were performed in the TwoSampleMR, MR PRESSO, and MVMR R software packages in R (version4.1.2).

2.5. Mice and interventions

C57BL/6 J and APP/PS1 transgenic mice (male, 16-week-old, 26–29 g) were purchased from Beijing Huafukang Biological Company. The mice were allocated to three groups: wild-type (WT) group (C57BL/6 J mice, n = 10), AD group (APP/PS1 mice, n = 10), and high-dose vitamin D (HVD) diet-treated AD group (AD+HVD group, APP/PS1 mice, n = 10). The sample size was determined based on the resource equation approach and the previous studies [49–51]. Three groups of mice were fed with identical diets, except for the difference in vitamin D3 dose [the WT/AD group (normal diet, 1500 IU/kg vitamin D3) and the AD+HVD group (HVD diet, 7500 IU/kg vitamin D3)]. The food consumption of mice was monitored biweekly during 3-month dietary intervention.

2.6. Measurement of serum 25(OH)D level

Blood samples were collected from the retro-orbital sinus after 3-month dietary intervention. Serum components were obtained by centrifugation at 3000 rpm for 10 min. The serum 25(OH)D level was mea-

sured using an ELISA kit (MLBIO Biotechnology, China) according to the manufacturer's instructions.

2.7. MRI acquisition and analysis

The MRI data were performed on a 9.4 Tesla MRI scanner (Bruker BioSpec 94/30 USR, Germany). The 3D T2-weighted images were acquired using Turbo rapid acquisition relaxation enhanced sequence with the following parameters: rare factor = 12, repetition time (TR) = 1500 ms, echo time (TE) = 38 ms, field of view (FOV) = 18 mm × 18 mm × 7.5 mm, matrix size = 120 × 120 × 50, voxel size = 0.15 mm × 0.15 mm × 0.15 mm. Automatic segmentation of four hippocampal subregions including CA1, CA2, CA3 and dentate gyrus (DG) and NAc (please see the MVMR results below) were performed based on Turone Mouse Brain Atlas and Template (TMBTA) using SPM12 software. The total hippocampal volume was calculated by summing the volumes of all four subregions. Total intracranial volume was regarded as a nuisance variable to correct differences in intracranial size. The average volumes of the hippocampus, hippocampal subregions and NAc on both sides were used as the final metrics.

2.8. β-amyloid (Aβ) and vitamin D receptor (VDR) analyses

With regard to Aβ immunofluorescence staining, brain tissue was first acquired and coronal sections were prepared using a cryostat. The sections were then incubated with an anti-Aβ primary antibody (1:200, Abcam) followed by a secondary antibody (1:100, Abcam). Images were subsequently obtained using a Pannoramic digital scanner (3D HISTECH, Hungary).

In terms of Aβ and VDR western blotting analysis, the hippocampal tissues were first acquired and proteins were extracted. Equal amounts of protein were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were incubated with anti-Aβ (1:500, Abcam) and anti-VDR (1:500, Abcam) at 4 °C overnight, followed by incubation with secondary antibodies for 1 hour at room temperature. The Aβ and VDR protein bands were visualized, and their expression levels were analyzed using ImageJ software.

2.9. Biosafety assessment

Blood samples and major organs (brain, heart, liver, spleen, lung and kidney) were collected to evaluate the biosafety of dietary intervention. Hematological parameters, including white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), platelet (PLT), alanine aminotransferase (ALT), aspartate transaminase (AST), total bilirubin (TBiL), albumin (ALB), blood urea (Urea), creatinine (Cr), calcium (Ca) and phosphorus (P) levels were measured by a hematology analyzer (Mindary BC-2800 Vet, China) and an automated chemistry analyzer (Mindary BC-240 Vet, China). The major organs were fixed in 4 % paraformaldehyde for 24 h, and paraffin-embedded sections were prepared for Hematoxylin/eosin (H&E) staining. The stained sections were then visualized using a Pannoramic digital scanner (3D HISTECH, Hungary).

2.10. Statistical analyses of animal experiment

The experimental design included blinding procedures. All analyses were carried out by researchers who were blinded to the diet groups. Statistical analyses of animal experiments were performed using SPSS software (Version 25). The Shapiro-Wilk test was conducted for normality of data distribution. All data were expressed as mean ± SD. Significant differences among three groups were assessed by one-way analysis of variance (AVOVA) followed by Tukey's post hoc test (equal variances) or Dunnett's T3 post hoc test (unequal variances). The p value less than 0.05 was considered as statistically significant.

3. Results

3.1. IVs selection and quality control

After removing the ineligible IVs according to our established criteria, SNPs for NDs risk were selected as eligible IVs, and the details of the SNPs are presented in Supplementary Table 4–6. The F statistics values for genetic IVs ranged from 44.42 to 157.33, implying no potential weak instrument bias. Additionally, the IVs could explain 0.27 %–5.05 % of the variance of exposure.

3.2. Causal effects of genetic liability to NDs and subcortical morphology

We identified genetically predicted AD risk had significant negative effects on the volume of three subcortical structures, NAc (β= −0.09; $p = 5.13 \times 10^{-5}$), amygdala (β= −0.07; $p = 8.44 \times 10^{-4}$) and hippocampus (β = −0.07; p = 0.001) surpassing Bonferroni correction (Fig. 2a). Namely, doubling the odds of genetic predisposition to AD was linked to a volumetric reduction in the NAc by 0.09 standard deviation (SD) (95 % CI: −0.129, −0.045), in the amygdala by 0.07 SD (95 % CI: −0.114, −0.030) and in the hippocampus by 0.07 SD (95 % CI: −0.112, −0.028), respectively. However, there was little evidence supporting the causal effects of the other three NDs (PD, MS, and ALS) and subcortical volume (Supplementary Table 7).

3.3. Causal effect of AD on serum vitamin D level

Since genetic liability for AD was shown to have a causal effect on the volume atrophy of three subcortical structures using two-sample MR analysis, the MVMR approach was then applied to investigate the mediating role of serum vitamin levels (vitamins C, D, and E (α-tocopherol and γ-tocopherol)) in this causal effect. After removing pleiotropic SNPs (rs11767557, rs73223431, rs7412, and rs867230; Detailed information on these pleiotropic SNPs in Supplementary Table 8) using the RadialMR method, we found that genetically predicted AD risk was negatively associated with serum vitamin D level (β = −0.02; $p = 6.84 \times 10^{-6}$). Specifically, each doubling of the probability of genetic predisposition to AD was associated with a 0.02 SD reduction in serum vitamin D level (95 % CI: −0.032, −0.013) (Fig. 2b). No significant causal association was found between AD and circulating concentration of vitamin C or E (Supplementary Table 9).

3.4. Mediating effect of serum vitamin D level on AD-subcortical morphology

Based on the above MR analysis results, only serum vitamin D levels were identified as a potential mediator of the causal effect of AD risk on the volume reduction of the NAc, amygdala, and hippocampus. Therefore, only serum vitamin D levels were included in the subsequent mediation analysis. Using the two-step MVMR method (Supplementary Table 10), we observed that low serum vitamin D level played a mediating role in the effect of AD risk on the volume reduction of the hippocampus and NAc, but not on amygdala. By applying the product-of-coefficients method, low serum vitamin D levels were found to explain 3.99 % (95 % CI: −0.006 to -5.82×10^{-5}) of the effect of AD risk on hippocampal volume reduction and 3.97 % (95 % CI: −0.007 to -2.94×10^{-4}) of the effect of AD risk on NAc volume reduction, respectively (Fig. 2c).

3.5. MR sensitivity analyses

The directions of the estimates from the other three MR methods (weighted median, weighted mode and MR-Egger approaches) were consistent with the IVW estimates. The p value for the MR-Egger intercept test in each MR analysis was greater than 0.05, revealing no significant horizontal pleiotropy that could bias the causal estimates. The Cochran's Q statistics did not show any evidence of heterogeneity.

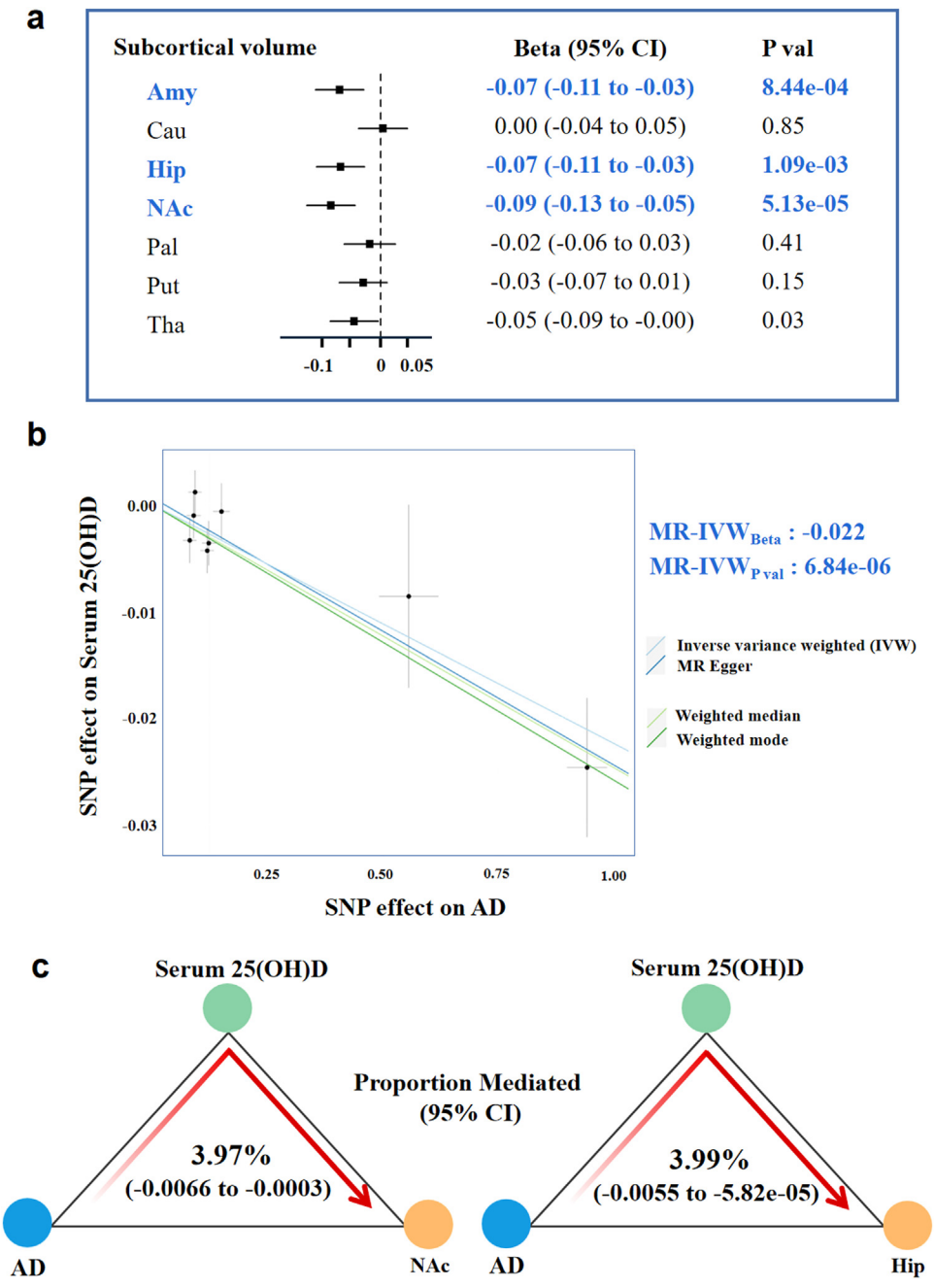

Fig. 2. The results of MR analyses. a. Forest plot shows results of the causal effects of AD on subcortical volume in the IVW method. The x-axis shows beta and 95 % CI. b. Scatter plot shows the relationship between AD and Serum 25(OH)D. The x-axis represents the effect of SNP on exposure (AD) and the y-axis represents the effect of SNP on outcome (Serum 25(OH)D). A slope less than 0 means that the exposure is negatively associated with the outcome. c. Mediation proportion of serum 25(OH)D in the causal association between the risk of AD and volume of hippocampus and NAc. The mediation proportion (95 % CI) for serum 25(OH)D was obtained through both two-sample MR and two-step multivariable MR analyses. Abbreviations: AD, Alzheimer's disease; Amy, amygdala; Cau, caudate nucleus; CI, confidence interval; Hip, hippocampus; MR, Mendelian randomization; NAc, nucleus accumbens; Pal, pallidum; Put, putamen; SNP, single nucleotide polymorphism; Tha, thalamus.

MR-Steiger test and reverse MR analyses supported no evidence of the causal effect of subcortical morphology on any ND risk. Meanwhile, the leave-one-out plot suggested that no single SNP had an exorbitant influence on the overall estimates. Overall, the sensitivity analyses confirmed the robustness of the results by the IVW method. The results of the sensitivity analyses are shown in Supplementary Table 11 and Figure S1–2.

3.6. Hippocampal and NAc volume alteration in AD mice treated by HVD diet

Encouraged by our MR results, it is worth exploring whether increasing serum vitamin D levels through dietary intervention could delay hippocampal and NAc atrophy in AD mice (Fig. 3a). Notably, although there were no significant differences in the average daily food intake among the three groups, the average daily vitamin D intake of the mice in the AD+HVD group was significantly higher than that of the mice in the WT group ($\eta^2 = 0.976$, $p = 0.002$, Figure S3) and the AD group ($\eta^2 = 0.976$, $p = 0.006$, Figure S3) on a normal diet. Compared with the WT group, the serum 25(OH)D level ($\eta^2 = 0.779$, $p = 0.030$), hippocampal volume ($\eta^2 = 0.547$, $p = 0.010$), and NAc volume ($\eta^2 = 0.438$, $p = 0.075$) in the AD group were significantly decreased (Fig. 3b-e). This indicates vitamin D deficiency and atrophy of the hippocampus and NAc in AD mice. The serum 25(OH)D level was dramatically elevated in AD mice after 3 months of HVD diet treatment ($p = 0.012$), approximately 7.49 ng/mL higher than that in the AD group (Fig. 3b). Not surprising, the total hippocampal and NAc atrophy was significantly alleviated after 3 months of by 3-month HVD diet treatment. The total hippocampal volume ($p = 0.029$) and NAc volumes ($p = 0.039$) increased 1.80 and 0.09 respectively in the AD+HVD group compared to those in the AD group (Fig. 3c-e). Each unit increase in serum 25(OH)D level was associated with a 0.24 unit increase in total hippocampal volume and a 0.01 unit increase in NAc volume. Furthermore, the increase in total hippocampal

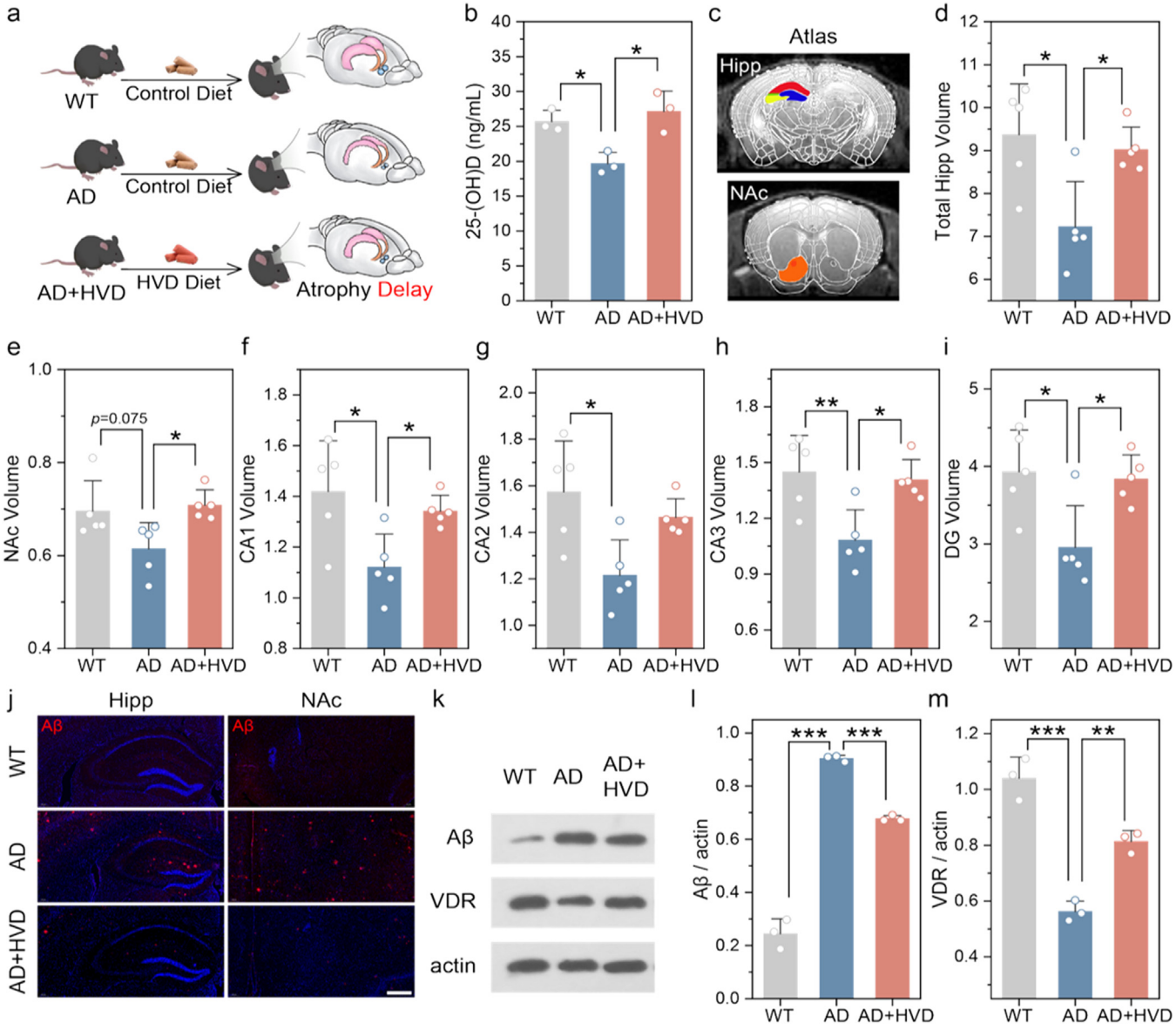

Fig. 3. Hipp, NAc volume and Aβ, VDR expression alteration in AD mice treated by HVD diet.
a. Schematic and potential effect of dietary interventions. b. Serum 25(OH)D level after interventions in three group mice. c. Representative MRI images overlaid with coronal atlas of mouse brain annotated with Hipp subregions (Red: CA1; Green: CA2; Yellow: CA3; Blue: DG) and NAc regions (Orange). d-i. Volume analysis of total Hipp, NAc and Hipp subregions after interventions in three group mice. j. Immunofluorescence images of Aβ aggregates in Hipp and NAc. Scale bar: 500 μm. k-m. Representative immunoblots and quantitative analysis for Aβ and VDR. Data are presented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. one-way ANOVA followed by Tukey's post hoc test. Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; DG, dentate gyrus; Hipp, hippocampus; HVD, high-dose vitamin D; NAc, nucleus accumbens; VDR, vitamin D receptor; WT, wild type.

volume was mainly mediated by three hippocampal regions (CA1, CA3 and DG) (Fig. 3f-i).

3.7. Aβ deposit and VDR expression alteration in AD mice treated by HVD diet

The red fluorescence intensity of Aβ staining in the hippocampus and NAc in the AD group was greater than that in the WT group (Fig. 3j), but the intensity of Aβ staining was decreased in the AD+HVD group compared to the AD group. Most importantly, western blotting analysis showed that Aβ expression in hippocampus was significantly increased in the AD group compared with the WT group ($\eta^2 = 0.990$, $p = 9.60 \times 10^{-4}$), but it was significantly decreased in the AD+HVD group ($p = 0.0004$) compared to the AD group (Fig. 3k, l). Furthermore, VDR expression in hippocampus was significantly decreased in the AD group compared with the WT group ($\eta^2 = 0.953$, $p = 8.40 \times 10^{-4}$), but it was significantly increased in the AD+HVD group ($p = 0.003$) compared to the AD group (Fig. 3k, m).

3.8. Biosafety evaluation of dietary interventions

No significant changes in hematological parameters (RBC, HGB, PLT, ALT, AST, TBiL, ALB, Urea, Cr, Ca, and P) were observed among the three groups after three months of dietary interventions (Figure S4–5). The increased WBC count in the AD group may be associated with AD-related systemic inflammation, a phenomenon commonly reported in both AD patients and mice [52,53]. H&E staining showed no notable damage in the main organs (brain, heart, liver, spleen, lung, and kidney) (Figure S6).

4. Discussion

This is the first application of MR mediation analysis to study the mediating role of serum vitamin level in the causal effect of NDs risk on subcortical volume. We only observed that genetic AD predisposition led to low serum vitamin D level and atrophy in NAc, amygdala and hippocampus. More importantly, low serum vitamin D level mediated 3.99 % and 3.97 % of the causal effect of AD risk on hippocampal and NAc atrophy, respectively. Animal experiments further confirmed significant delay in hippocampal and NAc atrophy, significant reduction of Aβ deposit and increase of VDR expression in the AD mice with 3-month HVD diet compared to those with normal diet.

Hippocampal atrophy is widely recognized as a hallmark of AD and is responsible for episodic memory impairment. Researchers have found that genetic liability to AD is associated with reduced hippocampal volume [54,55]. Amygdala atrophy has also been well described in early stage of AD, with a similar magnitude to hippocampal atrophy [54]. This could be related to certain preclinical symptoms, such as olfactory deficits and/or emotional abnormalities [56]. A negative association between the polygenic risk score of AD and amygdala volume was also reported in an AD case-control cohort [54]. The NAc is a major component of the ventral striatum and displays strong interconnections with limbic structures such as the hippocampus and amygdala. As a significant element in the brain's reward system, the NAc plays a vital role in cognition and emotional behaviors. Several studies have reported sizable reductions in the volume of the NAc in individuals with AD [57,58], suggesting that NAc atrophy might also have a substantial impact on the clinical manifestations of AD. Recent MR studies on AD and subcortical atrophy have corroborated our findings to varying extents. Specifically, several studies have demonstrated a causal link between AD and atrophy of the hippocampus [21] and its subfields [22]. Also, the causal relationship between AD and reduced right ventral striatum volume [19] may support our findings, as the NAc is a key component of the striatum.

Among the four selected types of vitamins, we found that only genetically predicted AD risk was associated with low serum vitamin D levels in the MR analyses. Additionally, serum 25(OH)D levels were reliably lower in the AD mice compared to the WT mice. Some observational and MR studies support the finding that serum vitamin D deficiency is associated with an increased risk of dementia [59,60]. In our MR analyses, we found, in the reverse direction, high AD risk had a causal effect on low serum vitamin D level. It could be argued that potential impaired cognitive function in individuals with high AD risk is likely to lead to insufficient dietary intakes of vitamin D and/or reduced sunlight exposure, resulting in a subsequent decline in serum vitamin D concentration.

Most importantly, our two-step MVMR analyses confirmed that low serum vitamin D level partially mediated the causal effect of AD risk on the atrophy of hippocampus and NAc. Our animal experiments revealed significant delays in hippocampal and NAc atrophy in the AD mice fed a 3-month HVD diet compared to those fed a normal diet. Most cross-sectional and prospective studies have reported a strong association between vitamin D deficiency and hippocampal volume reduction, which coincides with our findings [61,62].

Vitamin D is critical to brain structure and function via bind to VDR through a number of potential mechanisms [15,63–65]. First, VDR is expressed throughout the brain, including the hippocampus. Vitamin D deficiency and VDR dysregulation in the context of AD exacerbate AD-like pathologies [66]. Second, there may be vascular mechanisms involved, as vitamin D has been associated with reduced thrombosis and regulation of the renin-angiotensin system. Third, vitamin D has been shown to have various neuroprotective actions, including neurotrophic, immunomodulatory, and regulation of intracellular calcium balance. Based on these theories, low vitamin D levels could partially contribute to hippocampal atrophy. In our animal experiments, a 3-month HVD diet increased VDR expression, effectively attenuated Aβ burden, and ultimately delayed hippocampal atrophy in AD mice. These findings are consistent with previous reports [67]. But to our knowledge, no previous studies have examined the association between serum vitamin D levels and NAc volume. This is most likely because the NAc is too small to measure accurately. However, several lines of evidence from animal models indicate that vitamin D may also have a neuroprotective effect on dopaminergic pathways in the brain. McGrath et al. discovered that developmental vitamin D deficiency was associated with subtle changes in the range of proteins in the NAc, which adversely impacted adversely on normal brain development [68]. Katayoun et al. found that regular vitamin D treatment can improve symptoms of chronic mild stress in the depression model rat, probably by regulating the effect of dopamine-related actions in the NAc [69].

While our findings imply that low vitamin D level plays a mediating role in the relationship between AD risk and hippocampal and NAc atrophy, over 90 % of the effects remain unaccounted for. AD is a multifactorial disease with complex underlying mechanisms, and no single factor significantly contributes to the neuroanatomical alterations. Vitamin D has been implicated in mediating AD-related atrophy, but other potential mediators such as neuroinflammation and oxidative stress also play critical roles. Neuroinflammation, characterized by the activation of glial cells and increased secretion of pro-inflammatory cytokines, can exacerbate neuronal damage and contribute to brain atrophy [2]. Similarly, oxidative stress, resulting from an imbalance between reactive oxygen species and antioxidant defenses, leads to cellular damage and further promotes neurodegenerative processes [70]. These mediators, along with vitamin D deficiency, likely interact in complex ways to drive the pathophysiology of AD-related atrophy. In sensitivity analyses, the lack of a significant causal effect of subcortical structure volume change on the risk of AD appears to rule out the possibility of reverse causation. Additionally, although observational studies have reported relationships between other three NDs and certain subcortical structures [71,72], in our study, we found little evidence supporting causal effects of PD, MS, and ALS on subcortical volume. This discrepancy may indeed reflect distinct pathophysiological mechanisms underlying AD compared to PD, MS, and ALS. AD is primarily characterized by the accumulation of Aβ plaques and neurofibrillary tangles composed of hyperphosphorylated tau protein. These pathological hallmarks are known to affect the hippocampus, amygdala, and other medial temporal lobe structures, leading to significant atrophy and cognitive decline [2]. The significant negative effects observed in our study are consistent with these well-established mechanisms. PD is primarily a movement disorder characterized by the loss of dopaminergic neurons in the substantia nigra and the presence of Lewy bodies. Its primary pathological burden is in the basal ganglia [3], which may not directly impact on the subcortical structures to the same extent as AD. MS is an autoimmune-mediated disease characterized by demyelination and inflammation in the central nervous system. Its primary pathological burden is in white matter tracts, and while it can involve gray matter atrophy [4], the specific subcortical structures affected in our study may not be directly impacted by MS pathology. ALS is a neurodegenerative disease primarily affecting motor neurons in the brain and spinal cord. Its primary pathological burden is in the motor cortex and spinal cord [5], with less direct impact on the subcortical structures examined in our study.

Our study has several limitations. Firstly, our analysis only involved vitamins C, D, and E, as well as seven subcortical structures, which may bias some potential results. Secondly, the sample size for the GWAS of serum vitamin D encompassed 417,580 individuals, while subcortical structures comprised only 30,717 individuals. This has the potential to create uneven power dynamics for different traits or indices. Thirdly, as this study relied on data pooled from GWAS of European origin, it is necessary to validate the generalizability of these results in other ethnic groups. Such efforts would enhance the stability and generalizability of the findings and further advance significant progress toward using vitamin D supplements as a therapeutic option for AD-related brain atrophy across different ethnicities. Fourth, our study lacks longitudinal data. Future research should incorporate longitudinal brain imaging and vitamin D supplementation trials, which are crucial for a com-

prehensive understanding of the phenomenon being investigated. Fifth, future studies should emphasize insights gained from mapping intrinsic brain connectivity networks, in addition to examining brain structure alterations caused by AD, as these networks provide a potentially mechanistic framework for understanding various aspects of behavioral abnormalities associated with AD [73,74]. Last but not least, our study makes a strong case for vitamin D supplementation as a potential neuroprotective strategy, but caution should be exercised. Although no toxic effects were observed in our study after three months of HVD intervention, some studies have reported potential toxic effects of vitamin D that should not be overlooked [75,76]. The precise dosage of vitamin D supplementation has not been firmly established. Consequently, future research in this domain should approach the use of vitamin D with caution. More importantly, future randomized controlled trials are warranted to confirm these findings before making clinical recommendations.

5. Conclusion

Our findings suggest that increasing serum vitamin D level may modestly mitigate hippocampal and NAc atrophy in AD. However, most of the causal effect remains unexplained, highlighting the need for further research into additional modifiable factors.

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Ethical approval

All the human datasets in our study are publicly available GWAS summary statistics, and hence no additional ethical approval and participants' consent was required. Detailed ethical approval and participants' consent can be found in the original GWAS publications. All animal experiments were reviewed and approved by the Animal Care and Use Committee of the Institute of Radiation Medicine, Chinese Academy of Medical Sciences (SYXK2019–0002).

Data share statement

GWAS summary statistics of NDs, serum vitamin levels and seven subcortical structures were publicly available. Details of how to access the data and details of the data release schedule are available from Supplementary Tables.

Consent for publication

Not applicable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Haonan Li: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Meng Cheng:** Validation, Methodology, Investigation, Conceptualization. **Nannan Zhang:** Visualization, Validation, Methodology, Conceptualization. **Siqi Wang:** Software, Methodology, Formal analysis. **Caihua Ye:** Validation, Investigation, Formal analysis. **Haodong Li:** Validation, Methodology, Investigation. **Shengnan Wang:** Validation, Resources, Methodology. **Zirui Wang:** Methodology, Data curation, Conceptualization. **Xuan Yang:** Resources, Methodology, Data curation, Conceptualization. **Zhixuan Liu:** Software, Methodology, Data curation. **Xingyu Zhang:** Validation, Methodology, Investigation, Formal analysis. **Jiayuan Xu:** Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Qiang Xu:** Writing – review & editing, Supervision, Software, Methodology, Data curation, Conceptualization. **Junping Wang:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tjpad.2025.100155.

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