Vitamin B12 Levels Association with Functional and Structural Biomarkers of Central Nervous System Injury in Older Adults

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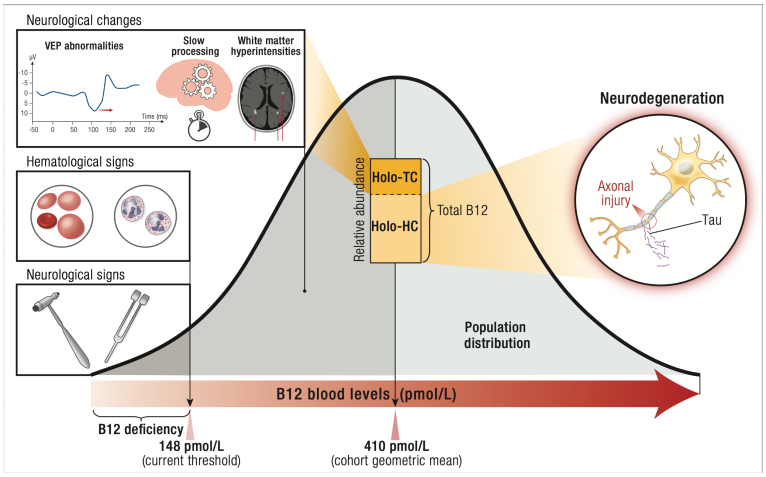

At low levels of B12, specifically when bound to transcobalamin for cellular uptake, evidence of slower conductivity in the brain could point toward impaired myelin. At high levels of B12, specifically when bound to the biologically inert

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transport protein haptocorrin, biomarkers of neurodegeneration appear in the serum, indicating neuroaxonal injury. The biological basis for this phenomenon has yet to be explored. Holo-HC = holo-haptocorrin; Holo-TC = holo-transcobalamin; VEP = visual evoked potentials.

Objective: Vitamin B12 (B12) plays a critical role in fatty- and amino-acid metabolism and nucleotide synthesis. While the association between B12 deficiency and neurological dysfunction is well-known, the exact threshold for adequacy remains undefined in terms of functional impairment and evidence of injury. The objective was to assess whether B12 levels within the current normal range in a cohort of healthy older adults may be associated with measurable evidence of neurological injury or dysfunction.
Methods: We enrolled 231 healthy elderly volunteers (median age 71.2 years old) with a median B12 blood concentration of 414.8 pmol/L (as measured by automated chemiluminescence assay). We performed multifocal visual evoked potential testing, processing speed testing, and magnetic resonance imaging to assess neurological status. Moreover, we measured serum biomarkers of neuroaxonal injury, astrocyte involvement, and amyloid pathology.
Results: Low (log-transformed) B12, especially decreased holo-transcobalamin, was associated with visual evoked potential latency delay (estimate = −0.04; $p = 0.023$), processing speed impairment (in an age-dependent manner; standardized $\beta = -2.39$; $p = 0.006$), and larger volumes of white matter hyperintensities on MRI ($\beta = -0.21$; $p = 0.039$). Remarkably, high levels of holo-haptocorrin (biologically inactive fraction of B12) correlated with serum levels of Tau, a biomarker of neurodegeneration ($\beta = 0.22$, $p = 0.015$).
Interpretation: Healthy older subjects exhibit neurological changes at both ends of the measurable "normal" B12 spectrum. These findings challenge our current understanding of optimal serum B12 levels and suggest revisiting how we establish appropriate nutritional recommendations.

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Cobalamin, or vitamin B12 (B12), is an essential vitamin normally acquired through absorption in the enteric system in mammals. Deficiency in humans can be caused by a lack of intake (eg, vegan diet) or by any disease or procedure of the digestive tract impairing the absorption of B12.[1] After depletion of B12 stores, signs of deficiency such as hematological and neurological symptoms may develop, including megaloblastic anemia and subacute combined degeneration of the spinal cord.[2] The latter presents as a spinal syndrome (sensory ataxia, paresthesia, weakness, etc.) involving multiple tracts, principally the posterior columns.[2,3] Qualitative pathological analyses of the tissue revealed a degeneration with vacuolization of the tracts included in the white matter (WM),[4–7] suggesting that vitamin B12 might be important for maintenance of intact myelination. However, the exact cellular substrate that is impacted by B12 deficiency or insufficiency has not yet been elucidated. In addition to affecting general cognition and memory, vitamin B12 deficiency may even lead to dementia and psychosis, suggesting a broader dependence of the brain on B12.[8–11] Nonetheless, the VITACOG study established that B vitamin supplementation including B12 in older adults with mild cognitive impairment (MCI) leads to both functional and structural benefits.[12,13] According to their foundational work, B12 supplementation slows the progression of brain atrophy in MCI and of clinical decline in multiple areas of cognitive testing. Moreover, B12 deficiency is associated with a higher burden of WM hyperintensities (WMH) in the brain, which could indicate microstructural changes (ie, ependymal disruption or chronic ischemic changes) reflecting overall brain health.[14–16]

In the United States, the cutoff value for B12 "deficiency" state is currently defined as below 148 pmol/L.[1] This value was simply calculated as 3 standard deviations below the U.S. population average, independent of clinical observations.[17] The American Society for Nutrition criticized this approach in 2010, arguing that more than 5% of patients who have a syndrome consistent with B12 deficiency and who respond to B12 supplementation have blood levels above that threshold.[18] Other studies demonstrated that B-vitamins supplementation was beneficial in people with clinical features of cobalamin deficiency, regardless of the measured levels in the blood.[13,19] Selecting a cutoff value based on clinical observations would better reduce disparities in B12 deficiency diagnosis and management.

Cases of biochemical B12 deficiency wherein suboptimal B12 levels have been reported without overt clinical manifestation have been reported as subclinical cobalamin deficiency (SCCD).[20] SCCD is most prevalent in the elderly and is associated with greater WMH burden

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and cognitive decline over time.[21,22] In this context of B12 insufficiency, age might act as a vulnerability factor, accentuating the deleterious effects of low B12. While defining a threshold for optimal B12 levels based on clinical findings is crucial to prevent SCCD, sensitive tools to detect subtle neurological changes have not yet been used. The recording of visual evoked potentials (VEP) is a validated and non-invasive tool to assess myelin function in the visual pathway,[23,24] but at the commencement of our study it had not yet been applied to a cohort of participants to study the effects of low B12.

Distribution of the fractions of B12 measured in the blood adds another layer of complexity to understanding the neurological manifestation of B12 deficiency. Once cobalamin is absorbed, the transport proteins haptocorrin (HC) and transcobalamin (TC) bind it with great affinity and act as transporters in circulation.[25,26] Since only TC has a specific receptor (CD320) for cellular uptake, Holo-TC[1] is usually considered to be biologically available for cells ("active"). On the other hand, Holo-HC hypothetically represents the fraction of B12 that is not immediately available to tissues ("inactive"); it can only bind non-specific asialoglycoprotein receptors on liver cells for reuptake, degradation and excretion in the bile.[27]

In this work, we hypothesized that lower total B12 levels within the specified normal range may still be associated with subtle functional and structural neurological deficits if the active fraction of plasma B12 (Holo-TC) is too low to sustain adequate cellular B12 needs. To that end, we evaluated the association of B12 concentrations with markers of myelin integrity (multifocal VEP; mfVEP), cognitive performance, blood biomarkers of neuronal and glial integrity, as well as quantitative brain MRI analyses across a spectrum of measurable B12 levels as well as the active fraction of the plasma B12.

Methods

Brain Aging Network for Cognitive Health (BrANCH) Cohort

Healthy elderly participants in the current study were recruited since August 15, 2008, through the Brain Aging Network for Cognitive Health (BrANCH) at the University of California, San Francisco (UCSF) Memory and Aging Center (Fig 1). The participants from the BrANCH study and its subcohorts gave written informed consent and were approved by the UCSF Committee on Human Research. The BrANCH study recruited a total of 861 healthy older participants without a history of neurological or major systemic conditions (ie, diagnosed diabetes, hypertension, and cardiovascular disease). Individuals were followed annually with extensive neurological testing, evaluation of cognitive performance, and magnetic resonance imaging. Upon recruitment, 231 of these participants agreed to join the cross-sectional Myelin and Aging subcohort, which included additional blood collection and measurements of serum total B12 and Holo-TC levels. Two patients fell below the 148 pmol/L threshold for deficiency. Removing them from our analysis did not significantly change our results. Furthermore, their blood levels of Holo-TC, methylmalonic acid, and homocysteine (sensitive biomarkers of B12 deficiency) were within the range that is considered normal.[1] Therefore, we included them in the statistical analyses.

Biochemical Analyses

Total B12 levels were measured in the serum by an automated chemiluminescence assay. Holo-TC was measured as previously described, by enzyme immunoassay (EIA; "Active-B12", Axis-Shield) according to the manufacturer's instructions.[28] The measure of Holo-HC was inferred by subtracting Holo-TC from the total measured B12.

Multifocal Visual Evoked Potentials

The mfVEP recordings were performed at the UCSF MS clinic. Volunteers were presented a combination of pattern reversal and flashes across 120 sectors in the visual field. The response of the visual cortex is then recorded through the scalp over the occiput. Each participant underwent an approximately 30-minute mfVEP (7 minutes per eye, recorded twice) during which the response of the visual cortex was continuously recorded with 4 standard electroencephalogram electrodes on the scalp while watching the visual stimulus.[24] The recorded brain produces a peak after the stimulus is presented, and the latency of that peak is recorded. For the purpose of this work, we focused on the latency delay of the averaged recording (VEP).

Assessment of Cognitive Performance

Participants completed a validated battery of 5 computerized, visual (non-verbal) choice reaction time tasks (Dots, Lines, Search, Shapes, Abstract Matching 1, Abstract Matching 2), which have been described in greater detail elsewhere.[29] Performance was standardized and averaged across all tests to derive a composite z-score of cognitive processing speed, which was used as a primary outcome in the present analysis. Higher scores indicate slower cognitive processing speed (worse performance). This processing speed metric has previously been validated as a sensitive marker of cognitive aging related to WM integrity and inflammation.[29–31]

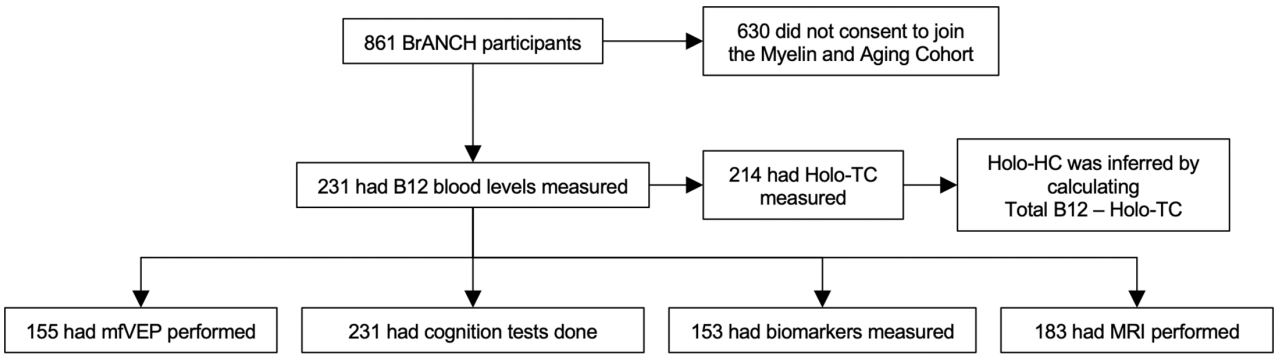


FIGURE 1: Flow chart of the participants' recruitment. The BrANCH cohort comprises 861 healthy elder recruited as controls, and 231 of them joined the Myelin and Aging subcohort to have their B12 blood levels measured. Holo-TC was measured in the serum of 214 of them, and Holo-HC values were calculated by subtracting Holo-TC from total B12. mfVEP was performed on 155 of these participants, 231 underwent cognitive tests, 153 had biomarkers of neurodegeneration measured in their serum, and 183 had MRI scans performed. BrANCH = Brain Aging Network for Cognitive Health; Holo-HC = holo-haptocorrin; Holo-TC = holo-transcobalamin; mfVEP = multifocal visual evoked potential; MRI = magnetic resonance imaging.

Single Molecule Assay

Serum and ethylenediaminetetraacetic acid (EDTA)-plasma were centrifuged for 15 and 10 minutes at room temperature, and 4°C, respectively. Aliquoted material was stored at −80°C until the biomarker assessment. Biomarker concentrations were measured on a Simoa HD-X analyzer (Quanterix, San Francisco, USA) according to the manufacturer's instructions. Neurofilament light chain (NfL), Tau, ubiquitin C-terminal hydrolase L1 (UCH-L1), and glial fibrillary acidic protein (GFAP) were measured in serum using the Neurology-4 plex A. Amyloid-beta 1–40 and 1–42 peptides were measured using the Aß40 and Aß42 kits from EDTA-plasma, respectively. Samples were measured in duplicate by researchers blinded to the clinical assignment and B12 values. Duplicates with coefficient of variation (%CV) < 20% were included in the statistical analysis. The cardiovascular risk factor (CVRF) is a value designed by authors to represent the presence of cardiac risk factors (diabetes, hypertension, hyperlipidemia, a history of coronary artery disease, and a history of cerebrovascular disease), as cardiovascular disease was shown to provoke a Tau increase in the serum, most likely from neuronal microinjury secondary to cardiovascular etiology.[32,33]

Magnetic Resonance Imaging

Participants included in the cohort performed magnetic resonance imaging (MRI) with a Siemens Trio 3 T scanner at the UCSF Neuroscience Imaging Center. The analysis included examination of the following sequences acquired sagitally: T1-weighted scan (3DT1 Mprage) for volumes measurement and 3D fluid attenuated inversion recovery (3DFLAIR) for WMH quantification, as previously described.[34] We used the fluid attenuated inversion recovery and the T1-weighted images to measure areas of hyperintensities in mm^3. In particular, the pipeline used for WMH assessment was *xsec_supervised_trio*. Manual segmentation was performed to ensure accuracy.

Statistical Analyses

Descriptive statistics including mean, median and normality plots with tests were used to describe the different variables. Those that did not meet criteria for normal distribution were log transformed for further analysis. A quadratic relationship was found between the serum total B12 levels and the mfVEP latencies. Consequently, the cohort was stratified between groups above and below the geometrical mean (408 pmol/L) of total B12. The study was designed to have 80% power to detect a significant ($p < 0.05$) association between B12 blood levels and the selected outcomes. We used a mixed linear model to measure the association between serum B12 levels and mfVEP latency where the eye (right eye [OD], left eye [OS]) was considered a random factor to account for the variability between eyes of the same person. Linear regression tested the associations between B12 and processing speed. To study the interaction with age, an interaction term between age and Holo-TC was added to the model. Linear regressions were also performed to study the relationship of B12, its fraction Holo-TC and Holo-HC, with biomarkers of neurodegeneration and with structural changes on MRI. All models corrected for age, sex at birth, CVRF, body mass index (BMI), apolipoprotein E (APOE) ε4 allele, hemoglobin (Hb)A1C and education. Analyses were performed with the SPSS software (version 28.0.1.1) and R software (4.2.0).

Results

Cohort Description

Main demographics for the BrANCH cohort compared with the Myelin and Aging cohort are provided in the supplementary material (Table S1). The clinical criteria of the included participants are provided in Table 1. A subset of 231 healthy elder adults had their total B12 blood levels measured, which had a median of 414.8 pmol/L with an interquartile range (IQR) of [282.7–557.0] pmol/L. Holo-TC was measured on 214 of these participants and had a median of 92.9 pmol/L, with an IQR of [63.5–123.6] pmol/L. Holo-HC was calculated with a median (IQR) of 297.9 [194.5–417.3] pmol/L.

Low Available B12 Associates with mfVEP Latency Prolongation

To test the association between mfVEP latency and total serum B12, we compared a linear with a polynomial regression model. The polynomial model showed a better fit ($R^2 = 0.023$) than the linear model ($R^2 = 0.007$), suggesting a non-linear association. This hypothesis was reinforced by plotting the data, in which a quadratic relationship can be seen (Fig 1). Due to the asymmetric residual distribution in the linear regression assessing the correlation between B12 and VEP, we evaluated the association in cases with high vs. low B12 based on the geometric mean of 408 pmol/L, value at which the estimated inflection point falls in Fig SS1.

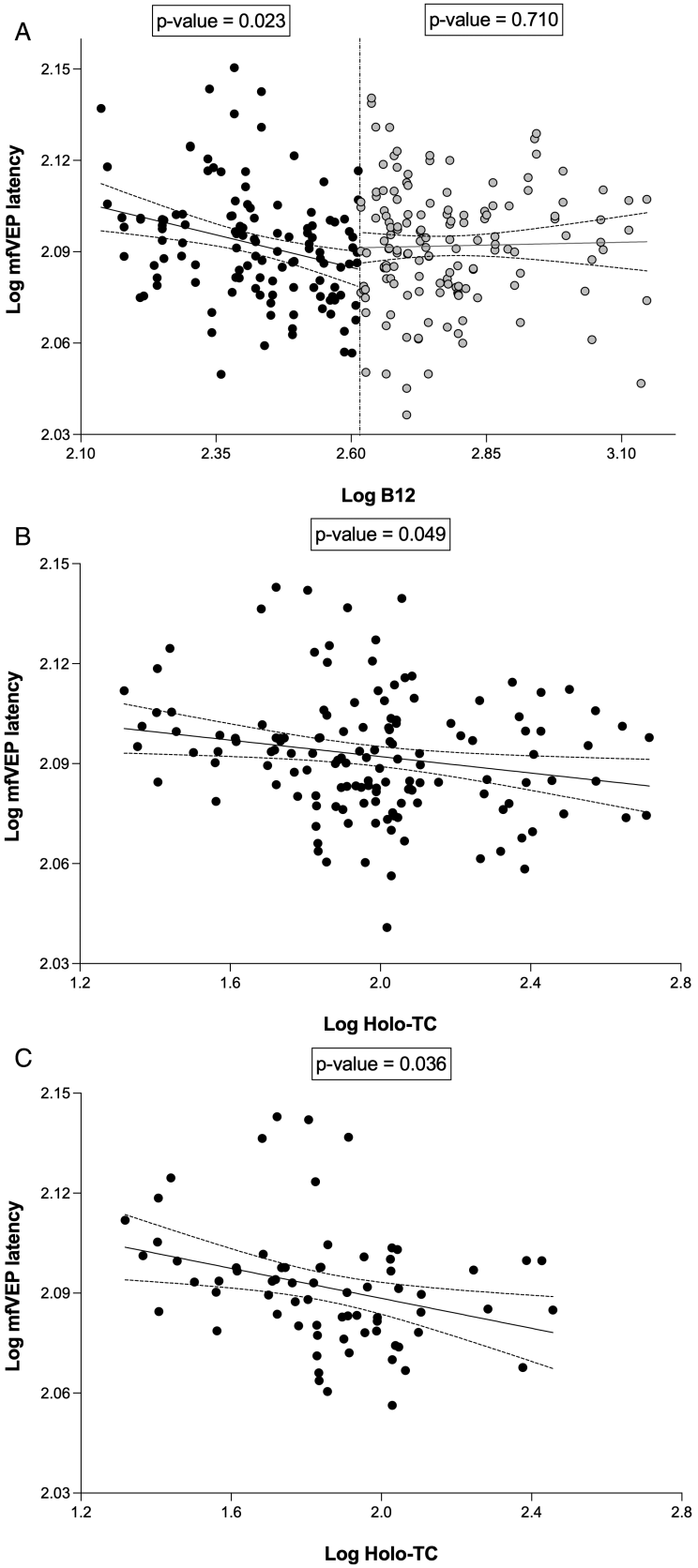
Therefore, and since we postulated that people at the lower ranges of B12 may show signs of abnormalities in their mfVEP recordings, we stratified the cohort for B12 levels above and below the mean (geometrical mean = 408 pmol/L). Albeit these B12 levels were considered normal, patients with lower levels of B12 showed a significant inverse association with mfVEP latency (estimate = −0.04; $p = 0.023$; Fig 2A) in a model correcting for age, sex at birth, CVRF, BMI, APOEε4 allele, HbA1C, and education. Thus, a measured total B12 below the mean, or below 408 pM, significantly associates with a delay in mfVEP latency, representing slower conductivity in the brain. Two cases had missing HbA1C, but removing them from the analysis did not change the results, thus we attributed them the median HbA1C value.

To confirm that the delay in mfVEP is due to a low B12 availability, we explored the relationship between

TABLE 1. Description of the Cohort's Characteristics at Baseline

Characteristics[a]	Myelin and Aging Cohort ($N = 231$)	Subcohort with VEP and B12 ($N = 155$)	Subcohort with Biomarkers and B12 ($N = 153$)
Age (years)	71.2 [67.3–76.4]	70.0 [66.0–74.0]	71.2 [67.0–75.2]
Females at birth, *N* (%)	121 (52.4%)	86 (55.5%)	93 (60.8%)
BMI (kg/m^2)	25.2 [22.9–27.4]	25.2 [22.9–27.2]	25.1 [22.8–27.5]
Education (years)	18 [16–20]	18 [16–20]	18 [16–19]
APOEε4 allele, *N* (%)	58 (25.2%) ($N = 230$)	36 (23.4%) ($N = 154$)	38 (25.0%) ($N = 152$)
Hemoglobin A1C (%)	5.5 [5.3–5.8] ($N = 229$)	5.5 [5.3–5.8] ($N = 154$)	5.5 [5.3–5.8] ($N = 151$)
Clinical Dementia Rating (CDR) scale[b], N (0/0.5/1)	0 [0–0]	0 [0–0]	0 [0–0]
B12 blood level (pmol/L)	414.8 [282.7–557.0]	419.9 [270.0–557.2]	414.8 [282.7–557.0]
Holo-transcobalamin (pmol/L)	92.9 [63.5–123.6] (N = 214)	97.0 [67.0–126.4] (N = 145)	98.0 [72.0–126.0] (N = 138)
Homocysteine (μmol/L)	8.1 [6.4–10.0] ($N = 228$)	8.4 [6.4–10.3] ($N = 153$)	7.8 [6.2–10.0] ($N = 152$)
Methylmalonic acid (μmol/L)	0.11 [0.00–0.15] ($N = 123$)	0.10 [0.00–0.14] ($N = 96$)	0.10 [0.00–0.14] ($N = 86$)

APOEε4 = apolipoprotein E ε4 allele; BMI = body mass index; VEP = visual evoked potential.
[a]Values are reported as median [25th percentile − 75th percentile] unless specified otherwise.
[b]CDR: 0 = normal.



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mfVEP and the Holo-TC level alone. Indeed, only available B12 on transcobalamin showed an inverse association with mfVEP (estimate = −0.01; $p = 0.049$; Fig 2B), while there was no association between unavailable Holo-HC and mfVEP (Fig S2). This analysis supports our hypothesis that the lack of available B12 induces a delay in conduction speed through the brain. In fact, the association between the biologically active Holo-TC levels and mfVEP latency increased (whole cohort: cohen $f^2 = 0.04$; low B12 strata: cohen $f^2 = 0.12$) when selecting for patients with a total measured B12 below the mean (estimate = −0.02; $p = 0.036$; Fig 2C). We included homocysteine, eGFR, LDL, HDL, and systolic blood pressure respectively in our model but the association between mfVEP and B12 remained significant. Overall, these results indicate that in a healthy population with B12 blood levels within the currently defined normal range, there is an inverse relationship between the biologically available B12 on transcobalamin and the latency of mfVEP.

The Association of Vitamin B12 Blood Levels and Cognitive Tests Is Age-Dependent

Previous studies have shown that B12 deficiency can cause cognitive impairment.[35] To further explore the impact of lower B12 levels on cognition, a battery of reaction time tests sensitive to subclinical cognitive aging were performed. Our goal was to see if lower B12 blood levels included within the current normal range, and in patients without overt neurocognitive deficits, could be associated with changes in cognitive processing speed.

Adjusting for sex, age, BMI, CVRF, education, HbA1C, and APOEε4, the relationship between total B12 blood levels and processing speed approached statistical significance (standardized $\beta = -0.11$; $p = 0.080$) such that higher total B12 related to lower processing speed z-scores, or faster performance (Fig S3A). After repeating the analysis on the Holo-TC and the Holo-HC fractions of B12, only Holo-TC showed a similar association with processing speed ($\beta = -0.11$; $p = 0.121$; Fig 3B). To determine whether the link between Holo-TC and processing speed was dependent on age, an interaction term between age and Holo-TC was added to the linear regression model. Age significantly moderated the relationship between Holo-TC and processing speed ($\beta = -2.39$; $p = 0.006$, Fig 3) such that the negative association between Holo-TC and processing speed strengthened with older age, suggesting that the impact of low available B12 on the processing speed is age-dependent.

High Unavailable B12 Associates with an Elevation in Biomarkers for Neurodegeneration

The demographic and clinical characteristics as well as the included biochemical parameters are summarized in Table 2. The number of samples included in the analyses for each biomarker is detailed in Table S3.

As expected, NfL, GFAP, and Aß40 levels correlated weakly with age ($rho_s = 0.31$, 0.33, 0.23, respectively, $p < 0.001$, <0.001, and 0.014, respectively).[36,37] NfL and Tau correlated inversely with BMI ($rho_s = -0.21$, −0.18, $p = 0.008$ and 0.032, respectively). In addition, NfL and Aß42 concentrations increased with higher serum creatinine ($rho_s = 0.24$ and 0.23, $p = 0.003$ and 0.016, respectively). Median GFAP concentrations were higher in females compared to males (213.9 vs 197.2, $p = 0.017$). None of the other biomarkers showed any association with sex.

Surprisingly, in a linear regression model correcting for age, sex, BMI, CVRF, education, APOEε4 status, HbA1C, and creatinine, there was a positive association between Tau concentration (z-score adjusting for the factors stated above) and B12 ($\beta = 0.25$, $p = 0.005$, $\Delta R^2 = 0.052$, Fig 4A), but not any of the other biomarkers (Table 3). To further investigate this association between Tau and B12, we investigated the correlation between B12 levels and blood B12 components adjusting for the factors mentioned above. While no association could be found between Holo-TC and any of the included biomarkers (Fig 4B), Tau and UCHL-1 increased with higher Holo-HC ($\beta = 0.22$ and 0.28, $p = 0.015$ and 0.022, respectively, Fig 4C,D). Both associations remained significant after bootstrapping ($p = 0.012$ and 0.042 for Tau and UCHL-1, respectively).

Low Available B12 on Holo-TC Associates with Higher Burden of WMH

On MRI, B12 deficiency can be associated with WMH, suggesting possible microstructural tissue disruption and/or increased vascular risk.[14,15] Here, we investigate

FIGURE 2: Low available serum indicators of B12 status (total B12 and Holo-TC) associate with mfVEP latency prolongation. (A) Mixed linear model of mfVEP latency with log B12 as the independent variable, correcting for age (years), sex at birth, education (years), BMI, CVRF, and APOEε4, as well as accounting for the eye as a random factor. The relationship between B12 and mfVEP latency was significant for people with log-transformed B12 values under the mean of 2.61 (dashed line). Black = B12 values below the mean; gray = B12 values above the mean. (B) Mixed linear model of mfVEP latency with Holo-TC as the independent variable, correcting for age (years), sex at birth, education (years), BMI, CVRF, HbA1C, and APOEε4, as well as accounting for the eye as a random factor for people with log-transformed B12 values under the mean. APOEε4 = apolipoprotein E ε4 allele; BMI = body mass index; CVRF = cardiovascular risk factor; HbA1C = hemoglobin A1C; Holo-HC = holo-haptocorrin; Holo-TC = holo-transcobalamin; mfVEP = multifocal visual evoked potential.

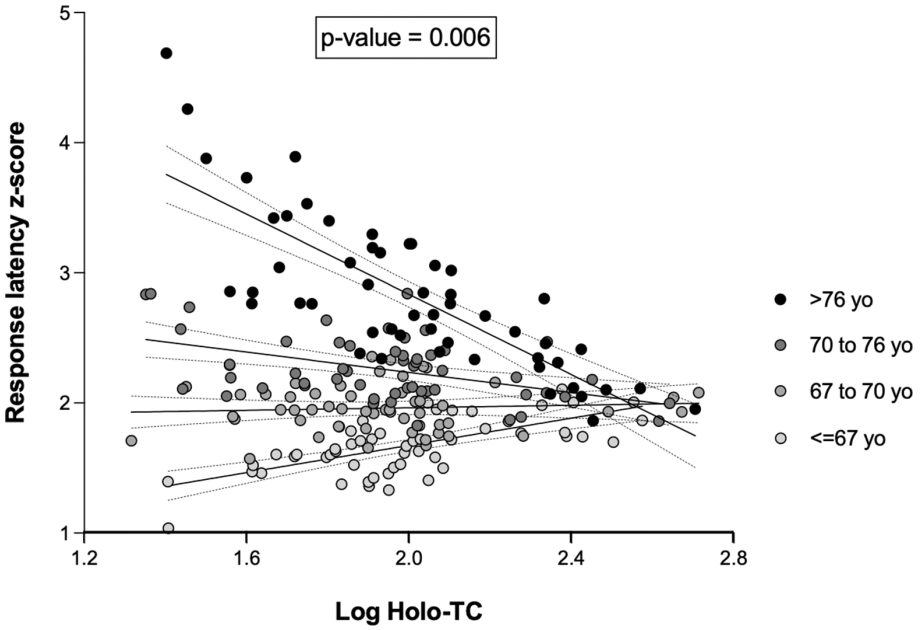


FIGURE 3: The association of vitamin B12 blood levels and cognitive tests is age dependent. The association between processing speed (response latency z-score) and Holo-TC appears with higher age. Linear regression model evaluating the association between Holo-TC and processing speed with an interaction term with age (*p*-value = 0.006), correcting for age, BMI, CVRF, sex, education level, HbA1C, and APOEε4 status. On this graph, the predicted regression for processing speed is represented for each quartile by age (hinges: 67, 70, and 76 years old; light shade is youngest; dark shade is eldest). APOEε4 = apolipoprotein E ε4 allele; BMI = body mass index; CVRF = cardiovascular risk factor; HbA1C = hemoglobin A1C; Holo-TC = holo-transcobalamin.

whether low B12 levels within the currently defined normal range also associate with WMH or other ultrastructural anomalies. In a linear regression correcting for age, sex, and the presence of CVRFs, no association was found between the volume of WMH and serum total B12 levels ($\beta = -0.03$, $p = 0.734$, Fig 5A). On the other hand, the volume of WMH associates with Holo-TC levels ($\beta = -0.21$, $p = 0.039$, Fig 5B). Plotting the absolute values for WMH volume (mm^3) and Holo-TC (pmol/L) also demonstrates that the participants with the highest WMH burden all had Holo-TC in the lower range (Fig 5C). There was no association between gray or WM volumes and total B12, Holo-TC, or Holo-HC blood levels that could reflect large scale and/or widespread neurodegeneration (Fig 4). These analyses suggest that, even within the currently defined normal range of values, lower levels of available B12, measured as Holo-TC, associate with larger volumes of WMH.

TABLE 2. Demographic, Clinical Characteristics, and the Included Biochemical Parameters

Biomarker	Median [25–75 Percentiles], Mean
NfL (pg/ml), % CV	23.5 [17.0–34.4], 4.5%
Tau (pg/ml), %CV	0.89 [0.59–1.3], 7.6%
UCH-L1 (pg/ml), %CV	17.4 [13.7–25.5], 8.8%
GFAP (pg/ml), %CV	206.2 [164.5–288.7], 5.7%
Aß42 (pg/ml), %CV	6.6 [5.6–7.5], 4.4%
Aß40 (pg/ml), %CV	205.0 [177.0–245.0], 2.2%
Aß42-Aß40 ratio	0.033 [0.029–0.035]

Aß, amyloid-beta; CV = coefficient of variation; GFAP, glial fibrillary acidic protein; NfL, neurofilament light chain; UCH-L1, ubiquitin C-terminal hydrolase L1.

Discussion

Our results suggest that in a cohort of healthy elderly individuals, lower B12 associates with a delay in VEP latency, and that it is specific to the biologically available fraction of serum B12 bound to transcobalamin (Holo-TC). Low levels of Holo-TC also associate with slowed spatial processing speed in an age-dependent manner. On MRI, participants with lower Holo-TC have a higher burden of T2 WMH. Remarkably, we find that high levels of Holo-HC, the biologically unavailable fraction of B12, associate with an increase in the levels of T-Tau proteins in the serum, a biomarker for neurodegeneration. These findings suggest that current parameters for defining adequate B12 levels may be inappropriate when considering

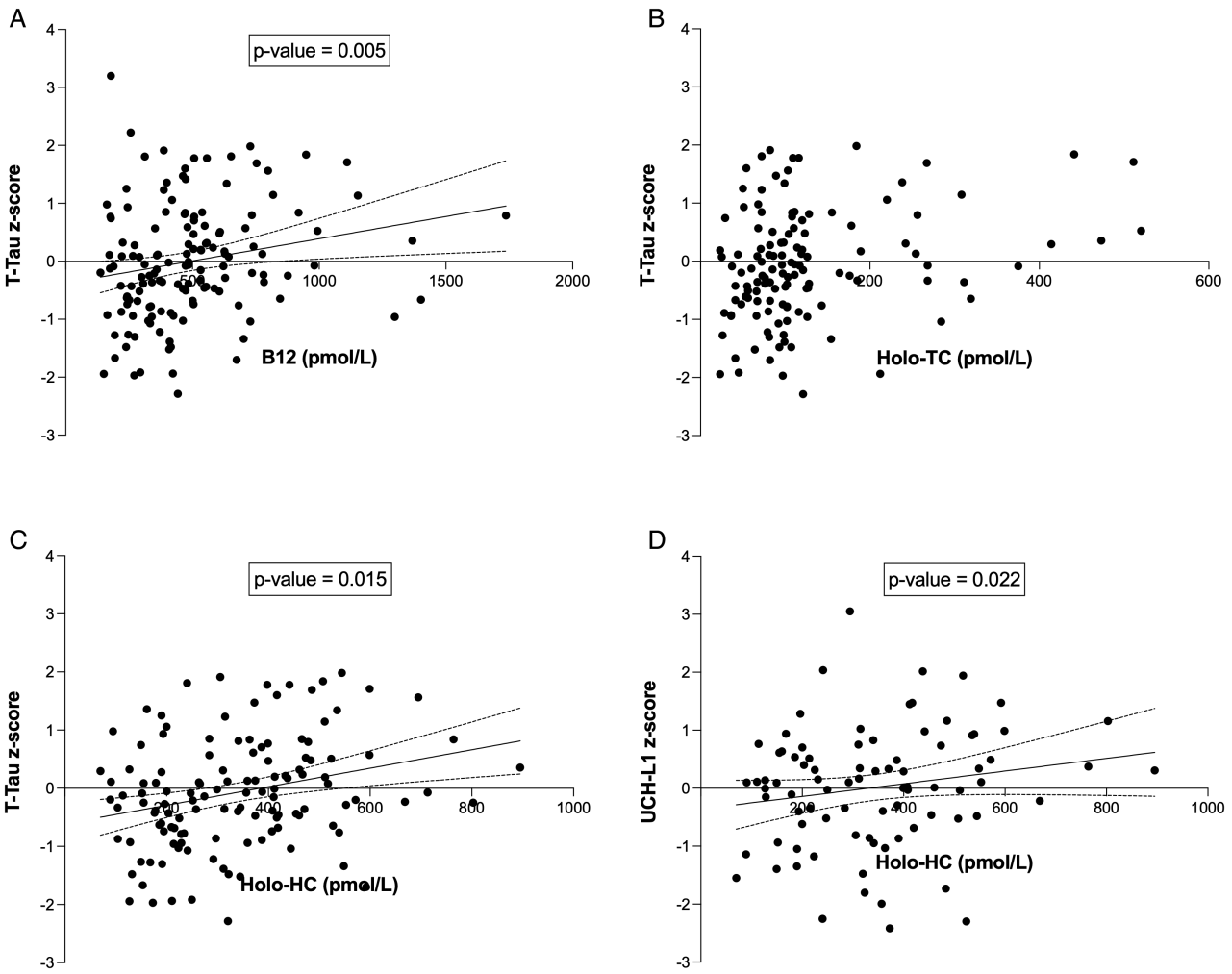


FIGURE 4: High unavailable B12 associates with an elevation in biomarkers for neurodegeneration. (A) There is a significant positive association between the levels of total measured B12 and the levels of the protein T-Tau (z-score adjusted for age, sex, BMI, CVRF, education, HbA1C, APOEε4 status and creatinine). (B) No correlation was found between T-Tau and Holo-TC. (C) A significant positive association exists between T-Tau and derived Holo-HC. (D) Another biomarker of neuroaxonal injury, UCH-L1, correlated positively with the levels of derived Holo-HC. BMI = body mass index; CVRF = cardiovascular risk factor; HbA1C = hemoglobin A1C; Holo-HC = holo-haptocorrin; Holo-TC = holo-transcobalamin; UCH-L1 = ubiquitin carboxy-terminal hydrolase L1.

neurophysiological, neuropsychological, serological, and neuroradiological outcomes.

Vitamin B12 deficiency results in prominent WM injury of the posterior columns in the central nervous system (CNS) of humans[2,6] as well as in animal models of B12 deficiency.[38–41] The mfVEP provides a non-invasive assessment of the functional integrity of a well-demarcated WM tract in the central nervous system: the visual pathway.[24] Our lab and other groups previously demonstrated VEP as a sensitive marker for myelin integrity in animal models as well as in humans.[23,42–44] Therefore, we postulate that the delay in mfVEP latency in the participants of our study reflects neurological impairment related to myelin integrity. The reported association between delayed VEP recordings and low B12 levels – especially the biologically available fraction bound to transcobalamin (Holo-TC) – suggests the necessity for sufficient blood B12 levels to maintain CNS health, particularly that of myelinated tracts. In line with the proposed association with WM integrity, the higher volume of WMH in participants with lower Holo-TC levels may relate to microstructural changes affecting vascular permeability and to oligodendrocyte loss.[45] Of note, the current study cannot determine whether the effect of B12 measured on WMH and all other outcomes directly results from the low B12. Many other studies have shown an association with high homocysteine in the blood, which is a by-product of B12 insufficiency.[12,46–48] Indeed, large-scale cohort studies demonstrated that low B12 and high homocysteine can have profound impacts on brain

TABLE 3. Association between the Cohort's Characteristics and B12 with Serum Levels of NfL, Tau, and UCH-L1, and with Plasma Levels of GFAP and Aß40/42

	NfL^{+}		Tau^{+}		UCH-L1^{+}		GFAP^{+}		Aß40^{+}		Aß42^{+}		Aß42/Aß40	
Parameter	**β**	***p***	**β**	***p***	**β**	***p***	**β**	***p***	**β**	***p***	**β**	***p***	**β**	***p***
Model 1 (R^2)	0.254		0.108		0.040		0.190		0.099		0.134		0.165	
Sex	0.142	0.070	0.050	0.567	0.036	0.771	0.264	0.001	0.056	0.618	0.185	0.089	0.193	0.078
Age^{+}	0.359	<0.001	0.212	0.016	−0.101	0.377	0.354	<0.001	0.177	0.083	0.124	0.199	−0.035	0.726
BMI^{+}	−0.236	0.002	−0.232	0.008	0.030	0.792	−0.126	0.114	−0.117	0.270	−0.077	0.448	0.027	0.794
CVRF status	−0.043	0.570	0.008	0.924	0.113	0.302	−0.012	0.877	−0.055	0.585	−0.134	0.169	−0.109	0.273
Creatinine^{+}	0.276	<0.001	0.070	0.425	0.073	0.527	0.074	0.361	0.146	0.176	0.268	0.010	0.159	0.131
Education	−0.016	0.832	−0.067	0.428	0.031	0.786	0.080	0.302	−0.065	0.516	−0.048	0.616	0.044	0.652
APOE	0.076	0.304	0.131	0.121	0.115	0.300	0.173	0.026	0.073	0.466	−0.097	0.310	−0.253	0.010
HbA1C^{+}	−0.005	0.949	−0.046	0.588	−0.027	0.811	−0.034	0.659	0.167	0.102	0.090	0.349	−0.133	0.181
Model 1 + B12 (R^2)	0.261		0.160		0.063		0.190		0.099		0.135		0.167	
B12^{+}	0.087	0.275	**0.251**	**0.005**	0.172	0.151	−0.021	0.797	−0.003	0.974	−0.038	0.707	−0.056	0.590

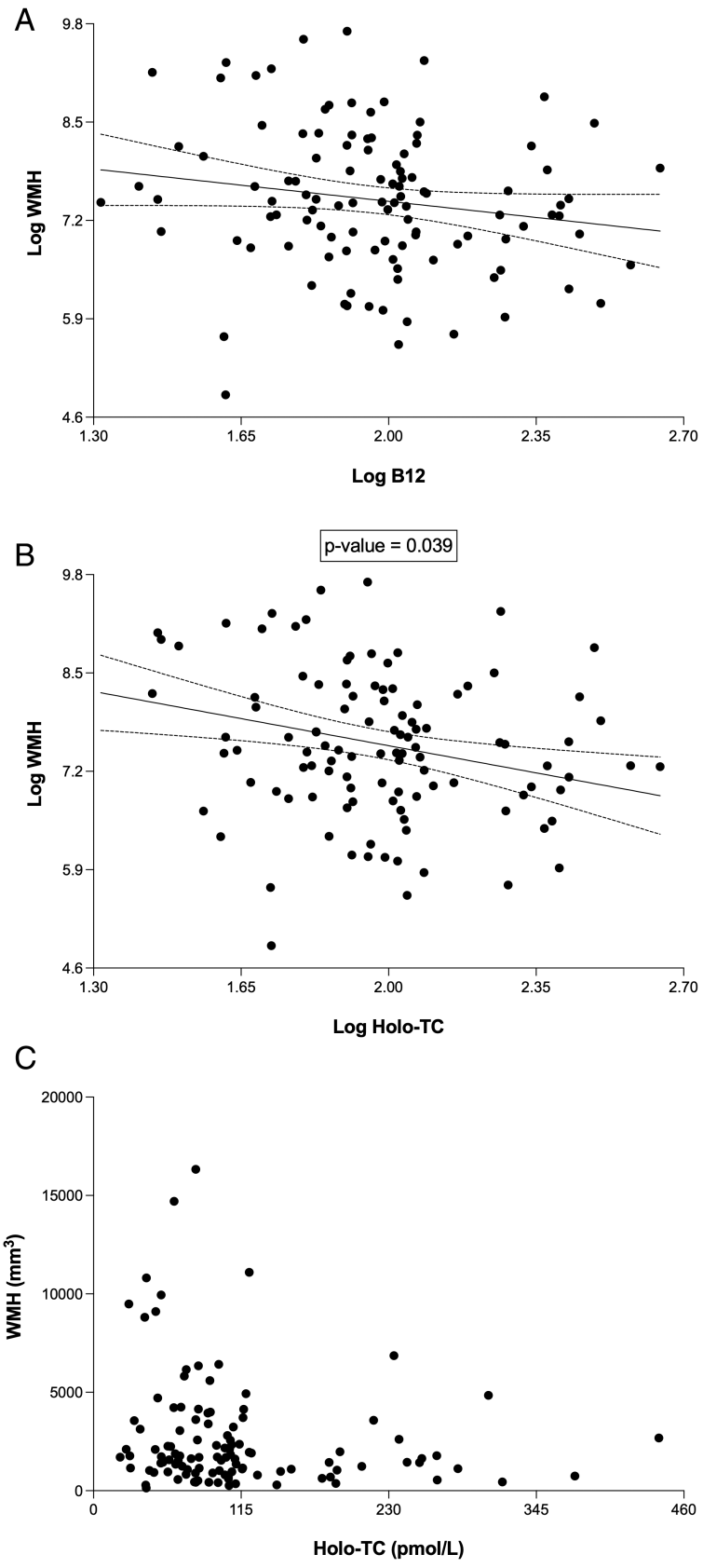
$^{+}$ Log transformed.

Aß = amyloid-beta; APOE = apolipoprotein E; BMI = body mass index; CVRF = cardiovascular risk factors; GFAP = glial fibrillary acidic protein; HbA1c = hemoglobin A1C; NfL = neurofilament light chain; UCH-L1 = ubiquitin C-terminal hydrolase L1.

structure[21,49]; our study aiming at lower but not deficient levels of B12 did not detect such overt changes. Our study has the additive value of showing a "precursor" stage at which microstructural changes are present and already impairing neurological function. In addition, older participants in our cohort exhibit a functional correlation with involvement of low B12 in maintaining myelin integrity. This is evident by the association between slower cognitive processing speed and low Holo-TC. Previous studies have also argued for a particular dependency of processing speed in elderly individuals on WM integrity.[29,50,51] This could reflect a specific vulnerability of elders to lower levels of Holo-TC, or that the cognitive tests used to measure processing speed lacks the sensitivity to detect changes in younger individuals.

Of note, the reported associations between B12 and indirect markers of myelin integrity in our study could be multifaceted. All used methods are not unequivocally specific for myelin and could partially reflect neuroaxonal and glial involvement. Nevertheless, we find no association between low B12 and putative markers of neuroaxonal injury and glial activation (eg, NfL, Tau, UCHL1 and GFAP respectively) in our population. Therefore, we think that the observed detrimental effect of low B12 on the CNS is likely related to myelin integrity. However, the specific involvement of B12 in the biological pathway of myelin synthesis and the cell-autonomous nature of the relationship between B12 and oligodendrocytes has not yet been defined.

Surprisingly, we found that the neurodegeneration biomarker Tau increases with the levels of B12 and Holo-HC in our cohort. The clinical relevance and biological basis of this finding have yet to be established in larger cohorts. Nevertheless, this association does not relate to biologically active B12 levels (Holo-TC), but is instead mediated by Holo-HC, which represents the major fraction of total blood B12 levels and the biologically unavailable B12 bound to the circulating protein haptocorrin. It is not clear whether this represents a deleterious neurological consequence of higher B12 or Holo-HC in the blood, or if it is an incidental finding related to an uncorrelated problem. Vitamin B12 and homocysteine have a known association with liver disease like metabolic-associated fatty liver disease and steatohepatitis, which are common in the obese population.[52–54] Indeed, liver disease was shown to impair Aβ clearance and to predispose to cognitive impairment in mice.[55] Criteria for patient inclusion in the study were strict for the evaluation of neurological diseases and major systemic conditions, and no participant suffered from severe obesity, but subclinical



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diseases such as elevated liver enzymes were not part of the screening process. Metabolic syndrome and in particular insulin resistance have been linked to biomarkers of low vitamin B12.[56,57] To account for such confounders, we included HbA1C, BMI, and the presence of CVRFs in our reported models; none of these co-variables had a significant effect on our outcomes of interest. Kidney disease represents another key factor when studying metabolites, since a reduced excretion rate may increase the serum concentration and precipitate heart failure by increasing homocysteine.[46] As creatinine was included in our measurements, we ensured no participant had kidney failure, and that kidney disease did not interfere with our results. On the other hand, we cannot exclude that high Holo-HC could reflect a non-canonical and neurotoxic cellular response from what it carries, for example, analogs of B12.[58] Our examinations exclusively identified cobalamin in the plasma samples from the patients, failing to detect any of the recognized analog forms of B12. It is important to note that this does not rule out the possibility that certain analogs might exist in extremely low concentrations or were inadvertently lost during the extraction and analysis procedures. Moreover, it remains plausible that unidentified analogs may also be present, considering that our methodology relies on identifying combinations of ions with known masses. The plaques seen in Alzheimer's disease contain hyperphosphorylated Tau proteins, and B12 plays a role in preventing these aggregations by inhibiting the fibrillization of Tau.[59,60] Therefore the retention of vitamin B12 by haptocorrin may decrease the bioavailability of B12 in the brain thus favoring Tau-mediated neuronal injury. Another element of the underlying mechanism for our observed effects may lie in the genetic variability for B12-related proteins, for example, the transcobalamin 1 (*TCN1*) and 2 (*TCN2*) gene variants, which may affect the levels of B12 measured in the blood between individuals and affect cognitive outcome as previously shown.[61] As mentioned, our small sample size limits the conclusions we can draw from these findings; larger cohort studies focusing on Holo-HC and neurological outcomes would help to further support their association. Longitudinal follow-up studies should also aim at evaluating the long-term consequences of potentially harmful amounts of B12 at both ends of the spectrum. As with most observational research, we could not obtain complete data from all participants, which may affect the generalizability of our results. The samples collected were in some cases insufficient to measure methylmalonic acid (MMA) and liver function tests (LFTs), which would have been a great addition to our study. However, the B12 levels of our cohort remained above the threshold at which MMA elevation appears. Moreover, patients with reported liver disease were not included in our cohort; elevated LFTs would have represented incidental findings. Of note, our sample exhibited a bias toward highly educated individuals. While this bias might work in our favor, given that educated populations generally follow healthier diets, it may lead to an underestimation of the observed effects.

Our findings are of critical importance for rethinking the "biologically sufficient" B12 levels. The population-based studies that defined healthy micronutrient levels may have missed the subclinical manifestations of low or high B12 at the extremes of the population distribution that can affect people without causing overt symptoms. While the clinical features of unambiguous B12 deficiency are well defined as predominantly hematological (most conspicuously macrocytic anemia) and neurological, the condition can manifest with either hematological or neurological syndromes in isolation.[27] Without sensitive tools to detect subtle neurological deficits, the heterogeneous nature of the symptoms remained unexplained. Notably, during and following B12 repletion therapy, patients often request higher dosing of B12 to treat their neurological symptoms, even after their hematological symptoms have resolved.[62] Our findings support the idea that subtle neurological deficits manifest at higher levels than the current threshold defined for deficiency, and most importantly, it could provide an explanation for the often-reported discrepancy between hematological and neurological symptoms.[63,64] When B12 intake or absorption is impaired, the levels of Holo-TC initially decrease in the blood, which can be first compensated by releasing the B12 in storage.[65,66] This slow progression toward deficiency could be better described by a sliding scale of insufficiency, wherein the tissue levels decline until Holo-TC falls below what is necessary to

FIGURE 5: Low available B12 on Holo-TC associates with a higher burden of WMH. (A) Linear regression model of log WMH with log B12 as the independent variable. The variables did not significantly associate after correcting for age, sex, BMI, education, total intracranial volume, HbA1C, APOEε4 and cardiovascular risk factors. (B) There is a significant negative association between log WMH and log Holo-TC after correcting for the same covariates as in (A). (C) Graph representing the absolute volume of WMH and the absolute Holo-TC values. APOEε4 = apolipoprotein E ε4 allele; BMI = body mass index; Holo-TC = Holo-transcobalamin; WMH = white matter hyperintensity.

supply critical cells for biochemical reactions in the nervous system, and then the bone marrow. Revisiting the definition for healthy B12 levels could promote earlier intervention and prevention of cognitive decline, especially in the elderly carrying increased risk for B12 malabsorption and insufficiency.[67,68] A study on an aging population previously argued for a broadening of the reference range for B12 levels in elder people, and our results further support such a shift to account for age.[69] The elderly population may be particularly vulnerable to the effects of declining B12 availability and may benefit from a change in guidelines for what is currently defined as healthy levels of vitamin B12.

The theoretical model we are proposing with this work is that inadequate amounts of vitamin B12 could induce neurological deficits at a threshold that is higher than the one in current use (Graphical abstract). Moreover, we should work on defining a cutoff for higher B12 and study how haptocorrin might be associated with or could induce neuro-axonal damage. Accordingly, our current understanding of optimal serum B12 may have to be revisited to account for the clinical manifestations of B12 inadequacy at both ends of the spectrum.

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Author Contributions

A.B.R., A.A., J.W.M., R.G., and A.J.G. contributed to the conception and design of the study. A.B.R., A.A., R.S., S.S., S.C.M., F.C.O., C.C., N.J., K.A., M.C., S.J., S.S., A.S., C.S., M.W., J.W.B., J.K., B.M., J.W.M., R.G., and A.J.G. contributed to the acquisition and analysis of the data. A.B.R., A.A., R.S., A.G., and A.K. contributed to drafting the text or preparing the figures.

Potential Conflicts of Interest

The authors have no conflicts of interest to report.

Data Availability

Data will be provided upon request to ensure replication of results.

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