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Circulating klotho is higher in cerebrospinal fluid than serum and elevated among *KLOTHO* heterozygotes in a cohort with risk for Alzheimer's disease

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Abstract

Background: Klotho is a longevity and neuroprotective hormone encoded by the *KLOTHO* gene, and heterozygosity for the KL-VS variant confers a protective effect against neurodegenerative disease.

Objective: Test whether klotho concentrations in serum or CSF vary as a function of *KLOTHO* KL-VS genotype, determine whether circulating klotho concentrations from serum and CSF differ from one another, and evaluate whether klotho levels are associated with Alzheimer's disease risk factors.

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Conflict of Interest/Disclosure Statement

The authors have no conflict of interest to report.

Methods: Circulating klotho was measured in serum (n = 1116) and cerebrospinal fluid (CSF; n = 183) of cognitively intact participants (aged 62.4 ± 6.5 years; 69.5% female). *KLOTHO* KL-VS zygosity (non-carrier; heterozygote; homozygote) was also determined. Linear regression was used to test whether klotho hormone concentration varied as a function of KL-VS genotype, specimen source, and demographic and clinical characteristics.

Results: Serum and CSF klotho were higher in KL-VS carriers than non-carriers. Klotho concentration was higher in CSF than in serum. Females had higher serum and CSF klotho, while younger age was associated with higher klotho in CSF.

Conclusion: In a cohort enriched for risk for Alzheimer's disease, heterozygotic and homozygotic carriers of the KL-VS allele, females, and younger individuals have higher circulating klotho. Fluid source, KL-VS genotype, age, and sex should be considered in analyses of circulating klotho on brain health.

Keywords

Klotho; KL-VS; serum; cerebrospinal fluid; Alzheimer's disease

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease [1]. Its etiology and potential avenues for its disruption are multifactorial. Factors that confer resilience to AD (i.e., delayed symptom onset despite presence of pathology) may contribute to stem the rising burden of the disease. The *KLOTHO* gene is one such genetic factor. Heterozygosity for the functional *KLOTHO* haplotype KL-VS [2] confers a protective effect against AD pathology [3-6], cognitive decline [6,7], and conversion to MCI or AD among apolipoprotein ε4 (*APOE* ε4) carriers [8].

KLOTHO encodes the klotho hormone, which is produced in the kidney and brain and circulates throughout the body following cleavage from its transmembrane form. Circulating klotho in blood [9-13] and cerebrospinal fluid (CSF) [14-16] is inversely associated with age, circulating inflammatory factors, deficit following neuronal injury, cerebrovascular disease, and neurological disease. Elevating klotho also enhances cognition in mice [17-21] and associates with better cognition in humans [13,18,22-24]. Although KL-VS alters the klotho protein [2], few studies have reported the association between *KLOTHO* genotype and circulating klotho *in vivo* [12,18] and none have reported the effect of KL-VS on CSF klotho. Given associations between KL-VS carriage and outcomes related to AD, elucidating the effects of *KLOTHO* genotype on circulating concentrations would move the field toward understanding how the KL-VS mutation exerts its influence. Sex, age, the *APOE* ε4 allele, and cardiovascular disease also influence risk for AD [25,26] and may interact with circulating klotho.

This study measured circulating blood and CSF klotho concentrations and KL-VS genotype in a sample at risk for AD. The aims were to 1) test whether klotho concentrations in serum or CSF vary as a function of KL-VS genotype, 2) determine whether circulating klotho concentrations from serum and CSF differ from one another, and 3) evaluate whether klotho

levels vary as a function of AD risk factors including age, sex, *APOE* ε4, and cardiovascular disease risk profile.

2. Materials and methods

2.1 Participants

One thousand and thirty participants from the Wisconsin Registry for Alzheimer's Prevention (WRAP) and Wisconsin Alzheimer's Disease Research Center Investigating Memory in People At risk, Causes and Treatments (WADRC IMPACT) cohorts were included in this study. These cohorts are enriched for risk for AD by family history and the *APOE* ε4 allele [27,28]. Participants were characterized as cognitively normal by standardized and multidisciplinary consensus conferences on the basis of performance on a comprehensive neuropsychological test battery, absence of functional impairment, and absence of neurologic or psychiatric conditions that would impair cognition [27]. Participants were selected for the present analyses on the basis of having undergone genotyping for *KLOTHO* KL-VS and a venipuncture or lumbar puncture for sampling of serum or CSF klotho. This yielded samples of 1116 participants with KL-VS genotype and serum klotho data, 183 participants with KL-VS genotype and CSF klotho data, and 169 with both serum and CSF klotho data. The University of Wisconsin Institutional Review Board approved all study procedures, and each participant provided written informed consent prior to participating.

2.2 Genotyping

DNA was extracted from blood using the PUREGENE DNA Isolation Kit (Gentra Systems, Inc, Minneapolis, MN). DNA concentrations were quantified using ultraviolet spectrophotometry (DU 530 Spectrophotometer, Beckman Coulter, Fullerton, CA). Single nucleotide polymorphisms for *APOE* (rs429358 and rs7412) and *KLOTHO* (rs9536314 for F35V and rs9527025 for C370S) were genotyped by LGC Genomics (Beverly, MA) by competitive allele-specific PCR-based KASP genotyping assays. Quality control procedures have been previously published [5,29] and were deemed satisfactory. As expected based on HapMap and existing literature [2,18], *KLOTHO* rs9536314 and rs9527025 were in perfect linkage disequilibrium. *APOE* ε4 status was classified as carrying at least one copy of the *APOE* ε4 allele (i.e., *APOE* ε4 non-carrier or *APOE* ε4 carrier). KL-VS genotype was classified as KL-VS non-carrier ($KL\text{-}VS_{NC}$), heterozygote ($KL\text{-}VS_{HET}$), or homozygote ($KL\text{-}VS_{HOM}$) based on variants in the *KLOTHO* single nucleotide polymorphisms specified above.

2.3 Specimen collection

2.3.1 Serum—Venipuncture was conducted the morning after a 12-hour fast from food, caffeine, and alcohol. Blood was collected into a 9mL vacutainer containing no anticoagulant or additive. The sample was allowed to clot for no more than 30 minutes and was then centrifuged at 3000rpm for 10 minutes at 4°C. Serum was then aliquoted into cryovials and stored at −80°C and was not thawed until assays were run.

2.3.2 Cerebrospinal fluid—Lumbar puncture was performed the morning after a 12-hour fast from food, caffeine, and alcohol. CSF samples that were drawn at the time of blood collection were not available for all participants; the mean time between CSF and blood collection was 0.39 ± 1.91 years. A Sprotte 24- or 25- gauge spinal needle was inserted into the L3-4 or L4-5 vertebral space. 22mL of CSF was collected by gentle extraction into polypropylene syringes. Within 30 minutes of collection, CSF was combined, gently mixed, and centrifuged at 2000g for 10 minutes. Supernatants were transferred in 0.5mL aliquots to polypropylene tubes and stored at −80°C.

2.4 Assays

Soluble α-klotho was measured using a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA; Immuno-Biological Laboratories, Takasaki, Japan) [30] as described [18,31]. In the original report of the ELISA, measured klotho was 92.6% of the expected value [30]. Briefly, serum or CSF was diluted four-fold with the immunoassay buffer, a standard curve was created by serial dilution of recombinant human α-klotho protein, and diluted serum or CSF was loaded in duplicate onto a plate pre-coated with affinity-purified anti-human klotho (67G3) mouse IgG monoclonal antibody. Control samples were included as references for each plate to enable accurate interplate comparisons. Following incubation for 1 hour at room temperature, the plates were washed five times with washing buffer, horseradish peroxidase-conjugated anti-human klotho (100 μl, 91F1) mouse IgG monoclonal antibody was added, plates were incubated for 30 min at room temperature, and then plates were washed five times. The reaction was visualized by addition of 100 μl of chromogenic substrate for 30 min at room temperature and then stopped with 100 μl of 1 n H_2SO_4. The absorbance at 450 nm was measured on a Spectramax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA), and α-klotho levels were calculated using the SoftMax Pro software (Molecular Devices). Samples with coefficient of variation above 10% were re-run. Final assay values had an average coefficient of variation of 2.8% for serum and 1.8% for CSF.

2.5 Clinical assessment

Atherosclerotic cardiovascular disease (ASCVD) 10-year risk was defined according to updated versions of the American College of Cardiology/American Heart Association equations for estimating risk of myocardial infarction, stroke, or coronary heart disease death [32] and calculated using the ‘PooledCohort’ package in R. The equations take age, race, sex, systolic blood pressure, smoking status (yes/no), total cholesterol, high-density lipoprotein (HDL) cholesterol, anti-hypertensive medication (yes/no), and diabetes status (yes/no) as inputs. These data were obtained at study visits for the WRAP or WADRC IMPACT cohorts. To verify that the klotho measurements (especially in serum) were not affected by underlying kidney dysfunction, estimated glomerular filtration rate (eGFR) was calculated from the Chronic Kidney Disease Epidemiology Collaboration equation [33] using the ‘nephro’ package in R. The equation utilized serum creatinine concentration measured at WRAP or WADRC IMPACT study visit, age, race, and sex as inputs.

2.6 Statistical analysis

Statistical analyses were conducted in R version 4.1.0. Differences on background characteristics as a function of KL-VS genotype were evaluated using one-way ANOVA (continuous variables) or χ^2 tests (categorical variables). A series of general linear models were fit to ascertain the effect of KL-VS genotype on serum and CSF klotho concentration that included terms for KL-VS genotype (i.e., KL-VS$_{NC}$, KL-VS$_{HET}$, or KL-VS$_{HOM}$), age, and sex. Follow-up unadjusted pairwise comparisons were calculated to evaluate between-group differences. To test whether serum and CSF klotho concentrations differed from each other, a linear model was fit that included terms for specimen source (i.e., serum or CSF), age at the respective sample collections, and sex. These models were then stratified by KL-VS genotype and refit to test effects of specimen source on klotho concentration within each genotype. To additionally evaluate agreement between serum and CSF klotho concentrations, we fit Pearson's correlation and two-way mixed effects intraclass correlation with agreement. Next, a series of linear regressions were fit to ascertain the effect of sex, age, *APOE* ε4 genotype, and ASCVD risk. Each of these variables of interest was treated categorically (i.e., sex = male versus female, age = split at median of 62.9 years for serum or 64.7 years for CSF sample, *APOE* = ε4 non-carrier versus carrier, ASCVD = split at 7.5% 10-year risk i.e., the threshold at which statin therapy is recommended [34]) and models were adjusted for sex and age where appropriate. These models were then stratified by KL-VS genotype and refit to evaluate whether observed effects differed across KL-VS genotype. These stratified models were not conducted for KL-VS$_{HOM}$ when there were fewer than two cases in any cell defined by sex, age, *APOE* ε4, or ASCVD. A sensitivity analysis repeated the above regressions after excluding outliers, defined as any value laying outside 1.5 times the interquartile range in serum or CSF klotho, respectively. One-way ANOVA (continuous variables) and χ^2 tests (categorical variables) were employed to evaluate whether the cases identified as outliers differed significantly from the remainder of the cohort on relevant background characteristics. Findings with a two-tailed p value ≤ .05 were considered significant.

3. Results

3.1 Participant characteristics

Table 1 details the background characteristics of the participants in the entire sample and stratified by KL-VS genotype. The majority of participants were female (69.5%), mean age was 62.4 ± 6.5 years, and 38.5% were *APOE* ε4 carriers. Mean education was 15.8 ± 2.7 years and most participants had family history of dementia (72.6%). Participants had an average ASCVD 10-year risk of 7.5 ± 7.5%, which is at the threshold for intermediate predicted risk of an atherosclerotic cardiovascular event and clinically recommended initiation of statin therapy; low and borderline risk are < 7.5%, while high risk is > 20% [34]. Participants were mostly overweight with a BMI of 29.3 ± 6.4 kg/m^2 and had normal eGFR levels (80.9 ± 14.3 mg/dL). As expected based on prior studies [5,12,18,35], our sample was comprised of 73.2% KL-VS$_{NC}$, 25.0% KL-VS$_{HET}$, and 1.8% KL-VS$_{HOM}$. Background characteristics did not differ among these three genotypes (all p's > .18).

The 38 outliers identified from serum analyses differed from the whole cohort demographically, consisting of a higher proportion of females (92.1% of outliers versus 69.5% of whole sample; $\chi^2 = 8.39$, $p = .004$) and having a younger average age (60.3 ± 7.0 versus 62.5 ± 6.5 years; $p = .040$). However, KL-VS genotype distribution was not different in outliers compared to the remaining cohort ($\chi^2 = 2.90$, $p = .234$). There were insufficient outliers (n = 3) in the CSF sample to conduct meaningful analyses to determine whether those differed from the whole cohort. Outliers for both serum and CSF were included in the primary analyses but were excluded in sensitivity analyses.

3.2 KLOTHO KL-VS genotype and circulating klotho

3.2.1 Serum—Mean serum klotho was 814.7 ± 285.1 pg/mL. Mean serum klotho values stratified by KL-VS genotype were 804.4 ± 266.2 pg/mL in KL-VS_{NC}, 833.3 ± 319.3 in KL-VS_{HET}, and 974.1 ± 444.6 in KL-VS_{HOM}. Results of the linear model controlling for age and sex revealed that the adjusted mean serum klotho concentrations were not significantly different between KL-VS_{HET} and KL-VS_{NC} (β [SE] = 28.9 [19.6], $p = .140$; Figure 1A). Serum klotho in KL-VS_{HOM} was higher than in KL-VS_{HET} (β [SE] = 160.1 [64.0], $p = .012$) and KL-VS_{NC} (β [SE] = 131.3 [65.5], $p = .045$). In sensitivity analyses that excluded the 38 outliers (Figure 1B), serum klotho concentration was significantly higher in KL-VS_{HET} than in KL-VS_{NC} (β [SE] = 29.2 [14.7], $p = .048$). Differences were not significant between KL-VS_{HOM} and KL-VS_{HET} (β [SE] = 53.4 [51.0], $p = .295$) or KL-VS_{NC} (β [SE] = 82.6 [50.0], $p = .099$).

3.2.2 Cerebrospinal fluid—Mean CSF klotho in the analytic sample of 183 was 998.0 ± 191.9 pg/mL. Mean CSF klotho values stratified by KL-VS genotype were 932.2 ± 153.2 pg/mL in KL-VS_{NC}, 1154.9 ± 173.3 pg/mL in KL-VS_{HET}, and 1272.4 ± 251.6 pg/mL in KL-VS_{HOM}. Results of the linear model controlling for age and sex revealed that adjusted mean CSF klotho concentrations in KL-VS_{HET} (β [SE] = 225.8 [25.1], $p < .0001$) and KL-VS_{HOM} (β [SE] = 327.8 [75.5], $p < .0001$) were significantly higher than in KL-VS_{NC} (Figure 2A). KL-VS_{HOM} and KL-VS_{HET} did not differ on CSF klotho (β [SE] = 102.0 [77.5], $p = .190$). Results were unchanged in the sensitivity analyses that excluded the three outliers (Figure 2B).

3.2.3 Serum versus cerebrospinal fluid—Among the subsample of 169 participants with both serum and CSF klotho data, mean serum klotho was 838.0 ± 275.8 pg/mL and CSF klotho was 999.4 ± 195.0 pg/mL. The mean absolute difference in time from CSF to serum sample collection was 0.39 ± 1.91 years. Results of the linear model controlling for time between specimen collection revealed that adjusted mean klotho concentration was significantly higher in CSF than in serum (β [SE] = 162.4 [25.3], $p < .0001$; Figure 3A). This pattern was also observed in analyses stratified by KL-VS genotype (KL-VS_{NC}: β [SE] = 96.2 [29.8], $p = .001$; KL-VS_{HET}: β [SE] = 309.2 [40.3], $p < .0001$; KL-VS_{HOM}: β [SE] = 578.4 [152.9], $p = .019$; Figure 3B). Statistical significance was unchanged in the analyses excluding outliers (Supplemental Figure). There was a weak correlation between serum and CSF klotho (r = .142, $p = .066$; Figure 3C). Agreement between values from the two specimen sources was low (ICC [CI] = 0.109 [−0.024, 0.243], $p = .055$).

3.3 Effects of demographic and clinical characteristics on circulating klotho

3.3.1 Sex—Mean serum klotho concentration in females was 837.5 ± 307.4 pg/mL and 762.7 ± 217.9 pg/mL in males. Age-adjusted analysis of sex on serum klotho revealed that females had significantly higher levels (β [SE] = 74.0 [18.5], $p < .0001$; Figure 4A). This effect was significant among KL-VS_{NC} (β [SE] = 77.2 [20.1], $p = .0001$) but not KL-VS_{HET} (β [SE] = 56.5 [41.5], $p = .174$) or KL-VS_{HOM} (β [SE] = −49.0 [284.1], $p = .865$). Results were unchanged in analyses excluding outliers.

Mean CSF klotho concentration in females was 1036.4 ± 175.9 pg/mL and 923.2 ± 201.2 pg/mL in males. Age-adjusted analysis revealed that females had significantly higher CSF klotho than males (β [SE] = 103.6 [28.8], $p = .0004$; Figure 4A). This pattern was also observed within KL-VS_{NC} (β [SE] = 119.0 [26.6], $p < .0001$) but was not statistically significant in KL-VS_{HET} (β [SE] = 98.2 [49.0], $p = .051$) or KL-VS_{HOM} (β [SE] = 554.3 [462.8], $p = .443$). These findings were unchanged in the analyses excluding outliers.

3.3.2 Age—Median age in the serum analytic sample was 62.9 years. Mean serum klotho concentration in younger participants (i.e., < 62.9 years) was 826.9 ± 275.2pg/mL and was 802.5 ± 294.4 pg/mL in older participants (i.e., ≥ 62.9 years). Older age did not predict serum klotho level while controlling for sex (β [SE] = −20.4 [17.0], $p < .229$; Figure 4B). Stratification by KL-VS genotype also revealed no effect of age within any KL-VS genotype (KL-VS_{NC}: β [SE] = −26.7 [18.5], $p = .150$; KL-VS_{HET}: β [SE] = −4.2 [38.2], $p = .913$; KL-VS_{HOM}: β [SE] = 174.3 [238.5], $p = .475$). These results were maintained in the analysis excluding outliers.

Median age in the CSF sample of 183 participants was 64.7 years. Mean CSF klotho concentration in younger participants (i.e., < 64.7 years) was 1044.0 ± 191.8 pg/mL and was 952.5 ± 181.9 pg/mL in older participants (i.e., ≥ 64.7 years). Older participants had significantly lower CSF klotho than younger counterparts while controlling for sex (β [SE] = −76.5 [27.2], $p = .005$; Figure 4B). This pattern was also observed within KL-VS_{NC} (β [SE] = −54.1 [24.8], $p = .031$) and was not statistically significant in KL-VS_{HET} (β [SE] = −25.2 [49.6], $p = .613$); sample size was insufficient for analysis in KL-VS_{HOM}. In the analyses excluding outliers, statistical significance was maintained in the whole CSF sample but was diminished among KL-VS_{NC} (β [SE] = −45.4 [23.6], $p = .057$), while KL-VS_{HET} remained non-significant.

3.3.3 *APOE* ε4—Mean serum klotho concentration was 803.1 ± 256.5 pg/mL in *APOE* ε4 non-carriers and 833.2 ± 325.1 pg/mL in carriers. *APOE* ε4 carriage did not predict serum klotho while controlling for age and sex (β [SE] = 27.0 [17.5], $p = .122$; Figure 4C). Analyses stratified by KL-VS genotype also revealed no effect of *APOE* ε4 within any KL-VS genotype (KL-VS_{NC}: β [SE] = 13.8 [19.1], $p = .469$; KL-VS_{HET}: β [SE] = 47.5 [39.2], $p = .227$; KL-VS_{HOM}: β [SE] = 327.4 [232.6], $p = .178$). Statistical significance was unaltered in analyses excluding outliers.

Mean CSF klotho concentration in *APOE* ε4 non-carriers was 997.9 ± 194.2 pg/mL and 998.1 ± 189.4 pg/mL in carriers. Demographic-adjusted analysis revealed that *APOE* ε4 did not predict CSF klotho while controlling for age and sex (β [SE] = −4.6, SE = 28.2,

p = .870; Figure 4C). Stratified analyses within KL-VS$_{NC}$ and KL-VS$_{HET}$ also revealed no effect of *APOE* ε4 (KL-VS$_{NC}$: β [SE] = 11.0 [25.5], p = .666; KL-VS$_{HET}$: β [SE] = −3.6 [53.3], p = .946). Sample size was insufficient for analysis in KL-VS$_{HOM}$. These findings were similar in analyses excluding outliers.

3.3.4 Atherosclerotic cardiovascular disease risk—It was not possible to calculate ASCVD for 18 participants due to missing data (e.g., total cholesterol), resulting in an analytic sample of 1098 participants for serum models and 182 for CSF models. Mean serum klotho concentration in participants with < 7.5% 10-year risk of ASCVD was 828.5 ± 291.3 pg/mL and 788.4 ± 271.4pg/mL in those with ≥ 7.5% risk. ASCVD risk category did not predict serum klotho while controlling for age and sex (β [SE] = −9.4 [22.2], p = .673; Figure 4D). Analyses stratified by KL-VS also revealed no effect of ASCVD risk on serum klotho (KL-VS$_{NC}$: β [SE] = −3.8 [24.2], p = .877; KL-VS$_{HET}$: β [SE] = −51.4 [49.6], p = .301; KL-VS$_{HOM}$: β [SE] = 290.5 [255.3], p = .272). Statistical significance was unchanged in analyses excluding outliers.

Mean CSF klotho concentration in participants with < 7.5% 10-year risk of ASCVD was 1021.4 ± 202.7 pg/mL and 954.6 ± 164.4 pg/mL in those with ≥ 7.5% risk. ASCVD risk category did not predict CSF klotho while controlling for age and sex (β [SE] = 9.2 [36.8], p = .803; Figure 4D). Analyses stratified by KL-VS also revealed no effect of ASCVD risk on CSF klotho (KL-VS$_{NC}$: β [SE] = 51.4 [33.2], p = .124; KL-VS$_{HET}$: β [SE] = −94.8 [66.5], p = .162). Sample size was insufficient for analysis in KL-VS$_{HOM}$ and statistical significance was unaltered when outliers were excluded.

4. Discussion

The main findings indicate that in a cohort enriched for AD risk, carrying one or two copies of the *KLOTHO* KL-VS allele results in higher circulating klotho concentration in serum and CSF in comparison to being a non-carrier. Additionally, mean circulating klotho concentration is higher in CSF than in serum regardless of KL-VS genotype. Female sex is associated with higher circulating klotho in both serum and CSF, while younger age is associated with higher klotho in CSF but not in serum. Ours is the first study to directly compare circulating klotho concentrations from two distinct specimen sources in a cohort at risk for AD and the largest to evaluate the effect of KL-VS genotype on serum or CSF klotho.

Carrying one copy of the KL-VS allele, but not two, has repeatedly been associated with longevity [2,35,36], cardiovascular function [35], enhanced cognition and brain health [18,24], and protection from the effects of AD [4-6,8]. It is possible that the KL-VS allele changes the processing [37] of the klotho protein, changes its activity, increases its levels, or some combination.

Evidence supports a relationship between the KL-VS allele and higher systemic levels of the klotho protein. Both the KL-VS allele and higher serum klotho associate with greater gray matter volume [12], and higher serum klotho associates with functional brain connections [12] and better cognition [23]. It stands to reason that KL-VS may exert its influence

via the systemic protein hormone shed by the transmembrane protein it encodes. It is important to note that although KL-VS genetic variation affects the klotho protein isoform, our ELISA measurements of klotho, either by serum or CSF, did not allow distinction between the wildtype klotho protein and a klotho protein altered by coding of the KL-VS allele. However, klotho is detected in all KL-VS genotypes reported in this study, including homozygotes. Thus, important questions that remain are whether wildtype klotho or KL-VS-coded klotho differ in their distribution among KL-VS_{HET} and KL-VS_{HOM} carriers, and whether the different klotho protein types separately associate with clinical, biomarker, and pathological measures in aging and neurodegenerative disease.

In our primary analysis, adjusted mean serum total klotho in KL-VS_{HET} was not significantly different than in KL-VS_{NC}. The lack of a difference is in contrast with prior results [12,18] indicating that KL-VS_{HET} have higher serum klotho. Our study included a substantially larger sample size and a wider range of klotho values than those studies, which may have contributed to the discrepancy of the present findings with those prior. However, the primary analysis also included 38 outliers, mostly in the KL-VS_{NC} group. The outliers comprised a significantly higher proportion of females and younger participants than the main cohort, which may have increased the mean klotho estimate given that female sex (and younger age, though not significantly) is associated with higher klotho. After truncating the non-representative outlier observations, we found that serum klotho was higher among KL-VS_{HET} compared with KL-VS_{NC}. This finding is in agreement with prior reports [12,18]. However, in those studies, adjusted mean differences between KL-VS_{HET} and KL-VS_{NC} were approximately 105–111 pg/mL and average ages were approximately 10 years greater than in our cohort, in which the observed difference between KL-VS_{HET} and KL-VS_{NC} was approximately 29 pg/mL. This raises the possibility that the effect of KL-VS genotype on serum klotho [23] is more pronounced in older age. Also in contrast with prior findings from our group [12], we found that serum klotho was highest in KL-VS_{HOM}. Considering evidence that KL-VS_{HOM} have worse health outcomes [24,35] it would initially appear counterintuitive that serum klotho is higher in KL-VS_{HOM}. However, the mutant klotho isoform produced by the KL-VS allele is thought to have diminished function compared to the protective wildtype klotho [2,17]. Thus, regardless of its concentration, the mutant klotho may be responsible for worse health outcomes observed in KL-VS_{HOM}. Because of the limited sample size of 20 KL-VS_{HOM} participants, definitive interpretation of this result will require follow-up investigation. However, 10 of the 20 KL-VS_{HOM} participants had serum klotho below the mean of KL-VS_{NC} participants, and the prior study [12] included only four KL-VS_{HOM} participants; it is possible that the randomly sampled participants in that study had low klotho concentration by chance. In our study, higher serum klotho in KL-VS_{HOM} was no longer observed when outliers were removed, and further investigation in this population will be needed.

CSF klotho was higher among KL-VS_{HET} and KL-VS_{HOM} compared to non-carriers. In one of the few other studies of CSF klotho with regard to klotho genotype, Zimmerman and colleagues reported that KL-VS_{HET} had higher CSF klotho concentration than KL-VS_{NC} among patients with Parkinson’s disease [16]. That finding in KL-VS_{HET} aligns with ours, while their study excluded KL-VS_{HOM} due to small sample size. In light of evidence that the klotho variant expressed by the KL-VS allele does not demonstrate altered shedding or half-

life [37], other mechanisms that impact klotho concentration may be at play. For example, higher CSF klotho among KL-VS$_{HET}$ could result from a compensatory effect whereby the functionally deficient klotho protein results in a positive feedback mechanism promoting its expression [16]. Why this positive feedback loop would be more robust in CSF than in serum is unclear but could result from differences between expression in distinct tissues (i.e., choroid plexus versus kidney). Mean CSF klotho concentration among healthy control participants are variable in the literature, ranging as low as 541.6 pg/mL [15] to as high as 1265.8 pg/mL [38], and our mean was within that range. While various patient populations, including AD [14], may have lower circulating CSF klotho than healthy individuals, our results are derived from an as-yet healthy population at risk for neurodegenerative disease.

Mean klotho in CSF was higher than in serum for the subset of participants with data on both specimen sources. This pattern was also observed within each KL-VS genotype. Circulating klotho detected in this study originates from shedding of the extracellular domain of the transmembrane alpha-klotho protein, which is primarily found in the choroid plexus of the brain and in the kidney. The majority of circulating klotho in CSF originates from the choroid plexus [39]. In fact, there is evidence that targeted intervention can alter CSF klotho while leaving serum klotho unchanged [40]. The difference between CSF and serum klotho in the present study is thus not surprising as the circulating hormone may arise from different sources. However, our data contrast with the finding in children that CSF klotho is lower than in plasma [39], possibly because of differences in study population studied (children versus older adults) or specimen source (serum versus plasma). Although CSF and serum samples in our study were not always collected on the same day within individuals, the mean difference of 0.39 years was controlled for in this comparative analysis. While serial klotho measurements are stable over the course of one day [14], further investigation will be needed to evaluate variability over longer periods of time within individuals. The lack of a significant correlation between serum and CSF klotho in our study contrast with other recent findings [41] of a significant positive correlation between serum and CSF klotho. However, the study by Kundu et al. included participants with diagnosed cognitive impairment and AD, which may diminish CSF klotho [14], possibly bringing them closer to serum levels if the neurodegenerative effect is stronger on CSF than serum. The present finding that CSF and serum klotho were not significantly correlated suggest that in our population klotho from these compartments may be independent of each other and are not interchangeable.

Sex and age are two factors thought to affect circulating klotho [14,23,30,42]. In the present study, concentrations in both serum and CSF were higher in females than in males. These results align with a report [23] that participants with high plasma klotho (> 669 pg/mL; the median in that study) were more likely to be female, and another in which serum klotho was higher in females [41]. In contrast, other previously published data indicate higher CSF concentrations among males than females [14] or no significant difference in serum klotho between sexes [22,43]. Furthermore, detection of sex differences may vary by the type of immunoassay performed [44]. Nonetheless, klotho is neuroprotective [17] and females have a higher lifetime risk of AD than males [1]. It is possible klotho is upregulated particularly among females in response to the elevated AD risk in our cohort. If true, this could account for the difference between ours and some prior reports. Alternatively, it is also possible that

higher klotho levels in females reflects female longevity in AD [45], which contributes to their higher lifetime risk compared to men.

Regarding the effect of age on klotho, older age was significantly associated with lower CSF klotho but not serum. Our findings in serum are in contrast with prior reports that older age is associated with lower serum klotho concentration [12,23,43] and one of no association between CSF klotho and age [16]. However, mean ages in those studies were approximately 74 and 75 years, respectively, while mean age in the present study was lower at 62.4 years and with only 12 participants aged 75 or older. It may be that participants in our cohort have experienced less age-related decline in serum klotho than in those prior studies, given the relatively younger age of our sample. In contrast, CSF klotho was lower in participants older than our median, suggesting that the effect of age on circulating klotho may take hold earlier in CSF than blood.

APOE ε4 and cardiovascular disease are important risk factors for AD but were not shown to impact serum or CSF klotho concentrations in this study. This was unexpected given that KL-VS_{HET} can attenuate the effects of *APOE* ε4 on amyloid [5] and age on tau [4] accumulation and conversion to AD [8] although not all reports agree [46]. KL-VS_{HET} is also associated with reduction in cardiovascular risk factors such as systolic blood pressure and HDL cholesterol [35]. However, the lack of a difference does not preclude the possibility that serum or CSF klotho concentrations may interact with *APOE* ε4 carriage or cardiovascular disease to influence brain health outcomes.

A limitation to the present findings is the demographic composition of the cohorts. Most participants were white and highly educated and thus do not accurately represent the broader US population. Further work in more diverse samples will be essential to generalize findings on *KLOTHO* genotype and circulating concentrations. Regarding findings in KL-VS_{HOM}, the sample sizes for serum (n=20) and CSF (n=4) were small. Additionally, the cross-sectional nature of these data precludes inference regarding the trajectory of changes in circulating klotho concentration as a function of KL-VS genotype and other characteristics. This limitation is addressable given that the WRAP and WADRC cohorts are prospectively followed, thus affording future opportunities for longitudinal analyses. The ELISA used in this study may not be directly comparable to other klotho assays (e.g., immunoprecipitation-immunoblot). Finally, owing to unavailability of biological samples, our study did not address whether klotho protein isoform affects quantification by ELISA, a question that awaits future experimentation.

In a cohort enriched for risk of AD, circulating klotho is higher in CSF than in serum, and is higher in serum and CSF among heterozygotic and homozygotic carriers of the *KLOTHO* KL-VS allele. Sex and age modulate circulating klotho concentration. Klotho specimen source, age, and sex should be considered in analyses of circulating klotho on brain health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Data Availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request and approval by the Wisconsin Alzheimer's Disease Research Center study scientific committee.

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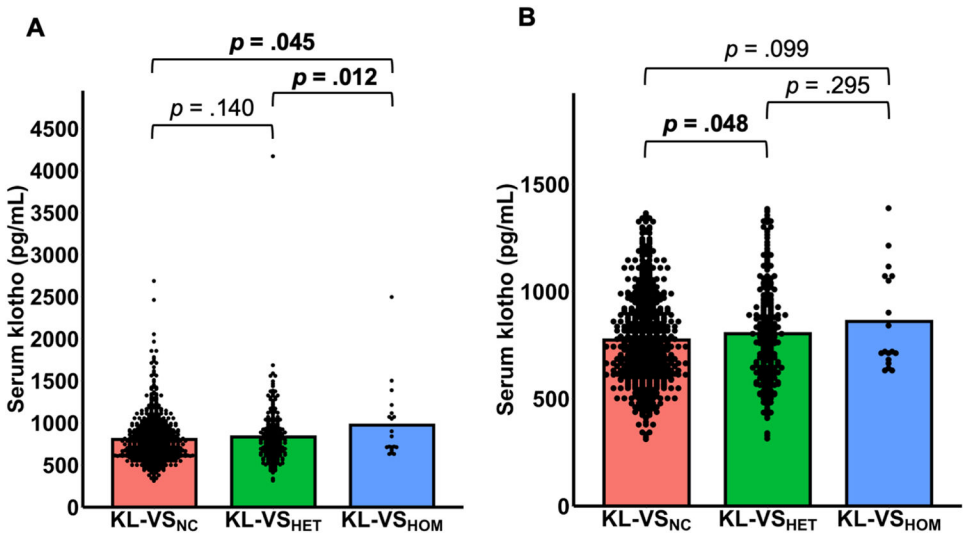

Figure 1. Serum klotho among *KLOTHO* KL-VS genotypes.

Serum klotho concentration by KL-VS genotype among all participants (n = 1116) with serum data (A) and those remaining after exclusion (n = 1078) for outliers (B). Linear regression was used to test the effect of KL-VS genotype on circulating serum klotho, adjusting for sex and age. Homozygotic carriers of *KLOTHO* KL-VS had elevated serum klotho in the whole-group analysis, while heterozygotic carriers had elevated serum klotho after exclusion of unrepresentative outliers. Outliers were selected on the basis of serum klotho values outside 1.5 times the interquartile range. Abbreviations: *KLOTHO* KL-VS allele non-carrier ($KL\text{-}VS_{NC}$); *KLOTHO* KL-VS allele heterozygote ($KL\text{-}VS_{HET}$); *KLOTHO* KL-VS allele homozygote ($KL\text{-}VS_{HOM}$).

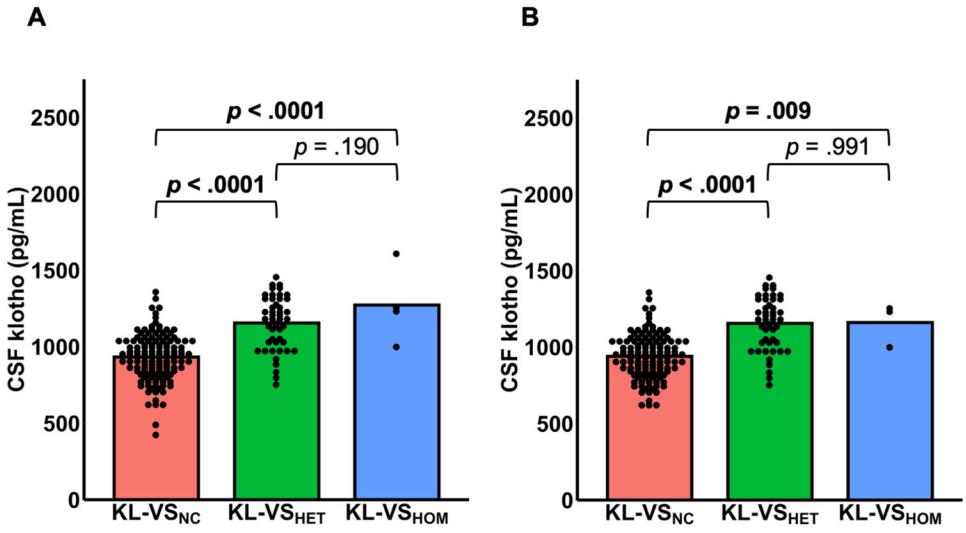

Figure 2. Cerebrospinal fluid klotho among *KLOTHO* KL-VS genotypes.

Cerebrospinal fluid klotho concentration by KL-VS genotype among all participants (n = 183) with cerebrospinal fluid data (A) and those remaining after exclusion (n = 180) for outliers (B). Linear regression was used to determine the effect of KL-VS genotype on circulating cerebrospinal fluid klotho, adjusting for sex and age. Klotho in cerebrospinal fluid was significantly higher among heterozygotic and homozygotic carriers of *KLOTHO* KL-VS than non-carriers in the whole-group analysis. This result held after exclusion of outliers. Outliers were selected on the basis of CSF klotho values outside 1.5 times the interquartile range. Abbreviations: *KLOTHO* KL-VS allele non-carrier ($KL\text{-}VS_{NC}$); *KLOTHO* KL-VS allele heterozygote ($KL\text{-}VS_{HET}$); *KLOTHO* KL-VS allele homozygote ($KL\text{-}VS_{HOM}$).

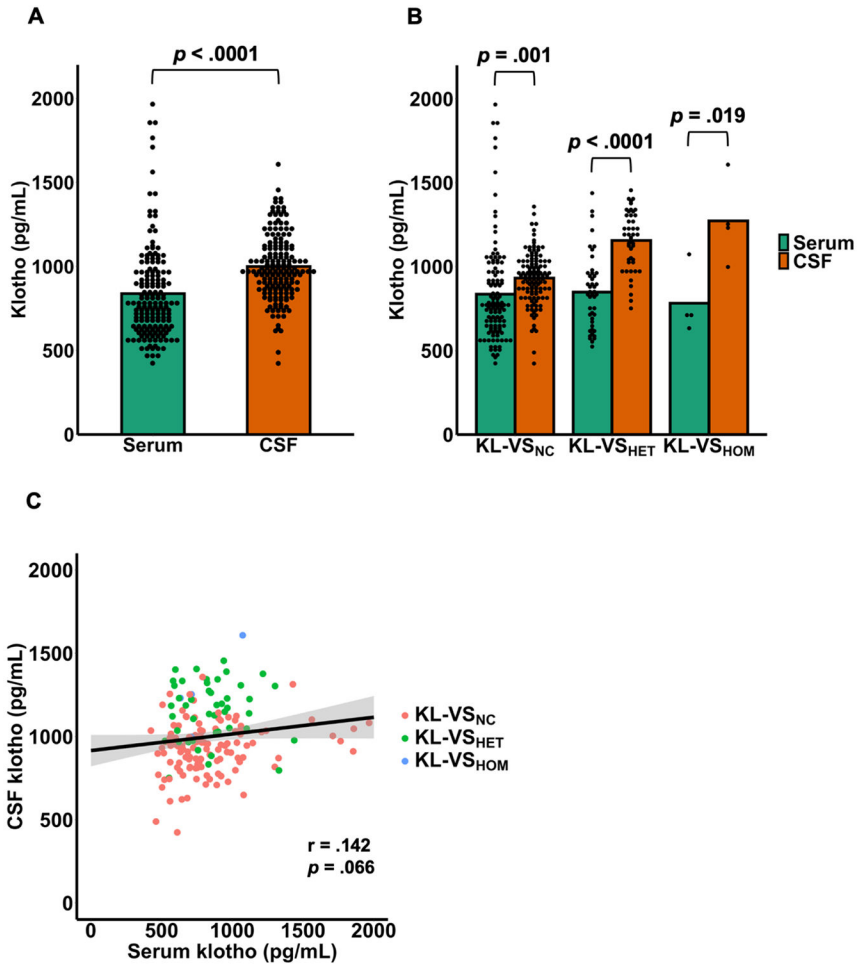

Figure 3. Circulating klotho concentration in serum versus cerebrospinal fluid.

Mean concentrations of klotho in serum and cerebrospinal fluid in the entire sample (n = 169) with data in both specimens (A) and stratified by KL-VS genotype (B). Linear regression was employed to determine the effect of specimen source (i.e., serum versus cerebrospinal fluid) on klotho concentration. Klotho concentration was significantly higher in cerebrospinal fluid than in serum in the whole sample and within each KL-VS genotype. Pearson correlation between klotho values from the two specimen sources (C). Values from the two sources were weakly correlated. Abbreviations: cerebrospinal fluid (CSF); *KLOTHO* KL-VS allele non-carrier ($KL\text{-}VS_{NC}$); *KLOTHO* KL-VS allele heterozygote ($KL\text{-}VS_{HET}$); *KLOTHO* KL-VS allele homozygote ($KL\text{-}VS_{HOM}$).

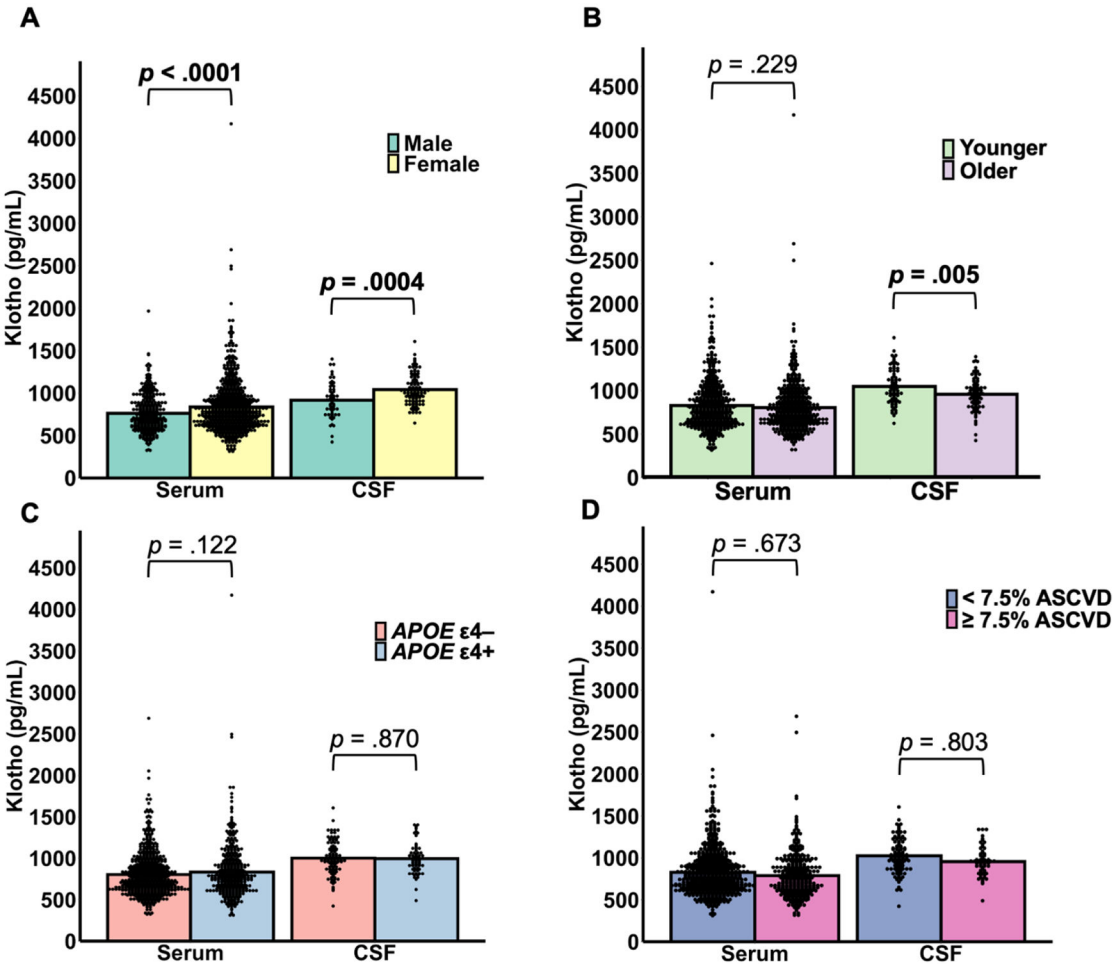

Figure 4. Circulating klotho concentration as a function of demographic and clinical characteristics.

Circulating klotho concentration in serum (n = 1116) and cerebrospinal fluid (n = 183) by sex (A), age (B), *APOE* ε4 carriage (C), and 10-year atherosclerotic cardiovascular disease risk (D). Linear regression was used to determine the effect of age, sex, *APOE* ε4, and 10-year atherosclerotic cardiovascular disease risk on circulating klotho concentration, adjusting for sex and age where appropriate. Klotho was significantly elevated among females in both serum and cerebrospinal fluid and was higher among younger participants in cerebrospinal fluid but not in serum. Circulating klotho did not differ by *APOE* ε4 or 10-year atherosclerotic cardiovascular disease risk. Age was dichotomized at the median in a sample-specific manner: serum analyses were split at the median age of 62.9 years and cerebrospinal fluid analyses were split at the median age of 64.7 years. *APOE* ε4 carriage was based on carrying at least one copy of the *APOE* ε4 allele. 10-year atherosclerotic cardiovascular disease risk was split at 7.5% 10-year risk based on the threshold at which statin therapy is recommended [34]. Abbreviations: cerebrospinal fluid (CSF); apolipoprotein E epsilon 4 allele carrier (*APOE* ε4+); apolipoprotein E epsilon 4 allele non-carrier (*APOE* ε4−); 10-year atherosclerotic cardiovascular disease risk (ASCVD).

Table 1.

Participant characteristics

	Overall	KL-VS$_{NC}$	KL-VS$_{HET}$	KL-VS$_{HOM}$	*p*
n (% of overall)	1130 (100.0%)	827 (73.2%)	283 (25.0%)	20 (1.8%)	-
Age, years	62.4 (6.5)	62.4 (6.6)	62.6 (6.5)	60.6 (5.6)	.37
Female, n (%)	785 (69.5)	572 (69.2)	197 (69.6)	16 (80.0)	.58
Education, years	15.8 (2.7)	15.7 (2.7)	16.0 (2.5)	16.5 (3.4)	.18
APOE ε4+, n (%)	435 (38.5)	316 (38.2)	112 (39.6)	7 (35.0)	.87
BMI, kg/m^2	29.3 (6.4)	29.2 (6.3)	29.5 (6.8)	29.1 (5.5)	.81
eGFR, mL/min/1.73 m^2	80.9 (14.3)	81.4 (14.3)	79.7 (14.2)	79.8 (17.3)	.22
ASCVD 10-year, % risk	7.5 (7.5)	7.6 (7.8)	7.5 (6.8)	4.9 (3.4)	.30

Data are mean (standard deviation) for continuous variables.

p-values result from χ^2 test for categorical variables or one-way ANOVA for continuous variables.

eGFR calculated from the Chronic Kidney Disease Epidemiology Collaboration equation [33].

ASCVD calculated from equations from Yadlowsky et al. [32].

Abbreviations: *KLOTHO* KL-VS allele non-carrier (KL-VS$_{NC}$); *KLOTHO* KL-VS allele heterozygote (KL-VS$_{HET}$); *KLOTHO* KL-VS allele homozygote (KL-VS$_{HOM}$); apolipoprotein E epsilon 4 allele carrier *(APOE* ε4+); body mass index (BMI); estimated glomerular filtration rate (eGFR); atherosclerotic cardiovascular disease risk (ASCVD).