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Low plasma ergothioneine levels are associated with neurodegeneration and cerebrovascular disease in dementia

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ABSTRACT

Ergothioneine (ET) is a dietary amino-thione with strong antioxidant and cytoprotective properties and has possible therapeutic potential for neurodegenerative and vascular diseases. Decreased blood concentrations of ET have been found in patients with mild cognitive impairment, but its status in neurodegenerative and vascular dementias is currently unclear. To address this, a cross-sectional study was conducted on 496 community-based participants, consisting of 88 with no cognitive impairment (NCI), 201 with cognitive impairment, no dementia (CIND) as well as 207 with dementia, of whom 160 have Alzheimer's Disease (AD) and 47 have vascular dementia (Martinez-Vea et al., 2006) [1]. All subjects underwent blood-draw, neuropsychological assessments, as well as neuroimaging assessments of cerebrovascular diseases (CeVD) and brain atrophy. Plasma ET as well as its metabolite L-hercynine were measured using high sensitivity liquid chromatography tandem-mass spectrometry (LC-MS/MS). Plasma ET concentrations were lowest in dementia ($p < 0.001$ vs. NCI and CIND), with intermediate levels in CIND ($p < 0.001$ vs. NCI). A significant increase in L-hercynine to ET ratio was also observed in dementia ($p < 0.01$ vs. NCI). In multivariate models adjusted for demographic and vascular risk factors, lower levels of ET were significantly associated with dementia both with or without CeVD, while ET associations with CIND were significant only in the presence of CeVD. Furthermore, lower ET levels were also associated with white matter hyperintensities and brain atrophy markers (reduced global cortical thickness and hippocampal volumes). The incremental decreases in ET levels along the CIND-dementia clinical continuum suggest that low levels of ET are associated with disease severity and could be a potential biomarker for cognitive impairment. Deficiency of ET may contribute towards neurodegeneration- and CeVD-associated cognitive impairments, possibly via the exacerbation of oxidative stress in these conditions.

1. Introduction

Dementia is a major cause of disability in late-life and is associated with high costs for public healthcare systems [2]. In light of the increased life expectancy and significant growth in elderly populations worldwide, the number of patients suffering from dementia is expected to increase dramatically over the next decades [2]. At present, the two commonest forms of dementia in the elderly are Alzheimer's disease (AD) and vascular dementia [1], with the former being a progressive neurodegenerative condition characterized by cortical accumulation of

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intercellular amyloid plaques and intracellular neurofibrillary tangles, and the latter predominantly a sequela of chronic, small vessel cerebrovascular diseases (CeVD) [3]. AD and vascular dementia, the latter part of a continuum of disease called vascular cognitive impairment (VCI) [4] share multiple common risk factors including aging, hypertension, diabetes and cardiovascular diseases [5–7]. Accumulating evidence suggests that AD- and CeVD-associated pathologies often coexist and may exert additive or synergistic effects that increase the risk and/or severity of cognitive impairment and dementia [8,9]. However, the underlying pathogenic mechanisms for AD and VCI, as well as how they may interact, are not fully understood, resulting in obstacles towards identification of clinically useful biomarkers and therapeutic targets.

Of the many disease processes implicated in AD and VCI, there is increasing focus on oxidative stress as a key pathogenic factor in both conditions [10–13]. Extensive oxidative damage in the brain can result from increased generation of harmful reactive oxygen species under various conditions, as well as reduced antioxidant potential, aberrant protein aggregation and endothelial injury, which in turn aggravate existing brain dysfunction [10,11,14]. Furthermore, it has become increasingly clear that oxidative stress is involved in the propagation of neuronal injury that occurs early in the course of these diseases. Accordingly, a wide range of oxidative damage markers such as stable oxidized products of lipids, proteins and nucleic acids, are elevated in the brain and cerebrospinal fluid (CSF) of patients with mild cognitive impairment (MCI), a pre-dementia phase comparable to CIND, AD and VaD [12,15–17]. However, in addition to markers of oxidative stress, it may also be worthwhile to investigate anti-oxidation processes in disease conditions.

Recent studies suggest that ergothioneine (ET), a naturally occurring amino-thione with strong antioxidant and other cytoprotective properties, may be neuroprotective [18–20]. In mammals, ET is derived solely from dietary sources such as mushrooms [21,22]. Unlike many other diet-derived micronutrients, ET can accumulate in blood and various body tissues (including the brain) due to the presence of a specific transporter, organic cation transporter novel-type 1 (OCTN1, also known as ergothioneine transporter, ETT), which transports ET into cells and tissues [19,23–26]. This specific transport, along with low urinary excretion and metabolic turnover, may explain the persistence of elevated blood ET as long as four weeks after oral administration in humans [27]. In several experimental models, ET exhibited cytoprotective effects against cardiovascular diseases [28], inflammation [29] and neuronal injury [30–32]. Furthermore, a recent longitudinal study showed that higher plasma ET is an independent predictor for decreased cardiovascular disease and mortality risks [33], suggesting a protective role of ET in vascular pathologies. In neurodegenerative diseases, serum ET was significantly lower in patients with Parkinson's disease (PD) compared to age-matched healthy subjects [34]. Similarly, decreased blood ET was reported in patients with MCI [18,35]. Taken together, these results suggest a potential involvement of ET in both neurodegeneration and vascular pathologies. L-Hercynine is thought to be a major oxidation product of ET [36] (see Supplementary Fig. S1) and has previously been detected in human blood with high correlations to ET concentrations [27]. However, the status of blood ET and L-hercynine in AD subjects with and without concomitant CeVD is at present unknown. In this study, we investigated associations of plasma ET and L-hercynine with clinical and neuroimaging parameters, namely (a) cognitive impairment and dementia (both AD and VaD); (b) CeVD; and (c) neurodegeneration.

2. Methods

2.1. Study population

Details of the inclusion criteria and assessment of the clinical cohort utilized in this retrospective case-control study have previously been described [37]. A total of 618 participants were recruited from memory clinics and the community in Singapore from August 2010 to July 2019. Only individuals with available blood plasma samples and gradable MRI scans (n = 496) were included in this study. All subjects underwent standard physical, clinical, blood tests, and neuropsychological as well as neuroimaging assessments at the National University of Singapore. The study received ethics approval from the National Healthcare Group Domain-Specific Review Board (reference 2010/00017; study protocol DEM4233) and was conducted in compliance with the guidelines in the Declaration of Helsinki. Written informed consent was obtained from all participants or their next-of-kin in their preferred language prior to their enrolment into the study.

2.2. Diagnostic criteria

The diagnoses of all study participants were discussed and confirmed at weekly consensus meetings attended by study clinicians and neuropsychologists, during which detailed clinical features, neuropsychological testing and brain imaging data were reviewed. Patients who had no objective cognitive impairment on formal neuropsychological test battery or functional loss were designated as no cognitive impairment (NCI). Details of the component tests used to assess the seven cognitive domains (Executive Function, Attention, Language, Visuomotor Speed, Visuoconstruction, Verbal Memory, Visual Memory) have been described previously [[38], also see Supplementary Table S1]. Participants with cognitive impairment no dementia (CIND) were defined as having impairment(s) in at least one domain of the neuropsychological tests (that is, scoring at least 1.5 standard deviations below the established education-adjusted cut-off values on any test), but otherwise did not meet the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSMIV) criteria for dementia. Diagnoses of Alzheimer's disease (AD) followed the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [39]. Vascular dementia was diagnosed according to the National Institute of Neurological Disorders and Stroke and Association Internationale pour la Recherché et l'Enseignement en Neurosciences (NINDS-AIREN) criteria [40]. For further analyses, individuals were classified into two groups based on their cognitive impairment status, with NCI as 'cognitively unimpaired' while CIND and Dementia (AD and VaD) were combined as 'cognitively impaired'.

2.3. Demographic and other risk factors assessments

A detailed questionnaire was administered to all participants to collect information on age, sex, race and highest attained education. Education status was categorized as low (not exceeding primary school education) or high (beyond primary school education). Physical examination included height, weight, and blood pressure measurements. Risk factors, such as hypertension, hyperlipidemia, diabetes mellitus, and cardiovascular diseases were ascertained from clinical interviews and medical record searches, and classified as present or absent. Hypertension was defined as systolic blood pressure of 140 mm Hg or more and/or diastolic blood pressure 90 mm Hg or more, or history of antihypertensive medication use. Diabetes mellitus was defined as glycated haemoglobin (HbA1c) of 6.5% or more, or on diabetic medication. Hyperlipidemia was defined as total cholesterol levels of 4.14 mM or more, or on lipid lowering medication. Cardiovascular disease was classified as a previous history of atrial fibrillation, congestive heart failure, and myocardial infarction. Apolipoprotein E (APOE) genotyping was performed as described previously [41], and APOE ε4 carrier status was defined as having at least one ε4 allele. Body mass index (BMI) was calculated as mass (kg)/height squared (m^2).

2.4. Neuroimaging

Magnetic resonance imaging [42] scans were performed on a 3-T Siemens Magnetom Trio Tim scanner, using a 32-channel head coil, at the Clinical Imaging Research Center, National University of Singapore. Subjects with claustrophobia, contraindications for MRI, or those who were unable to tolerate the procedure were excluded. All MRIs were graded by one radiologist and two clinicians blinded to the neuropsychological and clinical data. The sequences included T1-weighted Magnetization Prepared Rapid Gradient Recalled Echo, Fluid Attenuated Inversion Recovery, T2-weighted, and Susceptibility Weighted Imaging sequences.

MRI markers of CeVD were defined based on the Standards for Reporting Vascular Changes on Neuroimaging (STRIVE) criteria [43]: (a) Cortical infarcts were defined as hypodense lesions interrupting the cortex grey/white junction; (b) Lacunes were defined by the presence of hypodense focal lesions measuring ≥3 mm and <15 mm; (c) WMH were defined as hyperintense on T2 and FLAIR sequences and hypointense on T1 weighted images and were graded using the Age-Related White Matter Changes (ARWMC) scale [44]. Significant CeVD was defined as the presence of cortical infarcts and/or ≥2 lacunes, and/or confluent WMH (ARWMC score ≥8) in 2 regions of the brain, as described previously [45]. Additionally, quantitative analyses of brain atrophy as neurodegeneration markers on MRI were performed using an automated segmentation procedure at the Department of Medical Informatics, Erasmus University Medical Center, Netherlands (FreeSurfer, v.5.1.0) on T1 weighted images (TR = 7.2 ms, TE = 3.3 ms, matrix = 256 × 256 × 180 mm^3). Image preprocessing and the tissue classification algorithm have been described elsewhere [46]. Brain atrophy was indicated by two parameters: (a) Global cortical thickness, measured as the shortest distance between grey/white matter boundary and pial surface at each vertex, with cortical thickness averages converted to millimeters (mm); (b) Hippocampal volumes, calculated for left and right hemispheres separately, with average volumes were then converted into milliliters.

2.5. Plasma ergothioneine and L-hercynine measurements

Blood was drawn from study participants into ethylenediaminetetraacetic acid (EDTA) tubes and processed by centrifugation at 2000 × *g* for 10 min at 4 °C, followed by extraction of the upper plasma layer and storage at −80 °C until use. ET, deuterated ET-d_9, L-hercynine, and deuterated L-hercynine-d_9 standards were provided by Tetrahedron Scientific Inc. (Paris, France). 10 μl of plasma was mixed with 105 μl methanol containing ET-d_9 and L-hercynine-d_9. Samples were vortexed and incubated at −20 °C for 2 h, followed by centrifugation and drying of supernatant under a stream of nitrogen gas. Dried samples were resuspended in 100 μl of ultrapure water and transferred to silanized vials for analysis. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed using an Agilent 1290 UPLC coupled to a 6460-QQQ mass spectrometer (Agilent Technologies, CA, USA). Sample aliquots were kept at 10 °C in the autosampler, then injected (2 μl) onto a Cogent Diamond-Hydride column (4 mm, 150 × 2.1 mm, 100 Å; MicroSolv Technology Corporation, NJ, USA) at 30 °C. The mobile phase consisted of 100% acetonitrile (solvent A) and 0.1% formic acid (solvent B). Chromatographic separation was achieved using a gradient elution at 0.4 ml/min from 25% solvent B at 1 min to 40% solvent B over 3 min to elute ET (retention time of 3.6 min). MS was carried out under positive ion, electrospray ionization mode, using multiple reaction monitoring for quantification of specific target ions (see Supplementary Table S2). Capillary voltage was 3200 V, and gas temperature was kept at 350 °C. Nitrogen sheath gas pressure for nebulizing samples was kept at 50psi, with gas flow rate of 12.5 L/min. Ultra-high purity nitrogen was used as collision gas. Compounds were quantified with calibration standards (at least 8 concentrations encompassing the sample range), using the Agilent Mass Hunter software (Agilent Technologies, CA, USA).

2.6. Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics version 26 (IBM Co., Armonk, NY, USA) and R software version 4.0.2 (R Core Team). One-way analyses of variance (ANOVA) with Bonferroni *post hoc* test, Kruskal–Wallis test with Dunn's *post hoc* tests and Chi-square tests were used to detect group differences for normally distributed continuous variables (age and BMI), skewedly distributed continuous variables (ET, L-hercynine and L-hercynine/ET ratio), and binary variables (gender, APOE4 status, education level, hypertension, hyperlipidemia, diabetes and cardiovascular diseases), respectively. Blood biomarkers (ET, L-hercynine and L-hercynine/ET ratio) evaluated were not normally distributed, and were therefore log-transformed before inclusion as determinants in subsequent regression analyses. Binary logistic regression models were constructed with odds ratios (OR) and 95% confidence intervals (CI) to determine the associations between blood biomarkers with CIND and dementia. Unadjusted models were first assessed (Model 1), followed by adjustments for age, gender, education, APOE ε4 carrier status, hypertension, diabetes and cardiovascular diseases as covariates (Model 2). Further regression analyses were performed for both CIND and dementia stratified by significant CeVD on MRI. To examine the relationships between blood biomarkers with CeVD, two analytical approaches were adopted. First, lacunes and cortical infarct counts, as well as ARWMC scores, were used to stratify participants into those with or without significant CeVD based on previously described cut-offs ([45] also see above under *Neuroimaging*), then analyzed using binary logistic regression. Second, Poisson regression and negative binomial regression models (with measure of association as rate ratios (RRs) and corresponding 95% CIs) were constructed for counts of cortical infarcts and lacunes, respectively, while linear regression models with mean differences (β) and 95% CI were used for the ARWMC visual scores for WMH, as residuals fulfilled the normality and homoscedasticity assumption of a linear regression model. Models were assessed with no adjustments (Model 1) and subsequent adjustments for covariates including age, gender, hypertension, diabetes, cardiovascular disease and cognitive status (Model 2). Similarly, linear regression models with β and 95% CI were performed for associations between the blood biomarkers and imaging markers of neurodegeneration (global cortical thickness and hippocampus volume). Here the models were unadjusted (Model 1); adjusted for covariates including age, gender, education, APOE, hypertension, diabetes, cardiovascular diseases, intracranial volume, cognitive status (Model 2) and lastly, additionally adjusted for WMH (Model 3). P values of <0.05 were considered statistically significant.

3. Results

3.1. Group descriptions

Amongst the 496 study participants, 88 (17.7%) individuals were NCI controls, 201 (40.5%) were CIND and 207 (41.8%) were dementia patients. Of the dementia group, 160 (32.3%) had AD and 47 (9.5%) had VaD. Demographic and clinical characteristics of study participants are presented in Table 1. Patients with CIND and dementia were older ($p < 0.001$ for both), had lower education level ($p < 0.05$ and $p < 0.001$, respectively) and with higher APOE ε4 carrier frequency ($p < 0.01$ and $p < 0.001$, respectively) compared to NCI. The prevalence of vascular risk factors such as hypertension ($p < 0.05$ and $p < 0.001$, respectively) and diabetes ($p < 0.01$ and $p < 0.001$, respectively) were also significantly higher in CIND and dementia groups compared to NCI. No significant difference was observed in nutritional status (as indicated by BMI) among groups. Furthermore, whilst age

Table 1
Baseline characteristics of the study cohort (n = 496).

Characteristics	NCI (n = 88)	CIND (n = 201)	Dementia (n = 207)	P value
Age, mean (SD), y	68.7 (7.0)	73.0 (8.0)[a]	74.9 (7.5)[a]	<0.001
Female, no., %	46 (52.3)	98 (48.8)	120 (58.0)	0.172
Education ≤ elementary, no., %	26 (29.5)	90 (44.8)[b]	140 (67.6)[b,c]	<0.001
APOE ε4 carrier, no., %	12 (13.6)	60 (29.9)[b]	69 (33.3)[b]	0.002
BMI, mean, kg/m^2	24.4 (3.6)	23.9 (3.8)	23.0 (4.1)	0.198
Hypertension, no., %	50 (56.8)	138 (69.0)[b]	160 (78.0)[b,c]	0.001
Diabetes, no., %	18 (20.5)	76 (37.8)[b]	89 (43.0)[b]	0.001
Cardiovascular diseases, no., %	5 (5.7)	27 (13.4)	33 (16.0)	0.059
Hyperlipidaemia, no., %	59 (67.0)	159 (79.1)	152 (73.4)	0.084
Ergothioneine, median (IQR), nM	1197 (1093)	798 (954)[d]	588 (633)[d,e]	<0.001
L-Hercynine, median (IQR), nM	16.2 (16.8)	10.8 (15.1)[d]	10.4 (18.5)[d]	0.005
L-Hercynine/ET ratio, median (IQR)	0.011 (0.015)	0.012 (0.017)	0.017 (0.018)[d]	0.005

CIND = cognitive impairment no dementia, NCI = no cognitive impairment, BMI = body mass index, no. = number of cases, SD = standard deviation, IQR = interquartile range.
BMI was missing for 2 CIND and 6 dementia patients, hypertension data were was missing for 1 CIND and 2 dementia patients, cardiovascular disease data were missing for 1 NCI and 1 dementia patient.
Bold texts indicate statistically significant p values for tests by one-way ANOVA (age and BMI); Chi-Square (gender, education level, APOE status, hypertension, diabetes, cardiovascular diseases, hyperlipidaemia); Kruskal-Wallis H test (ergothioneine, L-hercynine and L-hercynine/ergothioneine ratio).
[a] Significantly different from NCI tested by one-way ANOVA with *post-hoc* Bonferroni tests (p < 0.05).
[b] Significantly different from NCI tested by Chi-Square test (p < 0.05).
[c] Significantly different from CIND tested by Chi-Square test (p < 0.05).
[d] Significantly different from NCI tested by Kruskal-Wallis H test with *post-hoc* Dunn's test (p < 0.05).
[e] Significantly different from CIND tested by Kruskal-Wallis H test with *post-hoc* Dunn's test (p < 0.05).

was significantly different amongst the clinical subgroups, the observed age range is not known to be associated with significant differences in ET levels [18].

3.2. Plasma ergothioneine (ET) and L-hercynine in a cohort of AD and VCI subjects

Compared to NCI controls, there was a significant decrease in plasma ET concentrations in CIND patients (p < 0.001 vs. NCI), and a further decline in dementia (p < 0.001 vs. CIND and NCI) (Fig. 1A). Within the dementia group, there was no significant difference between AD and VaD (AD, median [IQR]: 577 [638]; VaD, median [IQR]: 615 [669]; p = 0.879). There were significant reductions of plasma L-hercynine, a putative oxidation product of ET, in CIND and dementia compared to NCI controls (p < 0.01 for both, Fig. 1B). However, there was no significant difference in L-hercynine between the CIND and dementia groups. As expected, there was a significant positive correlation between ET and L-hercynine in all three diagnostic groups (Spearman's rho = 0.419, 0.395, and 0.562 for NCI, CIND and dementia respectively; p < 0.001 for all). The L-hercynine to ET ratio is thought to be an indicator of ET oxidation rate, and this ratio was significantly increased in the dementia group compared to NCI (p < 0.01, Fig. 1C), suggesting a higher rate or extent of ET oxidation in dementia. No differences in the L-hercynine/ET ratio were observed between NCI and CIND or between CIND and dementia.

Investigating the associations between plasma ET concentrations and demographic characteristics and vascular risk factors showed that age had a weak, negative correlation with ET levels (Spearman correlation coefficient rho = −0.174, p < 0.001). Lower ET levels were also found in male participants, patients with lower educational levels and in APOE ε4 carriers (p < 0.001, p = 0.001, p = 0.002, respectively, Mann-Whitney U tests). Plasma ET levels were significantly lower in patients with hypertension, diabetes or cardiovascular diseases (p = 0.006, p = 0.019, p < 0.001 respectively, Mann-Whitney U tests), but not with hyperlipidaemia. L-Hercynine/ET ratio was higher in male participants (p = 0.001) and significantly increased in those with hypertension, diabetes or cardiovascular diseases (p < 0.05 for all). L-Hercynine was decreased in patients with lower educational levels (p = 0.019) but not associated with other demographic characteristics or vascular risk factors. The significant associations of several demographic and disease factors with plasma biomarkers warranted the inclusion of these factors as covariates in subsequent multivariate analyses, which found lower levels of ET significantly associated with CIND (Odds ratio, OR: 0.20; 95% CI: 0.07–0.56; p = 0.002) and dementia (OR: 0.06; 95% CI: 0.02–0.20; p < 0.001) after adjustment for covariates (see Table 2). In contrast, lower L-hercynine concentrations were only associated with dementia (OR: 0.29; 95% CI: 0.13–0.69; p = 0.005) but not with CIND (OR: 0.59; 95% CI: 0.27–1.30; p = 0.191), while no significant association was observed in L-hercynine/ET ratio with CIND or dementia (Table 2, p = 0.215 and p = 0.114, respectively).

Model 1. Unadjusted.

Model 2: Adjusted for age, gender, education, APOE4, hypertension, diabetes, and cardiovascular diseases.

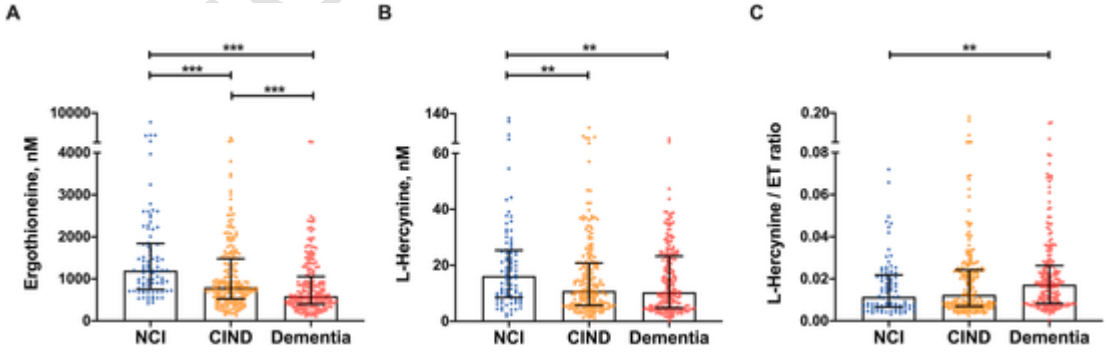

Fig. 1. Plasma ergothioneine (ET), L-hercynine, and L-hercynine/ET ratio in cognitive impairment and dementia. Data are presented separately for ergothioneine (A), L-hercynine (B), and L-hercynine/ET ratio (C). In the scatter plot the central horizontal line shows the median concentration, and the error bars show the 25th and 75th percentiles, respectively. p values of pairwise comparisons are indicated with asterisks: **p < 0.01, ***p < 0.001 using Kruskal-Wallis ANOVA with Dunn's *post hoc* tests.
CIND = cognitive impairment no dementia, NCI = no cognitive impairment.

Table 2
Associations between plasma biomarkers and diagnoses of CIND and dementia.

	CIND OR (95% CI) (n = 201)	Dementia OR (95% CI) (n = 207)
Ergothioneine*		
Model 1	0.13 (0.05–0.32)	0.03 (0.01–0.09)
Model 2	0.20 (0.07–0.56)	0.06 (0.02–0.20)
L-Hercynine*		
Model 1	0.39 (0.19–0.77)	0.03 (0.01–0.09)
Model 2	0.59 (0.27–1.30)	0.29 (0.13–0.69)
L-Hercynine/ET ratio*		
Model 1	1.55 (0.75–3.19)	2.96 (1.40–6.27)
Model 2	1.71 (0.73–4.00)	2.11 (0.84–5.34)

CIND = cognitive impairment no dementia, CI = confidence interval, OR = odds ratio.
* log transformed

Bold values represent statistically significant associations at $p < 0.05$.

Interpretation: Significant OR < 1 (bold text) indicates that each 10-fold (1 unit of $\log_{10}$ [plasma biomarker]) increase in plasma biomarker level was associated with (1-OR)*100% lower chance of getting the respective cognitive outcome. Significant OR > 1 (bold text) indicates that each 10-fold (1 unit of $\log_{10}$ [plasma biomarker]) increase in plasma biomarker level was associated with OR times more likely getting the respective cognitive outcome.

3.3. Associations between plasma biomarkers and CeVD

To explore potential associations between the measured plasma biomarkers and neuroimaging markers of CeVD, we first compared plasma biomarker values among participants stratified by clinical diagnoses and presence vs. absence of significant CeVD (Fig. 2). No significant difference is observed in plasma biomarkers levels among the NCI –CeVD vs. NCI + CeVD subgroups. Hence, we combined the two NCI subgroups in subsequent analyses. ET levels were significantly lower in the CIND subgroup only in the presence of CeVD, while decreased ET was detected in AD with or without concomitant CeVD, and also in VaD (Fig. 2). These results from pairwise comparisons were borne out in subsequent regression analyses. As shown in Table 3, after correcting for covariates, ET levels were significantly associated with all subgroups with significant CeVD, that is CIND + CeVD (OR: 0.09; 95% CI: 0.02–0.37; p = 0.001), AD + CeVD (OR: 0.09; 95% CI: 0.02–0.39; p = 0.001), and VaD (OR: 0.13; 95% CI: 0.02–0.88; p = 0.036). However, in the absence of significant CeVD, ET levels were only associated with AD (OR: 0.04; 95% CI: 0.01–0.19; p < 0.001), but not with CIND (OR: 0.41; 95% CI: 0.12–1.40; p = 0.149). In contrast, L-hercynine was not associated with CIND, AD or VaD subgroups, irrespective of CeVD status. For L-hercycine/ET ratio, associations with clinical subgroups were weak, and statistical significance was lost when adjusted for covariates (see Table 3 Model 2) except for the association with AD (OR: 4.81; 95% CI: 1.37–16.87; p = 0.014).

AD = Alzheimer's disease, CeVD = cerebrovascular disease CIND = cognitive impairment no dementia, NCI = no cognitive impairment, VaD = vascular dementia.

3.4. Associations between plasma ET and specific CeVD markers

Fig. 3 shows that plasma ET concentrations were significantly lower in subjects with cortical infarcts (p = 0.010), ≥2 lacunes (p = 0.034), and confluent WMH measured by ARWMC scores ≥8 (p < 0.001, Fig. 4A–C). Decreased L-hercynine concentrations were found in patients with confluent WMH (p = 0.023, Fig. 3D–F). No significant changes were observed for L-hercynine/ET ratio for any of the measured CeVD markers (p > 0.05 for all, Fig. 3G–I). In multivariate analyses, lower ET concentrations were significantly associated with significant WMH (Table 4A, OR: 0.46; 95% CI: 0.24–0.88; p = 0.018) and higher ARWMC scores (Fig. 4 and Table 4B, Mean Difference β: −1.48; 95% CI: −2.66 to −0.29; p = 0.015). In contrast, significant associations between L-hercynine or L-hercynine/ET ratio and any of the CeVD markers were lost after adjustment for covariates (Model 2, Tables 4A and 4B).

WMH = white matter hyperintensities, ARWMC = age-related white matter changes [Wahlund et al. ref [43]].

NCI = no cognitive impairment, CIND = cognitive impairment no dementia, ARWMC = age-related white matter changes [Wahlund et al. ref [43]].

3.5. Associations between plasma biomarkers and neurodegeneration

Plasma ET and L-hercynine levels were positively correlated with both cortical thickness (p < 0.001 and p = 0.002, respectively) and hippocampal volume (p < 0.001 for both), as shown in Fig. 5. L-Hercynine/ET ratio was negatively correlated with cortical thickness (p = 0.007) but not with hippocampal volume (p = 0.896). ET continued to show strong associations with global cortical thickness (β: 0.08; 95% CI: 0.03–0.13; p < 0.001) and hippocampal volume (β: 0.61; 95% CI: 0.28–0.95; p < 0.001) even after adjustments for demographics, vascular risk factors, cognitive status and intracranial volume (Model 2, Table 5). Since WMH is currently also regarded as a risk factor for brain

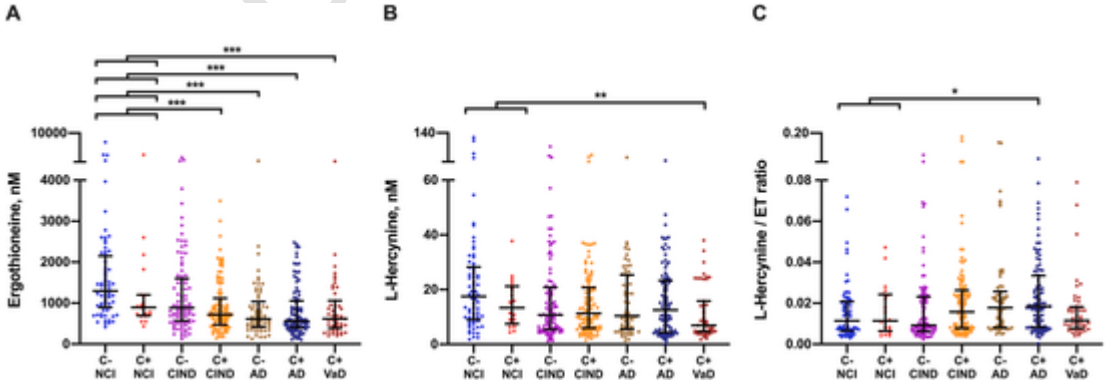

Fig. 2. Plasma ergothioneine (ET), L-hercynine, and L-hercynine/ET ratio in dementia and cognitive impairment with concomitant CeVD. Data are presented separately for ergothioneine (A), L-hercynine (B), and L-hercynine/ET ratio (C). In the scatter plots the central horizontal lines show the median concentrations, and the error bars show the 25th and 75th percentiles, respectively. *p < 0.05, **p < 0.01, ***p < 0.001 indicate significant pairwise comparisons using Kruskal-Wallis ANOVA with Dunn's *post hoc* tests. Absence and presence of significant CeVD indicated as "C-" and "C+" respectively.

Table 3
Associations between plasma biomarkers and concomitant CeVD in dementia and VCI.

	Significant CeVD				
	Absence		Presence		
	CIND OR (95% CI) (n = 96)	AD OR (95% CI) (n = 61)	CIND OR (95% CI) (n = 105)	AD OR (95% CI) (n = 99)	VaD OR (95% CI) (n = 47)
Ergothioneine*					
Model 1	0.24 (0.09–0.67)	0.02 (0.01–0.10)	0.05 (0.02–0.16)	0.03 (0.01–0.09)	0.02 (0.01–0.11)
Model 2	0.41 (0.12–1.40)	0.04 (0.01–0.19)	0.09 (0.02–0.37)	0.09 (0.02–0.39)	0.13 (0.02–0.88)†
L-Hercynine*					
Model 1	0.40 (0.18–0.88)	0.38 (0.15–0.96)	0.36 (0.16–0.82)	0.37 (0.17–0.80)	0.13 (0.04–0.39)
Model 2	0.60 (0.24–1.50)	0.55 (0.18–1.70)	0.68 (0.26–1.82)	0.65 (0.22–1.94)	0.25 (0.06–1.00)†
L-Hercynine/ET ratio*					
Model 1	0.97 (0.42–2.23)	4.33 (1.57–11.95)	2.46 (1.06–5.72)	3.33 (1.43–7.78)	1.40 (0.46–4.28)
Model 2	0.96 (0.34–2.68)	4.81 (1.37–16.87)	2.79 (0.99–7.84)	2.44 (0.72–8.28)	0.72 (0.15–3.40)†

AD = Alzheimer's disease, CeVD = cerebrovascular disease, CI = confidence interval, CIND = cognitive impairment no dementia, OR = odds ratio, VaD = vascular dementia.
Model 1: Unadjusted.
Model 2: Adjusted for age, gender, education, APOE4, hypertension, diabetes, and cardiovascular diseases.
* Log transformed.
† Corrected using Firth's bias-reduced logistic regression due to separation where hypertension perfectly predicts VaD.
Bold values represent statistically significant associations at $p < 0.05$.
Interpretation: Significant OR < 1 (bold text) indicates that each 10-fold (1 unit of $\log_{10}$ [plasma biomarker]) increase in plasma biomarker level was associated with (1-OR)*100% lower chance of getting the respective cognitive outcome. Significant OR > 1 (bold text) indicates that each 10-fold (1 unit of $\log_{10}$ [plasma biomarker]) increase in plasma biomarker level was associated with OR times more likely getting the respective cognitive outcome.

atrophy [47], we extended the multivariable model with additional adjustments for WMH. The results showed that significant associations exist between ET and atrophy markers independent of WMH (Model 3, Table 5, $p = 0.004$ and $p < 0.001$ for global cortical thickness and hippocampal volume, respectively). Furthermore, L-hercynine showed a positive association with hippocampal volume (β: 0.35; 95% CI: 0.10–0.60; $p = 0.007$) but not with global cortical thickness (β: 0.03; 95% CI: −0.01 – 0.07; $p = 0.115$), while L-hercynine/ET ratio was not associated with either quantitative atrophy marker (Table 5).

4. Discussion

The present study showed that decreased plasma ET levels were associated with dementia and VCI independent of age, sex and cardiovascular risk factors. These results are in line with a much smaller previous study which found decreased plasma ET concentrations in patients with MCI [18], and extend these earlier findings to show that ET is further reduced in demented patients compared to the prodromal stages of dementia. The incremental deficits of plasma ET along the clinical continuum suggest that ET may be associated with disease severity and is a potential peripheral biomarker for early detection of prodromal dementia. Furthermore, this is the first study investigating associations between plasma ET and the clinico-pathological features of neurodegeneration and CeVD, two of the major causes of cognitive impairment, and showed that low plasma ET levels strongly associate with neuroimaging markers of brain atrophy and WMH.

Oxidative stress is considered a central mechanism underlying the pathogenesis of dementia and is associated with both neurodegenerative and cerebrovascular changes that lead to loss of cognitive function. Modulation of oxidative stress levels and its related risk factors remains a therapeutically useful but challenging aim in dementia [12,14,48,49]. Recent evidence suggests that ET is one such compound that can offer antioxidant protection and other cytoprotective mechanisms against deleterious effects of oxidative stress [20,27]. ET exists in a wide variety of foods but is especially abundant in mushrooms which are capable of biosynthesizing this compound [21]. ET manifests a natural antioxidant property in that it reacts rapidly with a range of free radicals as well as chelates transition metal ions [22,50]. By virtue of its unique tautomeric structure and physiological predominance towards the thione form, ET is more stable and has a slower turnover compared to other antioxidants [21]. Moreover, in 2005 the specific ET transporter, ETT was found to mediate the avid uptake of ET by cells and the accumulation of ET in tissues [24]. ETT is also expressed in the brain [20,26], which allows ET to enter and accumulate in the brain [51–53]. The presence of a specific transport system suggests important physiological roles for ET, particularly in the brain. Indeed, numerous *in vitro* and *in vivo* studies have established the cytoprotective properties of ET against oxidative stressors and toxins through antioxidant and other mechanisms as previously detailed [19,20,22].

However, thus far, very few studies have investigated the association between ET and dementia, and it is at present unclear whether ET is linked to the core pathological features of AD, VaD or other dementia disorders. CeVD is considered as the main cause of vascular-related cognitive impairment and is observed to be highly prevalent in AD as well as in non-demented elderly [7,9,54]. Increased oxidative stress and altered antioxidant status have previously been found to be associated with CeVD [10,55,56]. The present data support this hypothesis by showing significant associations between decreased ET levels and white matter lesions (WML, as indicated by WMH neuroimaging features). Although the etiology of WML is not fully understood, it is often presumed to be of an ischemic or demyelinating origin, and appears to be associated with increased risk of stroke, dementia as well as being a predictor of accelerated cognitive decline [57,58]. The postulated mechanisms underlying WML include blood-brain barrier disruption, hypoperfusion, inflammation and other processes, all of which are closely related to increased vascular oxidative stress [59–61]. Evidence from *in vitro* studies suggested that ergothioneine may prevent neurovascular diseases by protecting the integrity and function of brain vascular endothelium [62,63]. Decreased ET in patients with significant WMH supports the role of a deregulated antioxidant defense system in the pathophysiological mechanisms leading to WML. Several animal studies have also reported a protective role of ET in cerebrovascular endothelia, with ameliorating effects against several vascular risk factors, including hypertensive disorders [64], diabetes [62] and ischemia/reperfusion-induced cardiovascular injury [65]. Furthermore, a longitudinal association between higher ET and lower risk of cardiovascular disease and mortality has been demonstrated in a cohort of 3236 participants in Sweden with a median follow-up time of 21 years [33]. These findings thus support a potential role for declining ET levels as a predictor for, and possibly a mediator of, peripheral vascular diseases. The association between ET and WMH remained robust even after controlling for several vascular risk factors, suggesting that ET could also be an independent biomarker for specific CeVD markers like WMH. Notably, these associations were not found between ET and infarcts or lacunes, suggesting the presence of a mechanistic link between ET and WMH which remains to be elucidated.

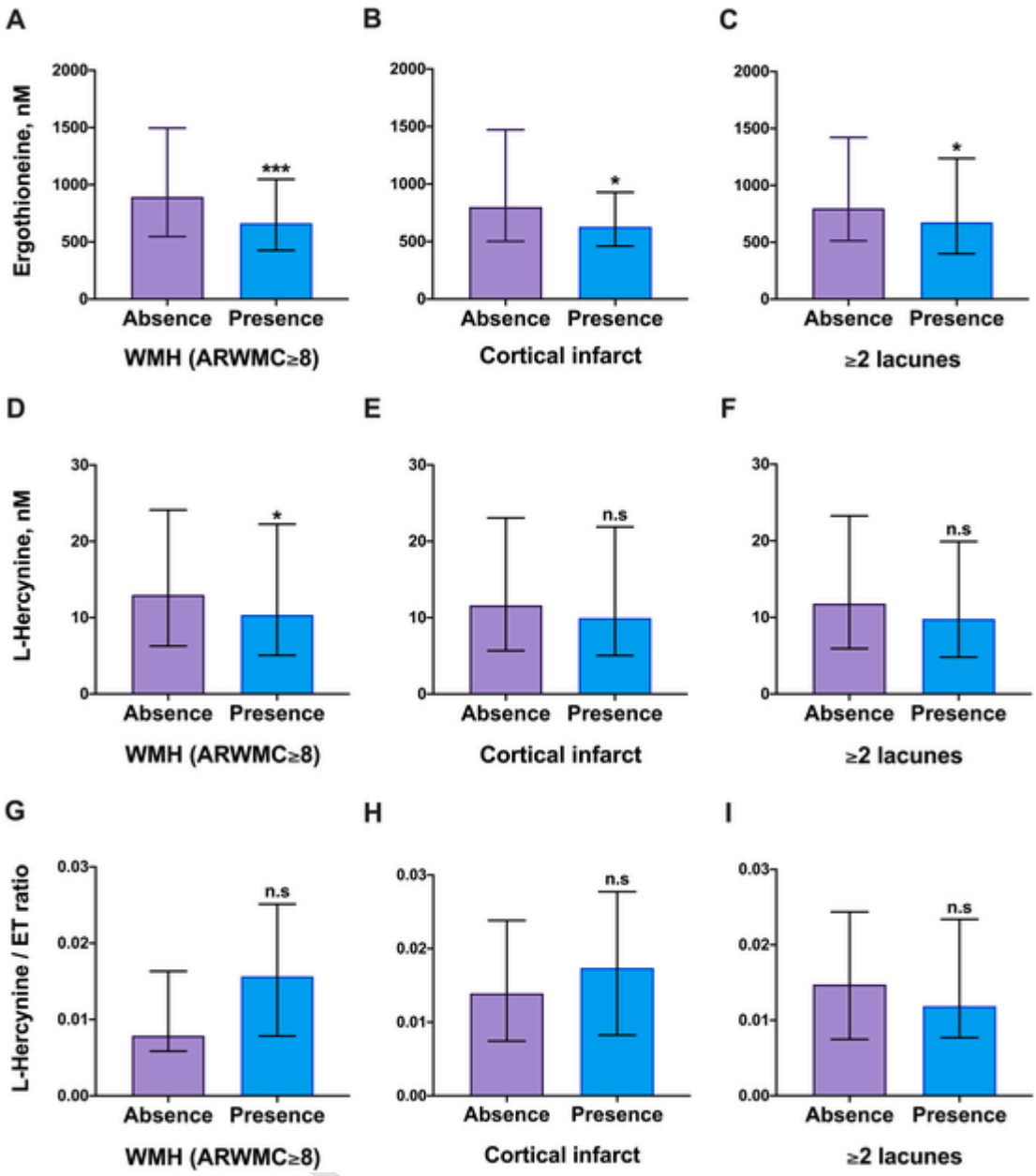

Fig. 3. Plasma ergothioneine, L-hercynine and L-hercynine/ET ratio in a cohort with dementia and VCI stratified by presence of specific CeVD markers. Cut-offs of counts/scores for definitions of significant CeVD have been previously described [45]. Bar graphs show medians and interquartile ranges (IQR). Mann-Whitney U tests were performed, with p values indicated with asterisks: *$p < 0.05$, ***$p < 0.001$, n.s: not significant.

Besides CeVD, ET may also have a potential role in neurodegeneration, the leading pathophysiological basis of dementias like AD. A wide range of *in vitro* and *in vivo* studies have demonstrated the neuroprotective potential of ET, probably achieved through the inhibition of neuroinflammation, oxidative stress, apoptosis and mitochondrial dysfunction amongst other mechanisms. It has been shown in several animal models that oral administration of ET protected against neuronal dysfunction and cognitive impairment induced by various neurotoxicants including β-amyloid peptides [32], cisplatin [30] and D-galactose [31]. In humans, lower blood levels of ET have been found in patients with other neurodegenerative disorders, such as PD [34] or mild cognitive impairment [18]. However, to the best of our knowledge, this study is the first to investigate associations between ET and the severity of neurodegeneration. The hippocampus and cortex are the brain regions where atrophy is most prominent in dementia patients [66–68]. In the present work, we show that lower ET levels were significantly associated with cortical thinning and decreased hippocampal volumes. These results provide further support for the hypothesis that declining peripheral ET levels may be associated with severity of neurodegeneration. The finding that ET changes are significant in CIND with CeVD (but not in CIND without CeVD), and also in AD with or without CeVD as well as in VaD, suggest that both neurodegenerative and cerebrovascular diseases contribute to ET depletion in an additive manner, but at severe disease stages (AD or VaD), this additive effect is no longer observed, suggestive of a floor effect for ET levels. Taken together, the above observations suggest that decreased plasma ET levels found in our patients with cognitive impairment and dementia may result from AD-related neurodegenerative or vascular mechanisms, or both. Indeed, ET reduction may reflect a depleted antioxidant capacity to counteract increased oxidative stress-associated cellular and tissue injury found in both AD and CeVD, and the presence of both neurodegenerative and cerebrovascular disease processes exacerbates these deficits. This postulate should

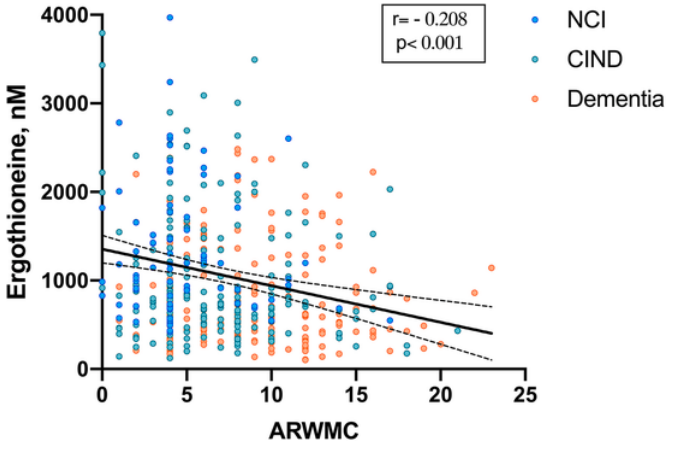

Fig. 4. Correlation between plasma ergothioneine and white matter hyperintensities. Scatter plot of plasma ergothioneine concentrations against ARWMC scores. Solid line indicates linear regressed best-fit curve while dashed lines indicate 95% confidence intervals. r = Spearman correlation coefficient (r) with associated two-tailed p value.

be further investigated by looking into associations of plasma ET with other peripheral oxidative stress biomarkers known to be linked to CeVD and neurodegeneration.

L-Hercynine is often regarded as a major oxidation product of ET. A significant increase in L-hercynine/ET ratio was observed in patients with dementia, consistent with some depletion of ET being related to oxidative stress. However, this association was not seen in dementia patients with CeVD, suggesting a complex relationship between ET oxidation and mixed pathologies in dementia. There were no significant associations between L-hercynine or L-hercynine/ET ratio with MRI markers of CeVD and atrophy. Other mechanisms which account for the early decrease of plasma ET in CIND and in WMH as well as neurodegeneration should be explored in future work.

There are several limitations in the present study. First, the effects of other potential confounders, such as differences in dietary intake of ET and the activity of its transporter (ETT), on decreased ET levels observed in patients with CIND or dementia are unclear, although an earlier study showed that the decrease in blood ET in MCI patients was unlikely to be due to the variation in ET intake from mushrooms, the major source of dietary ET [18]. Besides, in the current study there were no significant differences in BMI, a nutritional status indicator, in patients with CIND or dementia compared to NCI controls, suggesting comparable calorie intake amongst different diagnostic groups. Indeed, the potential links between long term habitual diet lacking in ET and cognitive impairment or dementia seem very important to explore, e.g., would supplementation of the diet with ET, even if given at the CIND/MCI stages, ameliorate disease features or change the course of disease? These questions will need to be addressed through longitudinal studies and clinical trials.

The adsorption and accumulation of ET in human tissues, including the brain, is also mediated by the transport efficacy of ETT [26,53,69], which might be impaired under disease conditions. Further study will be needed to determine if there are variable expression or functional activity of the ETT as well as differences in long term diet patterns between or within groups. Furthermore, ET levels in the brain were not examined and plasma ET levels may not necessarily reflect brain concentrations. Although studies in mice have revealed that ET levels in the brain change in a similar manner to blood values [52], this has not been validated in humans. Moreover, since the present study utilized a cross-sectional design, we were unable to investigate the temporal relationship between ET and the progression of WMH and brain atrophy. Lastly, the associations between ET and other important pathological hallmarks of AD like Aβ or tau burden in the brain have not been deter-

Table 4
Associations of plasms biomarkers with specific CeVD neuroimaging markers.

A: CeVD binary outcome variables using binary logistic regression			
	WMH (ARWMC≥8) OR (95% CI) (n = 217)	Presence of cortical infarct OR (95% CI) (n = 70)	Presence of ≥2 lacunes OR (95% CI) (n = 75)
Ergothioneine*			
Model 1	0.28 (0.15–0.49)	0.37 (0.17–0.82)	0.44 (0.20–0.96)
Model 2	0.46 (0.24–0.88)	0.83 (0.33–2.12)	1.12 (0.46–2.74)
L-Hercynine*			
Model 1	0.56 (0.35–0.88)	0.82 (0.43–1.55)	0.57 (0.31–1.06)
Model 2	0.67 (0.40–1.10)	0.97 (0.49–1.92)	0.66 (0.34–1.28)
L-Hercynine/ET ratio*			
Model 1	1.37 (0.83–2.26)	1.75 (0.87–3.55)	0.97 (0.49–1.92)
Model 2	1.08 (0.63–1.85)	1.09 (0.51–2.34)	0.56 (0.27–1.17)
B: CeVD continuous outcome variables using Poisson or linear regressions			
	WMH by ARWMC scores β (95% CI)	Number of cortical infarcts RR (95% CI)	Number of lacunes RR (95% CI)
Ergothioneine*			
Model 1	−2.79 (−3.93–−1.66)	0.42 (0.23–0.76)	0.32 (0.20–0.52)
Model 2	−1.48 (−2.66–−0.29)	0.89 (0.47–1.69)	0.64 (0.37–1.09)
L-Hercynine*			
Model 1	−0.83 (−1.79–0.12)	0.96 (0.59–1.56)	0.65 (0.44–0.96)
Model 2	−0.64 (−1.57–0.29)	1.11 (0.69–1.79)	0.74 (0.48–1.12)
L-Hercynine/ET ratio*			
Model 1	1.28 (0.24–2.33)	1.93 (1.14–3.25)	1.44 (0.96–2.16)
Model 2	0.78 (−0.25–1.81)	1.24 (0.73–2.13)	0.97 (0.62–1.50)

ARWMC = age-related white matter changes, CeVD = cerebrovascular disease, CI = confidence interval, MRI = magnetic resonance imaging, OR = odds ratio, WMH = white matter hyperintensities, RR = rate ratio, β = mean difference. WMH was missing for 1 CIND patient.
¶ Cut-offs of counts/scores for definitions of significant CeVD have been previously described [45].
∗Log transformed.
Model 1: Unadjusted.
Model 2: Adjusted for age, gender, hypertension, diabetes, cardiovascular diseases and cognitive status.
Bold values represent statistically significant associations at $p < 0.05$.
Interpretation (A): Significant OR < 1 (bold text) indicates that each 10-fold (1 unit of $\log_{10}$ [plasma biomarker]) increase in plasma biomarker level was associated with (1-OR)*100% lower chance (OR% higher chance when OR > 1) of getting the respective cognitive outcome.
Interpretation (B): 1. Linear regression models with mean differences (β) and 95% CI were used for the ARWMC visual scores for WMH grading: Significant β (bold text) < 0 indicates that for each 10-fold (1 unit of $\log_{10}$ [plasma biomarker] increment) increase in plasma biomarker level the outcome variable will decrease (increase when β > 0) by β value. 2. Poisson regression models and negative binomial regression models with rate ratios (RR) and 95% CI were constructed for the count of cortical and lacunar infarct, respectively: Significant RR (bold text) indicates that a patient with each 10-fold (1 unit of $\log_{10}$ [plasma biomarker]) increase in plasma biomarker level will have on average RR times as many cortical infarcts or lacunes on MRI compared to a person with lower plasma biomarker levels.

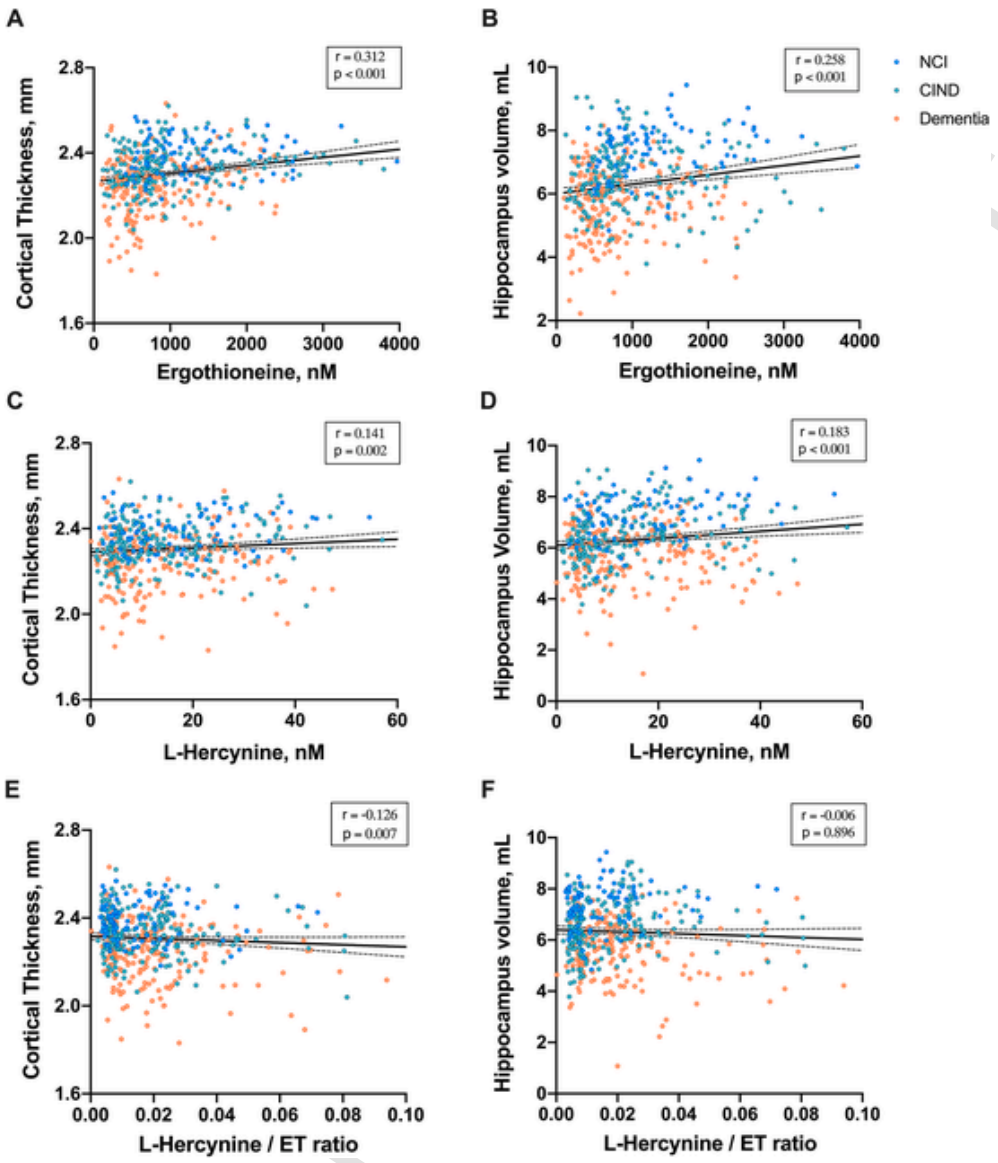

Fig. 5. Correlations between plasma ergothioneine, L-hercynine, L-hercynine/ET ratio with cortical thickness and hippocampal volume. Scatter plots of plasma biomarkers against cortical thickness (A,C,E) and hippocampal volumes (B,D,F). Solid lines indicate linear regressed best-fit curves while dashed lines indicate respective 95% confidence intervals. r = Spearman correlation coefficients with associated two-tailed p values.

mined, although numerous studies have shown that ET may be protective against Aβ [32,70] and hence declining ET levels may further accelerate disease pathology. Further studies are therefore needed to investigate the neuropathological correlates of ET alterations.

5. Conclusions

Our findings suggest that low plasma ET levels might be an early biomarker for WMH- or neurodegeneration-related cognitive impairment and dementia. The deficiency of ET may have deleterious effects on cognition through diminished antioxidant protection of vasculature and/or neurons in the brain, although multiple other mechanisms of cytoprotection could be involved [20,71]. Given that blood ET may be elevated via dietary supplementation, this highlights a potential therapeutic agent for cognitive impairment.

Author contributions

LYW and MKPL wrote the main manuscript text. IKC and JYT performed the mass spectrometry assays. SH, HV and CPC provided neuroimaging and clinical data. LYW, IKC, JRC, YLC, SH and HV analyzed the data. LYW prepared the figures. CPC, BH and MKPL contributed to the design and implementation of the experiments. All authors reviewed and revised the manuscript. All authors approved of the manuscript text.

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Declaration of competing interest

All authors declare no conflicts of interest related to the work presented in this manuscript.

Table 5
Associations of plasma biomarkers with quantitative atrophy neuroimaging markers.

	Global cortical thickness		Hippocampal volume	
	β (95% CI)	p	β (95% CI)	p
Ergothioneine*				
Model 1	0.13 (0.09–2.05)	<0.001	1.01 (0.66–1.35)	<0.001
Model 2	0.08 (0.03–0.13)	<0.001	0.61 (0.28–0.95)	<0.001
Model 3	0.07 (0.02–0.12)	0.004	0.61 (0.27–0.94)	<0.001
L-Hercynine*				
Model 1	0.05 (0.01–0.09)	0.008	0.62 (0.33–0.91)	<0.001
Model 2	0.03 (−0.01–0.07)	0.115	0.35 (0.10–0.60)	0.007
Model 3	0.03 (−0.01–0.07)	0.150	0.35 (0.10–0.60)	0.007
L-Hercynine/ET ratio*				
Model 1	−0.04 (−0.08–0.00)	0.056	−0.06 (−0.38–0.26)	0.707
Model 2	−0.02 (−0.06–0.02)	0.407	0.00 (−0.27–0.27)	0.999
Model 3	−0.02 (−0.06–0.03)	0.439	0.00 (−0.28–0.27)	0.996

β = mean difference, CI = confidence interval. Hippocampus volume and global cortical thickness were available for 465 participants.
∗ Log transformed.
Model 1: Unadjusted.
Model 2: Adjust for age, gender, education, APOE, hypertension, diabetes, cardiovascular diseases, intracranial volume and cognitive status.
Model 3: Further adjusting for WMH.
Interpretation: Linear regression models with mean differences (β) and 95% CI were used for global cortical thickness and hippocampus volume: Significant β (bold text) > 0 indicates that for each 10-fold (1 unit of $\log_{10}$ [plasma biomarker]) increase in plasma biomarker level the outcome variable will increase by β value.

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Abbreviations

AD Alzheimer's disease
ANOVA Analyses of variance
APOE Apolipoprotein E
ARWMC Age-Related White Matter Changes scale
β Mean differences
BMI Body mass index
CeVD Cerebrovascular disease
CI Confidence interval
CIND Cognitive impairment no dementia
CSF Cerebrospinal fluid
DSM-IV Diagnostic and Statistical Manual of Mental Disorders, 4 th edition
EDTA Ethylenediaminetetraacetic acid
ET Ergothioneine
ETT Ergothioneine transporter
FLAIR Fluid Attenuated Inversion Recovery
HbA1c haemoglobin A1c
IQR Interquartile range
LC-MS/MS Liquid chromatography-tandem mass spectrometry
MCI Mild cognitive impairment
MMSE Mini-Mental State Examination
MRI Magnetic resonance imaging
NCI No cognitive impairment
NINCDS-ADRDA National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association
NINDS-AIREN National Institute of Neurological Disorders and Stroke-Association Internationale pour la Recherché et l' Enseignement en Neuroscience
OCTN1 Organic cation transporter novel-type 1
OR Odds ratio

PD Parkinson's disease
RR Rate ratio
SD Standard deviation
STRIVE Standards for Reporting Vascular Changes on Neuroimaging
VaD Vascular dementia
VCI Vascular cognitive impairment
WMH White matter hyperintensities
WML White matter lesions

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2021.10.019.

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