**Title**: Selenium speciation in human serum and its implications for epidemiologic research: a cross-sectional study

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**Short title**: Selenium species in human serum

**Abstract:**

Observational studies addressing the relation between selenium and human health, particularly cancer risk, yielded inconsistent results, while most recent randomized trials showed a fairly consistent pattern suggesting null or adverse effects of the metalloid. One of the most plausible explanations for such inconsistencies is inadequate exposure assessment in observational studies, commonly carried out by measuring total Se content without taking into account the specific exposure to the individual chemical forms of the metalloid, whose toxic and nutritional properties may vary greatly. Data on the distribution of these species in human blood are very limited, as is their correlation with overall selenium levels.

The concentrations of organic and inorganic selenium species was analyzed in serum of fifty subjects sampled from the general population of the municipality of Modena, northern Italy, aged from 35 to 70 years. Samples were collected during a 30-month period, and determinations of selenium species were carried out using high pressure liquid chromatography coupled with inductively coupled plasma dynamic reaction cell mass spectrometry.

The majority of selenium was found to be present as organic species, but the inorganic forms showed higher levels than expected. These species showed limited correlations with age, sex and body mass index, while the organic forms increased in subjects consuming selenium-containing dietary supplements and decreased in smokers. The length of the sample storage period strongly influenced the distribution of selenium compounds, with a clear tendency towards higher inorganic and lower organic selenium levels over time. In multivariate analysis adjusting for potential confounders, total serum selenium correlated with human serum albumin-bound selenium and, in males, with two organic species of the metalloid (selenocysteine and glutathione peroxidase-bound selenium), while little association existed with the other organic forms and the inorganic ones.

These findings highlight the potential for exposure misclassification of observational epidemiologic investigations based on overall selenium content in blood and possibly other tissues, and the critical role of the storage conditions for speciation analysis.

**Keywords**: selenium, selenium species, serum, exposure, cross-sectional study, assessment

**Introduction**

The relation of metalloid selenium (Se) with human health, and particularly with cancer, is puzzling and still not entirely defined. It encompasses both the possibility of beneficial and adverse effects, the latter being supported by the most recent results of the Selenium and Vitamin E Cancer Prevention trial [[1-3](#_ENREF_1)]. There is a clear consensus about its safe range of exposure being very and unusually narrow, but opinions considerably diverge about the upper and lower safe Se limits as well as the specific outcomes linked to altered Se status, particularly for chronic exposure [[4-7](#_ENREF_4)]. Currently, a marked reassessment of the relation of Se with cancer risk is in progress, since the expectation of a beneficial effect of selenium supplements has vanished [[7](#_ENREF_7),[8](#_ENREF_8)] and indications of an excess risk for skin and prostate cancer [[9-12](#_ENREF_9)] have emerged. Such a trend is also occurring for cardiovascular and metabolic diseases [[13](#_ENREF_13),[14](#_ENREF_14)] } and neurological and cognitive disorders [[15](#_ENREF_15),[16](#_ENREF_16)].

In particular, improvements in the assessment of Se exposure retains a critical position in observational epidemiologic research in order to correctly define its effects on human health. This issue has long attracted the attention of investigators, in order to identify indicators of long-term selenium exposure, such as its concentration in blood, urine, hair and toenails, as well as its average dietary intake through foods and rarely through drinking water [[4](#_ENREF_4),[6](#_ENREF_6),[7](#_ENREF_7),[17](#_ENREF_17)]. Inadequate exposure assessment, particularly long-term, in addition to unmeasured confounding due to life-style and environmental factors and to genetic factors including selenoprotein polymorphisms, has been suggested to explain the conflicting results yielded by observational studies, and may have severely hampered the possibility to reliably identify the health effects of the metalloid. Among the potential pitfalls of the exposure assessment methods, a key role is played by the inability of overall tissue Se levels to adequately reflect exposure to specific selenium compounds [[8](#_ENREF_8),[18](#_ENREF_18)] }. In fact, this element is usually present in environmental sources and in living organisms in various inorganic and organic forms, having considerable and even extreme variations in both toxicological and physiological properties [[4](#_ENREF_4),[15](#_ENREF_15),[19-27](#_ENREF_19)] }, and little is known in the human about the relation of these Se species to each other and more generally with the overall Se level.

In the present study, we aimed to investigate the relation between blood levels of overall Se and individual Se species in a population-based sample from an Italian community, in order to test the adequacy of the exposure assessment based on the former biological indicator.

**Methods**

## Study participants

We selected the study participants from the population of the municipality of Modena, Northern Italy (around 180,000 inhabitants), aiming to recruit a representative sample of 50 individuals. To do so, we randomly sampled eligible subjects from each sex- and age-specific subgroup of Modena residents aged between 35 and 70 years. Fifty-one out of the 150 contacted individuals agreed to participate in this investigation (34% response rate, substantially similar in all subgroups except for a lower one in males <50 years). After we had obtained their written informed consent, the participants were invited to a Modena National Health Unit Center in the morning, to give a fasting venous blood sample. The sample was collected in a plastic tube, immediately centrifuged for 10 min at 3000 rpm and serum aliquots of 1 ml were stored at -15°C until use.

In addition, each participant completed a questionnaire collecting detailed information on education, occupational history, height and current weight, consumption of dietary supplements and smoking habits. . In particular, we asked them to detail specific intake (product, quantity/day, duration) of any dietary product, supplement or drug containing selenium taken at the blood sampling time for at least six months continuously. Assessment of commercial supplements containing Se and their metalloid content was done on the basis of a systematic search on all over-the-counter products marketed in Italy, with the help of the Reggio Emilia Drug Documentation Service. The recruitment of subjects to be included in the study, which was approved by the Modena province Ethical Committee, lasted 30 months.

*Laboratory analysis*

A 1 ml serum aliquot for each study subject was transported by air courier deep frozen in dry ice to the Munich laboratory, and kept continuously frozen until use. We slowly thawed samples in a refrigerator at 4 oC, vortexed and subsequently analyzed them. Suprapure grade chemicals were used throughout. Selenite (Se(IV)), selenate (Se(VI)), selenomethionine (Se-Met), selenocysteine (Se-Cys), thioredoxin reductase (EC 1.8.1.9.)-bound selenium (Se-TrxR), glutathione peroxidase (EC 232-749-6)-bound selenium (Se-GpX), human serum albumin (HSA) and Tris buffer were from Sigma-Aldrich, Deisenhofen, Germany. We purchased certified Se and Rh stock standards (1000 mg/l) from CPI International, Santa Rosa, CA, USA, and we obtained ammonium acetate (NH4Ac) and acetic acid (HAc) from Merck, Darmstadt, Germany. Arliq and methane (99.999% purity) were purchased from Air Liquide, Kleeve Germany. We prepared stock solutions of Se(IV) and Se(VI) at a concentration of 1000 mg Se/l by dissolving in Mili-Q water (18.2 MΩ cm, Milli-Q system, Millipore, Bedford, MA, USA). HSA was prepared at a concentration of 1000 mg/l. Preparation of HSA-Se was performed by mixing 10 mg Se/l selenite with this stock solution and incubating for at least 14 days. Working standards of Se species were prepared daily from their stock standard solutions by appropriate dilution with Milli-Q H2O. Selenoprotein P (SePP) is not commercially available as a standard compound, but it can be prepared from serum using affinity chromatography (AFC). We purified the AFC-SePP fraction by a mass-calibrated size exclusion chromatography (SEC) column, where the SePP fraction eluted at an RT calculated for 62kDa. The complete preparation process of SePP by AFC + SEC and purity checking by anion exchange chromatography (IEC) coupled with inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) is detailed in Solovyev et al. [[19](#_ENREF_19)].

We determined total Se and the Se species Se (IV), Se(VI), Se-Met, Se-Cys, Se-TrxR, Se-GpX, SePP and HSA-Se in serum samples using anion exchange chromatography (IEC) coupled with inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) according to methodologies previously established for biological matrices, [[19](#_ENREF_19),[21](#_ENREF_21)]. In general the IEC separation followed Xu et al. [[28](#_ENREF_28)] but was slightly modified by flattening the elution gradient (see below) for complete peak resolution.

We connected a Knauer 1100 Smartline inert Series gradient HPLC system to an anion exchange column AG 11 (precolumn 50 x 4 mm) + AS 11 (analytical column 250 x 2 mm I.D.) from Thermo (Dionex Idstein, Germany) for species separation. The sample volume was 100 µl. The mobile phases were: eluent A: 10 mM Tris-HAc, pH 8.0; and eluent B: A + 500 mM NH4Ac, pH 8.0. Gradient elution expressed as %-eluent A: 0–3 min 100%; 3–10 min 100–60%; 10–23 min 60–45%; 23–26 min 45–43%; 26–28 min 43–0 %; 28–52 min 0%; 52-60 min 100%. The flow rate was 0.70 ml/min. For internal standardization we mixed the column effluent with 1 µg/l Rh (final concentration, Rh flow rate: 0.1 ml/min) and directed to ICP-MS.

The experimental settings chosen for ICP-DRC-MS (Perkin Elmer NexIon) after optimization were: radio frequency power: 1250 W, plasma gas flow: 15L Ar/min auxiliary gas flow: 1.05L Ar/min, nebulizer gas flow: 0.94 L Ar/min, daily optimized, dwell time 300 ms, ions montored:78Se, 80Se, 103Rh, DRC reaction gas: CH4 reaction at 0.58 ml/min, DRC rejection parameter q: 0.6.

We analyzed total serum Se by graphite furnace atomic absorption spectrometry based on the method of the MAK collection-biomonitoring methods [[29](#_ENREF_29)].

We performed peak quantification from chromatograms by comparing peak areas with peak area calibration curves. We used the standard addition method for standard-retention-time matched identification of Se species and as QC means in quantification. Species identity was further confirmed using a 2-D approach of IEC-capillary electrophoresis (CE)-ICP-DRC-MS as described earlier [[19](#_ENREF_19)]. Species identification was regarded as acceptable when the species matched the standard compounds with both chromatography/electrophoretic techniques (match in first and second technique). Rh and Se data files were exported from the NexIon software and processed with PeakfitTM software for peak area integration. For each sample (or standard) we calculated a quotient of Se-peak area to Rh-peak area and we took it as the result corrected for the internal standard (Rh). The limit of detection for all Se species in serum was 0.02 µg/l.

Quality control was applied to total Se determination by analyzing the control materials ‘human serum’ and ‘urine’ from RECIPE, Munich, Germany. Control materials 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 use. Se concentrations were determined as 63±4 µg/l (serum, n=3) and 22±2 µg/l (urine, n=3), being close to the manufacturer´s target mean values of 62 and 23 (range: 16-30) µg/l, respectively.

In a previous inter-laboratory comparison our SePP quantification was compared to the immuno assay method for SePP determination by Hollenbach and coworkers [[30](#_ENREF_30)]. There the reliability of our SePP determination was verified since the immunologically determined SePP concentration in a pooled CSF sample was 0.54 nM/l (± 9.5%) with SePP being expressed as total protein. According to literature data regarding SePP (10 Se atoms per SePP and molecular weight MW(SePP) = 61 kDa [[31](#_ENREF_31)]) this SePP-protein concentration refers to 0.43 ± 0.04 µg Se/l at SePP corresponding well to our value at 0.41 ± 0.01 µg Se/l at SePP peak. Thus our method was verified by an independent reference method.

For recovery determination during IEC-ICP-DRC-MS individual Se species at a concentration of 10 µg Se/l were analyzed and peak Se concentrations were quantified and related to the injected Se amounts (=100%). Se-species recoveries were 105±12% for GPx, 99±7% for selenite, 103±3% for selenate, 101±6% for TrxR, 87±11% for Se-HSA, and 94±10% for SePP, Similarly, serum samples were quantified for total Se (=100%) before injection and compared to the sum of eluted and quantified chromatographic peaks. Recoveries ranged between 83 and 104 %.

*Data analysis*

We analyzed the percentile distribution of the overall selenium content and individual selenium species in the study population and in selected subgroups. Since we found a subject showing an extremely high and implausible level of inorganic hexavalent Se(VI), 88.7 µg/l, we considered this sample as an outlier and removed the entire subject from data analyses.

When concentration of a Se compound was lower than the detection limit of 0.02 µg/l, we inputted in the database a value of 0.01 µg/l [[32](#_ENREF_32)]. We summed up the organic and inorganic Se species to form the categories of total organic and inorganic selenium, leaving apart the HSA-Se and the unknown Se forms, which were grouped together to form individual categories.

We tested the differences of Se species among the various subgroups by computing P values with the “nonparametric equality-of-medians” test in Stata statistical software (ver. 13.1 - Stata Corp., College Station, TX, 2015). We computed the correlation between total serum Se level and concentrations of individual Se species or their category (organic Se, inorganic Se, HSA-Se, and unknown Se forms) in both crude and multivariate regression analyses. The latter analysis was carried out including in the model the concentration of total Se and of the individual Se species under examination, as well as a few potential confounders: age, sex, BMI, Se supplement use, smoking, sample storage time. When we computed a more complex regression model by adding educational attainment and occupation in the multivariate analysis, estimates did not substantially change and were more statistically unstable (data not shown). Storage time was considered as number of months elapsed from sampling time until analytical determinations, and body mass index (BMI) was computed as weight/height2.

## Results

Table 1 summarizes the characteristics of study subjects. Age ranged from 35 to 70 years, and men and women were almost equally represented. Nine subjects reported a current ongoing consumption of dietary supplements containing selenium, corresponding to an average Se daily additional intake of 28.6 µg.

Distributions of overall serum Se and individual Se species in all subjects and selected subgroups of study population are reported in Table 2. Ninety % of the study subjects showed Se levels ranging from 95 to 161 µg/l, with a median value of 118.5 and a mean value of 122.5; concentrations were lower in the youngest individuals (< 50 years) compared with the older ones, in females compared with males, and in subjects with BMI≥ 25 compared with leaner subjects. Most Se was organic-bound, around three times higher than overall inorganic Se, though this was not entirely true in the oldest participants, who exhibited median levels of inorganic Se of around 50% compared with organic Se. A substantial part of the metalloid was HSA-bound, and levels of HSA-Se tended to exceed those of inorganic Se. Taking into account the individual inorganic and organic Se species, we observed much higher levels of Se(IV) compared with Se(VI), while the majority of organic Se was composed by SePP and, to a lesser extent, Se-GpX. Considering consumption of dietary Se supplements, we found higher levels of Se in supplement users compared with non-users. The increase in total Se in supplement users, around 20%, was exclusively due to an increased amount of organic Se (specifically SePP, Se-GpX and Se-TrxR) and to a lesser extent HSA-Se, while levels of the inorganic species were substantially similar. Current smoking was associated with lower levels of total Se compared with non-smokers, due to the decreased concentrations of organic Se and in particular of SePP and Se-GpX,and despite the higher levels of inorganic Se, as a consequence of higher Se(IV) content. Ex-smokers showed comparable Se to non-smokers, though inorganic Se – mainly Se(IV) - was higher and levels of organic Se were lower (mainly due to a SePP decrease), while HSA-Se content were the highest found in this stratified analysis according to smoking status. In multivariate regression analysis adjusted for confounders, age and BMI showed little correlation with total serum Se and individual Se species (Table 3). BMI showed a slight inverse association with Se(IV) and HSA-Se and direct correlation with organic Se, in particular with Se-Met. Smoking showed little association with overall Se levels in the multivariate analysis, though a specific analysis for the various Se forms suggested a slight direct association with inorganic Se and a much stronger and inverse relation with organic Se. A detailed analysis showed conflicting associations between the individual organic Se species and smoking status, since such a relation was strong and inverse for SePP and Se-GpX while it was direct though slight for Se-Met. Concerning Se supplement use, we detected an independent direct association with total Se levels as well as organic Se, but not the inorganic one or HSA-Se. However, little independent association of any organic Se forms with supplement use emerged, with the partial exception of Se-GpX.

When we focused on the period elapsed from blood sampling until laboratory analyses, such storage time appeared to be strongly associated with total, organic and inorganic Se, as well as with some Se species, both in the crude and multivariate regression model after adjustment for all potential confounders (Figure 1). However, while the association with total and inorganic Se was direct, that with organic Se was inverse. Such associations were mainly due to an inverse relation of months of storage time with SePP in particular and with Se-GpX, while such a relation was positive with the inorganic forms, Se(IV) and Se(VI). Concerning HSA-Se and the unknown Se forms, these species also directly correlated with the storage time, and such a relation was particularly strong for HSA-Se.

Concerning the relation of total serum Se with the individual chemical species (Table 4), in unadjusted analysis we observed a direct association with inorganic Se (both Se(IV) and Se(VI)), HSA-Se and some organic species, namely Se-GpX and Se-Cys. In addition, there was a very weak indication of a direct association with Se-TrxR and an inverse association with SePP. These results were not confirmed in the multivariate model adjusted for potential confounders (age, sex, BMI, smoking, Se supplement use and storage time), where only direct associations emerged, specifically between total Se and HSA-Se as well as between total Se and organic Se considered overall, Se-Cys, Se-GpX and to a lesser extent Se-TrxR. In stratified analysis and after adjusting for confounders, such associations were observed in males but not in females (with the exception of HSA-Se, whose direct association was also detected in females), while no substantial difference emerged according to age, after splitting the study population according the 50 year cutpoint (data not shown).

**Discussion**

Methodology for exposure assessment of Se is a key issue in environmental epidemiology and more generally in public health and human medicine, owing to the powerful toxicity and the nutritional importance of this Janus-faced element [[4](#_ENREF_4),[33-35](#_ENREF_33)] and the difficulties in assessing its long-term low-level exposure in humans. The inadequacies in exposure assessment may have had a major role, in addition to confounding, in inducing the conflicting results of observational epidemiologic studies [[7](#_ENREF_7),[8](#_ENREF_8)], which frequently failed to predict the recent and fairly consistent results of randomized controlled trials [[10](#_ENREF_10),[36-40](#_ENREF_36)] which have substantially changed the approach to the Se and cancer relation, and raised concern about the adverse health effects of this metalloid [[3](#_ENREF_3),[12](#_ENREF_12)].

Most observational epidemiologic studies based their assessment of Se exposure on its overall content in biomarkers such as serum or plasma, hair, toenails and urine [[7](#_ENREF_7)]limitations of these approaches have been discussed in detail [[6-8](#_ENREF_6),[17](#_ENREF_17),[48](#_ENREF_48),[49](#_ENREF_49)], , including the general use of total selenium concentration instead of determination of individual Se species. This limitation, also a consequence of the analytical complexities and the high costs of speciation analyses, may have been a major source of exposure misclassification and thus explain the conflicting results of observational studies on the Se and cancer relation, as well as their partial failure in predicting the results of recent randomized trials [[10](#_ENREF_10),[36-40](#_ENREF_36)]..

In fact, it is well recognized that the various Se species show considerable differences and even opposite properties in their toxic and nutritional activity, as shown in several laboratory studies [[7](#_ENREF_7),[20](#_ENREF_20),[34](#_ENREF_34),[50](#_ENREF_50),[51](#_ENREF_51)]. Although a speciation issue exists not only for Se but also for other trace elements, it is undoubtedly of great importance in the determination of Se, and this favored the development of advanced and complex analytical techniques specifically addressing speciation of this metalloid [[21](#_ENREF_21),[28](#_ENREF_28)]. The potential for bias in epidemiologic research induced by lack of consideration of Se speciation has been clearly shown by a recent investigation on Se species in the cerebrospinal fluid of patients with amyotrophic lateral sclerosis and of controls [[50](#_ENREF_50)]. That study showed that relative risks of the disease computed according to the different forms of the metalloid were totally different, and that risk estimates based on total Se levels were opposite to those based on selenite, the most neurotoxic form of the element.

The results of the present study indicate that circulating levels of total Se, as estimated through overall serum levels, do not adequately reflect those of most Se chemical forms, particularly when the inorganic and some organic species of the metalloid are considered. Such lack of correlation may be more pronounced in females than in males, suggesting possible gender-specific patterns [[7](#_ENREF_7)]. These findings thus highlight the risk of misclassification of exposure to Se species on the basis of total Se levels, and therefore the potential for bias of observational studies, particularly when the health effects under investigation may be selectively or preferably linked to individual Se compounds. Lack of a relation between total Se and some Se species might clearly arise from a considerably variable ‘relative’ composition in Se species of the various foods [[54-58](#_ENREF_54)], the primary source of Se intake for most individuals, or drinking water [[42](#_ENREF_42),[59](#_ENREF_59)], and by individual differences in the metabolism of Se compounds [[22](#_ENREF_22),[60](#_ENREF_60),[61](#_ENREF_61)].

Inaccuracy of total Se in predicting Se species is of particular concern taking into account the biological relevance of some of the ‘unassociated’ species in the present study, Se(IV) and Se(VI) as well as two major organic forms, SePP and Se-Met. These species and particularly the inorganic ones are of toxicological interest[[49](#_ENREF_49)] due to their ability to replace sulfur in sulfhydryl groups [[62](#_ENREF_62),[63](#_ENREF_63)], induce oxidative stress and genotoxicity [[64-67](#_ENREF_64)]. SePP is of greater nutritional interest being an Se-transport protein as well as an antioxidant enzyme, confirmed by its capacity to increase following induction of free radical damage [[23](#_ENREF_23),[68-70](#_ENREF_68)], but it may also adversely affect glucose metabolism [[71](#_ENREF_71),[72](#_ENREF_72)] though its exact involvement in that is still unclear [[73](#_ENREF_73)]. Se-Met has been implicated in diabetes etiology on the basis of the adverse effects reported in the four randomized trials in which this outcome was investigated [[7](#_ENREF_7)], administering selenized yeast (mainly composed by Se-Met [[74](#_ENREF_74)]) or Se-Met alone.

On the contrary, the reasonable agreement in serum of total Se levels with Se-GpX and SePP indicates the substantial adequacy of the former indicator in predicting circulating levels of these two organic Se forms (and vice versa), at least in our study population and probably only in the absence of systematic disease or drug therapy affecting Se status and metabolism.

Our results also suggest that factors such as age, sex, obesity, smoking and particularly storage time, i.e. time elapsed from blood sampling until the implementation of laboratory analyses, may influence levels of some though not all Se species, though generally to a limited extent. The relation of these variables with Se compounds may also be due to unmeasured confounding, i.e. to concurrent variation in dietary habits, alcohol consumption and environmental exposures, or differences in Se metabolism. The study findings in smokers are not unexpected, since smoking has been frequently though not always associated with lower levels of Se in biomarkers [[75-77](#_ENREF_75)], and this may be surprising since tobacco smoke is a source of Se exposure [[43](#_ENREF_43)]. To explain this paradox, the possibility that heavy metals such as cadmium may favor Se elimination from the body has been suggested [[78](#_ENREF_78)]. In our study population, smoking appeared to determine lower content of total Se and particularly organic Se, while it appeared to be slightly associated with higher inorganic Se levels in smokers, a new and unexpected finding which needs further confirmation. We coded in the study smoking status with increasing values from one to three according to non-smoking, ex-smoking and current smoking, a choice which may have induced some exposure misclassification particularly for ex-smokers. Concerning the relation between BMI and Se levels, recently found to be inverse in two European studies [[14](#_ENREF_14),[79](#_ENREF_79)]}, our estimates were too statistically unstable to allow meaningful conclusions, though the different relations we observed for single Se species are interesting and appear to deserve further investigation.

Of interest is the unpredicted association between the relative distribution of the Se species and storage time, with a clear tendency towards higher inorganic Se and lower organic Se levels over time independently from confounding factors. The temperature at which the serum samples spent most of the storage time was -15 °C; no interruption of the sample storage due to technical faults or emergencies occurred during the study period in the laboratory, and the transfer in dry ice with the air courier (which took two days) is unlikely to have considerably increased the storage temperature and degraded the samples. So, we must consider the possibility that the organic Se compounds underwent some metabolic reactions, i.e. degradation and catabolism, leading to their conversion into inorganic forms or HSA-Se, as also suggested by our previous published [[21](#_ENREF_21)] and unpublished observations of ours[[22](#_ENREF_22),[80](#_ENREF_80)]. Results of our study suggest that the length of storage time should be minimal not exceed a few weeks, and that storage temperature should be considerably lower than that used in the present investigation, preferably at -80 oC or even in liquid nitrogen at -196 oC.

A few limitation of this study must be outlined. First, its limited statistical power, which was a consequence of the difficulties in finding volunteers available for the study from the general population and of the large amount of resources required for speciation analysis. This made in general statistically unstable the point estimates, though the coherence across age groups and sexes of some results and the association of serum Se species with dietary supplements and smoking status, as expected [[81](#_ENREF_81),[82](#_ENREF_82)] }, appears to confirm the reliability of study results.

The ability of the study sample to adequately represent the underlying general population of in another issue of concern. Though eligible subjects were randomly recruited from the Modena residents, acceptance of participation in the study may have been favored and hampered by some variables (education, gender, lifestyle factors).

A third *caveat* which needs to be outlined is that Se species concentrations may not be precise estimates of the biological activity of Se forms and Se-containing enzymes, which can be influenced by several factors, including the tissue redox status and the agonistic/antagonistic interactions with heavy metals and nutritional factors [[4](#_ENREF_4),[7](#_ENREF_7),[8](#_ENREF_8),[20](#_ENREF_20)]. Moreover, patterns of Se species observed in serum of study subjects may not necessarily correspond to their distribution in other body districts and tissues, neither to what might be observed with very different types and amounts of Se exposure. In fact, laboratory and human studies have shown that relative distribution of Se species may differ according to the amount and type of dietary Se, as well as the body tissue and district under investigation and the disease status [[50](#_ENREF_50),[56](#_ENREF_56),[85-90](#_ENREF_85)] }, and even the correlation of the various Se species found in different compartments may vary greatly [[19](#_ENREF_19),[50](#_ENREF_50)], thus highlighting the complexity of issues such as tissue-specific deposition and metabolism of the various Se species. Finally, it must be noted that, following ingestion of different Se species, retention of organic forms in body tissues is consistently higher than that of inorganic compounds [[91](#_ENREF_91)], and therefore levels of Se species in serum (or other tissues) may not necessarily reflect the relative intake of these species through diet and other sources, nor their toxicity and biological effects [[4](#_ENREF_4),[92](#_ENREF_92)].

In conclusion, our results suggest that in human serum some Se species are uncorrelated to each other, and they may not be adequately represented by the biomarker frequently used in epidemiologic observational studies for exposure assessment, total serum Se content. This suggests the need to take into account speciation analysis in such studies, to avoid severe misclassification of exposure to some Se compounds and its potential for bias.

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Table 1. Characteristics of study population

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | All subjects | | Males | | Females | |
|  | n | (%) | n | (%) | n | (%) |
| All subjects | 50 | (100) | 26 | (52) | 24 | (48) |
| Age  35-49 y  50-70 y | 23  27 | (46)  (54) | 12  14 | (46)  (54) | 11  13 | (46)  (54) |
| Body Mass Index  <20  20-24  25-29  ≥30 | 2  20  21  7 | (4)  (40)  (42)  (14) | 0  9  15  2 | (0)  (34)  (58)  (8) | 2  11  6  5 | (8)  (46)  (25)  (21) |
| Se Supplements users  Yes  No | 9  41 | (18)  (82) | 5  21 | (19)  (81) | 4  20 | (17)  (83) |
| Smoking habits  Never smoked  Ex-smokers  Current smokers | 26  15  9 | (52)  (30)  (18) | 13  8  5 | (50)  (31)  (19) | 13  7  4 | (54)  (29)  (17) |
|  |  |  |  |  |  |  |
| Storage time (months)  >36  24-36  <24 | 25  10  15 | (50)  (20)  (30) | 13  3  10 | (50)  (12)  (38) | 12  7  5 | (50)  (29)  (21) |

*aHealth, education, and business*

Table 2. Distribution of serum Se species median concentration (µg/l) in the all study subjects (n=50) and in selected subgroups. Inorganic Se includes Se(IV) and Se(VI); organic Se: SePP, Se-Met, Se-GpX, Se-Cys, and Se-TrxR.

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Percentile | | |  | Age (n) | | |  | Gender (n) | | |  | BMI (n) | | |  | Se Suppl. users (n) | | |  | Smoking habits (n) | | | |
| Species | 5th | 50th | 95th |  | <50(23) | ≥50(27) | *P1* |  | Males(26) | Females(24) | *P1* |  | <25(22) | ≥25(28) | *P1* |  | Yes(9) | No(41) | *P1* |  | Non (26) | Ex-(15) | Yes (9) | *P1* |
| Total Se | 95.00 | 118.50 | 161.00 |  | 109.00 | 127.00 | *0.002* |  | 122.50 | 114.50 | *0.089* |  | 115.00 | 124.50 | *0.023* |  | 138.00 | 118.00 | *0.269* |  | 121.50 | 121.00 | 113.00 | *0.171* |
| Inorganic Se | 4.60 | 21.15 | 67.20 |  | 11.10 | 29.80 | *0.156* |  | 20.00 | 23.75 | *0.571* |  | 23.10 | 18.35 | *0.569* |  | 19.30 | 21.60 | *0.713* |  | 14.50 | 22.40 | 23.80 | *0.225* |
| Se(IV) | 0.70 | 15.85 | 55.10 |  | 8.10 | 28.70 | *0.011* |  | 15.85 | 16.75 | *1.000* |  | 19.70 | 14.00 | *0.254* |  | 16.50 | 15.20 | *0.713* |  | 9.75 | 20.20 | 20.60 | *0.206* |
| Se(VI) | 0.01 | 3.05 | 10.00 |  | 2.20 | 4.60 | *0.047* |  | 2.85 | 3.30 | *0.571* |  | 2.45 | 4.60 | *0.254* |  | 2.70 | 3.70 | *0.066* |  | 2.85 | 2.90 | 3.70 | *0.543* |
| HSA-Se | 4.00 | 25.50 | 68.90 |  | 16.20 | 39.80 | *0.001* |  | 28.60 | 23.15 | *0.153* |  | 24.85 | 25.50 | *0.802* |  | 30.70 | 25.50 | *0.713* |  | 23.95 | 30.70 | 25.50 | *0.302* |
| Unknown | 0.01 | 0.01 | 12.70 |  | 0.01 | 3.00 | *0.006* |  | 0.01 | 0.01 | *0.608* |  | 0.01 | 0.01 | *0.707* |  | 0.01 | 0.01 | *0.750* |  | 0.01 | 1.10 | 0.50 | *0.077* |
| Organic Se | 8.43 | 66.51 | 105.40 |  | 78.21 | 57.31 | *0.002* |  | 75.66 | 60.60 | *0.258* |  | 62.81 | 68.76 | *0.569* |  | 82.21 | 66.41 | *0.713* |  | 79.56 | 57.31 | 67.31 | *0.302* |
| SePP | 1.00 | 26.95 | 80.80 |  | 44.70 | 10.60 | *0.011* |  | 27.80 | 24.20 | *1.000* |  | 25.05 | 26.95 | *1.000* |  | 29.40 | 24.50 | *0.713* |  | 39.25 | 19.00 | 4.20 | *0.515* |
| Se-Met | 0.01 | 0.01 | 7.60 |  | 0.01 | 0.01 | *0.896* |  | 0.01 | 0.01 | *0.333* |  | 0.01 | 0.01 | *0.585* |  | 0.01 | 0.01 | *0.342* |  | 0.01 | 0.01 | 0.01 | *0.708* |
| Se-Cys | 0.01 | 2.60 | 15.30 |  | 1.70 | 3.60 | *0.042* |  | 2.40 | 2.60 | *0.982* |  | 1.60 | 2.60 | *0.945* |  | 1.00 | 2.60 | *0.918* |  | 2.10 | 4.20 | 0.80 | *0.092* |
| Se-GpX | 2.60 | 17.10 | 49.00 |  | 17.80 | 15.20 | *0.395* |  | 17.85 | 13.00 | *0.571* |  | 17.20 | 16.60 | *1.000* |  | 18.90 | 15.20 | *0.269* |  | 17.70 | 15.60 | 16.60 | *0.847* |
| Se-TrxR | 0.01 | 5.95 | 20.90 |  | 6.60 | 5.60 | *0.395* |  | 6.15 | 5.95 | *1.000* |  | 6.30 | 5.80 | *0.569* |  | 9.20 | 5.70 | *0.269* |  | 5.35 | 7.20 | 9.40 | *0.431* |

*1P value of nonparametric equality-of-medians test*

Table 3. Crude and adjusted regression analysis of total serum Se and individual Se species in all subjects (n=50) in relation to age, sex, body mass index, smoking and Se supplement use. Multivariate analysis adjusted for all these variables as well as for storage time (smoking categorized as 0=non-smoker, 1=ex-smoker, 2=current smoker; Se supplement use as 0=no, 1=yes). Results expressed as regression coefficient (β), 95% confidence interval (CI) and P value (*P*).

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Crude |  |  |  | Adjusted |  |
|  | β | 95 % CI | *P* |  | β | 95 % CI | *P* |
| *Predictor variable: Age* | | | | | | | |
| Total Se | 0.94 | (0.45, 1.44) | *<0.001* |  | 0.31 | (-0.43, 1.05) | *0.406* |
| Inorganic Se | 0.71 | (0.23, 1.18) | *0.004* |  | 0.08 | (-0.66, 0.81) | *0.828* |
| Se(IV) | 0.57 | (0.13, 1.00) | *0.011* |  | 0.07 | (-0.60, 0.74) | *0.831* |
| Se(VI) | 0.14 | (0.04, 0.24) | *0.008* |  | 0.01 | (-0.15, 0.17) | *0.918* |
| HSA-Se | 0.90 | (0.35, 1.44) | *0.002* |  | -0.30 | (-1.02, 0.41) | *0.393* |
| Unknown | 0.14 | (0.00, 0.27) | *0.048* |  | -0.03 | (-0.24, 0.18) | *0.747* |
| Organic Se | -0.79 | (-1.61, 0.03) | *0.058* |  | 0.58 | (-0.52, 1.67) | *0.296* |
| SePP | -0.72 | (-1.44, -0.01) | *0.048* |  | 0.45 | (-0.62, 1.53) | *0.401* |
| Se-Met | 0.02 | (-0.07, 0.11) | *0.667* |  | 0.00 | (-0.15, 0.14) | *0.952* |
| Se-Cys | 0.12 | (0.00, 0.23) | *0.044* |  | 0.08 | (-0.11, 0.28) | *0.406* |
| Se-GpX | -0.22 | (-0.61, 0.16) | *0.246* |  | 0.02 | (-0.57, 0.61) | *0.951* |
| Se-TrxR | 0.02 | (-0.14, 0.18) | *0.842* |  | 0.03 | (-0.24, 0.29) | *0.832* |
| *Predictor variable: Body mass index* | | | | | | | |
| Total Se | -0.38 | (-1.65, 0.89) | *0.551* |  | -0.14 | (-1.21, 0.94) | *0.801* |
| Inorganic Se | -0.86 | (-1.99, 0.27) | *0.132* |  | -0.54 | (-1.60, 0.53) | *0.317* |
| Se(IV) | -0.98 | (-1.99, 0.02) | *0.055* |  | -0.73 | (-1.70, 0.25) | *0.141* |
| Se(VI) | 0.12 | (-0.12, 0.36) | *0.323* |  | 0.19 | (-0.04, 0.42) | *0.104* |
| HSA-Se | -1.51 | (-2.79, -0.23) | *0.022* |  | -0.84 | (-1.87, 0.20) | *0.110* |
| Unknown | -0.06 | (-0.37, 0.26) | *0.720* |  | 0.03 | (-0.28, 0.34) | *0.845* |
| Organic Se | 2.06 | (0.24, 3.87) | *0.027* |  | 1.22 | (-0.38, 2.81) | *0.131* |
| SePP | 0.84 | (-0.82, 2.50) | *0.314* |  | 0.16 | (-1.41, 1.72) | *0.842* |
| Se-Met | 0.21 | (0.01, 0.42) | *0.038* |  | 0.23 | (0.01, 0.44) | *0.038* |
| Se-Cys | 0.11 | (-0.16, 0.37) | *0.427* |  | 0.12 | (-0.16, 0.41) | *0.386* |
| Se-GpX | 0.66 | (-0.19, 1.52) | *0.125* |  | 0.48 | (-0.38, 1.34) | *0.263* |
| Se-TrxR | 0.23 | (-0.12, 0.58) | *0.187* |  | 0.23 | (-0.16, 0.61) | *0.239* |
| *Predictor variable: Smoking habits* | | | | | | | |
| Total Se | -2.79 | (-10.49, 4.91) | *0.470* |  | -2.23 | (-8.45, 3.99) | *0.474* |
| Inorganic Se | 3.72 | (-3.24, 10.68) | *0.288* |  | 4.05 | (-2.11, 10.21) | *0.192* |
| Se(IV) | 3.46 | (-2.8, 9.73) | *0.272* |  | 3.76 | (-1.87, 9.39) | *0.185* |
| Se(VI) | 0.26 | (-1.22, 1.74) | *0.728* |  | 0.29 | (-1.04, 1.62) | *0.665* |
| HSA-Se | -0.03 | (-8.24, 8.19) | *0.995* |  | 0.36 | (-5.61, 6.33) | *0.903* |
| Unknown | 1.62 | (-0.25, 3.48) | *0.087* |  | 1.72 | (-0.05, 3.48) | *0.056* |
| Organic Se | -8.05 | (-19.43, 3.34) | *0.162* |  | -8.30 | (-17.49, 0.89) | *0.076* |
| SePP | -7.05 | (-17.03, 2.93) | *0.162* |  | -7.35 | (-16.37, 1.66) | *0.107* |
| Se-Met | 1.22 | (-0.02, 2.45) | *0.053* |  | 1.22 | (-0.02, 2.46) | *0.054* |
| Se-Cys | -0.34 | (-1.97, 1.29) | *0.678* |  | -0.25 | (-1.89, 1.40) | *0.765* |
| Se-GpX | -3.11 | (-8.35, 2.12) | *0.238* |  | -3.24 | (-8.19, 1.72) | *0.195* |
| Se-TrxR | 1.24 | (-0.91, 3.38) | *0.252* |  | 1.31 | (-0.90, 3.53) | *0.238* |
| *Predictor variable: Se supplements use* | | | | | | | |
| Total Se | 17.41 | (2.86, 31.96) | *0.020* |  | 13.48 | (0.89, 26.06) | *0.036* |
| Inorganic Se | 2.91 | (-11.08, 16.89) | *0.678* |  | 0.69 | (-11.78, 13.15) | *0.912* |
| Se(IV) | 4.27 | (-8.30, 16.83) | *0.498* |  | 2.40 | (-8.99, 13.80) | *0.673* |
| Se(VI) | -1.36 | (-4.27, 1.56) | *0.355* |  | -1.71 | (-4.41, 0.98) | *0.206* |
| HSA-Se | -0.39 | (-16.72, 15.95) | *0.962* |  | -3.24 | (-15.32, 8.84) | *0.592* |
| Unknown | 2.39 | (-1.37, 6.15) | *0.207* |  | 2.20 | (-1.36, 5.77) | *0.219* |
| Organic Se | 12.48 | (-10.37, 35.32) | *0.278* |  | 13.79 | (-4.81, 32.39) | *0.142* |
| SePP | 4.62 | (-15.61, 24.85) | *0.648* |  | 5.54 | (-12.70, 23.77) | *0.544* |
| Se-Met | -0.56 | (-3.12, 1.99) | *0.659* |  | -0.38 | (-2.89, 2.13) | *0.763* |
| Se-Cys | 0.73 | (-2.52, 3.97) | *0.655* |  | 0.30 | (-3.02, 3.63) | *0.856* |
| Se-GpX | 5.91 | (-4.52, 16.35) | *0.260* |  | 6.33 | (-3.69, 16.35) | *0.210* |
| Se-TrxR | 1.78 | (-2.51, 6.07) | *0.409* |  | 2.00 | (-2.48, 6.48) | *0.372* |

Table 4. Regression analysis of serum total Se concentration with individual Se species in crude and multivariate analysis (adjusted for age, sex, BMI, smoking, Se supplement use, and sample storage time in the whole population, and in males and in females (unadjusted analysis only). Results expressed as regression coefficient (β), 95% confidence interval (CI) and P value (*P*).

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| All subjects  (n=50) |  | Crude |  |  |  | Adjusted |  |
| β | 95 % CI | *P* |  | β | 95 % CI | *P* |
| Inorganic Se | 0.42 | (0.13, 0.72) | *0.006* |  | 0.18 | (-0.13, 0.49) | *0.259* |
| Se(IV) | 0.47 | (0.14, 0.80) | *0.006* |  | 0.21 | (-0.13, 0.54) | *0.226* |
| Se(VI) | 0.95 | (-0.54, 2.44) | *0.207* |  | 0.08 | (-1.38, 1.53) | *0.913* |
| HSA-Se | 0.50 | (0.27, 0.73) | *0.000* |  | 0.44 | (0.14, 0.73) | *0.004* |
| Unknown | 1.21 | (0.09, 2.32) | *0.034* |  | 0.28 | (-0.82, 1.37) | *0.614* |
| Organic Se | 0.00 | (-0.19, 0.20) | *0.966* |  | 0.18 | (-0.02, 0.38) | *0.078* |
| SePP | -0.14 | (-0.36, 0.07) | *0.194* |  | -0.01 | (-0.23, 0.20) | *0.894* |
| Se-Met | -0.23 | (-1.98, 1.52) | *0.791* |  | -0.14 | (-1.70, 1.42) | *0.858* |
| Se-Cys | 1.52 | (0.21, 2.82) | *0.023* |  | 1.02 | (-0.12, 2.15) | *0.078* |
| Se-GpX | 0.31 | (-0.10, 0.72) | *0.138* |  | 0.44 | (0.07, 0.81) | *0.020* |
| Se-TrxR | 0.61 | (-0.41, 1.63) | *0.236* |  | 0.66 | (-0.19, 1.51) | *0.127* |
|  |  |  |  |  |  |  |  |
| Gender |  | Male(n=26) |  |  |  | Female(n=24) |  |
|  | β | 95 % CI | *P* |  | β | 95 % CI | *P* |
| Inorganic Se | 0.21 | (-0.22, 0.65) | *0.311* |  | 0.16 | (-0.40, 0.72) | *0.557* |
| Se(IV) | 0.18 | (-0.29, 0.66) | *0.425* |  | 0.29 | (-0.35, 0.93) | *0.351* |
| Se(VI) | 2.28 | (-0.29, 4.86) | *0.079* |  | -0.77 | (-2.79, 1.25) | *0.434* |
| HSA-Se | 0.45 | (-0.11, 1.01) | *0.110* |  | 0.36 | (-0.09, 0.81) | *0.106* |
| Unknown | -0.41 | (-1.86, 1.03) | *0.556* |  | 2.28 | (-0.12, 4.68) | *0.061* |
| Organic Se | 0.21 | (-0.08, 0.50) | *0.147* |  | 0.18 | (-0.16, 0.52) | *0.288* |
| SePP | -0.13 | (-0.45, 0.20) | *0.421* |  | 0.14 | (-0.20, 0.47) | *0.400* |
| Se-Met | -1.07 | (-3.44, 1.31) | *0.359* |  | 1.02 | (-1.44, 3.48) | *0.393* |
| Se-Cys | 2.09 | (0.18, 4.00) | *0.033* |  | 0.77 | (-0.93, 2.48) | *0.351* |
| Se-GpX | 0.66 | (0.28, 1.04) | *0.002* |  | -0.44 | (-1.36, 0.48) | *0.325* |
| Se-TrxR | 0.71 | (-0.60, 2.02) | *0.268* |  | 0.61 | (-0.72, 1.93) | *0.347* |

Figure 1. Regression analysis of total serum Se and individual serum Se species with storage time (months elapsed from blood sampling to analytical determinations). Dash line: crude analysis. Solid line: analysis adjusted for age, sex, body mass index, smoking and selenium supplement use. Regression coefficient (β) with its 95% confidence interval and P-value reported in each graph.