



## Research Paper

## Sex-specific and inter-individual differences in biomarkers of selenium status identified by a calibrated ELISA for selenoprotein P

Sandra Hybsier<sup>a</sup>, Torsten Schulz<sup>b</sup>, Zida Wu<sup>c</sup>, Ilja Demuth<sup>d,e</sup>, Waldemar B. Minich<sup>a</sup>, Kostja Renko<sup>a</sup>, Eddy Rijntjes<sup>a</sup>, Josef Köhrle<sup>a</sup>, Christian J. Strasburger<sup>c</sup>, Elisabeth Steinhagen-Thiessen<sup>d</sup>, Lutz Schomburg<sup>a,\*</sup>

<sup>a</sup> Institute for Experimental Endocrinology, Campus Virchow-Klinikum, Charité-Universitätsmedizin Berlin, Berlin, Germany

<sup>b</sup> ICI-immunochemical intelligence GmbH, Berlin, Germany

<sup>c</sup> Department of Endocrinology, Diabetes and Nutritional Medicine, Campus Charité Mitte, Charité-Universitätsmedizin Berlin, Berlin, Germany

<sup>d</sup> Research Group on Geriatrics, Charité-Universitätsmedizin Berlin, Berlin, Germany

<sup>e</sup> Institute of Medical and Human Genetics, Campus Virchow-Klinikum, Charité-Universitätsmedizin Berlin, Berlin, Germany

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## ABSTRACT

Selenoprotein P (SELENOP) is a liver-derived transporter of selenium (Se) in blood, and a meaningful biomarker of Se status. Se is an essential trace element for the biosynthesis of enzymatically-active selenoproteins, protecting the organism from oxidative damage. The usage of uncalibrated assays hinders the comparability of SELENOP concentrations and their pathophysiological interpretation across different clinical studies. On this account, we established a new sandwich SELENOP-ELISA and calibrated against a standard reference material (SRM1950). The ELISA displays a wide working range (11.6–538.4 µg/L), high accuracy (2.9%) and good precision (9.3%). To verify whether SELENOP correlates to total Se and to SELENOP-bound Se, serum samples from healthy subjects and age-selected participants from the Berlin Aging Study II were analyzed by SELENOP-ELISA and Se quantification. SELENOP was affinity-purified and its Se content was determined from a subset of samples. There was a high correlation of total Se and SELENOP concentrations in young and elderly men, and in elderly women, but not in young women, indicating a specific sexual dimorphism in these biomarkers of Se status in young subjects. The Se content of isolated SELENOP was independent of sex and age (mean ± SD: 5.4 ± 0.5). By using this calibrated SELENOP-ELISA, prior reports on pathological SELENOP concentrations in diabetes and obesity are challenged as the reported values are outside reasonable limits. Biomarkers of Se status in clinical research need to be measured by validated assays in order to avoid erroneous data and incorrect interpretations, especially when analyzing young women. The Se content of circulating SELENOP differs between individuals and may provide some important diagnostic information on Se metabolism and status.

## 1. Introduction

Selenium (Se) is an important micronutrient in our diet, essentially required for the biosynthesis of selenoproteins [1,2]. Selenoproteins comprise a number of important and essential members, implicated in the antioxidative defense and control of redox-dependent pathways [1,3,4]. The Se status currently is a poorly defined concept describing the degree of bioavailable Se for selenoprotein biosynthesis with the help of Se-responsive biomarkers [5]. Two hierarchical principles

govern selenoprotein biosynthesis in times of limited Se supply [6], safeguarding that the most essential selenoproteins are preferentially synthesized over so-called housekeeping selenoproteins [7], and that the survival-relevant tissues are protected from Se deficiency [8]. This serves to minimize adverse health consequences of an insufficient dietary Se supply so that brain and some endocrine organs (e.g. testes, bone or thyroid) are protected from Se loss and maintain a sufficiently high selenoprotein biosynthesis rate essential for their proper functioning. Relative to brain and endocrine tissues, the liver appears to reside

*Abbreviations:* Ab, antibody; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; GPX3, glutathione peroxidase 3; LLOQ, lower limit of quantification; LOD, limit of detection; LRP, lipoprotein receptor-related protein; mAb, monoclonal antibody; pAb, polyclonal antibody; Se, selenium; Sec, selenocysteine; SELENOP, selenoprotein P; TXRF, total reflection X-ray fluorescence spectroscopy; ULOQ, upper limit of quantification.

\* Corresponding author.

E-mail address: [lutz.schomburg@charite.de](mailto:lutz.schomburg@charite.de) (L. Schomburg).

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low in the hierarchical order of preferentially supplied tissues, despite being the principle site for converting nutritional organic and inorganic selenocompounds into an easily accessible and detoxified serum transport protein readily available to target tissues [9]. To this end, hepatocytes express high levels of selenoprotein P (SELENOP [10]; previously abbreviated as SEPP1, SELP or SePP), which is secreted into the blood and is taken up by target cells via interaction with members of the lipoprotein receptor-related protein (LRP) family, namely LRP2 (megalin) and LRP8 (APOER2) [9]. The open reading frame of human *SELENOP* mRNA contains 10 in frame UGA triplets specifying selenocysteine (Sec) insertion.

Insertion of Sec into the primary structure of selenoproteins during translation is accomplished by a tight interplay of several trans- and cis-acting factors; a) the UGA codon in the open reading frame, b) a characteristic stem-loop structure in the 3' untranslated region, which is called the Sec-insertion sequence (SECIS) element, c) a SECIS binding protein (SECISBP2), d) a specific elongation factor (EEFSEC), e) the Sec-specific tRNA (TRU-TCA1-1) and f) additional translation factors [1]. Inherited defects in the *SECISBP2* gene cause a multifaceted disease syndrome including reduced biosynthesis and concentrations of SELENOP [11].

The two different isoforms of the *TRU-TCA1-1* gene product tRNA<sup>[SerSec]</sup> are methylated, or not, at the anticodon wobble position U34. This influences the hierarchical principles governing selenoprotein biosynthesis in times of Se deficiency. Methylation occurs when Se supply is sufficiently high, while the non-methylated form prevails under Se-deficient conditions. The biosynthesis of non-essential selenoproteins is mainly supported by the methylated tRNA<sup>[SerSec]</sup>, which explains why their expression correlates with Se status [12]. The finding that liver does not belong to the preferentially supplied organs, and that SELENOP is not an essential selenoprotein, collectively explain why hepatic SELENOP biosynthesis and circulating SELENOP levels constitute a reliable Se-responsive biomarker of Se status over a wide range of Se intakes [13].

SELENOP concentrations in blood thus provide an insight into the systemic transport and Se status as well as the hepatic selenoprotein biosynthesis rate of an individual. However, quantification of SELENOP is challenging, and reported serum concentrations of SELENOP differ considerably between assays and research laboratories. Recently, the following concentrations of SELENOP in serum or plasma of healthy subjects have been reported: 6.7 (5.3–9.1) µg/L in a Turkish [14], 362.0 (252.5–694.5) µg/L in a South Korean [15], 5.3 ± 1.1 mg/L in a Japanese [16], and 52.3 ± 39.1 mg/L in a Chinese/Australian study [17]. These concentration ranges differ by almost 4 orders of magnitude, highlighting that there is an urgent need for a better characterization and calibration of SELENOP assays used in clinical analyses, and a need for a uniformly accepted reference material.

The majority of analytical studies have demonstrated that, depending on overall Se status, SELENOP accounts for the largest fraction of Se in blood. However, there are indications that the circulating SELENOP is not a homogenous protein. Variants of different molecular weight have been described [13,18], in relation to genotype, premature translational termination, limited posttranslational proteolysis or partial replacement of Sec by Cys [19–22].

To allow a more precise quantification and better characterization of SELENOP in human blood, we have developed a new immunoassay using monoclonal antibodies (mAb) and characterized the test according to highest standards of laboratory-developed assays including a calibration against the Standard Reference Material SRM1950 of the National Institute of Standards and Technology (NIST SRM1950). Our results indicate that gender and age affect the correlation between total Se concentrations in serum and circulating SELENOP concentrations. By using the new mAb for immuno-affinity purification, we succeeded in isolating SELENOP from a set of human serum samples and determined the average Se content, which differed between individuals

and was considerably below the predicted number of ten Se atoms per molecule of SELENOP.

## 2. Materials and methods

### 2.1. Human and mouse serum samples

The animal experiment from which samples were analyzed in this study has been described earlier [23]. The human serum samples were from participants of the Berlin Aging Study II (BASE-II), a multi-disciplinary project investigating medical, physical, cognitive, and social conditions related to “healthy” and “unhealthy” aging (cohort 2). The BASE-II study population consisted of about 2200 study subjects aged 60–80 years (elderly group) and 20–35 years (younger group) at baseline recruitment from the greater metropolitan area of Berlin, Germany [24,25]. All participants had given written informed consent, and the study had been approved by the Ethics Committee of the Charité- Universitätsmedizin Berlin, Germany (no. EA2/029/09). Additional high quality human serum samples from healthy subjects (cohort 1) were obtained from a commercial supplier (in.vent DIAGNOSTICA GmbH, Berlin).

### 2.2. Expression of recombinant human SELENOP

Recombinant human SELENOP was expressed in baculovirus-infected insect cells. High efficient biosynthesis was achieved after replacement of the Sec codons with cysteine codons via gene synthesis (Eurofins Genomics GmbH, Ebersberg, Germany). The synthetic reading frame was inserted into the baculovirus transfer vector pFastBac1 (Thermo Fisher Scientific, Karlsruhe, Germany), transformed into competent *E.coli* cells and recombinant bacmid DNA were isolated. Sf9 insect cells were transfected with bacmid DNA for obtaining a recombinant virus stock that was used to initiate SELENOP biosynthesis in “High Five” insect suspension cells. Cell culture supernatant was harvested 48 h after infection, and SELENOP was isolated by affinity chromatography on Ni-NTA agarose according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany).

### 2.3. Immunization and purification of antibodies

Monoclonal antibodies (Ab) were generated essentially as described [26]. In brief, three female BALB/c mice were immunized with an emulsion of purified recombinant SELENOP in TiterMax® Gold adjuvant (Sigma-Aldrich Corp., St. Louis, U.S.A.), followed by a second, third and fourth injection after 30, 60 and 61 days. The protocol had been approved by the local authorities (LAGeSo Berlin, permit No. H 0331/12). The antibody-titer of the mice was determined by an indirect ELISA on day 62 with immobilized recombinant SELENOP and pc rabbit anti-mouse Ab (Z109, Dako Deutschland GmbH, Hamburg, Germany) as detection antibody. The mouse showing the highest anti-SELENOP titer was killed, the spleen removed, lymphocytes isolated, and fused with immortalized murine myeloma cells (PAI, RRID: CVCL\_Z070) by PEG1500 (Roche Diagnostics GmbH, Penzberg, Germany).

A macrophage cell suspension was prepared from the abdominal cavity of a mouse as “feeder cells”. Fused hybridoma cells were cultivated first in HAT-DMEM-selection-medium followed by HT-DMEM-medium on a “feeder” cell layer. After ten days, the hybridoma culture supernatant was screened for Ab production, and productive hybridomas with high proliferation grade and Ab titers were expanded, cloned by limiting dilution and subcloned in DMEM medium containing 10% horse serum, 4 mM L-glutamine, 1 µg/mL fungizone, 100 U/mL penicillin, 100 µg/mL streptomycin and 1 mM pyruvate. Eight selected mAb were purified on a ProsepG-column (Merck Millipore, Darmstadt, Germany) and stored at 4 °C in PBS, pH 7.4.

#### 2.4. Development and validation of a SELENOP-ELISA

The eight candidate Ab were adsorbed to the surface of polystyrene plates as capture-Ab, and another aliquot was labeled with biotin for use as detection-Ab. All 8×8 possible sandwich-assay Ab combinations along with a SELENOP-specific polyclonal sheep Ab were tested with a serially-diluted human serum (Supplemental Table 1). Immuno-reactive SELENOP from the sample was bound by the capture-Ab. The detection Ab was added after a 3-fold wash step and bound to the capture-Ab-SELENOP complex. After washing, a Streptavidin-POD-conjugate (F. Hoffmann-La Roche AG, Basel, Switzerland) was incubated. The detection reaction was started by adding a tetramethylbenzidine containing substrate solution (Diarect AG, Freiburg, Germany) after a final wash step. The reaction was stopped by the addition of sulfuric acid, and the detection complex concentrations were quantified at 450 nm.

The assay calibrator was prepared by pooling of 30 individual sera of healthy U.S. citizens (Interstate Blood Bank Inc., Memphis, TN38134, U.S.A.). The sera were thawed, pooled in equal ratio and filtered through a 0.2 µm Supor® membrane (Pall GmbH, Dreieich, Germany). According to FDA guidelines, eight calibrators and three controls were generated. Two of the eight calibrators are anchor points which improve the 4PL-logistic-curve-fitting. These anchor points were above the highest (ULOQ – upper limit of quantitation; 793.7 µg/L) and below the lowest (LLOQ – lower limit of quantitation; 5.4 µg/L) standard concentration. The working range of the standard curve was covered by six calibrators with 12.0, 25.4, 51.7, 107.3, 215.5 and 398.4 µg/L SELENOP. Three controls were chosen from the upper (262.2 µg/L), middle (63.8 µg/L) and lower region (14.7 µg/L) of the quantification range. An international standard was used to ensure the traceability of the calibration procedure (NIST SRM1950 Standard Reference Plasma, National Institute of Standards and Technology, Gaithersburg, U.S.A.).

The assay performance parameters specificity, accuracy, precision, sensitivity, parallelism, and stability were determined with human serum. To determine the stability of the analyte, three serum samples were used directly and after dilution (1:21 in sample buffer). The samples and dilutions, respectively, were incubated at 2–8 °C (regular fridge stability), at 23 °C (bench-top stability), at 37 °C (body temperature stability) and at 50 °C over a period of up to 48 h. Aliquots (50 µL each) were incubated for 1, 2, 8, 24 and 48 h, and frozen at –80 °C at the end of the incubation period. All samples were then thawed and measured in parallel, in agreement with the guidelines of the Expert Working Group of the International Conference on Harmonisation (ICH). The limit of stability was set at 90–110% recovery of the starting concentration (“acceptance criteria”). The limit of stability is marked by the intersection of the 95% confidence interval with the recovery limit. The Table Curve® 2D software (Systat Software GmbH, Erkrath, Germany) was used to describe the non-linear mean- and confidence-interval trends. For curve-fittings, the simple non-linear equations with the best regression factor  $r^2$  were used. Stability upon freezing and thawing was determined with three undiluted serum samples. The samples were thawed at RT for 30 min, 50 µL were removed and transferred to an extra tube, labeled with “0” and frozen at –80 °C. After 24 h, the samples were thawed again, another set of 50 µL samples were taken, labeled and stored at –80 °C. This procedure was performed 5 times in total over a period of 5 days. All samples were finally analyzed by the SELENOP-ELISA in one run.

#### 2.5. HepG2 cell culture, RNA isolation, and real-time PCR

HepG2 cells were seeded at  $2 \times 10^6$  cells per well in 6-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and cultivated at 37 °C and 5% CO<sub>2</sub> in DMEM/F12 (Gibco, Thermo Fisher Scientific Inc.) +10% FCS (Biochrom GmbH, Berlin, Germany). The medium was exchanged at 80% confluency after 24 h, and cells were further cultivated for

24 h either in serum-free DMEM/F12 or DMEM/F12 containing 10% FCS. On the third day, a concentration range of sodium selenite (1 nM–10 µM, f.c.) was prepared in serum-free or FCS-containing medium, and treatment was started for additional 24 h by exchanging the medium. Then, cells were harvested, total RNA was isolated using Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories, Inc, Hercules, California, U.S.A.) and first-strand cDNA was synthesized using iScript™ cDNA Synthesis Kit (BioRad). Relative transcript levels of SELENOP (forward primer: 5′-TATGATAGATGTG GCCGTCTTG-3′; reverse primer: 5′-TGTGATGATGCTCATGATGGTA-3′) were determined using the Absolute QPCR SYBR Green Fluorescein Mix (Thermo Fisher Scientific) and beta actin (ACTB; forward primer: 5′-CACCACACCTTCTACAATGAGC-3′; reverse primer: 5′-CAGAGGCGTAC AGGGATAGC-3′) as housekeeper for normalization.

#### 2.6. LDH-measurement

As an indicator of cell damage, the release of lactate dehydrogenase (LDH) into the medium was determined using the Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific).

#### 2.7. Western blot analysis

SDS-PAGE was performed using 10% Bis-Tris gels in combination with a MOPS SDS running buffer as described in [27]. The samples were prepared in LDS sample buffer (Thermo Fisher Scientific) with DTT (Sigma-Aldrich). The proteins were blotted onto a 0.2 µm PVDF membrane (Thermo Fisher Scientific) in Bicine-Tris transfer buffer containing methanol. PonceauS (Sigma) was used for the visualization of the membrane-bound proteins. SELENOP was detected using anti-human SELENOP antibodies (InVivo, Hennigsdorf, Germany) and a polyclonal sheep anti-mouse IgG-HRP (GE Healthcare GmbH, Munich, Germany) in combination with the ECL Western Blotting Detection Reagents and Hyperfilm™ (GE Healthcare GmbH).

#### 2.8. Selenium analysis of blotted proteins

PonceauS-stained protein bands were excised using a 3 mm Harris Uni-Core™ stainless steel cutting tip (Agilent Technologies, Santa Clara, California, U.S.A.). Recovery of the proteins from the PVDF membrane was achieved by a detergent-based protocol according to Simpson et al. [28]. Each excised band was placed in a microtube, mixed with 50 µL elution buffer (50 mM Tris-HCl, pH 9.0, containing 2% SDS, 1% Triton X-100 and 0.1% DTT) and incubated overnight at 37 °C in a Thermomixer (Eppendorf AG, Hamburg, Germany). The tubes were centrifuged in a microfuge for 10 min at 10,000×g at ambient temperature, and the supernatants were analyzed by total reflection X-ray fluorescence spectroscopy (TXRF) using an S2 PICOFOX device (Bruker Nano GmbH, Berlin, Germany). Gallium (Alfa Aesar GmbH & Co KG, Karlsruhe, Germany) was used as internal standard, and the gallium-sample-mixtures were applied to quartz carriers and dried for 1 h at 50 °C before being analyzed. Detergent containing samples were applied to silicon coated carrier for the TXRF analysis.

#### 2.9. Affinity chromatography of human SELENOP

A SELENOP-specific antibody (clone mAb2, expanded by InVivo, Hennigsdorf) was coupled to aldehyde-activated agarose beads (AminoLink® Plus Coupling Resin, Thermo Fisher Scientific) according to the manufacturer's instructions (1.3 mg Ab2 per mL column bed). Serum or plasma samples, 35 mL each, were diluted 1:2 with binding buffer (PBS, pH 8.0) and filtered through a Supor® PES membrane with 0.8 µm pore size (Pall GmbH). The filtrate was incubated with the column bed overnight in batch at 4 °C using a rotary shaker, and then transferred to an empty column. The flow-through was collected, and the column bed was washed five times with 10 mL binding buffer.

Elution was achieved stepwise with citric acid (50 mM, pH 2.0), and eluates were neutralized by 1 M Tris, pH 9.0. The flow through, wash and the eluate were collected and analyzed by SELENOP-ELISA.

### 2.10. Immuno-affinity purification of SELENOP from human sera in a 96-well format

The SELENOP-specific antibody mAb2 (expanded and purified by InVivo) was coupled to agarose beads and used for immuno-affinity purification of SELENOP from a set of human samples. To this end, a 96-well sandwich combination consisting of a filter plate and a polypropylene plate was filled with 25  $\mu$ L column bed equivalent to 25  $\mu$ g antibody per well. Each purification step was performed for 10 min on an orbital shaker at 650 rpm followed by centrifugation at 22 °C and 1,000 $\times$ g for 1 min. Forty serum samples (à 100  $\mu$ L) from the BASE-II study plus a control serum were applied in duplicates and incubated overnight at 4 °C. Three wash steps à 200  $\mu$ L PBS, pH 7.4, followed. Bound SELENOP was eluted by 2 $\times$ 25  $\mu$ L citric acid (50 mM, pH 2.0). The combined eluate per sample (50  $\mu$ L) was neutralized by 5  $\mu$ L Tris (1 M, pH 9.0) and analyzed by TXRF and SELENOP-ELISA. The accuracy of the TXRF-measurement was verified by the trace element standard Seronorm™ Trace Elements Serum L-2 (Sero AS, Billingstad, Norway) on three different days. The measured Se concentration (mean  $\pm$  SD: 164  $\pm$  14.1  $\mu$ g/L) was within the required reference range (143–183  $\mu$ g/L) of lot number 0903107.

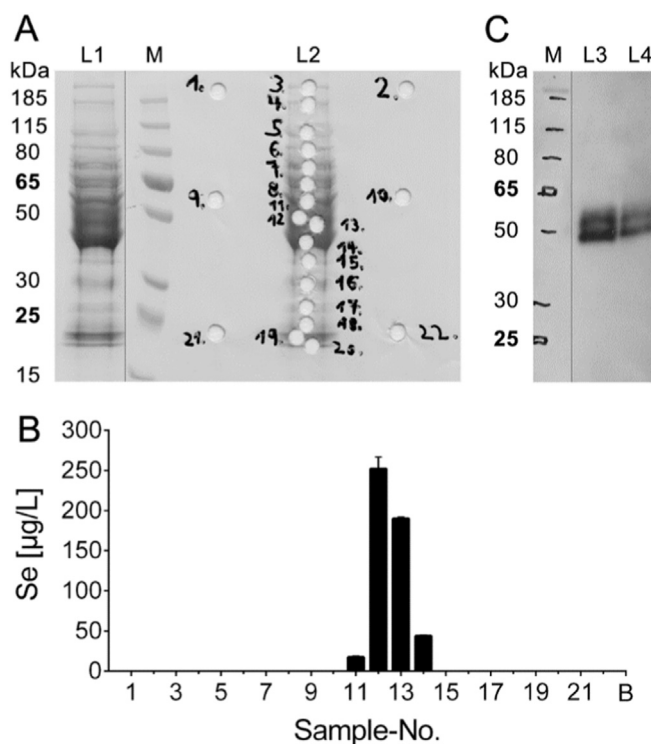
### 2.11. Statistical analysis

Biostatistical analyses were performed by the GraphPadPrism® Software Version 6.04 (GraphPad Software, Inc., San Diego, U.S.A.). The D'Agostino-Pearson omnibus test was used to test for normal distribution, and comparisons of normally-distributed values were achieved with an unpaired *t*-test or one-way ANOVA. Pearson's correlation coefficients were calculated to analyze associations between two parameters. IBM® SPSS® Statistics, Version 22 (IBM Corp., Armonk software) was used for linear regression analyses. The significance of the results was expressed as the P-value (two-tailed) which is interpreted as followed:  $P < 0.0001$  \* and  $P < 0.001$ \*\*\* are extremely significant,  $P < 0.01$ \*\* is very significant,  $P < 0.05$ \* is significant and  $P \geq 0.05$  is not significant. The software Table Curve® 2D Version 5.01 (Systat Software GmbH, Erkrath, Germany) was used for the precision profile (Fig. 4), and for the non-linear curve fitting in the stability results (Fig. 5).

## 3. Results

### 3.1. Establishment, characterization and calibration of a SELENOP-specific ELISA

Recombinant Sec-free human SELENOP was expressed in insect cells, purified and used for the immunization of mice. Hybridoma cells were generated essentially as described [26], and screened for SELENOP-reactive antibodies (Ab). Eight most productive and stable hybridoma cell lines were expanded and the monoclonal Ab were purified by protein chromatography. All of the eight newly generated Ab recognized human SELENOP in Western blot analyses without any cross-reactivity to the bovine selenoprotein present in FCS. For comparison, a formerly established SELENOP-specific polyclonal sheep Ab (pAb) and the reference assay (Ref) were included [29], and all nine Ab were tested in combination for signal strength and linearity in a sandwich-assay format (Supplemental Table 1). The highest sensitivity was achieved when the newly generated SELENOP-specific monoclonal Ab5 was immobilized as capture-Ab, and either mAb2, mAb3, mAb4, mAb7 or mAb8 were used as detection-Ab. For reasons of stability, affinity and yield after scale-up, finally mAb2 was selected as the detection-Ab of choice for the establishment of the novel



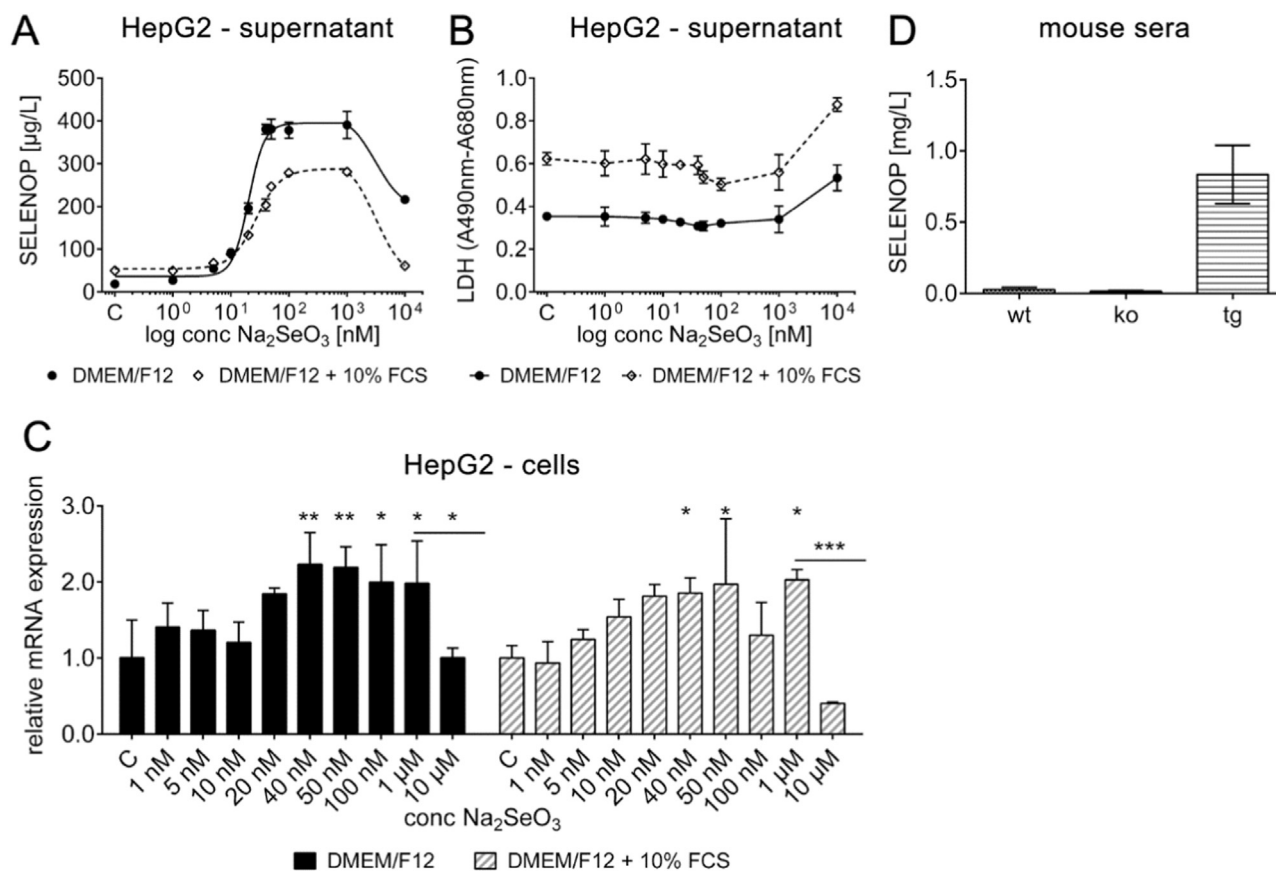
**Fig. 1.** Characterization of affinity-purified SELENOP by Western blot and trace element analysis. (A) PonceauS stained PVDF-membrane of a preparation of SELENOP from human serum after separation by SDS-PAGE (L1). Several discrete protein bands were visible, and were cut out along with membrane control samples as protein/PVDF membrane spots (L2). (B) Spots were eluted and analyzed by TXRF for quantification of Se contents (spots 1–22; B denotes the pure elution buffer); mean  $\pm$  SD, n=2 determinations per sample. (C) Western Blot of the affinity-purified SELENOP with the detection antibody mAb2 (L3; 3.0 ng, and L4; 1.5 ng of purified SELENOP).

### SELENOP-ELISA.

### 3.2. Characterization of the specificity of the antibodies used for SELENOP-ELISA

The ELISA signal for quantification requires the binding of detection mAb2 to its antigen SELENOP. To test whether mAb2 recognizes native human SELENOP directly in serum or plasma, an immuno-affinity column was prepared with mAb2 and used to purify SELENOP from human serum and plasma. The eluates were analyzed via SELENOP-ELISA, concentrated by acetone precipitation, and separated by SDS-PAGE. The preparations contained 8.3  $\mu$ g and 10.4  $\mu$ g SELENOP in total, respectively, and were transferred to a polyvinylidene difluoride (PVDF) membrane. After staining with PonceauS, different bands were visible and were excised with a cutter (Fig. 1A). The identity of the excised protein was determined by N-terminal Edman amino acid sequencing (Proteome Factory AG, Berlin, Germany). The human SELENOP-specific peptide sequence ESQDQS was identified at the molecular weight mark of 50 kDa, verifying the identity of the excised band and confirming the suitability of the antibody mAb2 for SELENOP purification by immuno-affinity chromatography.

Defined spots of the stained membranes were identified, cut out and isolated (Fig. 1A). Trace element analysis by total reflection X-ray fluorescence (TXRF) was performed as a second test for characterizing the immuno-affinity-purified SELENOP. Proteins were eluted from the excised membrane spots and Se contents were determined (Fig. 1B). In the SELENOP preparation from plasma, the highest Se concentration was identified at 50 kDa (not shown). Two separate immuno-reactive bands were visible in the preparation of SELENOP from serum after



**Fig. 2.** Specificity of the ELISA for human SELENOP expressed *in vitro* or in mice. All values are means  $\pm$  SD of biological triplicates (A–C) or 6 animals (D). (A) Increasing SELENOP concentrations are measured by the ELISA at 24 h after adding sodium selenite to the culture medium of HepG2 cells. Response curves and maximum levels differ between cells grown in the presence or absence of 10% FCS. (B) LDH activity in medium indicates cytotoxic effects at 10  $\mu$ M of selenite. (C) SELENOP mRNA concentrations increase moderately in response to selenite in the concentration range of 1.0 nM to 1.0  $\mu$ M. (D) The SELENOP-ELISA does not detect murine Selenop in wildtype (wt) or *Selenop*-knock out (ko) mice, but quantifies human SELENOP expressed from an albumin-promotor driven transgene (tg).

SDS-PAGE and blotting, migrating at 54 and 48 kDa in our SDS-PAGE system (Fig. 1C). The spots #12 and #13 displayed highest Se concentrations, corresponding in size to the immuno-reactive SELENOP bands in the Western blot analysis.

As a second test for specificity, HepG2 cells were incubated with increasing concentrations of sodium selenite ( $\text{Na}_2\text{SeO}_3$ ) in triplicates in order to induce human SELENOP biosynthesis in a reproducible *in vitro* system. Cells were cultivated under serum-free or fetal calf serum (FCS)-containing conditions, whereby the former tended to impair proliferation and the latter stimulated growth. Using the 4-parameter-logistic-curve-fitting model (4PL), the quantification of SELENOP resulted in a sigmoidal dose-response curve up to 1.0  $\mu$ M selenite, and a sharp decline at higher concentrations (10  $\mu$ M selenite).

The four parameters of the 4PL-curve fitting differed when determined for the two *in vitro* selenite supplementations (Fig. 2A). In serum-free medium, an eight-fold increase in SELENOP concentrations was observed between control (47.9  $\mu$ g/L) and cells exposed to 1.0  $\mu$ M of selenite (392  $\mu$ g/L). In FCS-containing medium, the analogous increase was only five-fold (from 56.8 to 287  $\mu$ g/L), and maximal SELENOP expression was slightly lower than under serum-free conditions.

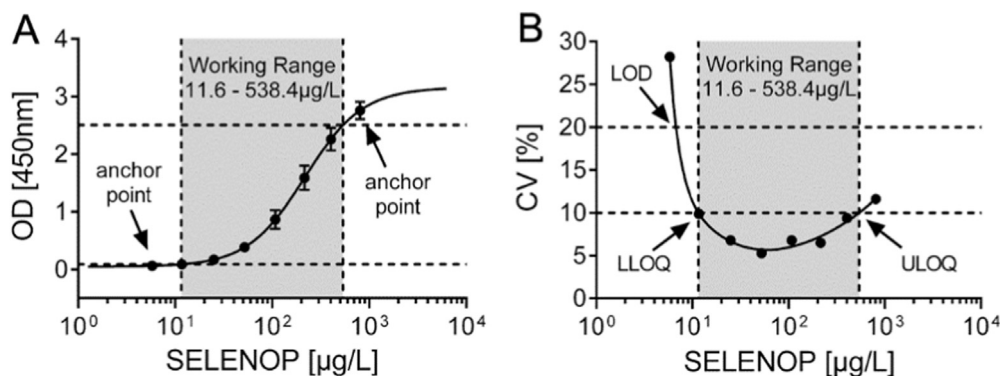
The Hill coefficient (Hill slope) describing the steepness of the curve is considerably steeper under serum free conditions than in the presence of FCS, and the half maximal effective concentration ( $\text{EC}_{50}$ ) of  $\text{Na}_2\text{SeO}_3$  for SELENOP induction is lower under serum free ( $\text{EC}_{50}=20.2$  nM) than under FCS-containing conditions ( $\text{EC}_{50}=27.1$  nM). The analysis of L-lactate dehydrogenase (LDH) activity for detecting cytotoxic effects indicated no cell damage up to 1.0  $\mu$ M of selenite (Fig. 2B). Elevated LDH-activity was

present in the medium at 10  $\mu$ M selenite, indicating that a cytotoxic concentration had been reached [30]. SELENOP transcript levels were about two-fold higher in selenite supplemented (at 1.0  $\mu$ M selenite f.c.) as compared to control cells (no added selenite) under both serum-free and serum-containing conditions (Fig. 2C).

In order to further test the specificity of the SELENOP-ELISA, serum samples from wildtype (*Selenop*<sup>+/+</sup>) and homozygous *Selenop* knockout mice (*Selenop*<sup>-/-</sup>) were analyzed along with samples from knockout mice specifically expressing a human SELENOP transgene in liver (*Selenop*<sup>-/-tg</sup>) [31]. Six samples per genotype (three male and three female mice) were analyzed, and immuno-reactive SELENOP was detected only in mice expressing the human transgene (0.8  $\pm$  0.2 mg/L SELENOP, mean  $\pm$  SD), but not in serum samples from *Selenop*<sup>+/+</sup> or *Selenop*<sup>-/-</sup> mice (Fig. 2D).

### 3.3. Determination of a typical standard curve and precision profile of the SELENOP-ELISA

The serum standards used in the SELENOP-ELISA were calibrated against the NIST Standard Reference Material SRM1950 [32]. Calibration was carried out on three days with two operators in four replications. The standards were evaluated using a serial dilution of SRM1950 in sample buffer of 1:8 to 1:1024. The SELENOP concentration (c SELENOP) of the reference plasma was calculated at 4.44 mg/L using the following equation:



**Fig. 3.** Standard curve and precision profile of the SELENOP-ELISA. (A) The intersections of the horizontal line of the precision profile at 10% CV limit the working range with the concentrations of the LLOQ and the ULOQ. Concentrations below or above are anchor points for curve fitting. (B) The intersection of the horizontal 20% CV-line generates the LOD; determined on separate days by three operators in two different laboratories.

$$c \text{ SELENOP} \left( \frac{\text{mg}}{\text{L}} \right) = \frac{c \text{ Se} \left( \frac{\mu\text{g}}{\text{kg}} \right) * \rho \text{ plasma} \left( \frac{\text{kg}}{\text{L}} \right) * \text{MW SELENOP} \left( \frac{\text{g}}{\text{mol}} \right)}{\text{MW Se} \left( \frac{\text{g}}{\text{mol}} \right) * \text{Se-atoms per SELENOP-molecule} * 1000}$$

For this calculation, the following data were used; a SELENOP-related Se concentration of 50.2 µg/kg (c Se), a plasma density of 1.028 kg/L (ρ plasma), an average molecular weight of SELENOP of 51,000 g/mol (MW SELENOP) and of Se of 78.96 g/mol (MW Se), and an average Se content of SELENOP of 7.5 atoms of Se per SELENOP, as determined in rat [33].

The precision profile (Fig. 3B) determines the dynamic (working) range of the standard curve (Fig. 3A). Three operators measured on three days in two different laboratories with two plates per day in four replications each standard of the standard series. The mean and the coefficient of variation (CV) of each concentration in these 18 determinations were calculated. SELENOP concentrations were plotted on the X-axis and the CV value on the Y-axis. A non-linear function with the highest regression factor was generated by the software TableCurve® and yielded the following result;  $r^2=0.996$ ; equation:  $y=a+bx+c/x+dx^2+e/x^2+fx^3+g/x^3$ ;  $a=-482.6$ ;  $b=182.2$ ;  $c=699.9$ ;  $d=-36.0$ ;  $e=-513.9$ ;  $f=3.36$ ;  $g=158.4$ . Fig. 3B shows the intersection points of the two horizontal lines at 10% and 20% CV with the precision profile. The intersection points at 10% and 20% CV were calculated by using the non-linear equation. The lower limit of quantification (LLOQ) was determined at a SELENOP concentration of 11.6 µg/L, and the upper limit of quantification (ULOQ) at 538.4 µg/L, thereby defining the working range at SELENOP concentrations between 11.6 and 538.4 µg/L. The intersection at 20% CV defines the limit of detection (LOD), and was reached at a SELENOP concentration of 6.7 µg/L.

### 3.4. Determination of the accuracy (trueness) and precision of SELENOP-ELISA

Three diluted control sera with different SELENOP concentrations in the lower (14.7 µg/L), middle (63.8 µg/L) and upper (262.2 µg/L) calibrator concentration range of the standard curve were determined by three operators with two plates per operator on three days. The controls were analyzed four times per plate. The average deviation of the closeness of the measured SELENOP concentrations to the nominal reference SELENOP concentration was +2.9% (operator 1; -0.4%, operator 2; +9.6%, operator 3; -0.4%).

The closeness of individual determinations of the same sample (precision) was measured at three levels. The repeatability within one plate (intra-assay precision), the reproducibility (inter-assay precision) and the intermediate precision (variation between two laboratories) were determined with three operators (2 plates per operator) on three days in two different laboratories. Six serum samples were analyzed six times per plate. The mean within one plate variation was determined at

6.2% CV (operator 1; 6.2%, operator 2; 6.6%, operator 3; 5.7%), the inter-assay-variation was 10.5% CV (laboratory 1; 11.1%, laboratory 2; 9.9%), and the variation between two laboratories were determined as 11.3% CV, resulting in an average precision of 9.3%.

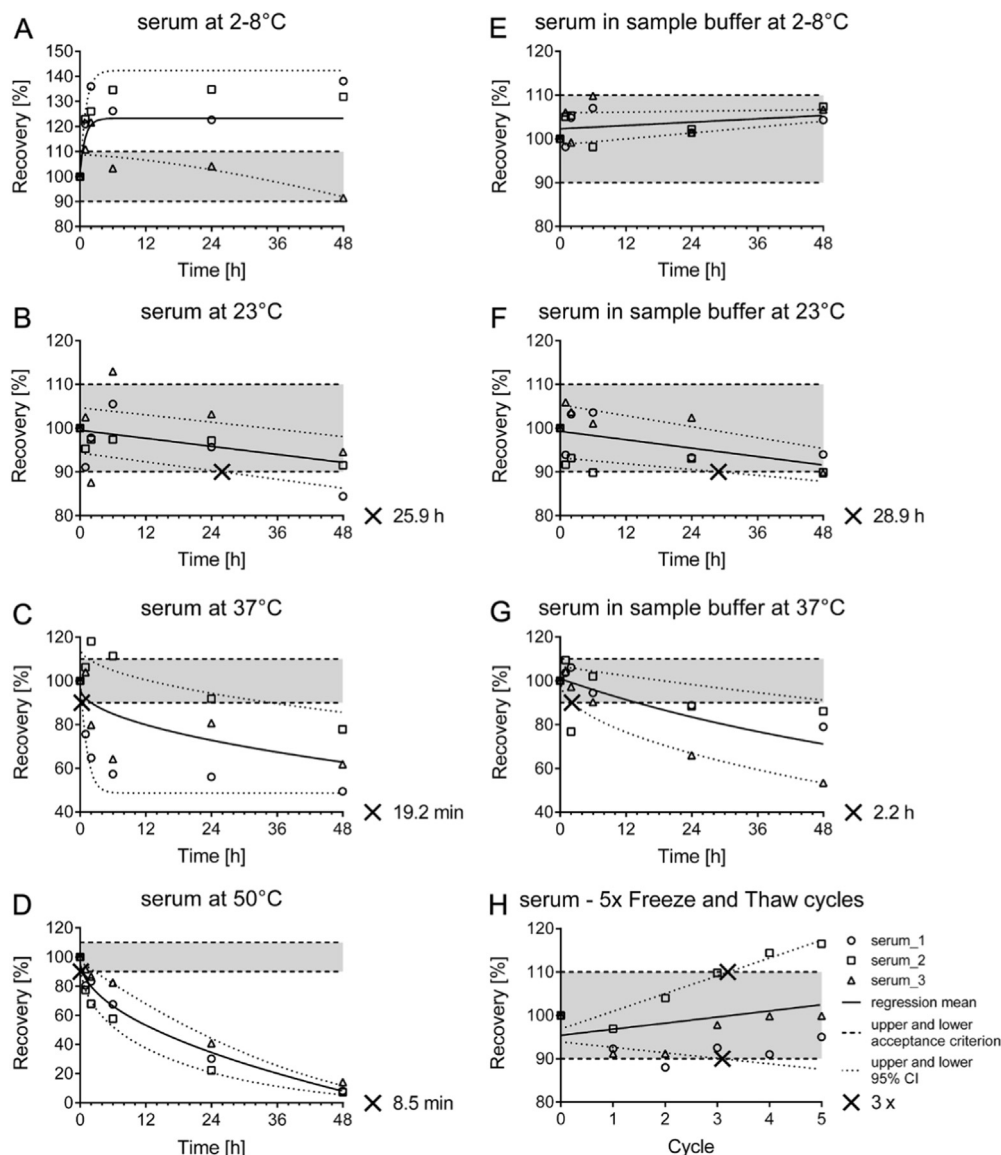
### 3.5. Parallelism and recovery of SELENOP in different human matrices

Serial dilutions in sample buffer within the working range of the standard curve of five matrix-matched serum as well as EDTA-, Na-citrate- and Li-heparin-plasma samples were tested in duplicates. The back-calculated concentrations were all within 20% of the nominal concentration (Supplemental Table 2). This result justifies the choice of the NIST reference plasma consisting of a Li-heparin matrix as a suitable calibration sample for the serum standards of the SELENOP-ELISA, and the recommendation to its acceptance as a universal reference material for calibrating quantification methods of SELENOP from human samples.

Next, the SELENOP concentrations in 54 matrix-matched serum and citrate-plasma samples were determined. A correlation analysis and an unpaired *t*-test indicate that the matrices yield highly correlating but different values (Pearson's correlation factor, two-tailed:  $r=0.909$ ,  $P < 0.0001$ ; mean ± SD, serum:  $4.0 \pm 0.9$  mg/L; versus citrate-plasma:  $3.4 \pm 0.8$  mg/L). Therefore, caution is advised when including samples of different matrices into the same analysis.

### 3.6. Stability of SELENOP in human serum

The stabilities of three serum samples and dilutions in sample buffer were compared over a period of 1, 2, 6, 24 and 48 h. Linear regression analyses were performed when 80% of the measured values fell into the 90–110% acceptance range. The recovery showed a positive trend upon storage at 2–8 °C (Fig. 4A). Within one hour at 2–8 °C, the confidence interval was surpassed to an unacceptable degree. Therefore, immediate storage of serum in the refrigerator for subsequent analysis in the SELENOP-ELISA is not recommended. The "benchtop" stability was determined at ambient temperature (Fig. 4B), and indicated that undiluted serum samples are stable for at least 24 h at 23 °C. At higher temperatures of 37 °C (Fig. 4C) or 50 °C (Fig. 4D), the measured recoveries intersected the confidence interval at 19.2 min (37 °C) and 8.5 min (50 °C), respectively, indicating a limited stability of SELENOP at elevated temperatures in undiluted serum. As the samples are routinely diluted prior to analysis in sample buffer, we tested SELENOP recovery in diluted samples (1:21, vol: vol) upon prolonged storage times at the different temperatures as described above. Both in the refrigerator (Fig. 4E) and at ambient temperature on the benchtop (Fig. 4F), the diluted samples showed an acceptable recovery of SELENOP during a time period of up to 48 h. In



**Fig. 4.** Stability of human SELENOP. Three serum samples were tested undiluted (A–D) and as dilutions in sample buffer (E–G). (A) Storage at 2–8 °C caused an increase in SELENOP recovery already after 1 h. (B) Recovery was within acceptable limits after storage at ambient temperature for 25.9 h. Incubations at higher temperatures of (C) 37 °C, or (D) 50 °C caused a rapid decline of recovery. (E) In comparison, diluted SELENOP samples were more stable and showed an acceptable recovery at 2–8 °C over the full time period. (F) At 23 °C, recovery was acceptable for up to 28.9 h. (G) At 37 °C, SELENOP recovery dropped more than 10% after 2.2 h. (H) Undiluted serum samples yielded acceptable SELENOP recovery after undergoing up to three freeze-thaw cycles.

comparison, recovery dropped below an acceptable level upon incubation at 37 °C for > 2 h (Fig. 4G). Human samples containing SELENOP can thus safely be stored for days in dilution buffer in the refrigerator before analysis. Finally, several rounds of freezing were tested. Despite yielding an increasing deviation from the starting concentration, SELENOP recovery stayed within acceptable limits even after three cycles of freezing and thawing (Fig. 4H).

### 3.7. Correlations of Se and SELENOP concentrations in male and female serum

Se and SELENOP concentrations in human serum do correlate well but not perfectly. In order to test the interrelation and obtain high quality serum samples, a blood donation was commissioned by a commercial provider. The processing of blood to frozen serum was performed within 4 h by close adherence to a defined pre-analytical procedure. Hereby, serum samples from 99 healthy Caucasian subjects (43 men, age range 18–64 y; 56 women, age range 18–55 years) were

obtained and analyzed (Table 1). The mean  $\pm$  SD concentrations of Se (male;  $77.5 \pm 14.1$  vs. female;  $80.0 \pm 12.5$   $\mu\text{g/L}$ ;  $P=0.422$ ) and SELENOP (male;  $4.3 \pm 1.0$  vs. female;  $4.2 \pm 0.7$   $\text{mg/L}$ ;  $P=0.432$ ) were not significantly different between the sexes. In both sexes, a significant positive correlation of Se and SELENOP was observed ( $P < 0.0001$ ). A linear regression model (model 1) was used to determine the sex-specific impact of Se on SELENOP concentrations. For men, 58% of the variance in SELENOP-concentrations were explained by Se, while it was 32% for women (adjusted  $R^2$ ). The regression coefficient B indicates the change of the dependent variable (SELENOP) by the factor (Se). Increasing Se by 10  $\mu\text{g/L}$  increased SELENOP by 0.52  $\text{mg/L}$  in men and by 0.35  $\text{mg/L}$  in women, respectively.

On this account, the influence of Se and age on the linear model was investigated (model 2). For men, age elicited no effect ( $P=0.664$ ), while for women, age was close to having a significant impact ( $P=0.068$ ). Se and age accounted for 35% of the variance in SELENOP concentrations in women, with Se (10  $\mu\text{g/L}$ ) being the stronger (standardized regression coefficient,  $\text{Beta}=0.569$ ) and age (10 years) the weaker factor

**Table 1**  
Linear regression analysis of Se and SELENOP in males and females.

| Cohort       | Sex    | N  | Model | Predictors | Adjusted R <sup>2</sup> | B (95% CI)            | Beta   | P     |    |
|--------------|--------|----|-------|------------|-------------------------|-----------------------|--------|-------|----|
| 1            | male   | 43 | 1     | Se         | 0.577                   | 0.052 (0.038, 0.066)  | 0.766  | 0     | #  |
|              |        |    | 2     | Se         | 0.568                   | 0.053 (0.039, 0.067)  | 0.771  | 0     | #  |
|              |        |    |       | age        |                         | 0.003 (-0.012, 0.019) | 0.045  | 0.664 | ns |
|              | female | 56 | 1     | Se         | 0.319                   | 0.035 (0.021, 0.048)  | 0.576  | 0     | #  |
|              |        |    | 2     | Se         | 0.349                   | 0.034 (0.021, 0.047)  | 0.569  | 0     | #  |
|              |        |    |       | age        |                         | 0.016 (-0.001, 0.033) | 0.203  | 0.068 | ns |
| 2Sample Load | male   | 20 | 1     | Se         | 0.909                   | 0.064 (0.054, 0.074)  | 0.956  | 0     | #  |
|              |        |    | 2     | Se         | 0.903                   | 0.064 (0.054, 0.075)  | 0.956  | 0     | #  |
|              |        |    |       | age        |                         | 0.000 (-0.007, 0.007) | -0.001 | 0.994 | ns |
|              | female | 20 | 1     | Se         | 0.361                   | 0.047 (0.018, 0.077)  | 0.628  | 0.003 | ** |
|              |        |    | 2     | Se         | 0.705                   | 0.049 (0.030, 0.069)  | 0.655  | 0     | #  |
|              |        |    |       | age        |                         | 0.015 (0.008, 0.022)  | 0.585  | 0     | #  |
| 2Eluate      | male   | 20 | 1     | Se         | 0.899                   | 0.126 (0.106, 0.146)  | 0.951  | 0     | #  |
|              |        |    | 2     | Se         | 0.893                   | 0.126 (0.105, 0.148)  | 0.954  | 0     | #  |
|              |        |    |       | age        |                         | 0.001 (-0.007, 0.008) | 0.015  | 0.851 | ns |
|              | female | 20 | 1     | Se         | 0.738                   | 0.118 (0.084, 0.151)  | 0.867  | 0     | #  |
|              |        |    | 2     | Se         | 0.742                   | 0.114 (0.080, 0.148)  | 0.84   | 0     | #  |
|              |        |    |       | age        |                         | 0.004 (-0.003, 0.011) | 0.136  | 0.27  | ns |

Investigating the influence of Se (model 1) and Se and age (model 2) on the linear dependence in serum (sample load) of both cohorts, and the eluate (SELENOP preparations) of cohort 2. The influence of Se on the variance of serum SELENOP-concentrations are higher in men than in women. The additional consideration of the impact of age on SELENOP showed an influence in female but not in male subjects. This finding was very pronounced in cohort 2, which had a focus on age. The age-dependent sexual dimorphism disappeared when SELENOP was isolated from serum (cohort 2), meaning that the Se content of the SELENOP-molecule is independent of age and sex (cohort 2, eluate).  $P < 0.0001$  (#);  $P < 0.01$  (\*\*); not significant (ns).

(Beta=0.203). An increase in Se by 10  $\mu\text{g/L}$  was associated with an increase in SELENOP by 0.34 mg/L (B=0.034), while an increase of 10 y of age were associated with an increase of 0.16 mg/L (B=0.016).

In order to test these findings in a second independent cohort with a closer examination of the influence of age, high quality serum samples were analyzed from young (22–26 y) and elderly (73–79 y) men and women (n=10 per group) participating in the Berlin Aging Study II (BASE-II) [24,25]. Again, compared to cohort 1 both Se (male;  $61.2 \pm 17.8$  vs. female;  $64.3 \pm 8.9$   $\mu\text{g/L}$ ;  $P=0.491$ ) and SELENOP (male;  $4.1 \pm 1.2$  vs. female;  $4.0 \pm 0.7$  mg/L;  $P=0.720$ ) concentrations did not differ between the sexes. The linear regression model 1 showed that 91% of the variance in SELENOP concentrations was attributable to Se in men (Beta=0.956,  $P < 0.0001$ ), but that only 36% of SELENOP variance were attributable to Se in women (Beta=0.628,  $P=0.003$ , Table 1). An increase in Se by 10  $\mu\text{g/L}$  were associated with a SELENOP increase of 0.64 mg/L in males, and 0.47 mg/L in females, respectively.

Including the cofactor age into the linear regression (model 2), a significantly higher proportion of SELENOP variance could be ascribed to Se in women (adjusted  $R^2=0.705$ ), whereas age had no effect in men. In women, Se was contributing to SELENOP variance with a standardized regression coefficient beta of 0.655 ( $P < 0.0001$ ), and age with 0.585 ( $P < 0.0001$ ). SELENOP increased by 0.49 mg/L (B=0.049) per increase of Se (10  $\mu\text{g/L}$ ), whereas 10 y of age were associated with an increase in SELENOP of 0.15 mg/L (B=0.015).

The relatively poor correlation of serum Se and SELENOP concentrations in young women may be due to a variable Se content per SELENOP molecule. To test this hypothesis, SELENOP was purified from male and female serum samples by affinity chromatography with mAb2- coated agarose beads. Per sample, 100  $\mu\text{L}$  of serum were incubated with 25  $\mu\text{L}$  of affinity column material, washed three times and eluted into a final volume of 55  $\mu\text{L}$  (Fig. 5). Se in the original serum (“sample load”) and the respective SELENOP preparation (“eluate”) correlated positively (Fig. 6A, Pearson  $r=0.804$ , 95% CI 0.658–0.892). In comparison, the SELENOP concentrations in sample load and eluate correlated to an even higher degree (Fig. 6B,  $r=0.929$ , 95% CI 0.868–0.962). Similarly, a high correlation is observed when comparing Se and SELENOP in serum (“sample load”) (Fig. 6C), and an even higher correlation when comparing both parameters in the eluates (Fig. 6D). One way ANOVA analysis of box and whiskers plots of Se (not shown)

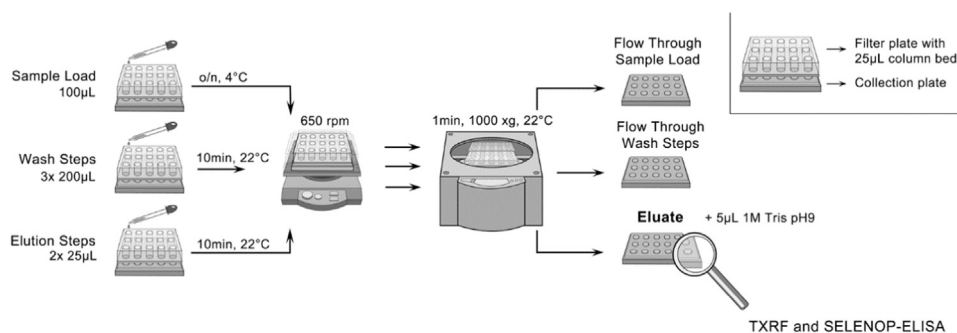
and SELENOP (Fig. 6E–F) showed no differences in concentrations between the groups in both the sample load and the eluate. To calculate the Se (MW; 78.96g/mol) to SELENOP (MW; 51,000g/mol) ratios, the mass concentrations were converted to their molar concentrations (nmol/L) using the specified molecular weights. The Se to SELENOP ratio highlights a significantly higher value in serum samples from young females as compared to the other three groups (Fig. 6G). Notably, this difference disappears in the eluates consisting primarily of SELENOP (Fig. 6H). These results were also confirmed in the linear regression analysis in Table 1. In the eluates, the influence of the female-specific cofactor age decreased to non-significant in the linear regression model 2 (adjusted  $R^2=0.742$ ,  $\text{Beta}_{\text{Age}}=0.840$ ,  $P_{\text{Se}} < 0.0001$ ,  $\text{Beta}_{\text{Age}}=0.136$ ,  $P_{\text{Age}}=0.270$ ). In men, the influence of age remained insignificant (adjusted  $R^2=0.893$ ,  $\text{Beta}_{\text{Se}}=0.954$ ,  $P_{\text{Se}} < 0.0001$ ,  $\text{Beta}_{\text{Age}}=0.015$ ,  $P_{\text{Age}}=0.851$ ).

The age-dependent sexual dimorphism is also reflected in the histogram of the frequency distribution, where the crude serum samples (Fig. 6I) indicate a positive skewed distribution ( $v=0.644$ ). In isolated SELENOP preparations, the ratios are less asymmetrically distributed ( $v=0.467$ ) and the major frequency peak resides at 5.0–5.6 atoms of Se per molecule of SELENOP (mean  $\pm$  SD:  $5.4 \pm 0.5$ ; min: 4.4; max: 6.7; Fig. 6J). A total of 43% of the examined sera reside within and > 50% outside of this range, independent of age or sex of the sample donor. Comparing the Se content of the SELENOP molecules in the eluate with the total Se to SELENOP ratio in the sample load results in  $48.3 \pm 5.4\%$  (mean  $\pm$  SD) of Se that is attributable to SELENOP in the young women analyzed. In the other groups, this value is significantly higher (young men;  $54.5 \pm 5.5$ , elderly men;  $56.1 \pm 8.8$ , elderly women;  $56.0 \pm 7.3\%$ ).

#### 4. Discussion

In this manuscript, we report on the generation, characterization, validation and calibration of a new ELISA, which quantifies human SELENOP. In addition, we describe a strategy for determining the Se content of SELENOP in clinical studies, and highlight its variability between healthy subjects. Specificity of the ELISA and the antibodies used was verified by finding a strong correlation of SELENOP to Se concentrations in human sera, supporting the notion that SELENOP is a valid biomarker of Se status [34]. Further proof for its specificity was





**Fig. 5.** Work-flow of the 96-well-affinity-chromatography procedure to isolate SELENOP from human serum samples for analysis. The samples were applied on the immuno-affinity material in a 96-well filter plate, washed and eluted. These steps were separated by a short centrifugation step for collecting the flow through, wash and elution fractions. The combined and neutralized eluates were analyzed by TXRF and SELENOP-ELISA in order to calculate the Se content per SELENOP.

obtained by monitoring sodium selenite-stimulated SELENOP biosynthesis from human hepatocytes. Final evidence was obtained by analyzing serum from mice expressing a human *SELENOP* transgene [31]. Comparing young and elderly male and female subjects, the correlations between Se and SELENOP in serum turned out to be particularly strong in young men, in elderly men and in elderly women, but not in young women. There were no indications of an influence of contraceptives on the circulating Se or SELENOP concentrations when separating the women into subjects using or not-using birth control pills. By isolating SELENOP, we realized that the Se content of SELENOP appeared lower than predicted, not uniform in different subjects, and independent of age and sex. Hence, it does not account for the relatively poor Se to SELENOP correlation in young females. These findings add a novel level of complexity to SELENOP biology and Se status analysis in clinical studies.

Unfortunately, until now there is no generally-accepted reference material for human SELENOP. In order to enable comparisons across laboratories, we have chosen the NIST reference plasma SRM1950 as standard as it has been thoroughly characterized for its Se-containing components [32]. With a total Se concentration of 108.5 µg/L and a calculated SELENOP concentration of 4.44 mg/L, it is well suited to serve as universal reference material as it lies in the same range that is determined in several human studies [16,35,36].

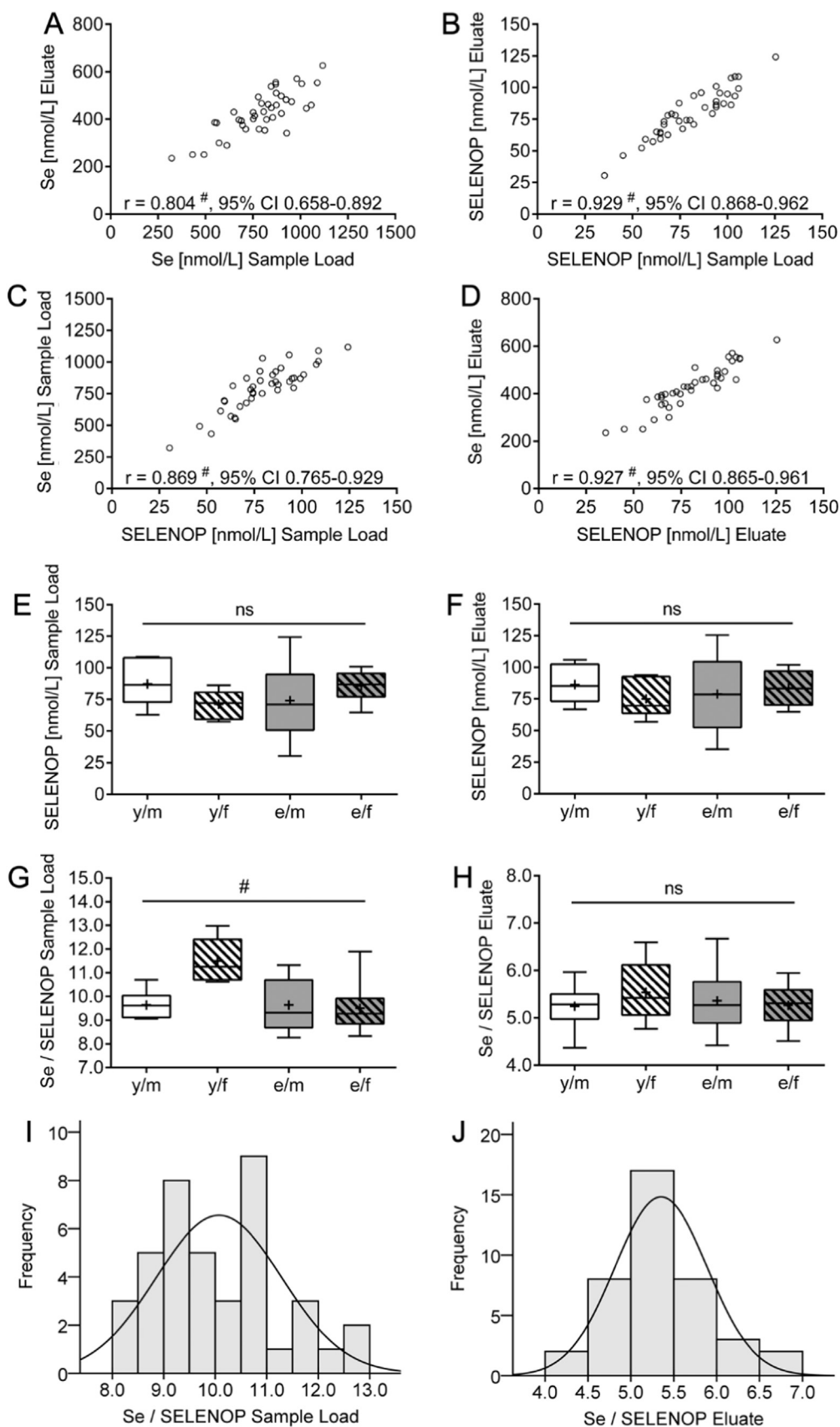
This finding, however, questions the validity of studies reporting grossly different SELENOP concentrations in humans, as mentioned in the introduction. It is of concern, that especially the reports on SELENOP being strongly up-regulated in patients with diabetes mellitus or in obese subjects used an uncharacterized commercial assay. These studies reported average SELENOP concentrations of 0.36 in healthy, 0.87 in prediabetic and 1.03 mg/L in diabetic subjects [15], or 14.5 in lean and 52.3 mg/L in obese subjects [17], i.e., outside a reasonable range in relation to other studies comparing the Se status of healthy and diabetic patients [37–40]. Even from a theoretical consideration, assuming 50% of serum Se attributable to SELENOP with an average of 7.5 atoms of Se per SELENOP molecule and a MW of 51 kDa, a SELENOP concentration of 0.36 mg/L or 52.3 mg/L would correspond to serum Se concentrations of as low as 8.8 µg/L and as high as 1280 µg/L, respectively. Both values are outside a reasonable dimension for healthy human subjects, especially in relation to the congruent results from experienced scientists, who have established quantitative tests for SELENOP determination like Hill and Burk [41], or Saito and Takahashi [19].

The metabolism of Se, the associations of Se status with disease and the effects of Se supplementation show some sex-specific differences [6,42]. It was therefore highly interesting to observe that the correlations of Se and SELENOP concentrations differ between the sexes, and that the correlation was poorest in young women. Differences in Se distribution to GPX3 and SELENOP and a varying Se-content in albumin were noted before in men and women in an epidemiological

study [43]. It is reported that GPX3 is more highly expressed in healthy females than in males [44], and that alcohol consumption increased total Se in women but not in men [45]. Until now, however, only few clinical studies report on both Se and SELENOP concentrations, such as a trial which compared healthy Chinese subjects and reported a higher correlation of Se and SELENOP in young boys as compared to adult men [41]. A small intervention study provided evidence for a sex-specific response of different SELENOP genotypes to Se supplementation [46]. Similarly, upon SeMet supplementation, females appeared to excrete higher amounts of Se in urine when compared to males, indicative of a higher amount of excretable small selenocompounds in blood [47]. Collectively, males and females differ in a number of Se-related parameters, and this notion has been corroborated in several respective animal experiments highlighting pre- and posttranscriptional processes as being responsible for some of the sex-specific differences [48,49].

Very few clinical studies have analyzed gender as separate factor in association analyses, or have specifically compared young and elderly women. More often, specific differences are found with respect to age, e.g., in the relation of Se and blood lipids [50]. However, menopausal state has been described as an important factor in relation to Se status [51]. GPX3 activity increases in parallel to estrogens during the female menstrual cycle [52], and females express higher serum GPX3 concentrations than males [44]. Further speciation studies with human serum are needed to better pinpoint the Se-containing fraction(s) responsible for the relatively poor correlation of Se and SELENOP in young females.

Another surprising finding relates to the average number of Se atoms per SELENOP molecule, which can be covered by a combination of ELISA and trace element quantification of purified SELENOP preparations. Theoretically, the SELENOP transcripts predict the co-translational insertion of 10 Sec residues per molecule. The number of UGA codons per transcript varies among the mammals, and the predicted numbers range from 7 Sec residues per SELENOP in guinea pig to 15 in dog [53]. Our data show that the theoretical predictions should not be taken for granted, as done in cases when genetic information is extrapolated to the proteins [54]. Recently, we have shown that not all theoretical positions are fully occupied by Sec in SELENOP isolated from healthy human subjects [20]. This can be considered as translational relaxation or translational errors, which have been shown to be of relevance for the function of selenoenzymes, e.g. in response to pharmacological treatments with antibiotics of the aminoglycoside type [55–58]. Here, we report that on average only  $5.4 \pm 0.5$  Sec residues per SELENOP molecule are present, and that a considerable inter-individual variation exists. Again, only few studies in the literature report on the Se content of SELENOP. In rat, an average ratio of 7.5 Se atoms per Selenop is reported [59], while in mice the ratio is around 5 Se atoms per Selenop molecule [60]. These results are in agreement with our analysis from human serum, as shown above.



**Fig. 6.** Analysis of Se and SELENOP status of young [y] and elderly [e] male [m] and female [f] participants. Forty samples (10 per group) of the BASE-II study were analyzed and subjected to affinity-chromatography via mAb for the isolation of SELENOP. (A) Se, (B) SELENOP and (C–D) Se and SELENOP concentrations were compared by Pearson correlation. Box-and-Whiskers plots present the mean values (“+”) and 5th–95th percentiles of the SELENOP concentrations in serum (E) or in SELENOP preparations (F). (G) The Se to SELENOP ratio in serum was significantly higher in young women as compared to the other groups. (H) This difference was not observed when analyzing the purified SELENOP preparations. (I) The total Se to SELENOP ratios show an asymmetric distribution across the samples, while (J) the Se to SELENOP ratios of the SELENOP preparations are less skewed distributed.

The molecular reasons for the lower than predicted Se content of SELENOP may be a premature translational termination at UGA codons within the reading frame giving rise to C-terminal shortened and Se-poor isoforms, as suggested by Ma et al. [21]. Accordingly, in the MS/MS-based analyses of the reference plasma SRM1950 of the U.S. population, three SELENOP molecules of 57, 49 and 45 kDa were identified [18]. Alternatively, slightly shortened SELENOP isoforms may result from a specific limited proteolysis within the Se-rich C-terminus [19]. Both mechanisms would explain the relatively low Se content of circulating SELENOP and need to be further analyzed in detail.

Alternatively, it has been shown before that the selenoprotein biosynthesis machinery is not always working very precisely and incorporation errors occur, e.g., in the presence of antibiotics [55–58]. A more relaxed precision rate would also nicely explain the overall phenomenon that such an inefficient and slow process as Sec insertion still proceeds relatively fast despite the consecutive hurdles that are given by a total of successive 10 UGA codons in a single reading frame, as is the case in human SELENOP [61,62]. Other endogenous, dietary or environmental factors may also act in a similar way to aminoglycoside antibiotics, causing incorporation errors specifically at UGA codons within reading frames, i.e., during Sec insertion, and leading to a relatively low Se content of SELENOP. Detailed analyses of purified SELENOP preparations are thus necessary to clarify this finding, which obviously is difficult as in our previous studies, only 4 out of the 10 potential Sec positions could be successfully identified and structurally determined by LC-MS/MS [20]. This finding may point to truncated SELENOP isoforms not reaching the concentrations necessary for successful identification in the MS analyses conducted.

In conclusion, our analyses indicate a specific sexual dimorphism of the correlation between Se to SELENOP in pre-menopausal women, and a considerable inter-individual variation in the Se content of human SELENOP. It remains to be tested which parameters elicit the most intense effect on the Se content of SELENOP, e.g., Se status, genotype, selenocompound intake, epigenetic mechanisms, other hepatotropic endocrine or inflammatory signals, nutritional components, etc. But more importantly, it remains to be tested whether a determination of the Se to SELENOP ratio in human serum offers additional diagnostic, predictive or other health-related information on top of the straight forward analysis of Se status by direct serum Se or SELENOP quantifications. To this end, respective clinical analyses with high-quality serum or plasma samples and employing validated quantification techniques with suitable standard reference materials are needed.

### Conflict of interest statement

T. Schulz is full-time employee of ICI-immunochemical intelligence GmbH, Berlin. The other authors declare that no competing financial interests exist.

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### Clinical trial registry number and website

The medical portion of this trial was registered in the German Clinical Trials Registry ([http://drks-neu.uniklinik-freiburg.de/drks\\_web/navigate.do?navigationId=start](http://drks-neu.uniklinik-freiburg.de/drks_web/navigate.do?navigationId=start)) as DRKS00009277.

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### Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2016.12.025.

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