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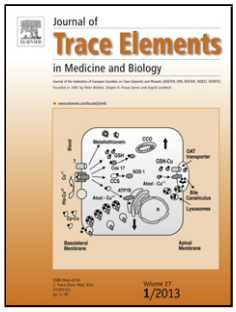

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Selenium speciation in paired serum and cerebrospinal fluid samples of sheep

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Short title: Selenium speciation in cerebrospinal fluid

Abstract

This study was performed to characterise selenium (Se) and Se species in cerebrospinal fluid (CSF) of sheep and its relation to the respective Se concentrations in serum. Paired samples from 10 adult sheep were used for the study. Five sheep were fed a diet with a marginal Se concentration of <0.05 mg Se/kg diet dry weight (dw, Se^{-}), and five animals were fed the same diet supplemented with sodium selenite revealing a concentration of 0.2 mg Se/kg diet dw (Se^{+}). The feeding strategy was conducted for two years; The results on metabolic effects were published previously. At the end of the feeding period, paired samples of serum and CSF were collected and analysed using ion exchange chromatography inductively coupled plasma – dynamic reaction cell – mass spectrometry (IEC-DRC-ICP-MS) technique for total

Se concentration and concentrations of Se species. Albumin concentrations were analysed additionally.

The feeding strategy caused significant differences ($p<0.01$) in total serum Se concentrations with 33.1 ± 5.11µg Se/l in the Se^- group and 96.5 ± 18.3 µg Se/l in the Se^+ group, respectively. The corresponding total Se concentrations in CSF were 4.38 ± 1.02 µg Se/l and 6.13 ± 1.64 µg Se/l in the Se^- and the Se^+ group, respectively, missing statistical significance ($p=0.077$). IEC-DRC-ICP-MS technique was able to differentiate the Se species selenoprotein P-bound Se (SePP), selenomethionine, glutathione peroxidase-bound Se (Se-GPx), selenocystine, thioredoxin reductase-bound Se, ovine serum albumin-bound Se (Se-OSA), SeIV and SeVI in ovine serum and CSF. Quantitatively, SePP is the main selenoprotein in ovine serum followed by Se-GPx. The CSF/blood ratio of albumin ($Q_{Albumin}$) reflected a physiological function of the blood-CSF barrier in all sheep. $Q_{Se\text{-}species}$ were higher than $Q_{Albumin}$ both feeding groups, supporting the hypothesis of local production of Se species in the brain. Significant positive regression lines for CSF vs. serum were found for albumin and Se-OSA only, suggesting a role of albumin to convey Se across the blood-CSF barrier. The ovine model, together with the IEC-DRC-ICP-MS technique to characterise the Se species, might be a worthwhile model for further studies as repeated samples collection as well as modification of the nutritional status is feasible and effective.

Keywords

Selenium; selenoproteins; sheep, cerebrospinal fluid, serum; IEC-ICP-DRC-MS

Introduction

Selenium (Se) is an essential trace element for mammals. Several regions of northern Europe as well as some parts of the US have low soil Se concentrations, and forage grown in these areas provide a marginal or even deficient Se supply for farm animals [1]. Therefore,

undernutrition with Se was found frequently also in sheep [2]. The main biological function of Se is believed to exist through its incorporation into selenoproteins, which happens mainly in the form of selenocystine residues as an integral constituent of reactive oxygen species detoxifying selenoenzymes such as glutathione peroxidases (GPx), thioredoxin reductases (TrxR) and selenoprotein P (SePP) [3]. Marginal Se supplementation leads to a decrease of the antioxydative enzyme activity, in the first instance obvious in cytosolic GPx activity [4], probably due to the low rank of GPx in the hierarchic regulation of selenoprotein expression [5].

Se and selenoproteins are also known to play an important role in brain function [6], however, little is known about the mode of exchange of Se or selenoproteins between the extracellular compartments blood and cerebrospinal fluid (CSF). CSF fills the larger spaces within and around the central nervous system (CNS) and regulation of the composition and the volume of the CSF is indispensable for proper brain functions [7]. At brain cell level the interstitial fluid (ISF) provides the environment for water and solutes to enter and to leave cells. ISF and CSF are very similar in composition, but differ significantly from that of blood plasma [7]. The responsible barriers are the blood-brain barrier separating blood and ISF within the parenchyma as well as the blood-CSF barrier. However, only the CSF is attainable for clinical laboratory diagnostics by puncture of the central spinal canal. The anatomical basis of the blood-CSF barrier is the arachnoid membrane, the pia mater, the ependyma lining the ventricles and the choroid plexus. The choroid plexus seems to be predominantly responsible for the CSF formation [7]. CSF is removed mainly by the arachnoid villus system, the lymphatic outflow system as well as by the perineural pathway [8,9].

Se concentration in the brain seems to be provided with high priority. Depletion studies in mice showed a strong decrease of Se in body tissues, but an only slight decrease in the brain [10]. Radiolabelling studies documented that the uptaken amount of ^{75}Se into the brain of rats depends on the Se status and shows a significant higher quantity in Se-deficient animals. The

authors assumed that the Se uptake is regulated by a brain region-specific expressed receptor and that the expression of the receptor is nutritionally regulated by the Se status. Within the CNS, CSF showed the highest uptake of the applied ^{75}Se (intraperitoneal injection) and the authors suggested a high turnover rate of this Se in the ventricular system [11].

There is also the demand to characterise Se species crossing the blood-CSF barrier. This topic was firstly approached by measuring Se species in paired samples (serum and CSF) of healthy human patients using SAX-ICP-DRC-MS as published recently [12]. The study presented here conducts this topic by Se speciation in paired ovine samples having sufficient vs. marginal nutritional Se supplementation levels. The results are a part of a comprehensive study with a main focus on metabolic changes comparing marginal and sufficient Se supplementation published previously [4].

Materials and methods

Chemicals and reagents

All chemicals and reagents used were of suprapure grade. The chemical list consists of the following: From CPI, Santa Rosa, USA: Certified selenium and rhodium (Rh) stock standards (1000 mg/l). From Sigma-Aldrich, Deisenhofen, Germany: Selenite, selenate, selenomethionine (Se-Met), selenocystine (Se-Cys), Se-TrxR (EC 1.8.1.9.), Se-GPx (EC 232-749-6), human serum albumin (HSA), TRIS buffer, protein standards for SEC mass calibration (α-2 macro-globuline, ferritin, γ-globuline, transferrin, glutamine synthetase, lysozyme, glutathione-disulfide, glutathione (reduced), citrate). From Merck, Darmstadt, Germany: Ammonium acetate and acetic acid. From Air Liquide, Gröbenzell, Germany: Ar_{liq} and methane (99.999% purity).

Preparation of SePP from human serum as a standard

SePP is not commercially available but its purification from human plasma using affinity chromatography is described in literature [13,14] and this method has been applied successfully for human samples in the past [12]. Briefly: 200 µl serum were separated at 1 ml/min with a Heparin-affinity column (Amersham, GE Healthcare Europe GmbH, Munich, Germany) using a linear 12 min gradient of A= 50 mM Tris, 10mM NH_4-acetate/acetic acid, pH 6.0 to buffer B =A but 800 mM NH_4-acetate, pH 8.5, and remaining at 100% B for another 8 min. SePP elution was monitored at 280 nm and Se was subsequently determined in fractions with FI-ICP-DRC-MS. For verification an aliquot of SePP fraction was analysed by SEC-ICP-DRC-MS, where it eluted at RT calculated for 60 kDa, which fits to literature data [15].

Animals and feeding strategy

The study was part of a comprehensive project, the general study design was presented previously [4]. Ten sheep were used for the examination presented here, briefly the study design was as follows: The adult Cameroon crossbreed sheep were fed either a total ration providing < 0.05 mg Se/ kg dry weight (dw, n=5, Se^-) representing a marginal Se supplementation or a total ration providing 0.2 mg Se/kg dw (n=5; Se^+) representing a sufficient Se supplementation for sheep. All other nutrients were adequately provided according to the dietary recommendations for sheep; the composition of the diets was monitored according to national standardised chemical analysis of animal feed. The feeding regime was applied for a biennial examination period. General inspection and recording of food intake was done daily. The sheep were weighed monthly.

Blood and CSF collections

Due to one main focus of the comprehensive study, all sheep underwent general anaesthesia to perform computed tomography (CT) of the lungs [16] at day 750 of the study. As it is

impossible to collect CSF by puncture of the ependymal canal in sheep without general anaesthesia due to resistance of the animals and subsequent risk of cerebrospinal damage, the anaesthetic stage (for the CT) was used to collect the CSF samples. The animals laid down in a prone position with the hips flexed and bending the hind legs in a cranial direction alongside the body. The skin in the lumbosacral area was shared and defatted with alcohol. The lumbosacral space was identified being at the midline depression between the last palpable dorsal lumbar spine and the first palpable sacral dorsal spine as described by [17]. A sterile needle (1.2 x 40 mm, Nipro Europe N.V., Zaventem, Belgium) was used to penetrate through the skin, subcutaneous tissue, the interarcuate ligament and the ligamentum flavum into the dorsal subarachnoid space. CSF welled up in the needle hub and was collected into a plain, sterile tube for serum samples (Sarstedt, Nümbrecht, Germany). The CSF was centrifuged at 10.000xg for 5 min, aliqoted in small tubes, shock frozen into liquid nitrogen and stored at -80°C until further analysis.

Serum was collected immediately before the collection of CSF from the Vena jugularis using a plain serum collection system (S-Monovette ®, Sarstedt, Nümbrecht, Germany). The serum was allowed to clot at room temperature for at least 30 min. Within one hour after collection the serum samples were centrifuged at 10.000xg for 10 min. The serum was obtained by careful aspiration using a standard 1000 µl pipette (Eppendorf AG, Hamburg, Germany). The serum was aliqoted and transferred to new tubes. Finally, the serum was shock frozen in liquid nitrogen and store at -80°C until further analysis.

Analysis of albumin

Albumin concentration in serum was analysed using the bromocresol green method (L+T, Eberhard Lehmann GmbH, Berlin, Germany; inter-assay coefficient of variation 3.2%, detection limit 2 g/l).

The CSF was centrifuged for 10 min at 200xg (Rotafix 32, Hettich GmbH und Co. KG, Tuttlingen, Germany) to eliminate cell debris. The supernatant was collected carefully using a pipette (Research® plus, Eppendorf, Hamburg, Germany). Subsequently, the CSF was concentrated by ultrafiltration (Vivaspin 500 10kDa MWCO, Sartorius StedimBiotech GmbH, Goettingen, Germany) and centrifugation at 15000xg (Thermo Scientific™ Heraeus™ Pico™ 17 Microcentrifuge, Heraeus Sepatech GmbH, Osterode, Germany) for 20 minutes. Albumin in the concentrated CSF was measured using gel electrophoresis (Elphoscan Mini Plus, Sarstedt, Nümbrecht, Germany).

HPLC-Parameters

Ion Exchange Chromatography (IEC)

IEC separation was performed as described by Solovey and colleagues [12]. In short terms, a Knauer 1100 Smartline inert Series gradient HPLC system (Knauer 1100 Smartline inert Series, Knauer, Berlin, Germany) was connected to an anion exchange column AG 11 (pre-column) + AS 11 (250 x 4 mm ID) from Thermo Scientific, Dreieich, Germany. The sample volume was 100 µl. The separation method used a gradient elution at 0.80 ml min^{-1} with 10 mM Tris-HAc, pH 8.0 (eluent A) and 10 mM Tris-HAc + 500 mM ammonium acetate, pH 8.0 (eluent B) as described previously [12]. The column effluent was mixed with 1 µg/l Rh (final concentration) as internal standard and directed to ICP-MS.

Size exclusion chromatography (SEC)

SEC was used to prepare mass-range characterised SEC fractions for a 2-D approach for species identification. SEC separation was achieved by two serially connected colums (Biobasic 300mesh column, Thermo Scientific, Dreieich, Germany, + TSK 40 S, Tosoh, Stuttgart, Germany, each 300 x 7.8mm ID). Tris-HAc (10 mM, pH 7.4) + 250 mM NH_4Ac was used as the eluent at a flow rate of 0.75 ml min^{-1}. Mass calibration was performed using

protein standards of defined molecular mass resulting in retention times vs. ln(kDa)= -0.557 x RT +14.551" (r^2=0.999). For subsequent analysis by the regular IEC-ICP-MS method the SEC column effluent from samples was fractionated by a Fraction Collector 100 (Pharmacia, Freiburg, Germany) and stored at -20 ºC. Retention time match of Se-compounds with respective Se-species standards for both methods serially applied was considered as identified Se species.

Inductively coupled plasma mass spectrometry

Total Se determination and peak quantification from chromatograms

Flow injection analysis ICP-DRC-MS was applied for total Se determination according to our previously published method [18]. In short terms: The Knauer 1100 Smartline inert Series binary HPLC system with a 100µl injection loop (Perkin Elmer, Rodgau-Jügesheim, Germany) was directly coupled to ICP-DRC-MS at 0.8 ml min^{-1} flow rate of 50 % eluent A and B, see above. Samples were diluted 0.8 + 1 by adding 1 µg/l Rh (final concentration) as internal standard.

From each FI-analysis and from each chromatogram (standards and samples) the quotient of Se vs. Rh was calculated. These Rh-normalized FI-signals or chromatograms were subject to peak area evaluation.

In preliminary experiments calibration curves for each available standard compound were generated and checked in comparison to the FI-ICP-DRC-MS quantification. Variables "b" and "a" of calibration equations (y=b*x + a) for the different Se species ranged between 17.35 and 18.21 for "b" and -2.26 and -2.88 for a, whereas the calibration equation elaborated by FI-ICP-DRC-MS was y=18.2x -2.29. Since only insignificant differences were found between species calibration curves (and respectively calculated concentrations) and additionally no significant difference was seen compared to the FI-calibration curve, the latter was used generally for quantification.

Peak quantification from FI-quantification and from chromatograms was done by comparing peak areas with peak area calibration curves from FI-ICP-DRC-MS.

Quality control

Quality control for total Se determination was performed by analysing samples additionally with our reference method for selenium, graphite furnace atomic absorption spectrometry, according to [19]. Additionally control materials "ClinCal Plasma Calibrator 9985" and "urine 8847" from Recipe, Munich, Germany were reconstituted as indicated on flask labels and the resulting solutions were diluted 1/50 (serum) or 1/10 (urine) with Milli-Q water before measurements. The ClinCal Plasma Calibrator 9985 was certified with 129 µg/l (mean value), we found 126 µg/l. The urine control was certified with 29.3 µg/l, whereas we found 30.2 µg/l. Furthermore we analyzed "NIST 1950 – human plasma certified reference material". The values for NIST 1950 were (certified / found) for total Se: 105.5 / 99.2 µg/l; SePP: 50.2 / 50.7 µg/l; Se-GPx: 23.6 / 16.7 µg/l; Se-HSA: 28.2 / 27.2 µg/l; Se (VI): no indication / 4 µg/l.

Additionally to standard retention time match, species identification was performed by a 2-D approach: A) mass range defined SEC fractions from CSF or serum samples were analysed with the regular IEC-ICP-DRC-MS method. Species identification was regarded as OK when species matched the standard compounds after serial analysis with both chromatographic techniques (match in first and second technique).

The inter-assay coefficient of variation for albumin analysis in serum was 3.2 % using an internal ovine serum standard. Additionally, two commercial reference serum samples (Human/ Human-high, Seronorm^{TM}, Billingstad, Norway) were used. Quality control in electrophoresis setup was done using commercial control material (Elphortrol normal/abnormal, Sarstedt, Nümbrecht, Germany).

Statistics

Data from total Se concentration in serum and CSF were tested for Gaussian distribution using the Shapiro-Wilk test and group differences were analysed using the student's t-test. These data are presented as mean ± standard deviation (SD). In some Se species, the analysed data were below the limit of quantification (LOQ) of 0.05 µg/l in serum as well as in CSF using IEC-ICP-DRC-MS. To include these data into a statistical evaluation the data below the LOQ were set uniformly to 50% of the LOQ (= 0.025 µg/l) and, consequently, the data set negated Gaussian distribution. Subsequently, these results were analysed using the Wilcoxon rank-sum test to compare the Se^{-} and the Se^{+} group for the respective parameter. This is permitted because this nonparametric test involves only the summation of ranks. These data were presented as median, interquartile ranges, minimum and maximum.

Linear regression analysis was used to analyse the relationship between selenoprotein P-bound Se and glutathion peroxidase-bound Se in serum vs. total Se in serum as well as to analyse the relationship between ovine serum albumin-bound Se and albumin in serum vs. CSF, respectively. Spearman`s correlation coefficients were evaluated according to non-Gaussian data distribution of Se species. The ratios of the respective parameters in CSF/serum were calculated and were given as quotients (Q_{analyt}).

All calculations were carried out using SAS 9.1 (SAS Institute Inc., Cary, North Carolina, USA). The study was approved by the Ethical Commission of the Lower Saxony State Office for Consumer Protection and Food Safety (AZ 33.12-42502-04-08/1579).

Results

General condition of the experimental animals

Detailed data of the experimental animals were published earlier [4,16]. In brief, during the whole experiment the sheep showed no abnormalities of behaviour and general health. They

showed physiological posture, movement, social behaviour and were alert. Furthermore, their food intake was normal as was the excretion of urine and faeces. There were no signs of hair loss or muscular dystrophy being common clinical Se deficiency symptoms in sheep. There was no significant increase or decrease in mean body weights when comparing both groups during the study. Final mean body weights were 39.4 ± 12.3 kg and 37.4 ± 8.8 kg for the Se^- and Se^+ group, respectively.

Total selenium in serum and CSF

The mean total Se concentration showed significant differences between the Se^- and Se^+ group whereas the mean total Se concentrations in CSF were concurrent but showed a tendency to be slightly higher in the Se^+ group ($p=0.07$). Data of the means per group for the total Se concentrations in serum and CSF are given in Tab 1. The mean $Q_{\text{total Se}}$ was 0.066 ± 0.026 and 0.136 ± 0.042 for the Se^+ and the Se^- group ($p<0.05$), respectively.

Concentrations of Se species in in serum and CSF

The IEC-ICP-DRC-MS was able to separate and detect up to 18 different Se species in serum and CSF, respectively. Not all of them, however, were present in each sample. Figure 1 shows examples for serum and CSF chromatograms. According to available standard compounds eight species could be assigned clearly to the following selenoproteins: selenoprotein P-bound Se (SePP), selenomethionine (Se-Met), glutathion peroxidase-bound Se (Se-GPx), selenocystine (Se-Cys), thioredoxin reductase-bound Se (Se-TrxR), selenium IV (SeIV), ovine serum albumin-bound Se (Se-OSA) and selenium VI (SeVI). For Se-Cys, more than 50% of data per group in serum and CSF were below the LOQ, the same situation was obtained for Se-Met in CSF. Therefore, theses data were not considered for further statistics. Significant group differences (Se^+ vs. Se^-: $p<0.05$) were found for SePP and Se-GPx in serum, respectively. Se-GPx in CSF showed a slight tendency ($p=0.094$) towards higher

concentrations in Se^+ group. Se-TrxR in CSF was measurable quantitatively in Se^+ animals, but was below the LOQ in CSF of all Se^- sheep ($p<0.05$). The median concentrations of the identified Se species are given in Tab 2.

Considering the total Se concentrations in serum and CSF, there was a percentage of non-identified Se species in serum of 62.2 % and 62.5% the Se^- and Se^+ group, respectively. In CSF the percentage of non-identified Se species were 46.8% in the Se^- group and 61.7 % in the Se^+ group.

Interrelationship of total Se vs. Se species within serum or CSF

In serum, there was an obvious interrelationship of the total Se concentration with the concentrations of SePP and Se-GPx (Fig. 2), respectively, considering all animals.

In CSF, regression analysis including all individuals resulted in no significant relationships between the total Se concentration and any Se species in the CSF.

Quotients and interrelationship of Se species between serum and CSF

The $Q_{Albumin*1000}$ was 6.13 ± 1.62 and 6.95 ± 1.64 for the Se^- and Se^+ group, respectively. A significant regression line was found plotting albumin in serum vs. albumin in CSF considering all animals (Fig. 3). There was also a significant result testing the regression of Se-OSA in serum vs. the Se-OSA in CSF as shown in Fig. 4. All other parameters tested for significant correlation between concentrations in CSF vs. serum were negative. Median and interquartile ranges of $Q_{Se\ species}$ and $Q_{Albumin}$ in the Se^+ and the Se^- group, respectively, are shown in Fig. 5.

Discussion and Conclusion

Methodical issues

Se speciation via IEC-ICP-DRC-MS is a highly sophisticated method to detect various Se species in very small quantities. The sensitivity of this method exceeds biochemical analysis of selenoproteins in biological fluids extensively due to limitations by the detection limits in biochemical methods. Initially, two other methods (graphite furnace atomic absorption spectrometry (SOLAAR M, ThermoFischer Scientific, Dreieich, Germany) and total reflection X-ray fluorescence (Picofox 2, Broker, Berlin, Germany) were tested to measure total Se concentration in CSF, but the concentrations were below the detection limit (data not shown). Beyond the accurate analysis of the total Se concentrations in serum and CSF, the Se speciation by IEC-ICP-DRC-MS satisfies by its independency from species specific variations in selenoprotein structures being a challenge in biochemical methods. For example, preliminary approaches to analyse SePP in serum of farm animals (pig, cattle, sheep) using a test established for human SePP in serum [20] failed (Renko and Humann-Ziehank 2013, not published data). Thus, this paper firstly describes data on ovine Se speciation in serum as well as in ovine CSF, completed by differentiation in marginal vs sufficient nutritional Se supplementation.

However, a distinct limitation is the small number of animals included into this study. This was caused by the study design of the main study, but additionally by the very high costs and efforts to analyse samples by IEC-ICP-DRC-MS. Nevertheless, the data give novel insights into Se and selenoproteins in paired ovine serum and CSF samples.

Se and Se species in serum

There were obvious group differences considering the mean Se concentrations in serum (Tab. 1). This reflects clearly the efficiency of the nutritional management leading to an obvious sufficient Se status in der Se^{+} group and a marginal Se status in the Se^{-} group. Further differentiation of selenoproteins is meaningful to characterise the metabolic outcomes of different dietary Se supplementations, but, generally, studies on selenoproteins (except for

GPx activity) in ovine serum are very rare. One early study [21] using the SDS-PAGE technique differentiated five selenoproteins in ovine serum allocating the 55.5-kDa protein to SePP and the 23.0-kDa protein to GPx.

The analysed Se species detected in the adequately Se supplemented group of our study showed a hierarchy in concentration (Se^+ group: SePP > Se-GPx > SeVI > Se-Met > Se-OSA > Se-TrxR ≈ SeIV). The hierarchy differs from the results found in humans serum [12]. However, this finding depicts that SePP is the main selenoprotein likewise in ovine serum followed by Se-GPx as shown for other species before [22,23]. The SePP concentration in human serum showed a leading relationship to the total serum Se concentration as long as the total Se was > 65 µg/l [12]. Our results from sheep suggest a similar pattern (Fig. 2), but separate regression analysis for data below and above 65µg/l was not satisfying due to the low number of animals included in our study. The percentage of SePP and Se-GPx in relation to the total Se concentration analysed by IEC-ICP-DRC-MS in serum of the Se^+ group comprised approximately 15.3 % and 9.61%, respectively. Compared to human SePP concentrations and GPx activity in serum analysed by ELISA and colorimetry [24], respectively, the ovine concentrations were just half of the human concentrations. That might be due, at least partially, to differences in the analytical methods. However, the relation SePP : Se-GPx was 1.6:1 in ovine serum being quite similar to the study [24] using human serum (1.7:1). Se-GPx in serum, which was mainly determined in former studies as GPx activity, was found previously to vary broadly over time comparing groups consuming the same feeding strategy as well as over time for individual animals [4]. An earlier study [25] suggested that about 25% of total serum Se is associated with GPx. Our results indicated that this proportion might be even lower (9.61% in Se^+ and 15.7 % in Se^-) being in accordance to 16.7 % reported previously using the SAX-ICP-DRC-MS method in human serum [12]. The occurrence of SeVI, Se-Met, Se-OSA, Se-TrxR and SeIV in ovine serum was likely, but was not analysed in this species before and was quantified here for the first time. However, the

quantity of Se species in serum not assignable to known selenoproteins was high (62.2 % and 62.5% the Se^{-} and Se^{+} group, respectively) being independent from the general Se status. This finding needs further investigation.

Only SePP and Se-GPx showed significant group differences (Tab. 2): The Se^{-} group showed only 17.4% of SePP concentration and 56.1 % of Se-GPx concentration compared with the Se^{+} group. Regarding Se-GPx, this finding is in accordance with former studies describing a higher GPx activity in serum of Se supplemented calves compared to marginal supplemented animals [26]. Moreover, effects on serum GPx activity were also found in the sheep of the current study as published earlier [4]. The obvious lower SePP concentrations in the Se^{-} group reflect the feeding strategy distinctly gaining attention for using SePP as a diagnostic parameter. Routine diagnostic approaches to analyse SePP in serum of farm animals (e.g. using ELISA technique) are not introduced yet, but might be an interesting tool prospectively. Obviously, our results underline the role of SePP in the hierarchy of selenoproteins in serum being paramount also in sheep as shown for other species before [27].

Se and Se species in CSF

The Se concentrations in CSF differ slightly comparing the individuals as well as comparing the calculated means of the Se^{-} and the Se^{+} group (Tab. 1). This result was in accordance with former studies [6,18,28] postulating a fairly independence of the total Se concentration in the CSF from the total Se concentration in serum. That underlines a high priority for Se uptake and retention in the CSF during marginal dietary Se supplementation. CSF was identified before as a key part of the brain Se homeostasis showing the highest uptake of the applied ^{75}Se in the brain, suggesting a high turnover rate of Se in the ventricular system [11]. However, there was a tendency to slightly lower mean Se concentrations in CSF of our Se^{-} animals. Considering the very long time period of marginal Se supplementation to the Se^{-} group in our study, one may speculate that permanent low Se status may progressively

overcome independency of the CSF Se concentrations from serum concentrations. That should be proved by repeated measurements in long term studies.

Among the selenoproteins in the Se^+ group, our experiment revealed SePP and SeVI as being main players in the CSF, followed by Se-OSA ≈ Se-GPx ≈ Se-TrxR > SeIV. Scharpf and colleagues [29] described SePP in human CSF as well as SePP mRNA in the brain tissue and postulated a Se-dependent antioxidative defence system for neurons and ependymal cells. While Solovyev and colleagues [12] also identified SePP in CSF being predominant, the SeVI concentration in their study was low and close to the detection limit. Whether the increased appearance of SeVI in our study is more related to some artefact, although the sample preserving cooling chain (-80 ^oC) was permanently maintained up to thawing directly before analysis, remains unclear. Presently, this finding is hard to explain. Interestingly, SeIV in CSF was found in the Se^- group in even higher concentrations compared to the Se^+ group suggesting an independency of SeIV from the general Se status of the animal.

The CSF/serum ratios

The mode of substances crossing the blood-CSF barrier still remains unclear for many analytes. Albumin, the main protein in mammalian serum, is synthesised by hepatocytes only, but can be found regularly in the CSF as well. Therefore, the ratio of albumin in CSF vs. albumin in serum ($Q_{Albumin}$) is a routinely used index for the integrity of the blood-CSF barrier. However, the CSF flow rate also seems to play an important role as a decrease of CSF flow rate was proposed to explain quantitatively the increase of CSF protein concentrations [30]. Human reference data are age-related impeding a general comparison with sheep, however, the $Q_{Albumin}$ was below 7.0 *1000 in both groups of sheep resembling to reference ranges of healthy human adults older than 40 years [31]. The $Q_{Albumin}$ of our experimental animals was nearly the same in the Se^+ and the Se^- group underlining an independency from Se supplementation. However, regression analysis of albumin in CSF vs. albumin in serum

showed a significant positive relationship (Fig. 3) supporting some mechanism for albumin transfer from serum into CSF.

It was suggested that megalin participates in brain Se/SePP uptake [32], as shown before for the SePP uptake by the kidney [33], however, that needs to be proven in prospective studies. General genetic inactivation of SePP compromises brain selenoprotein metabolism and Se retention leading to neurological defects, while liver-specific inactivation of SePP does not affect brain Se status, selenoprotein biosynthesis or Se retention. This study postulated an evidence for local SePP expression in brain to guarantee physiological function [34]. Our results, showing nearly identic median SePP concentrations in the CSF of the Se^- and the Se^+ group, are in accordance to the delineated view on SePP synthesis in the CNS.

The concentrations of Se bound to albumin were analysed in human serum [35] and CSF [12] before and were found in the presented study in sheep as well. Se-OSA was also found to be positive correlated in CSF vs serum (Fig. 4). As mentioned above, the needs of the CNS for Se to generate selenoproteins is generally accepted, the way of Se delivery is still unclear. As albumin is believed to cross the blood-CSF barrier this finding may suggest a prominent role of Se-OSA maintenance of Se delivery to the CSF. All other parameter tested for significant correlation between blood and CSF were negative.

$Q_{CSF\text{-}serum}$ calculated for the Se species (Fig. 5) were higher in Se^- sheep (except for Se-GPx) but high intra group variation impeded statistical significance. However, whereas $Q_{Albumin}$ showed comparable results to human reference values and the study of Solovyev and colleagues [12], the $Q_{Se\ species}$ were higher in both feeding groups in general. That may indicate a local production of these selenoproteins in the central nervous system as well as postulated for SePP [34] previously. However, there are no data published for sheep and, thus, these findings can serve for first orientation looking at Se species in ovine CSF vs. serum.

In conclusion, a modification of dietary Se supplementation leads to a distinctly higher total Se concentration in serum in sufficiently supplemented compared to marginal Se supplemented sheep, whereas the total Se concentration in CSF did not reflect the nutritional management. In addition to the total Se quantification, IEC-DRC-ICP-MS was able to differentiate the Se species SePP, Se-Met, Se-GPx, Se-Cys, Se-TrxR, SeIV, Se-OSA and SeVI in ovine serum and CSF. Quantitatively, SePP is the main selenoprotein in ovine serum followed by Se-GPx, both parameters being positively correlated with the total Se concentration. The $Q_{Albumin}$ reflected a physiological function of the CSF-blood barrier. $Q_{Se\ species}$ were higher than $Q_{Albumin}$ in both feeding groups in general, supporting the hypothesis of local production of selenospecies in the brain. Significant positive regression lines for CSF vs. serum regarding all animals were found for albumin and Se-OSA only, recommending an evaluation if albumin may play a main role to convey Se across the blood-CSF barrier, prospectively. The ovine model used here, together with a highly sophisticated analytical method (IEC-DRC-ICP-MS) to characterise the Se species at both sides of the functional blood – CSF barrier, might be a worthwhile model for further studies as repeated sample collection as well as modification of the nutritional status is feasible and effective.

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The authors declare that there are no conflicts of interest.

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Figure captions

Fig. **1** Example of chromatograms from paired serum and CSF samples.

Fig. 2 The relationship of SePP and Se-GPx in serum vs. the total Se concentration in serum without consideration of group (n=10); regression line SePP (—): $y = 0.1672x - 2.3035$, $R^2 = 0.6948$; $p<0.01$; regression line Se-GPx (----): $y = 0.1033x + 1.8488$, $R^2 = 0.4113$; $p<0.05$

Fig. **3** The relationship of albumin in serum vs. albumin in CSF considering all individuals (n=8); regression line: $y = 0.0084x - 0.053$. $R^2 = 0.6177$; $p<0.05$

Fig. **4** The relationship of Se-OSA in serum vs. Se-OSA in CSF considering all individuals (n=10); regression line: $y = 0.0762x + 0.1202$; $R^2 = 0.4651$; $p<0.05$

Fig. **5** Median and interquartile ranges of quotients ($Q_{CSF/serum}$) of Se and Se species and the mean quotients ($Q_{CSF/serum}$) of albumin (Se^- group: 0.006130 ± 0.00162; Se^+ group: 0.006950 ± 0.00164)

Table 1
Concentrations (mean ± standard deviation) of total Se in serum and CSF of the Se^- and the Se^+ group, respectively.

group	total Se serum [µg/l]	total Se CSF [µg/l]
Se^-	33.1 ± 5.11	4.38 ± 1.02
Se^+	96.5 ± 18.3[b]	6.13 ± 1.64[*]

[b] significant difference ($p<0.01$) between groups

[*] $p=0.077$

Table 2 Concentrations of the clearly identified Se species in serum and cerebrospinal fluid

		SePP [µg/l]		Se-GPx [µg/l]		Se-TrxR [µg/l]		SeIV [µg/l]		Se-OSA [µg/l]		SeVI [µg/l]		Se-Met [µg/l]	
		Se^-	Se^+	Se^-	Se^+	Se^-	Se^+	Se^-	Se^+	Se^-	Se^+	Se^-	Se^+	Se^-	Se^+
Serum	**median**	**2.58**	**14.85**[a]	**5.21**	**9.28**[a]	**<LOQ**	**2.44**	**0.516**	**2.53**	**1.45**	**3.26**	**1.92**	**5.32**	**2.01**	**4.49**
	min	1.24	4.10	4.079	7.06	<LOQ	<LOQ	<LOQ	<LOQ	0.716	1.29	0.154	0.187	<LOQ	<LOQ
	max	6.07	21.6	6.29	23.9	12.3	19.5	0.821	17.4	3.91	7.85	16.1	45.0	2.96	5.65
CSF	**median**	**0.603**	**0.617**	**0.255**	**0.47***	**<LOQ**	**0.228**[a]	**0.094**	**0.082**	**0.376**	**0.423**	**1.044**	**0.631**	ns	ns
	min	0.072	0.122	<LOQ	0.14	<LOQ	<LOQ	<LOQ	<LOQ	0.137	<LOQ	<LOQ	<LOQ	ns	ns
	max	0.731	3.20	0.393	1.07	<LOQ	2.838	0.936	1.039	0.408	0.834	3.553	2.27	ns	ns

[a] significant differences ($p<0.05$) between groups;

* tendency ($p=0.094$) for differences between groups;

ns = no statistics due to the facts that more than 50% of data in both groups were below the limit of quantification (LOQ)

< LOQ: analysed data were below the LOQ of 0.05 µg/l in serum/CSF. To include these data into a statistical evaluation the data were set uniformly to 50% of the LOQ (= 0.025 µg/l).

Table 2 Concentrations of the clearly identified Se species in serum and cerebrospinal fluid

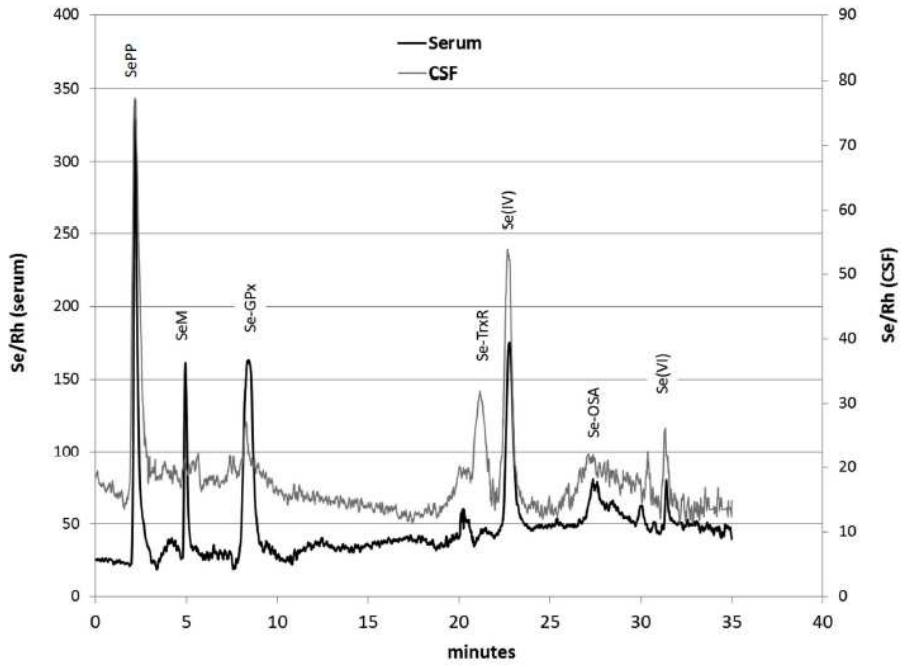
		SePP [µg/l]		Se-GPx [µg/l]		Se-TrxR [µg/l]		SeIV [µg/l]		Se-OSA [µg/l]		SeVI [µg/l]		Se-Met [µg/l]	
		Se^-	Se^+	Se^-	Se^+	Se^-	Se^+	Se^-	Se^+	Se^-	Se^+	Se^-	Se^+	Se^-	Se^+
Serum	**median**	**2.58**	**14.85[a]**	**5.21**	**9.28 [a]**	**<LOQ**	**2.44**	**0.516**	**2.53**	**1.45**	**3.26**	**1.92**	**5.32**	**2.01**	**4.49**
	min	1.24	4.10	4.079	7.06	<LOQ	<LOQ	<LOQ	<LOQ	0.716	1.29	0.154	0.187	<LOQ	<LOQ
	max	6.07	21.6	6.29	23.9	12.3	19.5	0.821	17.4	3.91	7.85	16.1	45.0	2.96	5.65
CSF	**median**	**0.603**	**0.617**	**0.255**	**0.47***	**<LOQ**	**0.228 [a]**	**0.094**	**0.082**	**0.376**	**0.423**	**1.044**	**0.631**	ns	ns
	min	0.072	0.122	<LOQ	0.14	<LOQ	<LOQ	<LOQ	<LOQ	0.137	<LOQ	<LOQ	<LOQ	ns	ns
	max	0.731	3.20	0.393	1.07	<LOQ	2.838	0.936	1.039	0.408	0.834	3.553	2.27	ns	ns

[a] significant differences ($p<0.05$) between groups;

* tendency ($p=0.094$) for differences between groups;

ns = no statistics due to the facts that more than 50% of data in both groups were below the limit of quantification (LOQ)

< LOQ: analysed data were below the LOQ of 0.05 µg/l in serum/CSF. To include these data into a statistical evaluation the data were set uniformly to 50% of the LOQ (= 0.025 µg/l).



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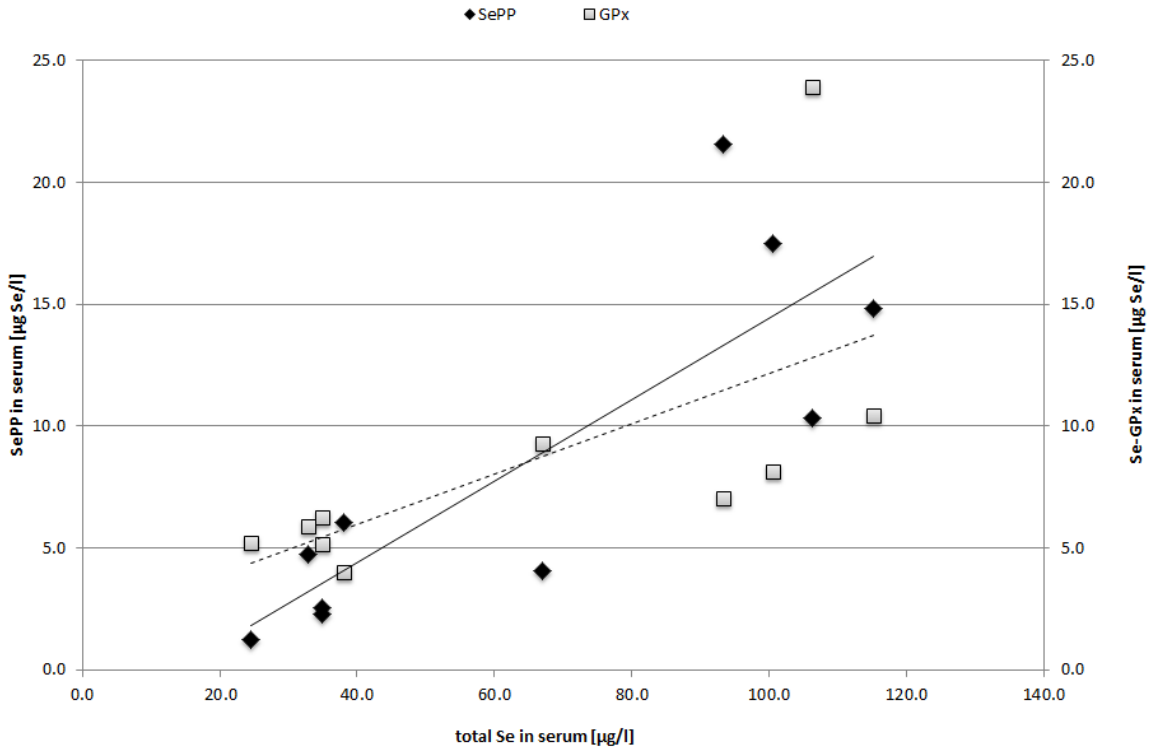

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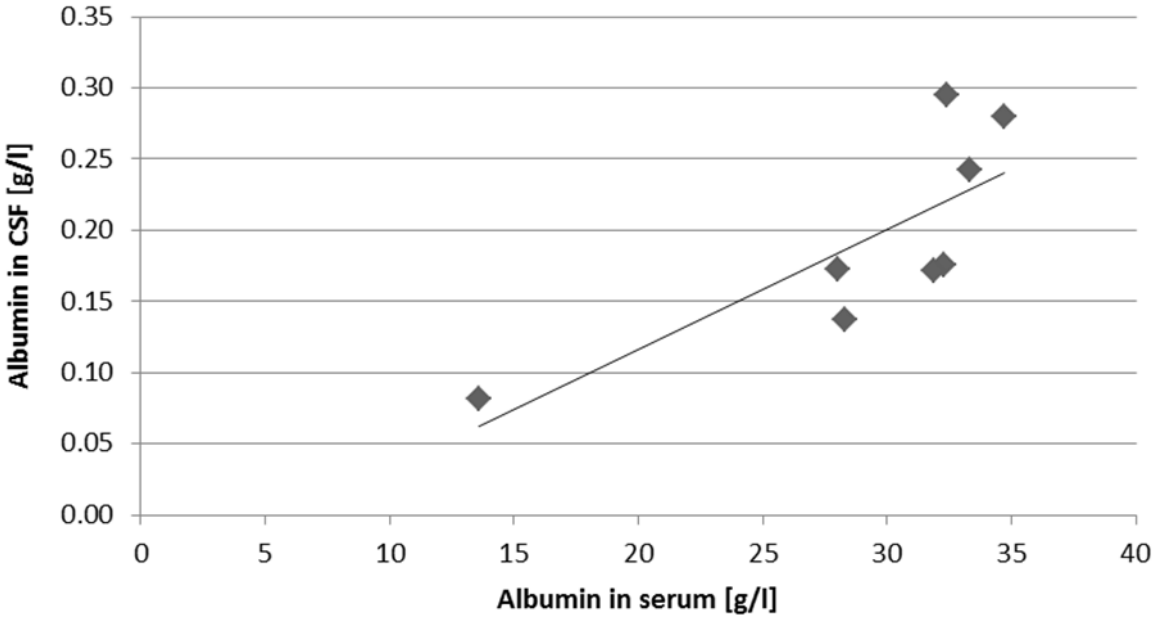

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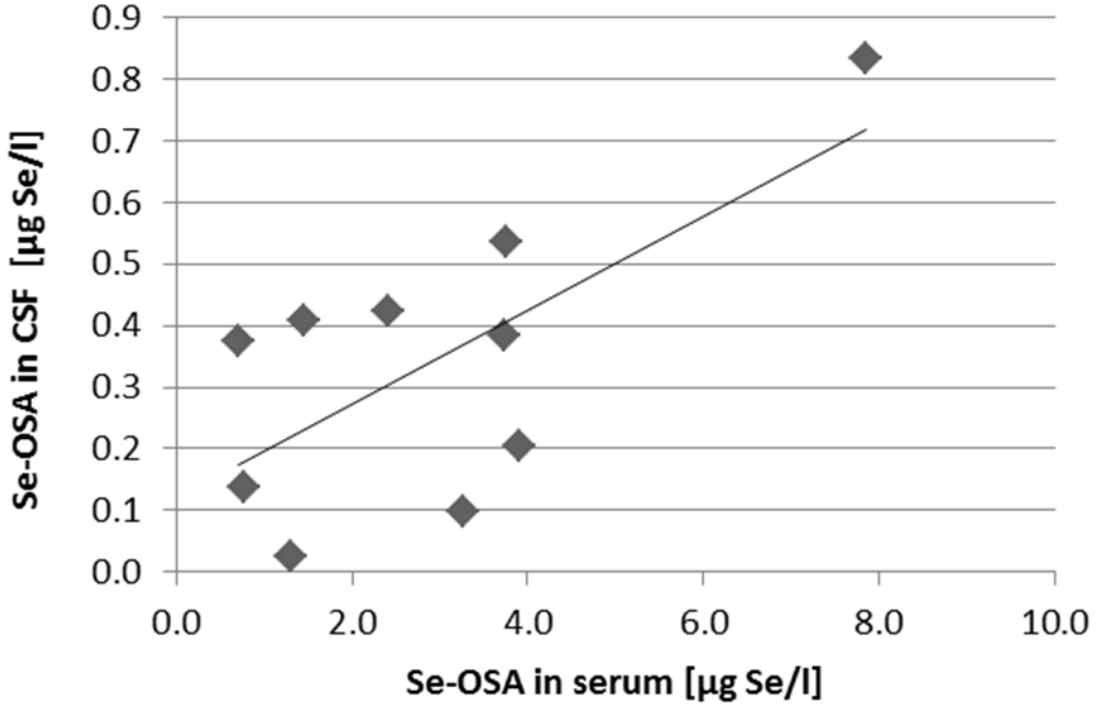

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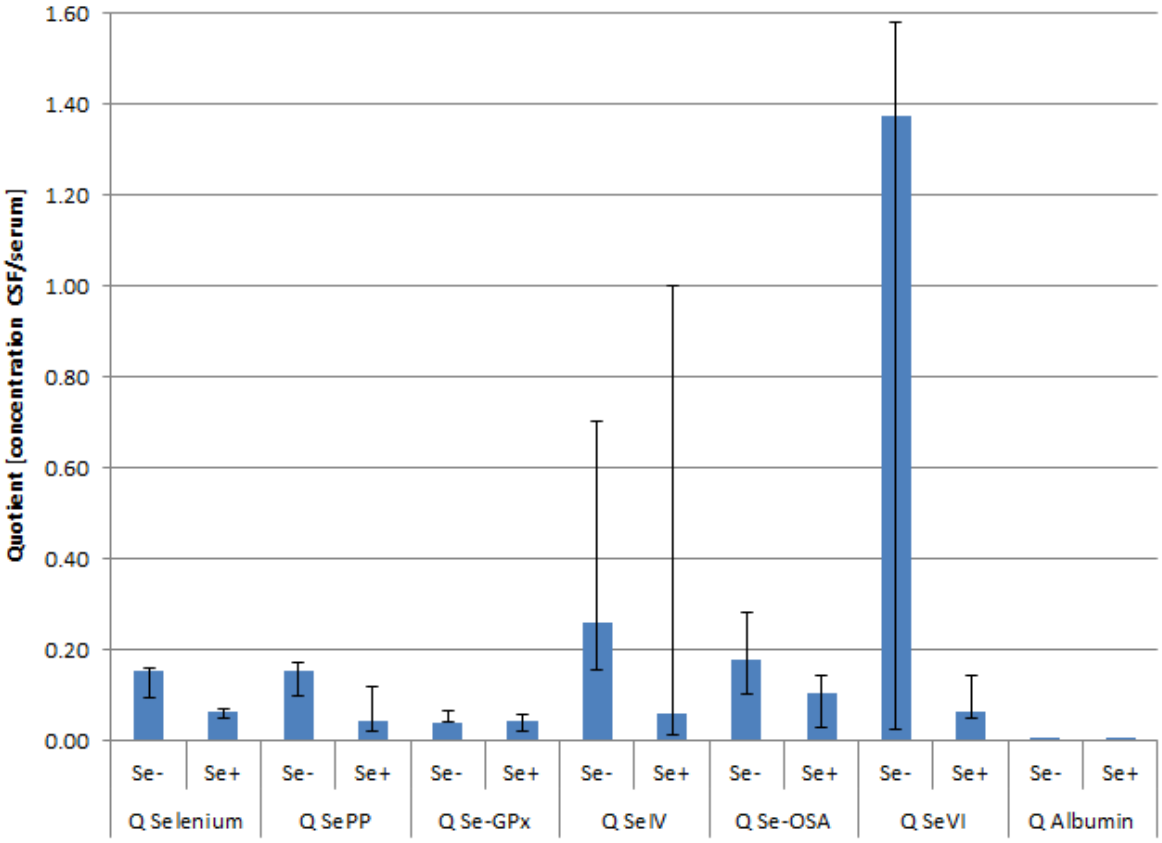

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