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Supranutritional sodium selenate supplementation delivers selenium to the central nervous system: results from a randomized controlled pilot trial in Alzheimer's disease

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Abstract:	Insufficient supply of selenium to antioxidant enzymes in the brain may contribute to Alzheimer's disease (AD) pathophysiology; therefore, oral supplementation may potentially slow neurodegeneration. We examined selenium and selenoproteins in serum and cerebrospinal fluid (CSF) from a dual-dose 24-week randomized controlled trial of sodium selenate in AD patients, to assess tolerability, and efficacy of selenate in modulating selenium concentration in the central nervous system (CNS). A pilot study of 40 AD cases were randomized to placebo, nutritional (0.32 mg sodium selenate, three times daily), or supranutritional (10 mg, three times daily) groups. We measured total selenium, selenoproteins, and inorganic selenium levels, in serum and CSF, and compared against cognitive outcomes. Supranutritional selenium supplementation was well tolerated and yielded a significant ($p < 0.001$) but variable (95% CI = 13.4 - 24.8 µg/L) increase in CSF selenium, distributed across selenoproteins and inorganic species. Reclassifying subjects as either responsive or non-responsive based on elevation in CSF selenium concentrations revealed a moderate improvement in Mini-Mental Status Examination (MMSE) scores (+3.2 points, $p = 0.03$). Pooled analysis of all samples revealed that CSF selenium could predict change in MMSE performance (Spearman's rho = 0.403; $p = 0.023$). High-dose sodium selenate supplementation is well tolerated and can modulate CNS selenium concentration, although individual variation in selenium metabolism must be considered to optimize potential benefits in AD.	
Response to Reviewers:	See attachment.	

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Response to Reviewers

Reviewer #1:

R1.1 ***"Currently, the widely accepted tolerable upper limit is in the range of 400 µg/day, the no observed adverse effect level at 800 µg/day and the lowest observed adverse effect level starts at around 900 µg/day, respectively. A recent report with 30 mg (!) Se/daily dose affected around 100 healthy subjects, developing hair and nail loss and a number of long-lasting side effects (Morris JS, Crane SB. Selenium toxicity from a misformulated dietary supplement, adverse health effects, and the temporal response in the nail biologic monitor. Nutrients. 2013;5(4):1024-57.) This report indicates that such high intakes of around 500-times the recommended daily intake of 60-70 µg/day do not directly cause fatalities but rather health problems. The authors used such overdoses but report no health problems which sound controversial to accepted knowledge."***

We appreciate the Reviewer pointing out concerns about the possible toxicity of the highest dose of selenate tested. We found that there were no serious adverse events that were emergent at this dose, and we note this in the Results. We reconcile our findings with the report of Morris and Crane (and associated reports) by noting the following:

1. The misformulated supplement exposed subjects to an average of ≈30 mg of elemental selenium per day. The highest dose we tested was 30 mg of sodium selenate per day in divided doses, which is equivalent to 12.5 mg of elemental selenium per day, i.e. less than half the dose of selenium in the misformulated supplement.
2. The selenium in the misformulated supplement was combined with many other bioactive ingredients including chromium at 17 times the RDA. This may have increased the toxicological burden.
3. The no observed adverse effect level that the Reviewer mentions would apply to normal people (e.g. in the context of supplementation), but in a chemotherapeutic

intervention trial such as ours, a certain degree of adverse effects can be acceptable. Since our trial of sodium selenate at 30 mg per day has been completed under controlled conditions, the data in our current manuscript are all the more important to examine whether this drug can be used safely in this disease context.

4. No deaths or hospitalizations were attributed to the misformulated supplement exposure, and the range of symptoms reported were mainly gastrointestinal complaints, mood changes, and skin and nails changes. These types of adverse effects may be considered acceptable in a successful chemotherapeutic intervention for a severe neurodegenerative disorder, such as Alzheimer's disease. Nevertheless, every effort must be made to mitigate them, with the selenium treatment dose being less than half that of the misformulated supplement, the range of treatment-emergent adverse events (TEAEs) found in our study is reassuring.

Nevertheless, to address the concerns about the high dose of selenium being used we have rewritten the final paragraph of the Results section (as follows), and added Discussion of the misformulated supplement reports (below that):

"Treatment emergent adverse events (TEAEs) were reported previously according to treatment group, with 90% reporting at least one TEAE [1]. To summarize, all TEAEs were reported as mild. The most common solicited TEAEs (incidence ≥ 20% in both placebo/nutritional and supranutritional groups) were fatigue, headache, and lethargy; with nausea, muscle spasms, and dizziness reported in the supranutritional group. One participant experienced a pre-syncopal serious adverse event that was resolved in 24 hours and continued in the study. Two supranutritional group participants withdrew due to TEAEs; one due to appearance of a skin rash of uncertain cause, and the other resulting from dysmorphic changes in toenails and fingernails. In the latter case, the participant had the 24-week observation and was not excluded from further analysis. On application of our responsive/non-responsive stratification, no relationship between the number of TEAEs and level of selenium uptake was observed ($p = 0.976$ for serum; $p = 0.900$ for CSF; Fisher's exact test)."

We also now discuss the implications of Morris and Crane's report [2], as well as the related article by MacFarquhar *et al* [3] in the Discussion:

"While significantly more subjects in the Supranutritional group (35%) experienced TEAEs compared to the placebo group (10%) [1], the adverse effect profile was similar to that associated with toxicity resulting from consumption of a misformulated nutritional supplement in the US in the late 2000s that contained selenium at ≈400-fold the recommended daily allowance (RDA) [2, 3]. In this incident, an unknown number of consumers inadvertently received a daily selenium dose in the order of 30 mg equivalent, as well as ≈30 mg of chromium and other substances. We note that the misformulated supplement exposed subjects to an average of ≈30 mg of elemental selenium per day. The highest dose we tested was 30 mg of sodium selenate per day in divided doses, which is equivalent to 12.5 mg of elemental selenium per day, i.e. less than half the dose of selenium in the misformulated supplement. Furthermore, the chromium in the misformulated supplement (17-fold the RDA), as well as many other bioactive ingredients in the mixture, may have potentiated the toxicological burden. In any case, since our trial of sodium selenate at 30 mg per day was completed under controlled conditions, the data are important to examine whether this drug can be used safely in this disease context. Our observations that 30 mg of sodium selenate per day for 24 weeks, being less than half that of the misformulated supplement, without serious adverse events is reassuring for the use of this dose as a chemotherapeutic, where the benefits may exceed the risk of mild TEAEs."

R1.2 ***"Line 112: The method should not only [be] referred to in another paper but at last shortly described."***

As we understand it, the reviewer could be referring to the analytical method used for selenium determination in the Seronorm standard by ICP-MS. This certified reference material was analysed using the conditions outlined. For clarity, we have elaborated this section of the Subjects and Methods to read:

"Determination of total selenium concentration in serum and CSF

Selenium concentration in serum and CSF was measured using inductively coupled plasma-mass spectrometry (ICP-MS). Neat serum and CSF were diluted in 1% nitric acid (1:20 and 1:3 respectively, to 300 µL final volume). Selenium was measured on

mass at *m/z* = 78 (^{78}Se; natural abundance = 23.8%) using an Agilent Technologies 7700x ICP-MS system (Agilent Technologies, Australia) fitted with 'cs' lenses and platinum cones. Hydrogen (4 mL/min) was used as a reaction gas to remove polyatomic interferences at *m/z* = 78. Values were the average of four technical replicates. Selenium concentrations were calculated by external calibration using multi-element standards (AccuStandard, USA) containing of 0, 0.1, 0.5, 1, 5, 10, 50, 100 µg/L of selenium. An internal standard solution containing 200 µg/L of yttrium (^{89}Y) was introduced online *via* a Teflon T-piece. Analytical validity was assessed using reconstituted lyophilized Seronorm™ Trace Elements in Serum (Sero AS, Norway) standard reference materials, which was prepared using the same protocol for serum samples. The measured analytical recovery of selenium in the Seronorm™ standard was within the acceptable range, per manufacturer's guidelines (measured serum = 153.89 ± 6.48 µg/L, *n* = 4; certified range = 95–176 µg/L)."

R1.3 "*Line 113: The authors add 1 % HNO3 to neat serum. Proteins will be denaturated and precipitated including selenoproteins. Wrong determinations are likely to occur.*"

1% nitric acid was used as a diluent for serum and CSF aliquots undergoing total selenium analysis by ICP-MS, as described above. Nitric acid (pH<1) is not a flocculent under these conditions, and we observed no precipitates. But, in any case, to confirm that the extraction method captured all the selenium present in the samples, we used a certified reference standard of serum with a known amount of selenium (Seronorm™), as described in our revised methods above. Our recovery was in accordance with the certified reference range, indicating that we had not unduly lost selenium content during processing.

R1.4 *"Line 119: The concentration of the internal standard should be in the concentration range of the analyte in the final measurement solution. 20µg/L is way too high."*

The ^{89}Y internal standard meets all necessary selection criteria for ICP-MS, specifically: i) monoisotopic; ii) absence of interferences; iii) no matrix effects; iv) comparable atomic mass to analyte (89 amu vs 78 amu); and v) not present in the samples analysed. It should be noted that concentration is not considered a criterion for internal standard selection for ICP-MS, as the wide linear dynamic range covers at least nine orders of magnitude [4]. The stated concentration of ^{89}Y of 20 µg/L is a reference value only; the final

concentration following online addition *via* narrow-bore 0.64 mm i.d. Tygon tubing and mixing in a T-piece results in a substantially lower concentration. Importantly, online addition, as opposed to sample spiking, provides better signal stability, which is essential for internal standard use in ICP-MS.

R1.5 ***'Line 124: Analytical quality control must be included in the paper and must be clearly shown. Please provide the data of recovery, accuracy and precision, not just "within acceptable range".'***

As mentioned above, we now include recovery, accuracy and precision of the Seronorm standard as the mean ± standard deviation of four replicate measures:

> "The measured analytical recovery of selenium in the Seronorm™ standard was within the acceptable range, per manufacturer's guidelines (measured serum = 153.89 ± 6.48 µg/L, n = 4; certified range = 95–176 µg/L)."

R1.6 ***"Line 130-144: Size exclusion chromatography is unable to really identify the seleno species. Roughly, a separation between se-proteins and small Se ligands may be possible. There will be no way to differentiate between Se (IV) and Se (VI). The assumption of the authors for the last peak being Se (IV) is supported by no data. Also the known additional retention effects in SEC cannot hold for Se (IV), if so more likely for small organic compounds."***

We agree that SEC does not have sufficient resolution to distinguish between oxidation states of selenium. We originally stated that the later-eluting peak was only provisionally assigned as selenite, but we have now clarified our discussion of this observation, as follows:

> "Two additional resolved peaks were also significantly increased ($p < 0.001$, Student's t-test) in serum from the supranutritional group, representing inorganic selenate and an unidentified selenium-containing compound, both eluting below the lower molecular weight limit of the SEC column. Interactions between the silica column and negatively charged inorganic selenium compounds are known to influence retention time in SEC-ICP-MS [5], thus we suspect that the peak following

the selenate standard (serum Peak 3, CSF Peak 4, Fig 4) might be selenite (SeO_3^{2-}). A previous analytical study of the stability of selenocompounds in human serum suggested selenite is not present in freshly-drawn serum and is an artifact of storage, but the report examined serum from only two donors, with total serum selenium at levels not commensurate with our supranutritional group [6]. Regardless of its chemical species, the appearance of this selenium-containing peak at 24-weeks is indicative of a specific response to supranutritional selenium supplementation."

R1.7 ***"Table 2: Please complete with data on Selenoproteins and inorganic Se compounds."***

We quantitated the selenium chromatograms produced using SEC-ICP-MS by signal intensity for each peak (as area under the curve) and have included this information as Table 3. This allows comparisons between treatment groups of areas under the curve for the 4 peaks identified.

R1.8 ***"Table 2: Even the placebo group has really high Se concentration in serum. How should the postulated (by authors) "redox deficiency" be explained when Se is already sufficiently high?"***

We wish to stress that at no point did we use the term 'redox deficiency'. We note, in the last paragraph of the introduction, that there were a host of apparent benefits in preclinical models of AD treated with selenate, specifically,

- reducing pathological tau hyperphosphorylation *via* activation of protein phosphatases 2A,
- down-regulation of BACE1 expression
- suppression of amyloid and markers of nucleic acid oxidation in APP/PS1 transgenic mice.

It was on the basis of these findings that sodium selenate proceeded to clinical trial testing for AD. However, selenium is involved in redox management within the brain, and this activity is regulated by selenoprotein synthesis in the central nervous system (CNS), independent of circulating selenium levels. Thus, we suspect that one additional potential mechanism of action of sodium selenate therapy might be to augment CNS selenoprotein

synthesis, which would be important in AD where levels of brain selenium are decreased compared to normal tissue. Why this happens in AD in the presence of normal serum concentrations of Se is not yet clear, but also not within the scope of this report. Changes in brain chemistry in disease are not always reflected in the periphery or blood. Nonetheless, we have now elaborated more on this in the Discussion.

> "… glutathione peroxidase 4 (GPx4) is the most abundant selenoprotein in brain, and has recently garnered attention as an important regulator of ferroptosis [7], a newly identified form of iron-dependent programmed cell death that causes aggressive lipid peroxidation [8, 9] thought to play a major role in AD pathology [10]. Since AD affected brain tissue has lower levels of selenium [11, 12], GPx4 expression and activity may suffer from insufficient selenium supply, and on this basis supplementation trials are worth exploring."

R1.9 ***"Figure 3: The authors compare slopes, being nearly the same and draw conclusions. However when looking on those data, the sample size is very small each and the respective slopes are practically defined from one point, which in both cases could also look like an outlier. All the other data points do not allow to come to a clear conclusion."***

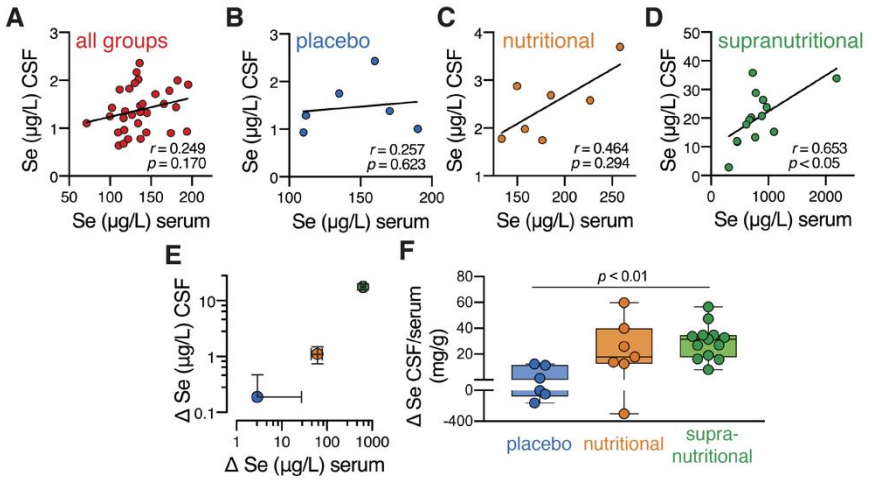
The Reviewer makes a good point, and we were also concerned about the small sample size, as we mentioned in the text. In this revision, we have deleted this panel and replaced it with a new analysis. Our point was to test whether the dimension of the change in CSF Se content after 24 weeks was commensurate with the change in serum Se content in our three treatment groups. To examine this, we have now expressed the results differently, although all the data points are still presented.

Figure 3E now shows the mean change in serum Se (x axis) and CSF Se (y axis) in matched subjects (where both serum and CSF samples were available and assayed at both baseline and 24 weeks of treatment). The inset shows the individual data points from each subject (with box and whisker analysis) expressed as the change in CSF Se per unit change in serum Se. This allows us to test whether the change in CSF Se is simply proportional to the change in serum. We find that this is roughly correct: that the increase in CSF Se is a muted reflection of the change in CSF Se. For every g increase in serum Se, there is a ≈30 mg increase in CSF Se, ie about 3% of serum Se boost is transduced into the CSF, following supranutritional Se supplementation.

The revised Results now reads:

"We examined whether the dimension of the change in CSF Se content after 24 weeks' treatment was commensurate with the change in serum Se content in our three treatment groups. We analysed the change in serum selenium matched to the change in CSF selenium in subjects where both samples were available and assayed at baseline and 24 weeks of treatment. The mean changes in each group indicated that the boost in serum selenium following supplementation was matched by a muted increase in CSF selenium. Of the increase in serum selenium in the two supplementation group, only ≈3% of the increase was transduced into the CSF (Fig. 3E&F). The change in CSF Se was approximately proportional to the change in serum in both the nutritional and the supranutritional selenate supplementation groups. In the supranutritional group, the increase in CSF selenium as a proportion of the change in serum selenium was significantly more than in the placebo group (≈3%, Dunn's test $p = 0.0019$, Fig. 3F), consistent with a small proportion of the boost in serum selenium surmounting the blood brain barrier with this high dosage regimen."

Figure 3 revised:



The Figure legend for Figure 3E&F now reads:

"E) Changes in serum selenium (x axis) and CSF selenium (y axis) in matched subjects (where both serum and CSF samples were available and assayed at both baseline and 24 weeks of treatment). Data are means ± SD, n = 6, 7 and 13 for placebo, nutritional and supranutritional groups, respectively. The axes are in log units to capture the large shifts in values as the doses are increased. F) Individual data points from each subject (with box and whisker analysis) expressed as the change in CSF Se per unit change in serum Se for each

matched subject. The supranutritional group exhibited a small boost in CSF selenium when normalized to the change in serum selenium (≈+3%, or ≈30 mg/g). P value is from Dunn's multicomparison's test."

R1.10 ***"Line 237: The retention time of SELENOP can be only roughly indicative with a column separation range of that big range and the short retention times. Furthermore, the authors assign the first peak to SELENOP and a second to Se-HAS, although Se-HAS is slightly bigger and thus must elute before (!) SELENOP. It seems that the peak assignments are wrong."***

The reviewer is correct, the peaks were incorrectly deduced for serum and CSF samples. Corrected text now reads:

> "…Selenate supplementation clearly increased binding of selenium into serum proteins (Fig 4A), with a large increase (+656%; $p < 0.001$, Student's *t*-test) in the earliest eluting peak (Peak #1, Fig. 4A). We have previously characterized the selenium content in this peak by SEC-ICP-MS and MS/MS bottom up proteomics, and confirmed the presence of both selenoprotein P (comprising ≈50% of this peak) and albumin that could not be discriminated by chromatography at this resolution [13]. Selenoprotein P has a molecular mass of 43 kDa, but is highly glycosylated. The molecular mass of albumin is 67 kDa, and Peak #1 has an apparent M_r of 75 kDa against size standards. Using this approach, we cannot discriminate between selenium incorporated as selenocysteine and that being transported *via* transient binding to free thiol groups on serum albumin [14]. Chemically inert buffers at physiological pH used for SEC-ICP-MS preserve the integrity of selenium thiol ligands, with the compromise being relatively low separation efficiency [15]. As such, we suspect that Peak #1 contains highly-abundant serum albumin followed closely (without resolution) by selenoprotein P.…"

<u>Reviewer #2</u>

R2.1 ***"…there is no sufficient evidence to support that Peak I in Fig 4 is selenoprotein P. Western blot with SelP antibody is suggested to confirm the increase of SelP levels."***

See response to **R1.10**, and the appropriate caveats that we introduce about peak assignment. We agree that we cannot confidently assign identities to Peak #1. However, our purpose was not specifically to study specific selenoprotein incorporation following selenate supplementation.

R2.2 "*…why there is no increase in the levels of Gpx4 and any other selenoproteins except SelP, since Gpx4 is the most abundant selenoprotein in the brain?"*

While Gpx4 is the most abundant selenoprotein in the brain, it is a membrane-associated protein that is not found in CSF[16] . Other selenoproteins are either membrane-resident or intracellular, and not expected in the biological fluids that we examined.

R2.3 *"…in Fig4-B, the levels of selenate (peak 3) in the CSF varied little before and after the supplementation of selenate at the high dose. Why? The author should give a discussion."*

We agree that this is interesting −selenium appeared to be thrust into proteins upon supplementation and the selenate peak changed little (although if you consider Peak #4 as well, then the amount of selenate + selenite might have doubled). With the addition of Table 3, specific numbers can be compared. We have rewritten this section of the Results as follows:

"In CSF samples, we again identified a marked increase in the low mass peaks, selenate (Peak #3 provisionally, +29.7%, $p < 0.001$) plus selenite (suspected for Peak #4), following supranutritional supplementation. The lower total selenium concentration in CSF reduced the effect of peak tailing, allowing selenoprotein P (likely to be Peak #2), which we have previously confirmed in CSF samples using a targeted proteomics approach [13], to be resolved from albumin (likely to be Peak #1), which is also present in CSF [17]. Selenium bound to albumin was markedly increased in the supranutritional group (Peak #1 in Fig 4B, +3022%, $p < 0.001$), as was the selenoprotein P peak following selenate treatment (Peak #2 in Fig 4B, $p < 0.001$; Table 3). Both selenium-binding proteins were below the limit of detection in untreated CSF, consistent with previous findings [16]. It was also apparent from the ratios of the selenium in the chromatographic protein peaks to the low mass peaks (Table 3), that selenate supplementation boosted selenoprotein production far more in the periphery (serum. Fig 4A) than in the brain (CSF, Fig 4B).

Thus, augmenting brain levels of selenoprotein P (and potentially other selenoproteins) may require higher levels of selenate precursor than peripheral organs."

R2.4 ***"The word "safely" in the title should be re-considered."***

Although the TEAEs presented by treated participants were mild in this study, we agree with the reviewer that further studies are needed to confirm the safety of high dose of sodium selenate. We have removed 'safely' from the manuscript title.

Reviewer #3

R3.1 ***"Sample sizes are relatively small and may therefore undermine the analyses involving ANCOVA especially given group comparability on subject factors."***

The Reviewer is quite correct. We apologize that there was an error in our description of the statistical test that we used for comparison between groups. We report differences over time, and to do so we actually used either Wilcoxon Signed Rank or paired Student's *t* tests. Log transformed values were calculates for change variables, that were used for comparison between responsive and non-responsive groups. The corrected text now reads:

"All statistical analyses were conducted on the intention to treat population. Data were included for all participants enrolled in the study who had complete data for this exploratory analysis. Baseline characteristics were compared between groups using ANOVA for continuous data and using Fisher's exact test when data were categorical. Longitudinal changes were assessed by Wilcoxon Signed Rank or paired Student's *t* tests. Correlation analyses were performed using Pearson's correlation coefficient or Spearman's rho, depending on the presence or absence of normal data distribution as assessed by Kolmogorov-Smirnov. When correlation analysis was performed using change variables, log transformed data was included instead of raw numbers. For SEC-ICP-MS chromatograms, peak areas were derived using the standard width-at-half-height method in Prism Version 6h (GraphPad, USA), normalized to total protein content, and compared against peaks with corresponding molecular mass using a paired Student's *t*-test."

R3.2 ***"Multiple comparisons limit the results, especially as reported without adjustment for multiple comparisons."***

See response to R3.1 above.

R3.3 ***"Changes in biomarkers and cognitive measures might also be examined with baseline value as covariate rather than change scores."***

We initially performed linear regression analysis using baseline selenium as covariate, but no effect was observed. We decided not to show this information as we were unpowered for multiple comparisons adjusted for covariates.

R3.4 ***"Lovell MA, Xiong S, Lyubartseva G, Markesbery WR. Organoselenium (Sel-Plex diet) decreases amyloid burden and RNA and DNA oxidative damage in APP/PS1 mice. Free Radic Biol Med. 2009 Jun 1;46(11):1527-33."***

We have added reference to this work in the Introduction:

"…It also down-regulates the expression of BACE1, a key enzyme involved in the AD-associated amyloid deposition [18], and reduces levels of amyloid and markers of oxidative damage to RNA and DNA in APP/PS1 transgenic mice [19]."

R3.5 ***"Xiong S, Markesbery WR, Shao C, Lovell MA. Seleno-L-methionine protects against beta-amyloid and iron/hydrogen peroxide-mediated neuron death. Antioxid Redox Signal. 2007 Apr;9(4):457-67."***

We have added reference to this work in the Discussion

"…Since AD affected brain tissue has lower levels of selenium [11, 12] and selenium supplementation has been shown to directly interdict amyloid and iron neurotoxicity by modulating GPx activity [20], GPx4 expression could thereby have insufficient selenium supply, and on this basis supplementation trials are worth exploring."

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Original research

Supranutritional sodium selenate supplementation delivers selenium to the central nervous system: results from a randomized controlled pilot trial in Alzheimer's disease

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Running Title

Sodium selenate delivers selenium to the brain

Abbreviations

AD, Alzheimer's disease; ApoE, apolipoprotein E; CNS, central nervous system; CSF, cerebrospinal fluid; GPx, glutathione peroxidase; ICP-MS, inductively coupled plasma-mass spectrometry; MMSE, Mini-Mental Status Examination; MRI, magnetic resonance imaging; Se, selenium; SeMet, selenomethionine; SeO_4^{2-}, selenate; SeO_3^{2-}, selenite; SAE, serious adverse event; SEC, size exclusion chromatography; TEAE, treatment emergent adverse event

Clinical Trial Registry number and website:

The Vel002 study is listed on the Australian and New Zealand Clinical Trials Registry (http://www.anzctr.org.au/), ID: ACTRN12611001200976

Abstract

Insufficient supply of selenium to antioxidant enzymes in the brain may contribute to Alzheimer's disease (AD) pathophysiology; therefore, oral supplementation may potentially slow neurodegeneration. We examined selenium and selenoproteins in serum and cerebrospinal fluid (CSF) from a dual-dose 24-week randomized controlled trial of sodium selenate in AD patients, to assess tolerability, and efficacy of selenate in modulating selenium concentration in the central nervous system (CNS). A pilot study of 40 AD cases were randomized to placebo, nutritional (0.32 mg sodium selenate, three times daily), or supranutritional (10 mg, three times daily) groups. We measured total selenium, selenoproteins, and inorganic selenium levels, in serum and CSF, and compared against cognitive outcomes. Supranutritional selenium supplementation was well tolerated and yielded a significant ($p < 0.001$) but variable (95% CI = 13.4 - 24.8 µg/L) increase in CSF selenium, distributed across selenoproteins and inorganic species. Reclassifying subjects as either responsive or non-responsive based on elevation in CSF selenium concentrations revealed a moderate improvement in Mini-Mental Status Examination (MMSE) scores (+3.2 points, $p = 0.03$). Pooled analysis of all samples revealed that CSF selenium could predict change in MMSE performance (Spearman's rho = 0.403; $p = 0.023$). High-dose sodium selenate supplementation is well tolerated and can modulate CNS selenium concentration, although individual variation in selenium metabolism must be considered to optimize potential benefits in AD.

Key words

sodium selenate, selenium, Alzheimer's disease, supranutritional selenium supplementation, randomized controlled trial.

Introduction

Selenium is essential for normal neurological function [1]. Insufficient selenium intake produces inactive selenoproteins, which increases vulnerability to oxidative stress. In the brain, this has been associated with cognitive decline [2, 3]. This may contribute to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease (AD).
Oxidative damage is a biochemical hallmark of AD [4], and therapies designed to reduce cellular oxidative load may have therapeutic potential [5]. The clinical trials of selenium supplementation in AD patients are small in number and power, and have produced inconclusive data (reviewed by Loef *et al.* [6]). Nevertheless, reported associations between selenium status and cognition from human *post mortem* findings, as well as animal studies, indicate a potential role for selenium deficiency in AD.

There is no consensus yet regarding changes in circulating selenium levels in AD [7], although several large cohort studies reported lowered levels [8, 9, 3]. We recently reported that *post mortem* temporal cortex samples from AD cases contain ≈14% less total selenium than age-matched healthy controls [10]. The apolipoprotein-E (*APOE*) ε4 allele, which is the major genetic risk factor for AD, was also associated with a decrease in selenium in these samples, and a redistribution of selenium in the tissue from the membrane-bound and insoluble fractions to the soluble fraction [10].

A small-scale randomized pilot trial of subjects with mild cognitive impairment (MCI) [11], supplemented daily with selenomethionine (SeMet)-rich Brazil nuts (*Bertholletia excelsa;* ≈290 µg selenium/day; ~75% as SeMet [12]) found improved verbal fluency and constructional praxis after six months compared to controls [13]. In contrast, the large-scale primary prevention PREADViSE study (ClinicalTrials.gov identifier NCT00040378) found

that long-term (5.4 ± 1.2 years of supplement use) daily supplementation with SeMet (200 μg/day) did not decrease the risk for dementia in cognitively healthy males over 60 years of age [14].

Selenium supplementation can also be achieved using inorganic forms, such as selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}). Selenate reduces pathological tau hyperphosphorylation common to AD *via* activation of protein phosphatases 2A both *in vitro* and in animal models of tauopathies [15-17]. It also down-regulates the expression of BACE1, a key enzyme involved in the AD-associated amyloid deposition [18], and reduces levels of amyloid and markers of nucleic acid oxidation in APP/PS1 transgenic mice [19]. Our group recently reported results of a Phase IIa exploratory trial of selenate in AD (Vel002) [20], and showed that despite a significant amelioration of brain structural deterioration, there were no significant effects on cognitive performance outcomes. However, it is not proven whether selenate effectively delivers selenium into the central nervous system (CNS). In this study, as exploratory analysis, we examined the selenium concentration in serum and cerebrospinal fluid (CSF) taken from patients participating in this Phase IIa trial to assess the degree to which 24-week sodium selenate (Na_2SeO_4) supplementation at the doses used increased serum and CSF selenium concentration, the latter indicative of selenium uptake by the CNS.

Subjects and Methods

Study participants

The Vel002 study (Australian and New Zealand Clinical Trials Registry ID: ACTRN12611001200976) recruited patients in four centers in Melbourne, Australia, who were diagnosed with probable AD according to NINCDS-ADRDA criteria [21]. Eligible

subjects were ≥ 55 years old; presented with a modified Hachinski score ≤ 4 and a 'mild' to 'moderate' degree of dementia, as defined by a Mini-Mental Status Examination (MMSE) score of between 14 and 26 at screening; were under treatment with an acetylcholine esterase inhibitor at a stable dose for at least four months; and had a documented volumetric MRI brain scan performed within 14 days of baseline that revealed no gross structural abnormality. Exclusion criteria were: contraindication for lumbar puncture; history of alcohol and/or other substance abuse; known sensitivity to selenium; presence of any other dementia syndrome or other neurological or psychiatric illness; significant medical disease not adequately controlled; history of epilepsy, diabetes, impaired renal, hepatic, or hematological function; known history of familial AD; current or recent (within six weeks of screening) treatment with lithium, NMDA receptor antagonists, steroids, or injectable non-steroidal anti-inflammatory drugs; current treatment with carbamazepine, digoxin, phenobarbitone, phenytoin, or warfarin; and consumption of dietary supplements containing more than 26 µg selenium/day [20].

Informed consent was obtained before the interview from all participants or their legally authorized representative, and the participant's caregiver. The study was approved by the Melbourne Health Institutional Ethics Committee.

Study protocol

Full details of the trial protocol can be found in Malpas *et al.* [20]. This was a double-blind, randomized, placebo-controlled pilot study. An initial screening visit was performed to confirm AD diagnosis by an experienced clinical neurologist or neuropsychiatrist. Following screening, 40 participants were randomly assigned to one of three study groups for 24 weeks' treatment: placebo, 'nutritional' (0.32 mg of sodium selenate, three times per day); and

'supranutritional' (10 mg of sodium selenate, three times daily). The randomization sequence was a 1:1:2 ratio, and this sample size was determined based on the early stage of investigation, i.e. Stage IIa as reported by Malpas *et al*. [20]. All investigators, participants, and caregivers remained blinded to randomization status until the conclusion of the trial [20]. A total of 36 participants completed the study. The participant CONSORT flow chart is shown in Fig 1. A list of the biofluid samples for this study is provided in Supplementary Table 1.

Determination of total selenium concentration in serum and CSF

Selenium concentration in serum and CSF was measured using inductively coupled plasma-mass spectrometry (ICP-MS). Neat serum and CSF were diluted in 1% nitric acid (1:20 and 1:3 respectively, to 300 µL final volume). Selenium was measured on mass at *m/z* = 78 (^{78}Se; natural abundance = 23.8%) using an Agilent Technologies 7700x ICP-MS system (Agilent Technologies, Australia) fitted with 'cs' lenses and platinum cones. Hydrogen (4 mL/min) was used as a reaction gas to remove polyatomic interferences at *m/z* = 78. Values were the average of four technical replicates. Selenium concentrations were calculated by external calibration using multi-element standards (AccuStandard, USA) containing of 0, 0.1, 0.5, 1, 5, 10, 50, 100 µg/L of selenium. An internal standard solution containing 200 µg/L of yttrium (^{89}Y) was introduced online *via* a Teflon T-piece. Analytical validity was assessed using reconstituted lyophilized Seronorm™ Trace Elements in Serum (Sero AS, Norway) standard reference materials, which was prepared using the same protocol for serum samples. The measured analytical recovery of selenium in the Seronorm™ standard was within the acceptable range, per manufacturer's guidelines (measured serum = 153.89 ± 6.48 µg/L, *n* = 4; certified range = 95–176 µg/L).

Chromatographic analysis of selenoproteins

Frozen aliquots of serum and CSF samples from the supranutritional group (baseline and 24 weeks) were brought to 4 °C and transferred into standard glass chromatography vials with polypropylene low-volume inserts. A 20 µL injection of neat serum/CSF was resolved using a BioSEC3 150 Å, 4.6 x 300 mm size exclusion chromatography (SEC) column (Agilent Technologies) with a molecular weight range of 500 to 150,000 Daltons (Da) on an Agilent Technologies 1200 Series liquid chromatography (LC) system equipped with a Peltier-cooled (4 °C) autosampler. A 200 mM ammonium nitrate buffer containing 10 µg/L cesium and antimony as online internal standards (see Lothian and Roberts [22]) was adjusted to pH 7.5-7.7 with 28% ammonium hydroxide and used as the isocratic mobile phase (0.4 mL/min flow rate) for all separations. The column was calibrated for molecular mass estimation using a standard mix of heteroatom and metal-containing proteins [23], and injections of sodium selenate (1.88 ppb) prepared in the chromatography buffer were used to estimate selenate retention time in serum and CSF samples. Selenium was measured with the same instrument configuration described above. The size exclusion chromatography (SEC) eluent was directly connected to the concentric nebulizer (Glass Expansion, Australia) of the ICP-MS *via* polyethyl ether ketone tubing. The LC and ICP-MS systems were controlled using Mass Hunter (Agilent Technologies) and all SEC-ICP-MS chromatographic traces were measured in time resolved analysis mode.

Cognitive testing

Cognitive testing was performed at baseline and week 24. Conventional ‘pencil-and-paper’ tests were administered, including the Alzheimer’s Disease Assessment Scale cognitive subscale (ADAS-Cog), MMSE, controlled oral word association test (COWAT), and the

category fluency test (CFT). Three tests were also administered from the CogState computerized battery (CogState Ltd, Melbourne, Australia). These included the one-card learning memory task (OCL), identification reaction time task (IDN), and the detection reaction time task (DET).

Statistical analysis

All statistical analyses were conducted on the intention to treat population. Data were included for all participants enrolled in the study who had complete data for this exploratory analysis. Baseline characteristics were compared between groups using ANOVA for continuous data and using Fisher's exact test when data were categorical. Longitudinal changes were assessed by Wilcoxon Signed Rank or paired Student's *t* tests. Correlation analyses were performed using Pearson's correlation coefficient or Spearman's rho, depending on the presence or absence of normal data distribution as assessed by Kolmogorov-Smirnov. When correlation analysis was performed using change variables, log transformed data was included instead of raw numbers. For SEC-ICP-MS chromatograms, peaks areas were derived using the standard width-at-half-height method in Prism Version 6h (GraphPad, USA), and compared against peaks with corresponding molecular mass using a paired Student's *t*-test.

Changes in selenium biomarkers (*i.e.* total selenium and chromatographically-separated selenoproteins) and cognitive tests were calculated as the difference between values at 24 weeks and baseline. Participants were classified as responsive or non-responsive to selenate treatment according to the difference in measured indicators of selenium status, with the responsive group presenting an increment in serum or CSF post treatment at least three times above the highest value at baseline. Comparisons between responsive and non-responsive

groups were performed using a Mann-Whitney *U* test. The Kruskall-Wallis followed by Dunn's post hoc test was conducted on the comparison of the changes in selenium levels in CSF normalized by change in serum in the three treatment groups. All statistical analyses were carried out using the Statistical Package for the Social Sciences software, version 22.0 (SPSS; IBM, USA), and figures were constructed in Prism version 6h (GraphPad, USA) and Adobe Illustrator CC 2018 (Adobe Systems, USA). Statistical significance was set at $p < 0.05$.

Results

Studied groups were similar for age, sex, MMSE score, and *APOE* ε4 allele frequency (Table 1), as well as for selenium biomarkers (Table 2). All but one participant were selenium sufficient at baseline according to the plasma selenium reference range of 84-100 µg/L recommended by Thomson [24] required to maintain adequate glutathione peroxidase (GPx) activity and selenoprotein P levels (Table 2). Both experimental groups receiving sodium selenate treatment showed significant increases in selenium concentration in serum (nutritional +45%, $p < 0.01$, Student's *t* test; supranutritional +504%, $p < 0.001$, Wilcoxon Signed Rank test) and CSF (nutritional +69.3%, $p < 0.05$; supranutritional +1680%, $p < 0.001$, Student's *t* test; Table 2; Fig 2) from baseline. One individual in the nutritional group had unchanged serum selenium concentrations and decreased levels in CSF, which we believe was due to poor compliance with treatment regime. As expected, there was a dose-dependent effect on serum selenium concentration, with the magnitude of change in serum selenium concentration in the supranutritional group showing an approximately ten-fold increase in serum concentration compared to the nutritional group at the study conclusion. CSF selenium was only moderately increased by nutritional supplementation (+56%, $p < 0.05$ *vs* baseline),

whereas supranutritional intake produced a more marked change (+1395%, $p < 0.001$ *vs* baseline) with high variance between participants.

As CSF represents the main export pathway from the brain, increased selenium concentration is indicative that more has entered the CNS. We therefore examined the correlation between serum and CSF selenium concentration to assess the neuro-bioavailability of selenate. At baseline, no correlation was observed between serum and CSF concentration (Fig 3A). Post-treatment, there was also no correlation between serum and CSF selenium concentrations for either the placebo or nutritional groups, although these sample sizes were small (Fig 3B, C). Post-treatment, only the high-dose supranutritional group showed a correlation between serum and CSF selenium ($r = 0.653$, $p < 0.05$, Spearman's rho; Fig 3D). We examined whether the dimension of the change in CSF selenium content after 24 weeks' treatment was commensurate with the change in serum selenium content in our three treatment groups. We analysed the change in serum selenium matched to the change in CSF selenium in subjects where both samples were available and assayed at baseline and 24 weeks of treatment. The mean changes in each group indicated that the boost in serum selenium following supplementation was matched by a muted increase in CSF selenium. Of the increase in serum selenium in the two supplementation group, only ≈3% of the increase was transduced into the CSF (Fig. 3E). The change in CSF selenium was approximately proportional to the change in serum in both the nutritional and the supranutritional selenate supplementation groups. In the supranutritional group, the increase in CSF selenium as a proportion of the change in serum selenium was significantly more than in the placebo group (Fig. 3F, ≈3%, Dunn's test $p = 0.0019$), consistent with a small proportion of the boost in serum selenium surmounting the blood brain barrier with this high dosage regimen.

According to the original trial data reported [20], the diffusion tensor imaging (DTI) analysis revealed that the supranutritional group had less of a reduction in white matter organization, evidenced by decreased mean, axial and radial diffusivity. This may suggest a measurable clinical benefit, as sodium selenate may have a direct effect on slowing white matter atrophy in the human brain. We analysed subsets of serum and CSF (n = 11, samples with sufficient volume left after other measurements) from the supranutritional-dose group by SEC-ICP-MS to assess treatment-induced changes in selenium-containing macromolecules and low molecular weight inorganic selenium species. Selenate supplementation clearly increased binding of selenium into serum proteins (Fig 4A), with a large increase (+656%; $p < 0.001$, Student's t-test) in the earliest eluting peak (Peak #1, Fig. 4A). We have previously characterized the selenium content in this peak by SEC-ICP-MS and MS/MS bottom up proteomics, and confirmed the presence of both selenoprotein P (comprising ≈50% of this peak) and albumin that could not be discriminated by chromatography at this resolution [25]. Selenoprotein P has a molecular mass of 43 kDa, but is highly glycosylated. The molecular mass of albumin is 67 kDa, and Peak #1 has an apparent M_r of 75 kDa against size standards. Using this approach, we cannot discriminate between selenium incorporated as selenocysteine and that being transported *via* transient binding to free thiol groups on serum albumin [26]. Chemically inert buffers at physiological pH used for SEC-ICP-MS preserve the integrity of selenium thiol ligands, with the compromise being relatively low separation efficiency [27]. As such, we suspect that Peak #1 contains highly-abundant serum albumin followed closely (without resolution) by selenoprotein P. Differentiating the proportional increase in selenium directly attributable to both proteins would require higher resolution chromatographic methods [28], though the effects of the denaturing conditions typically employed on albumin-selenium binding have not been characterized.

Two additional resolved peaks were also significantly increased ($p < 0.001$, Student's *t*-test) in serum from the supranutritional group, representing inorganic selenate and an unidentified selenium-containing compound, both eluting below the lower molecular weight limit of the SEC column. Interactions between the silica column and negatively charged inorganic selenium compounds are known to influence retention time in SEC-ICP-MS [29], thus we suspect that the peak following the selenate standard (serum Peak #3, CSF Peak #4, Fig 4) might be selenite (SeO_3^{2-}). A previous analytical study of the stability of selenocompounds in human serum suggested selenite is not present in freshly-drawn serum and is an artifact of storage, but the report examined serum from only two donors, with total serum selenium at levels not commensurate with our supranutritional group [30]. Regardless of its chemical species, the appearance of this selenium-containing peak at 24-weeks is indicative of a specific response to supranutritional selenium supplementation.

In CSF samples, we again identified a marked increase in the low mass peaks, selenate (Peak #3 provisionally, +29.7%, $p < 0.001$) plus selenite (suspected for Peak #4), following supranutritional supplementation. The lower total selenium concentration in CSF reduced the effect of peak tailing, allowing selenoprotein P (likely to be Peak #2), which we have previously confirmed in CSF samples using a targeted proteomics approach [25], to be resolved from albumin (likely to be Peak #1), which is also present in CSF [31]. Selenium bound to albumin was markedly increased in the supranutritional group (Peak #1 in Fig 4B, +3022%, $p < 0.001$), as was the selenoprotein P peak following selenate treatment (Peak #2 in Fig 4B, $p < 0.001$; Table 3). Both selenium-binding proteins were below the limit of detection in untreated CSF, consistent with previous findings [32]. It was also apparent from the ratios of the selenium in the chromatographic protein peaks to the low mass peaks (Table 3), that selenate supplementation boosted selenoprotein production far more in the periphery (serum. Fig 4A) than in the brain (CSF, Fig 4B). Thus, to augment brain levels of selenoprotein P

(and potentially other selenoproteins) may require higher levels of selenate precursor than peripheral organs.

In both the nutritional and supranutritional groups there was considerable variance in the response in serum and CSF selenium concentration to selenium supplementation. By stratifying the data into responsive (defined as change in serum and CSF three times above the highest baseline value) or non-responsive (all remaining samples), regardless of dose, we were able to reassess the effects of selenate supplementation in cognitive performance *per* the original trial outcomes report [20]. For the MMSE readout, according to the CSF marker, the unresponsive group deteriorated during the trial by -3.1±3.5 points ($p < 0.0049$; paired Student's *t* test), but the responsive group did not significantly deteriorate (-0.4±3.0 points; $p = 0.646$; paired Student's *t* test), with paired analysis indicating that the difference between these two groups was significant ($p = 0.03$; Mann-Whitney *U* test; Fig 5A). No differences were observed in any other measures of cognition (Fig 5B-G). Pooled CSF selenium concentration from all groups correlated with change in MMSE performance ($r = 0.403$; $p < 0.05$, Spearman's rho; Fig 5H), indicating that change in CSF selenium may be associated with improved cognitive performance following supplementation.

Treatment emergent adverse events (TEAEs) were reported previously according to treatment group, with 90% reporting at least one TEAE [20]. To summarize, all TEAEs were reported as mild. The most common solicited TEAEs (incidence ≥ 20% in both placebo/nutritional and supranutritional groups) were fatigue, headache, and lethargy; with nausea, muscle spasms, and dizziness reported in the supranutritional group. One participant experienced a pre-syncopal serious adverse event that was resolved in 24 hours and continued in the study. Two supranutritional group participants withdrew due to TEAEs; one due to appearance of a skin rash of uncertain cause, and the other resulting from dysmorphic changes in toenails and

fingernails. In the latter case, the participant had the 24-week observation and was not excluded from further analysis. On application of our responsive/non-responsive stratification, no relationship between the number of TEAEs and level of selenium uptake was observed ($p = 0.976$ for serum; $p = 0.900$ for CSF; Fisher's exact test).

Discussion

While the readouts of this pilot study of selenate supplementation in AD reported no significant benefit on cognitive performance [20], there was no stratification by biofluid selenium biomarkers in the initial report. Here, we find that the retention of selenium in serum and CSF was highly variable in this study. When the participants were stratified according to response in biofluid to the selenate supplementation, a significant arrest in cognitive deterioration on MMSE was noted. This was not corroborated by the other performance tests used. However, this pilot study was underpowered to detect cognitive changes. Our findings inform future trial design of selenium supplementation, supporting stratification of outcome measures by biofluid selenium changes.

Due to concern about the potential toxicity of inorganic selenium [33], intervention studies have tended to focus on organic compounds. For instance, a double-blind, randomized, placebo-controlled trial of selenium-enriched yeast supplementation [34], which contained SeMet at 54-60% of the total selenium (including inorganic species) found that a dose of 300 µg of selenium-enriched yeast per day over five years was well tolerated by older adults (mean age 66.1 years) during the 5 years of dosing. However, at a 10 year follow-up, this dose group exhibited increased all-cause of mortality (hazard ratio = 1.59, 95% CI = 1.02-2.46), especially for those who had baseline plasma selenium ≥ 82 µg/L (hazard ratio = 2.20, 95% CI = 1.16-4.17) [35]. While such studies do raise concerns regarding the possible long-

term toxic effects of the dose used in our trial (equivalent to 12.5 mg of elemental selenium per day, much higher than Rayman *et al.* [35]), our six month intervention did not report any life-threatening TEAEs. In contrast to this long-term supplementation study of healthy adults, we tested high dose selenate treatment as a chemotherapeutic disease-modification intervention.

While significantly more subjects in the Supranutritional group (35%) experienced TEAEs compared to the placebo group (10%) [20], the adverse effect profile was similar to that associated with toxicity resulting from consumption of a misformulated nutritional supplement in the US in the late 2000s that contained selenium at ≈400-fold the recommended daily allowance (RDA) [36, 37]. In this incident, an unknown number of consumers inadvertently received a daily selenium dose in the order of 30 mg equivalent, as well as ≈30 mg of chromium and other substances. We note that the misformulated supplement exposed subjects to an average of ≈30 mg of elemental selenium per day. The highest dose we tested was 30 mg of sodium selenate per day in divided doses, which is equivalent to 12.5 mg of elemental selenium per day, i.e. less than half the dose of selenium in the misformulated supplement. Furthermore, the chromium in the misformulated supplement (17-fold the RDA), as well as many other bioactive ingredients in the mixture, may have potentiated the toxicological burden. In any case, since our trial of sodium selenate at 30 mg per day was completed under controlled conditions, the data are important to examine whether this drug can be used safely in this disease context. Our observations that 30 mg of sodium selenate per day for 24 weeks, being less than half that of the misformulated supplement, without serious adverse events is reassuring for the use of this dose as a chemotherapeutic, where the benefits may exceed the risk of mild TEAEs.

Our data indicate that selenate supplementation promotes protein incorporation, supporting the possibility that inorganic selenium toxicity in the brain is mitigated by endogenous selenoprotein production [38]. Furthermore, it has been shown that while both SeMet and selenate are readily bioavailable (both > 90%), the half-life of selenate is considerably shorter [39]. It has been suggested that the dose-response for selenium intake and benefits to human health follows a U-shaped relationship, indicating that selenium supplementation to populations with adequate or high selenium status could cause adverse effects [40]. Further studies to clarify risk-benefit profiles for different selenocompounds are needed, particularly when being used as a treatment for a terminal condition such as AD.

To our knowledge, this is the first study to determine selenium concentration in paired serum and CSF samples before and after supplementation, which allowed us to evaluate the bioavailability of sodium selenate to the CNS and its ability to promote selenoprotein synthesis. Following supplementation, the distribution of selenium into selenoproteins as a proportion of total selenium in the biofluid was much greater for serum than for CSF (Fig 4), which confirms that the blood brain barrier may limit the entry of selenium into the brain.

Our findings that small molecular weight selenium species are the dominant forms in the CSF are at variance with those of Solovyev *et al*. [32], who found selenoprotein P was the major selenocompound in CSF of healthy individuals, followed by selenomethionine bound to albumin. Both Solovyev and colleagues' study and the present work used archived frozen samples, thus the discrepancy between the studies is unlikely to be due to selenite artifact from storage [30].

Our observations are consistent with two potential fates for sodium selenate: i) reduction to selenite, according to a pathway previously identified in the gut [41]; and ii) direct

incorporation into selenoproteins that cross the blood-brain barrier, which would support efficiency of this compound in promoting antioxidant activity in the CNS. While selenoprotein P is the master regulator of selenium delivery to the CNS [42], glutathione peroxidase 4 (GPx4) is the most abundant selenoprotein in brain, and has recently garnered attention as an important regulator of ferroptosis [43], a newly identified form of iron-dependent programmed cell death that causes aggressive lipid peroxidation [44, 45] thought to play a major role in AD pathology [46]. Since AD affected brain tissue has lower levels of selenium [10, 6] and selenium supplementation has been shown to directly interdict amyloid and iron neurotoxicity by modulating GPx activity [47], GPx4 expression and activity may suffer from insufficient selenium supply, and on this basis supplementation trials are worth exploring.

Conclusions

This pilot trial showed that sodium selenate supplementation at a high or supranutritional dose induced an increase in selenium uptake into the CNS. The elevation in CSF selenium induced by treatment varied considerably among participants, indicating that factors, such as genetics, influence selenium delivery to the brain. Analysis of selenoproteins in CSF suggested inorganic selenium could increase expression and incorporation of selenium into biomolecules. When stratifying the study groups as either responsive or non-responsive to selenate supplementation, we found subtle but significant, improvement in MMSE score was associated with selenium CSF. Although 24 weeks of treatment was well tolerated, the potential benefits of selenium supplementation for AD must be weighed against recent data reporting increased mortality in healthy elderly subjects after long term supplementation.

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Figure legends

Fig 1 Participant CONSORT flow chart. "Analyzed" refers to subjects who completed the protocol, where cognitive testing was completed at baseline and week 24, and where at least one biofluid sample was measured for selenium content.

Fig 2 Selenium concentrations in serum (A, B and C) and cerebrospinal fluid (D, E and F) at baseline and after 24 weeks. A and D: Placebo; B and E: Nutritional group; E and F: Supranutritional group. A, B, D, E, F: Student's paired *t*-test; C: Wilcoxon Signed Rank test. * $p < 0.05$; *** $p < 0.001$.

Fig 3 Correlation between selenium concentration in serum and CSF. A) Baseline ($n = 32$). B) Placebo group, post treatment ($n = 6$). C) Nutritional group, post treatment ($n = 7$). D) Supranutritional group, post treatment ($n = 14$). E) Changes in serum selenium (x axis) and CSF selenium (y axis) in matched subjects (where both serum and CSF samples were available and assayed at both baseline and 24 weeks of treatment). Data are means ± SD, n = 6, 7 and 13 for placebo, nutritional and supranutritional groups, respectively. The axes are in log units to capture the large shifts in values as the doses are increased. F) Individual data points from each subject (with box and whisker analysis) expressed as the change in CSF selenium per unit change in serum selenium for each matched subject. The supranutritional group exhibited a small boost in CSF selenium when normalized to the change in serum selenium (≈+3%, or ≈30 mg/g). P value is from Dunn's multicomparison's test.

Fig 4 Selenium trace of supranutritional group samples at baseline (blue) and post treatment (red) on LC-ICP-MS. Dashed line = sodium selenate (1.88 ppb). Selenium-containing peaks identified based on mass alone and should be considered approximations. A) Serum: Peak #1:

co-eluting albumin-associated selenium and selenoprotein P; Peak #2: selenate; Peak #3: inorganic selenium; B) CSF: Peak #1: albumin-associated selenium; Peak #2: selenoprotein P; Peaks #3 and #4: inorganic selenium. Data are average ± SEM, n = 11 matched serum or CSF samples from the same subjects; *** $p < 0.001$, Student's t-test of area under curve.

Fig 5 Cognitive performance changes in subjects categorized as either responsive or non-responsive to sodium selenate treatment, according to serum (n = 17 and 18 for responsive and non-responsive, respectively) and CSF (n = 12 and 14 for responsive and non-responsive, respectively) changes. Changes (Δ) were calculated as [post-treatment − baseline] scores. A) MMSE: Mini-Mental State Examination (* $p < 0.005$, Mann-Whitney U test). B) ADAS-Cog: Alzheimer's Disease assessment scale - cognitive subscale. C) CFT: Category fluency test. D) COWAT: Controlled oral word association test. E) DET: Detection reaction time task. F) OCL: One-card learning memory task. G) IDN: Identification reaction time task. H) Correlation between changes in CSF selenium concentration and in MMSE score in combined responsive and non-responsive groups (r = 0.403; $p < 0.05$, Spearman's rho).

Table 1. Cohort characteristics at baseline.

Parameter	All (n = 36)	Placebo (n = 9)	Nutritional (n = 8)	Supranutritional (n = 19)	p value[a]
Age (y)[b]	70.2 ± 7.5	68.7 ± 6.9	73.4 ± 5.5	69.5 ± 8.3	0.316[c]
Sex, % men	41.7	33.3	50.0	24.1	0.904[d]
MMSE[b, e]	20.0 ± 3.7	20.3 ± 5.2	19.5 ± 2.4	20.0 ± 3.5	0.965[c]
APOE ε4[e] carriers, %	69.4	66.7	75.0	68.4	0.999[d]

[a] p value for between-group comparison; [b] Data presented as mean ± standard deviation; [c] ANOVA; [d] Fisher's exact test; [e] *APOE* ε4, apolipoprotein E epsilon 4 allele carriers; MMSE, Mini-Mental Status Exam.

Table 2. Selenium concentrations in serum and CSF at baseline and after 24 weeks treatment with sodium selenate.

	Placebo				Nutritional				Supranutritional			
	t = 0 w[c]	t = 24 w	Change		t = 0 w	t = 24 w	Change		t = 0 w	t = 24 w	Change	
	Mean ± SD[c] (n)	Mean ± SD (n)	95% CI[c] (n)	p value	Mean ± SD (n)	Mean ± SD (n)	95% CI (n)	p value	Mean ± SD (n)	Mean ± SD (n)	95% CI (n)	p value
Serum selenium (µg/L)	135.8 ± 40.0 (9)	143.8 ± 34.3 (8)	-51.6, 51.3 (8)	0.996[a]	122.2 ± 26.3 (8)	176.7 ± 46.2 (8)	20.3, 88.7 (8)	0.007[a]	145.4 ± 28.8 (19)	858.3 ± 447.1 (19)	497.4, 928.3 (19)	<0.001[b]
CSF[c] selenium (µg/L)	1.3 ± 0.4 (9)	1.4 ± 0.5 (7)	-0.4, 0.7 (7)	0.538[a]	1.6 ± 0.6 (6)	2.5 ± 0.7 (7)	0.2, 1.6 (6)	0.026[a]	1.4 ± 0.5 (17)	20.2 ± 9.1 (14)	13.4, 24.8 (13)	<0.001[b]

[a] Student paired test; [b] Wilcoxon Signed Rank test; [c] 95% CI, 95% confidence interval; CSF, cerebrospinal fluid; SD, standard deviation; w, weeks. A breakdown of the samples used for analysis is found in Supplementary Table 1.

Table 3: Relative changes in major selenium containing species in serum and CSF following supranutritional supplementation (as area under the curve of ^{78}Se signal intensity from SEC-ICP-MS chromatograms).

	Serum				CSF			
	t = 0 w[b]	t = 24 w	Change		t = 0 w[b]	t = 24 w	Change	
	Mean ± SD[b] (n)	Mean ± SD (n)	95% CI[b] (n)	*p* value	Mean ± SD[b] (n)	Mean ± SD (n)	95% CI[b] (n)	*p* value
Peak #1	1496±673.2 (11)	11316±5098 (11)	8563, 11077 (11)	<0.0001	4.4±9.5 (11)	137.4±42.9 (11)	113.8, 152.1 (11)	<0.0001[a]
Peak #2	838.1±38.5 (11)	6211±674.2 (11)	5057, 5688 (11)	<0.0001	< LOD (11)	123.3±18.1 (11)	113, 136.1 (11)	<0.0001[a]
Peak #3	672.6±48.8 (11)	6232±213.7 (11)	5403, 5716 (11)	<0.0001	1347±451.3 (11)	1748±678.8 (11)	253.9, 546.7 (11)	<0.0001[a]
Peak #4	–	–	–	–	351.7±121.4 (11)	1551±339.6 (11)	1040, 1360 (11)	<0.0001[a]

[a] Student paired test; [b] 95% CI, 95% confidence interval; CSF, cerebrospinal fluid; SD, standard deviation; w, weeks.

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Figure 1

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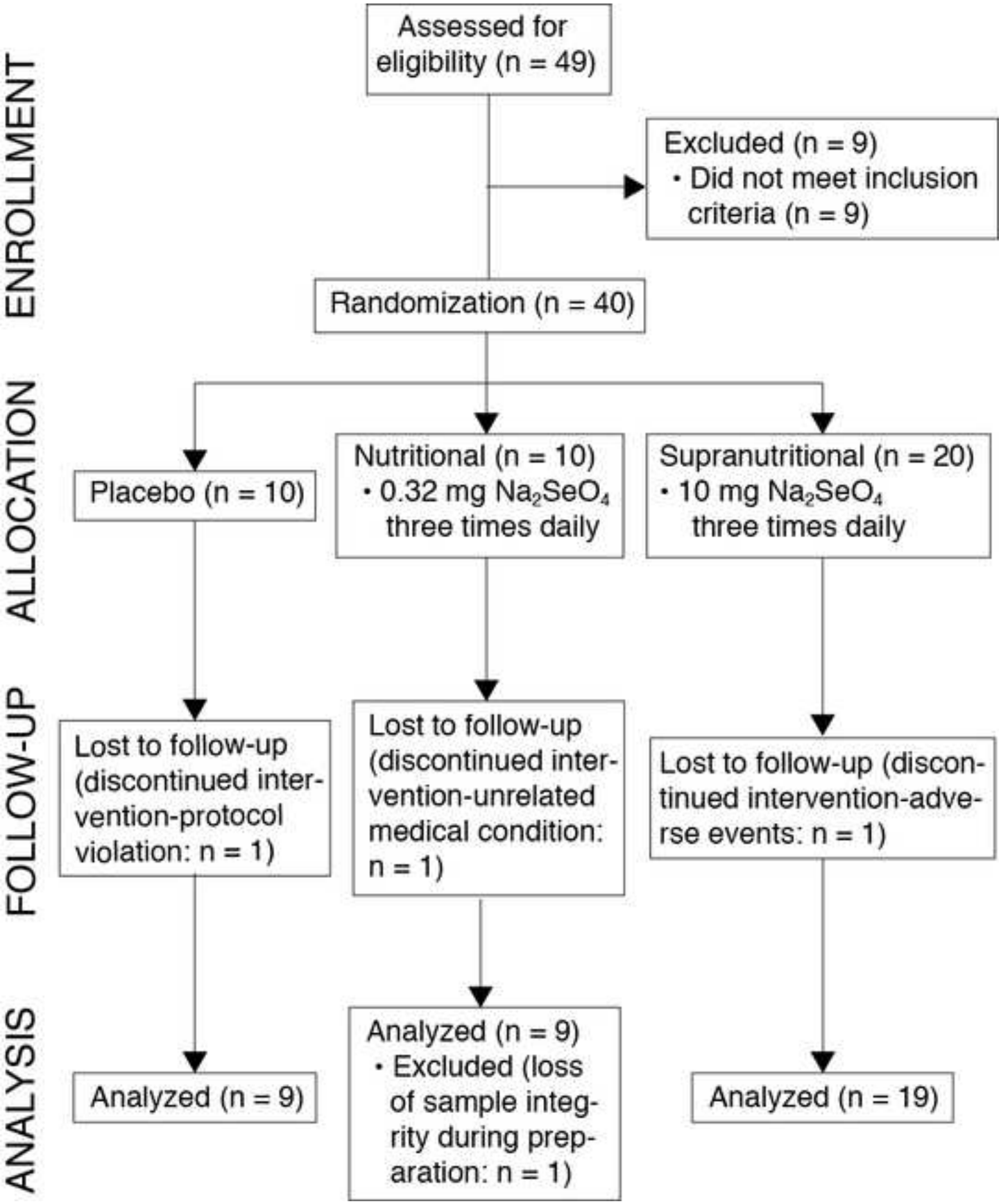

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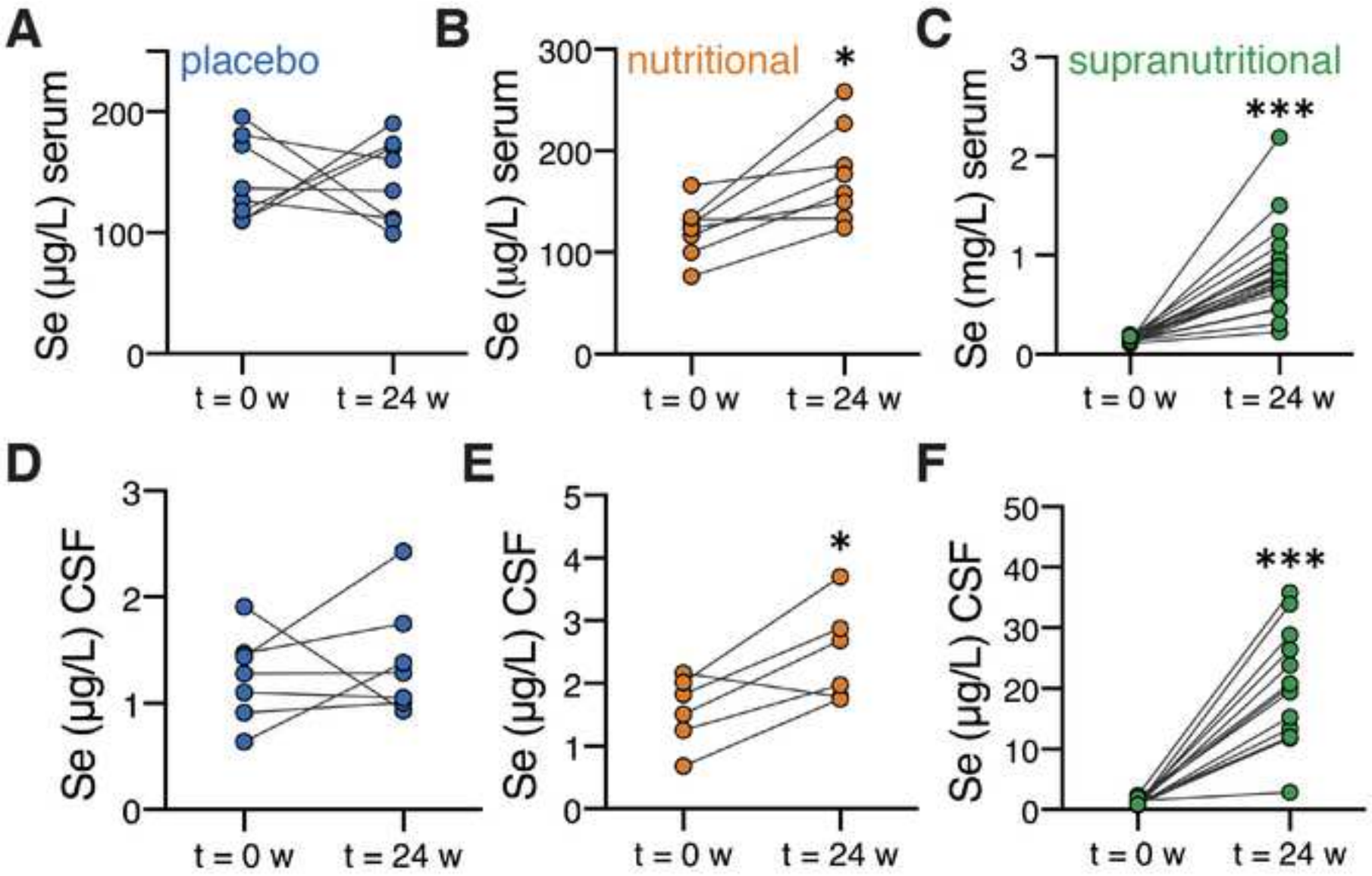

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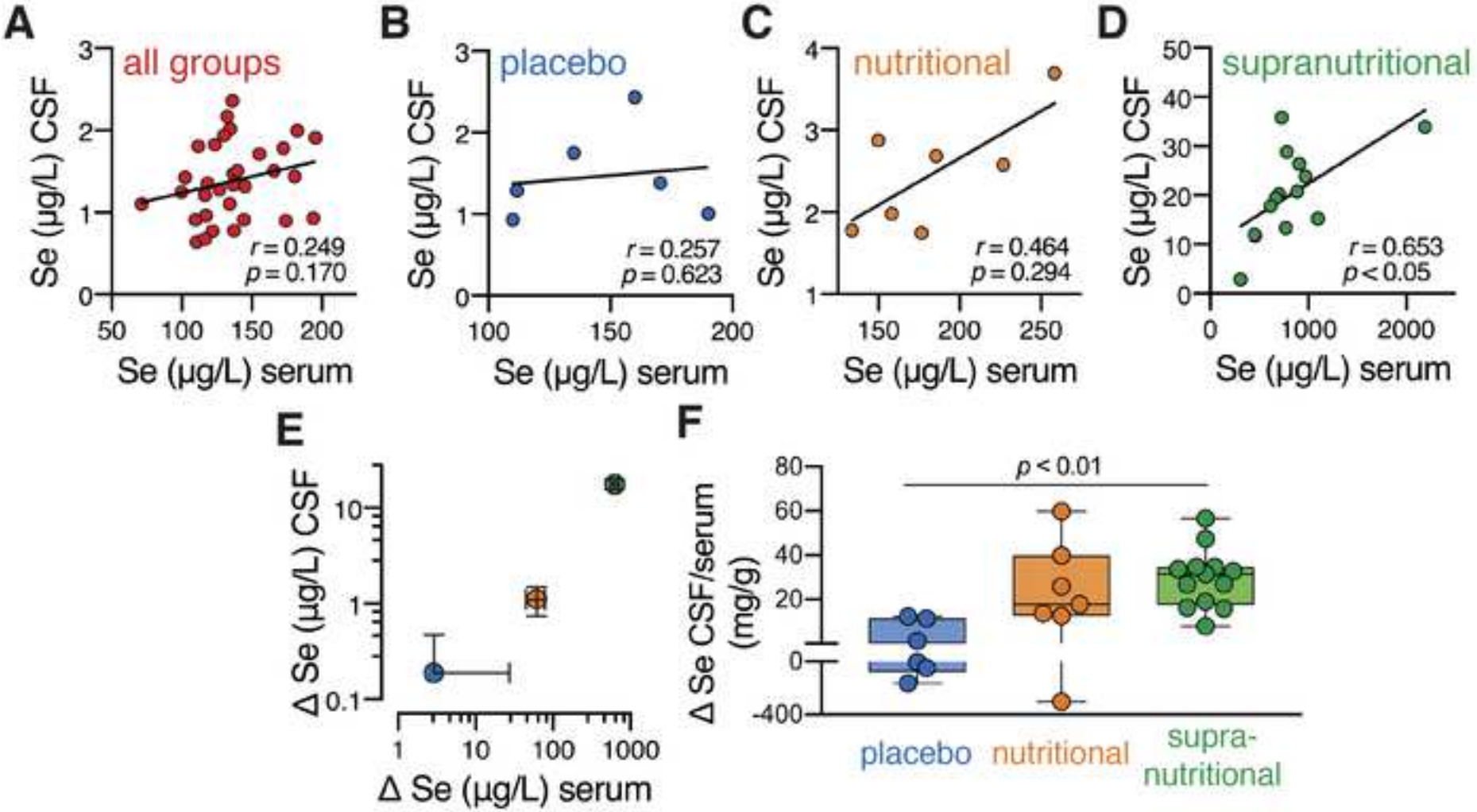

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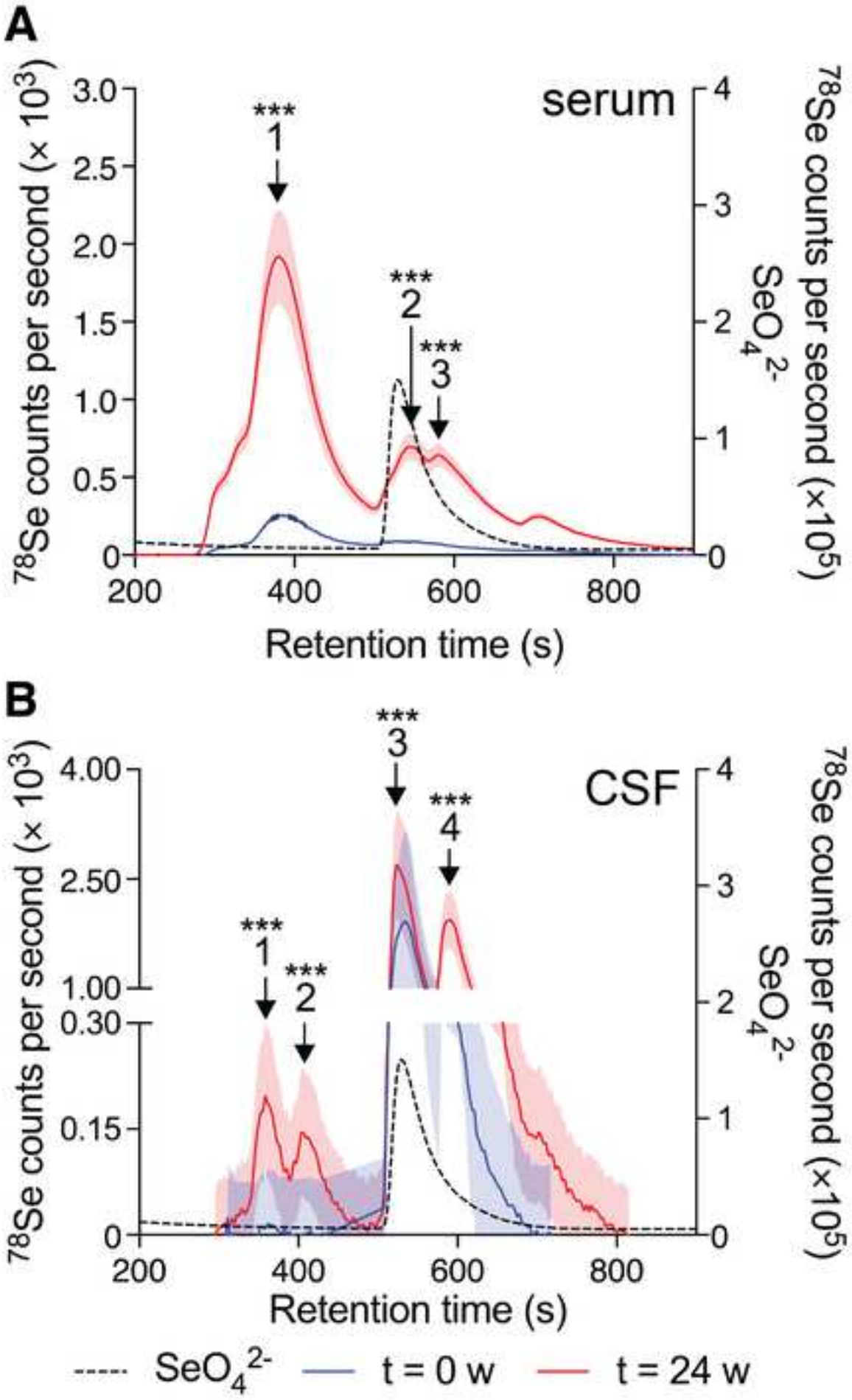

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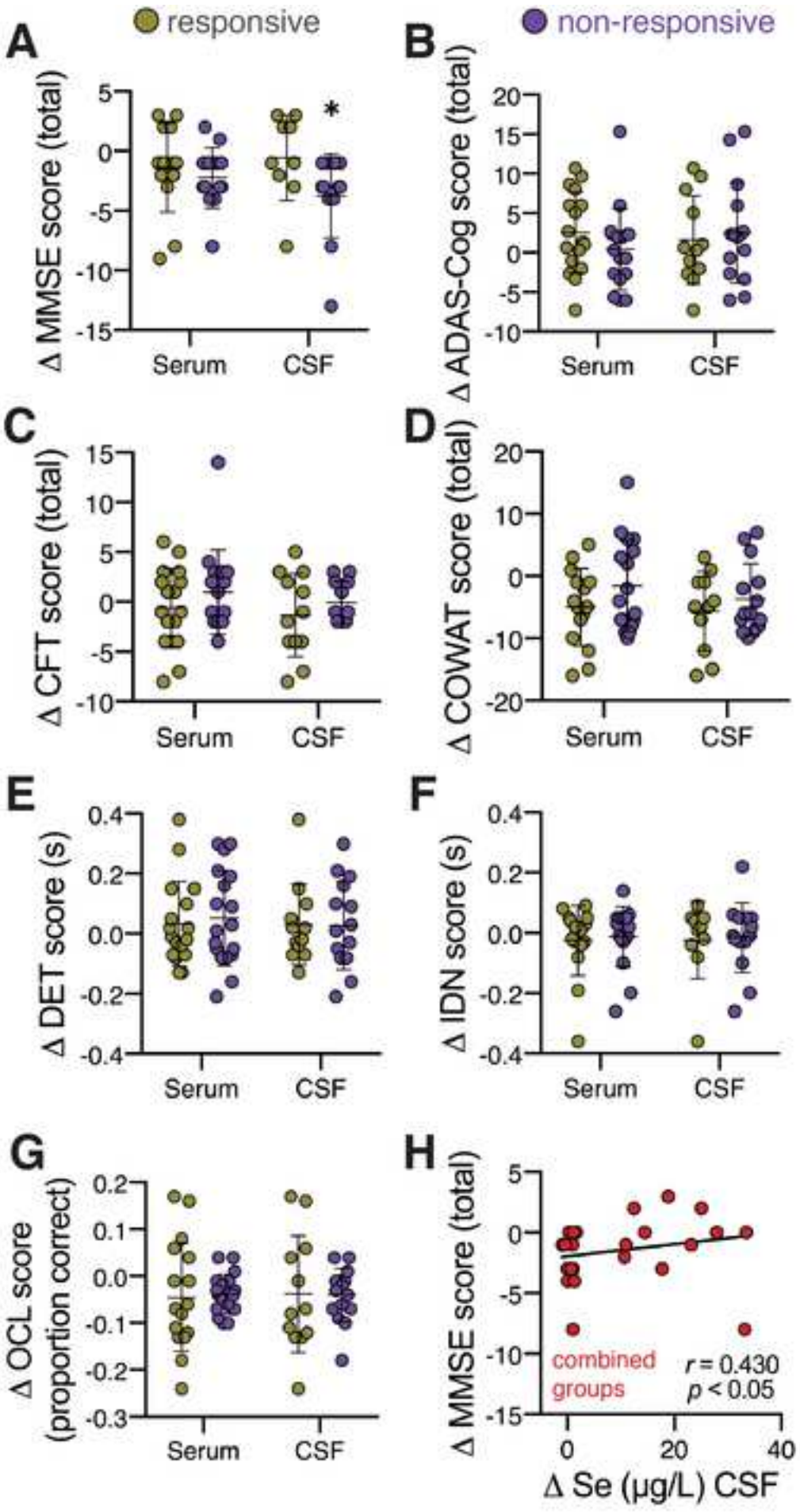

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