Insights into cancer and neurodegenerative diseases through selenoproteins and the connection with gut microbiota – current analytical methodologies

Running title: Selenium speciation in medicine

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Article key issues

- Selenium plays important roles in medicine, namely: inhibiting angiogenesis, controlling proliferation of malignant cells, immune system, thyroid function, fertility, as an antagonist of xenobiotics and controlling several diseases (Kaschin-Beck, Keshan, cardiovascular, cancer and neurodegenerative diseases).
- The essentiality or toxicity character is of selenium depends on both, a narrow range of concentration and the chemical specie involved. The chemical species present in the human body are selenoproteins, selenium containing proteins and selenometabolites.

- Due to the health benefits of selenium, nutraceuticals and functional foods enriched in this element have received considerable attention in recent years.

- Microbiota colonization is shaped by diet and dietary components, including selenium among other trace elements. It has been demonstrated that dietary selenium affects the composition and diversity as well as the microbial gut colonization, which, in turn, influence the host selenium status and selenoproteome expression.

- The use of ICP-MS allows quantifying selenium at very low detection limits, with high tolerance to matrix, large linearity range and multielemental capabilities. The ICP-MS can be coupled to different chromatographic systems for selenium speciation.

- IDA can be performed for quantification, with important advantages, namely: (i) absence of matrix effects; (ii) absence of instrumental drift; (iii) correction with dilution or preconcentration factors is not necessary; (iv) uncertainty only depends on the measurement of the relative abundances.

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Abstract

Introduction: Selenium plays many key roles in health especially in connection with cancer and neurodegenerative diseases, among others. However, it needs to be appreciated that the essentiality or toxicity action of selenium depends on both, a narrow range of concentration and the chemical specie involved. In this context, human selenoproteins are essential biomolecules against these disorders, mainly due to its antioxidant action. To this end, analytical methodologies may allow identifying and quantifying individual selenospecies in human biofluids and tissues.

Areas covered: This review focus on the role of selenoproteins in medicine, with special emphasis in cancer and neurodegenerative diseases, considering the possible link with gut microbiota. In particular, this article reviews the analytical techniques and procedures recently developed for the absolute quantification of selenoproteins and selenometabolites in human biofluids and tissues.

Expert commentary: The beneficial role of selenium in human health has been extensively studied and reviewed. However, severe challenges remain that are highlighted and discussed in this article; these include: (i) speciation of selenium (especially selenoproteins) in cancer and neurodegenerative disease patients; (ii) supplementation of selenium in humans using functional foods and nutraceuticals; (iii) the link between selenium and selenoproteins expression and the gut microbiota and (iv) analytical methods and pitfalls for the absolute quantification of selenoproteins and selenometabolites.

Keywords: cancer; ICP-MS; metallomics; microbiota; neurodegenerative diseases; selenium; selenoproteins; speciation.

1. Introduction

Selenium is a well-known essential element which plays key roles in medicine. The relationship between Se intake and status is not linear, but approximates more closely to a U-shape where adverse effects (*e.g.* on mortality and prostate cancer) are found at low and high Se intake [1]. Moreover, the bioaccessibility, essentiality or toxicity character depends not only on its narrow range of required concentration, but also on the chemical form. In this sense, there are three types of selenium containing biomolecules in the body, namely: (i) selenometabolites or selenospecies of molecular mass below 1500 Da (*e.g.* inorganic selenium, selenoamino acids, methylated selenium), (ii) selenium containing proteins, containing selenomethionyl residues (*e.g.* selenoalbumin (SeAlb)) [2] and (iii) selenoproteins, with selenocysteinyl residues incorporated by a specific codon and –SeH as the active center (*e.g.* glutathione peroxidase (GPx), selenoprotein P (SELENOP) and other selenoenzymes). Figure 1 illustrates the selenium metabolism in human body after intake. As can be seen, selenocysteine (SeCys) is co-translationally incorporated into certain proteins by an in-frame opal stop codon (UGA) in combination with specific stem-loop structures within the 3´-untranslated region of transcripts and a unique set of accessory factors. This aminoacid represents the 21st proteinogenic residue not included in the classical genetic code [3]. Selenide is a common intermediate metabolite in the synthesis of selenoproteins from inorganic or organic selenium precursors. Selenomethionine (SeMet) can be transformed into selenocystathionine and then to SeCys through the trans-selenation pathway (cystathionine β-synthase and cystathionine γ-lyase) to be later lysed by β-lyase to selenide. At the same time, SeMet can be used for the synthesis of selenium containing proteins by methionine-RNAt [4]. There are 25 genes encoding for about 25 human selenoproteins, the majority of them without a clear function defined. All of the selenoproteins that catalyses redox reactions belong to the family of

glutathione peroxidases (GPxs) [5], thyroid hormone de-iodinases [6] methionine-R-sulphoxide reductase [7] and thioredoxin reductase isoenzymes [8]. However, antioxidant activities have not yet been demonstrated for all selenoproteins.

In the bloodstream, selenoprotein P (SELENOP) accounts for the higher content of selenium and is a good biomarker of Se status in humans, but extracellular GPx and selenoalbumin (SeAlb) can also be used as complementary indicators [9].

Extracellular GPx (eGPx), which is the form present in plasma accepts both thioredoxin and glutathione as a reduction co-substrate. In addition, it is the most important selenoenzyme in the detoxification of reactive oxygen species (ROS) catalysing the reduction of extracellular hydrogen peroxide and lipid hydroperoxide [10]. SELENOP transports selenium from the liver to peripheral [11]. Besides transport, this selenoprotein acts as an antioxidant in the extracellular space, located in the endothelium, where it is bound to heparin and associated with carbohydrates [12], protects plasma proteins against oxidation and nitration [13], protects human astrocytes and endothelial cells from oxidative damage [14] and reduces phospholipid hydroperoxides by means of gluthathione or thioredoxin co-substrates.

In general, among the wide range of physiological functions of selenium, selenium is well known for its pivotal roles in health, for example in inhibiting angiogenesis [15], controlling proliferation of malignant cells [16], enhancing the immune response [17], inhibiting the activation of certain transcription factors [18], controlling of human Kaschin-Beck disease, Keshan disease, maintaining thyroid function, preventing cardiovascular disease, improving fertility, preventing cancer, preventing neurodegenerative diseases [1,19], as well as antagonizing the toxicity action of several elements being mercury the most known [20]. Table 1 shows the

selenoproteins determined in biofluids and tissues from cancer and neurodegenerative disease patients and model organisms or cells.

In this review, we discuss the roles of selenium in health, with an especial emphasis in cancer and neurodegenerative diseases, as well as its connection with gut microbiota. Current analytical approaches to determine chemical species of selenium in human biofluids and tissues are also reviewed.

2. The importance of selenium in nutrition and supplementation

Due to the health benefits of selenium, functional foods containing this element (*e.g.* Saccharomyces cerevisiae yeast (Baker's yeast)) and nutraceuticals (*i.e.* multivitamins and multimineral preparations containing inorganic Se) have received considerable attention in recent years. The selenized yeast (Se yeast) is particularly attractive due to its low cost, facility to grow under different conditions and its ability to assimilate up to 3000 mg g^{-1} of Se starting from sodium selenite added to the growth medium. However, other Se-rich supplements can be proposed, like microalgae [21].

The chemical specie of selenium ingested is very important since they have different essentiality character and bioaccessibility. Once ingested, selenomethionine is randomly incorporated into proteins at methionine positions [22]. However, as discussed below, selenium can only be recognized by the body after selenomethionine catabolization to be incorporated into selenoproteins, which explain that experimental exposure experiments usually feed this element in the inorganic form [23]. Otherwise, experiments reflect both the specific and the highly variable non-specific chemical forms of selenium which are difficult to correlate with specific selenium metabolic processes. On the other hand, it is well known that inorganic forms of Se are more

acutely toxic than organic forms such as selenized yeast, in which SeMet is the main specie (54–74%) of the total Se [24]. However, organic forms of Se have been reported to be more toxic during long-term consumption due to the rapid incorporation into tissue proteins rather than be excreted.

Dietary Se intakes depend on regional and food variability, but an adequate intake (AI) range from 15 µg/day for children (1-3 years) to 70 µg/day for adolescents aged 15–17 years and adults [25]. According to Food and Agriculture Organization (FAO) and World Health Organization (WHO), the recommended daily allowance (RDA) was set at 26 µg/day for women and 34 µg/day for men aged 18–65 years [25]. On the other hand, the tolerable upper intake level (UL) for selenium is 300 µg Se/day for adults. This value covers selenium intake from all sources of food, including supplements [26].

Due to the narrow Se concentration range considered beneficial and non-toxic [27], the Se bioavailability/bioaccessibility of Se from a food is crucial to guarantee the required assimilation [28], since usually only a fraction is absorbed and transformed into a biologically available form. Likewise, the upper limit of therapeutic Se dose (0.2 mg per day) is only a few times less than the potentially toxic dose (0.8–1.0 mg per day) [23]. Regarding the assimilation of the element in the body, the Se-bioavailable fraction can be considered as the quantity of Se absorbed through the intestinal barrier reaching later the systemic circulation. On the other hand, the Se-bioaccessible fraction is the quantity of Se that is soluble in the intestine and finally, the Se-bioactive fraction is that which is transformed into active selenometabolites [29]. It has been reported that organic compounds of Se are more bioavailable than the inorganic forms, and that other factors such as total protein, fat and the presence of heavy metals critically affect Se bioaccessibility in a food [30]. Likewise, approximately 80% of dietary Se is absorbed,

although it depends on the type of food consumed, the chemical specie and the Se status of the subject [30].

3. Selenoproteins in cancer

The anticancer metabolic mechanisms requires sturdy antioxidant systems against the progressive cell proliferation to protect normal cells from oxidative stress [31]. In this sense, selenium plays important roles in redox regulation suggesting that this element have chemopreventive properties inducing apoptosis in malignant cells without affecting normal cells [32]. On the other hand, some investigations have reported the role of selenium in cancer demonstrating that their supplementation decreases the mortality of different types of cancer [33,34] and that the risk of several cancers (*e.g.* lung cancer) decrease in populations with high levels of selenium [35,36]. As previously stated, the role of selenium heavily depends on its concentration and experiments with cells evidenced that low concentrations are absolutely necessary for cell growth, but moderate to high concentrations inhibit it. This inhibiting action is tumor-specific and selenium induces apoptosis in malignant cells at concentrations that do not affect the viability of normal cells [32].

One of the most important actions of several selenoproteins is the antioxidant activity to overcome the oxidative damage [37] and sometimes their altered expression has been linked to cancer risk [38]. Table 1 shows the selenoproteins determined in biofluids and tissues from cancer and neurodegenerative disease patients as well as model organisms or cells. Some specific selenoproteins might be involved in tumorigenesis, such as SELENOP required for the synthesis of other selenoproteins and implicated in selenium transport [39]. Some polymorphisms in SELENOP are associated with an increase on cancer susceptibility [40]. On the other hand, several

studies reported decreased levels of SELENOP in hepatocellular carcinomas, gastric adenocarcinomas, colorectal and prostate cancers [41], although their expression does not always diminished in all types of cancer as for example in metastatic melanoma in which they increased compared to normal melanocytes [42]. Moreover, a decrease in the risk of lung cancer has been reported and related to high levels of SELENOP [35].

The main family of selenoproteins that has been linked to cancer is GPx due to its antioxidant activity to overcome the oxidative stress caused by free radicals [37]. The family of Gluthathione Peroxidases (GPXs) are the most studied selenoproteins in the field of cancer because of their antioxidant capabilities. Among GPXs, GPx3, which is expressed in the kidney and secreted to plasma, presents the most specific functions as tumor-supressor and their downregulation is associated with poor prognosis in different types of cancer [43]. On the other hand, the overexpression of GPx3 triggers a decrease of metastasis in prostate cancer cells [44]. In the same way, the over-expression of GPx4, located in cellular membranes, decrease cancer growth in fibrosarcoma cells [45]. The polymorphisms of GPx have also been studied. For example, Moscow et al found an allelic variant of GPx1 gene, that is linked to an increase risk of lung cancer [46]. Moreover, the genotypes of GPX1, GPX4, TXNRD2 and SEP15 as well as total selenium have been associated with a higher risk of lung cancer [47]. Other preliminary studies also suggest that genetic variants in some of these selenoproteins may affect their function and cancer risk [48], but to date little information is available on the extent of genetic variability in selenoproteins with the possible impact on cancer risk [47]. In the case of GPx1, it has been suggested that the presence of variants affects cancer risk differently [49]. Likewise, the GPx1 allele with five Ala repeats is significantly associated with breast cancer risk [50] while

overexpression of GPx2 gene is associated with increased differentiation and proliferation in colorectal cancer [51].

On the other hand, Thioredoxin Reductases (TrxR) are important selenoproteins for redox homeostasis capable of reducing disufide bonds [41]. The expression of these selenoproteins can protect against malignant transformation [52] and inhibit the tumour progression and metastasis [41]. TXNRD1 promotes tumour growth, DNA replication, and tumorigenicity and its downregulation increases sensitivity of cancer cells to some chemotherapy drugs suggesting it potential use as target for anticancer agents [53].

Finally, the role of metals in cancer onset and progression is well known [54]. In this sense, the antagonistic action of selenium against the toxicity of mercury, arsenic and other metals have been described [55].

4. Selenoproteins in neurodegenerative diseases

Selenium also plays a key protective role against oxidative stress in neurodegenerative diseases. In this sense, it has been demonstrated a negative correlation between cognitive impairment, selenium levels and the activity of several selenoproteins in Alzheimer disease (AD) patients [56]. In general, the levels of selenium have been reported to be diminished in serum and plasma [57–60], while increased in brain tissue, with exceptions [61–64]. Selenium decreases with age, which can affect the neurological function in the elderly. In fact, a correlation between cognitive declination and decreased plasma selenium was found in a longitudinal study (subjects 60-71 age) [65], but also between low selenium concentration in nails and lower cognitive scores [66]. Moreover, although the level of selenium in brain is low, it has been reported that the brain and testes are special organs for the retention of selenium because even in the case of selenium deficiency, high level can be found in them [67–69].

Selenium speciation studies carried out in AD and Mild Cognitive Impairment (MCI) humans demonstrated that the total selenium and selenoproteins contents are increased in MCI and somewhat decreased in serum samples from AD, while selenometabolites are diminished in MCI (0.65-fold) and AD (0.72-fold) [70,71]. This fact is in good agreement with numerous studies regarding analysis of total selenium and the activity of selenoproteins [56] and may explain the consumption of selenometabolites to produce selenoproteins to combat the oxidative stress, almost in the first stages of the disease. Moreover, the inter-elements ratios between total concentrations, high (HMM) and low molecular mass (LMM) fractions of selenium and aluminium, significantly increase in AD patients against healthy controls. The ratio between Cu and Se (low molecular mass fraction) also increase in AD against healthy controls and decrease between Mn and Se in AD and MCI (total concentrations and low molecular mass fractions) against healthy controls. This fact may explain the protective effect of selenium against oxidative stress caused by other elements and the interconnected homeostasis. In addition, the lowered LMM/TOTAL ratios of selenium in AD may be a consequence of a regulatory mechanism to maintain the levels of essential selenoproteins by a decrease in the levels of LMM species [70,71].

Table 1 shows the selenoproteins determined in biofluids and tissues from cancer and neurodegenerative disease patients as well as model organisms or cells. In neurodegenerative disease animal models, selenium depletion is associated with decreased SELENOP and increased cell loss. This protein and its receptor (apolipoprotein E receptor 2, apoER2) are the responsible of the enhanced retention of selenium in brain against other organs. In fact, SELENOP uses the same receptor than ApoE, whose polymorphisms are a genetic risk of AD [72]. Moreover, selenium supplementation in diet have been demonstrated to restore the levels of GPx and

SELENOP [73] and reduce the Aβ plaque deposition in APP/PS1 mouse brain (Transgenic mice expressing human amyloid precursor protein “APP” and human presenilin-1 “PS1) [74]. The use of selenium as an antioxidant for reduction of oxidative stress in central nervous system and ultimately reduce the effects of AD has also been proposed by other authors [75]. In summary, SELENOP affects Aβ and hyperphosphorylated tau aggregation, as transport protein provides brain Se for the synthesis of antioxidant selenoproteins and possess signalling functions though neuronal ApoER2 [76].

Other important selenoprotein is SELENOK (11 kDa) which plays an important role in promoting the effective Ca^{2+} influx during the activation of immune cells [77,78]. Enhancing the migration and phagocytosis of microglial cells is of key importance to reduce the risk of neurodegenerative diseases, such as AD and Parkinson's disease (PD). A recent study indicate mice SELENOK can enhance the migration and phagocytosis of microglial cells [10,78].

5. The link between selenium and gut microbiota in medicine

The human gut microbiome, defined as the repertoire of microorganisms and their genomes inhabiting the human gut, is one of the key elements in the interplay between diet, including macro and micronutrients, and the host. Gut dysbiosis, an altered gut bacterial composition, is associated to both metabolic and inflammatory alterations, contributing, among others, to the development and maintenance of obesity and type-2 diabetes [79]. One of the mechanisms linking the gut microbiota with locally and systemic detrimental health effects is the low-grade inflammation associated to impaired intestinal barrier function and also, to the intestinal permeability [80].

Gut microbiota also have an important role in cancer risk and progression by influencing inflammation and genomic stability of host cells through deregulation of

different signals/pathways [81,82]. Microbial and archaeal composition differ between mucosal and fecal samples from colorectal cancer and healthy donors [83], with a higher presence of *Fusobacterium nucleatum* and Enterobacteriaceae and lower abundance of butyrate-producing bacteria, including Lachnospiraceae and *Clostridium* genus members in colorectal cancer compared to healthy controls [81,82]. Recently, a specific strain from Lachnospiraceae family showed a protective effect against colitis-associated death when administered to colitis-prone mice, identifying a disease-modulating microbe by microbe–phenotype triangulation method [84].

Lifestyle and metabolic diseases are well-known risk factors for colorectal cancer, while epidemiological studies demonstrate a link between gastrointestinal cancers and environmental factors such as diet. Epidemiological and clinical studies indicated that Se deficiency can contribute to the increase of inflammatory bowel diseases and cancer [85,86]. The specific link between gut microbiota, selenium status and cancer is difficult to establish and likely, multiple mechanisms may be involved in the complex interplay between microbiome, diet and human host. It has been demonstrated that dietary selenium affects both composition of the intestinal microbiota and colonization of the gastrointestinal tract, which, in turn, influence the host selenium status and selenoproteome expression [87]. Lower expression of different selenoproteins have been described in colorectal adenomas and cancer tissues [88], while higher SELENOP concentrations have been associated with a reduction in overall mortality from cancer [88].

Dietary levels of Se in mice affected the gut microbiota composition, with increased level of *Dorea* in Se-deficient mice [89] Increased *Dorea* abundance has been described in cancer patients [90]. Se-supplementation was associated to a decrease in *Parabacteroides* absolute abundance [87]; contrarely, *Bifidobacterium*, *Turicibacter*

and *Akkermansia* were increased in rodents fed with Se-supplemented diets [89,91], being these last genus associated to gut barrier protection, immune modulation, and metabolic regulation of the host [92–95]. Susceptibility to intestinal dysfunctions as inflammation or pathogen infection may also be enhanced by Se-deficient diets, probably through modulation of intestinal barrier function by gut microbiome metabolites [89] and alteration of selenoproteins expression as well as other antioxidant molecules [96].

Gut microbiota is also linked to plasma levels of selenium in mice [96], likely by the ability of microbiota to actively take up Se [97], but also by indirect mechanisms related to the modulation of the epithelial barrier [98] or by active metabolism of Se and Se-compounds [99].

Previous studies have shown the ability of Se and selenoproteins to impact inflammatory signaling pathways implicated in the pathogenesis of intestinal inflammation and cancer. In particular, two transcription factors, nuclear factor-κB (NF-κB), and peroxisome proliferator activated receptor (PPAR)γ, which are involved in the activation of immune cells, and are also implicated in various stages of inflammation and resolution, respectively, are impacted by Se status [100]. In fact, Se deficiency and inadequate selenoprotein expression impairs innate and adaptive immune responses, and especially at colonic level it has been reported an increase of inflammatory cytokines [101]. Especially, the effect of the gut microbiota on selenoproteins and other molecules linked to the redox homeostasis, and those linked to WNT/β-catenin signalling pathway, may have an impact in the regulation of oxidative stress, apoptosis, inflammation and immune response, which in turs appear to have a direct influence on cancer risk and development [102,103].

6. Analytical methodologies and pitfalls for selenoproteins determination in human biofluids and tissues

Among the human samples in which is possible to analyse selenium, serum is the most accessible biofluid as it responds quickly to changes in selenium status correlated with its dietary intake or physiological disorders. The concentration of selenium in human serum is about 90 ng g^{-1} and the relative abundance of the species is: SELENOP>SeAlb>GPx>SeO_3^{2-} [104].

For clinical purposes SELENOP has usually been characterized and quantified using antibody-based enzyme immunoassays such as ELISA [68]. However, those assays often suffer a lack in selectivity and results are usually affected by high standard deviations. On the other hand, it is possible to find in the literature data about enzymatic activities of several selenoproteins, but the absolute quantification is rarely reported. However, there is a lack of reference analytical methods capable of providing results traceable to the international systems of units (SI) for many of these protein biomarkers. These methods are of great interest for the development of certified matrix reference materials or calibration standards for biological species, which are indeed required for the standardization of chemical measurements [105]. For this purpose, undoubtedly, the use of inductively coupled plasma mass spectrometry (ICP-MS) allows quantifying selenium at very low detection limits, with high tolerance to matrix, large linearity range and multielemental capabilities [20,106]. Moreover, isotopic dilution analysis (IDA) can be performed for quantification, which has several important advantages, namely: (i) absence of matrix effects; (ii) absence of instrumental drift; (iii) correction with dilution or preconcentration factors is not necessary; (iv) uncertainty only depends on the measurement of the relative abundances [107]. Species-unspecific isotope

dilution mode (SUID) is especially useful either for untargeted analysis of chemical species of an element or when the isotopically labelled specie is not commercially available [107]. In the particular case of selenium, the use of ICP-MS with reaction/collision cell is highly recommended due to the polyatomic interferences of $^{40}Ar^{2+}$ and $^{79}Br^{1}H^{+}$ on ^{80}Se signal, which usually are overcame using hydrogen as reaction gas in an octopole reaction system (ORS), especially in human serum samples, which contains high levels of bromide [9,108]

On the other hand, as above stated, the speciation of selenium offers important information besides the total concentration measurement. To this end, it is necessary to combine orthogonal chromatographic systems with ICP-MS detection and several chromatographic methods have been proposed for the separation of selenoproteins in human plasma or serum, based on size exclusion chromatography [109] (SEC), anion exchange chromatography [108–110] (AEC) and affinity chromatography [108] (AFC). However, due to the poor resolution of SEC, precision of the quantitative results for selenoproteins are not good and usually overlapping is present in the obtained chromatogram [109,111,112]. Although, AEC provides good recoveries of analytes, chromatographic resolution is neither acceptable [108]. Moreover, when AFC is used, the weakly-retained eGPx co-elutes with non-target matrix components and selenometabolites, which cause difficulties for the accurate quantification of the different selenium species. To overcome this problem, a column switching method based on the use of in series three dimensional chromatography: size exclusion, affinity and anion exchange high performance liquid chromatography (3D/SE-AF-AEC-HPLC), using different columns of each type and hyphenated to inductively coupled plasma-(quadrupole) mass spectrometry (ICP-qMS) was proposed for the absolute and accurate quantification of eGPx, SELENOP, SelenoAlb and Selenometabolites [104]. This

method was satisfactory applied to human serum [20] and mice organs and serum [113]. Of course, the combination of inorganic (ICP-MS) and organic mass spectrometry, especially using high resolution spectrometers (*e.g.* quadrupole time of flight, Orbitrap) is of great interest for the unequivocal identification of selenium containing species. Recently, an interesting method has also been proposed for the accurate quantification of SELENOP in plasma using isotopically enriched selenopeptides and species specific isotopic dilution with HPLC coupled to ICP-MS/MS [105].

In general, the analysis of selenoproteins in cancer patients is scarce, but published papers describes the use of inmunoassays [35,114–116]. Concretely, techniques such as radioinmmunoassay [115], inmmunolunometric sandwich assay [117], Enzyme-Linked ImmunoSorbent Assay (ELISA) [35] and colorimetric enzyme-linked immonoassay [116] have been used to determine the concentration of SELENOP in lung [35] breast [115], colorectal [116] and prostate cancers [117].

7. Expert opinion and five-year view

Although the key roles of selenium are well known, there is a lack of information about the function of many of the selenoproteins. The main chemical species of selenium involved in medicine are selenoproteins, selenium containing proteins and selenometabolites. However, the function of many selenoproteins is still unknown, and for this reason, an effort should be performed in this sense especially conditioned to the development of new analytical methodologies and protocols. In this context, new analytical methods and procedures are completely necessary for the development of certified matrix reference materials or calibration standards for these protein biomarkers. In this way, advanced analytical approaches based on the use of

highly sensitive and selective techniques are claimed, as well as multifaceted instrumental couplings for the comprehensive analysis of the human selenoproteome and selenometabolome in complex biological samples. To this end the coupling of a highly sensitive detector such as ICP-MS to different orthogonal chromatographic systems allows the absolute quantification of selenoproteins and selenometabolites in human tissues and biofluids at very low levels and with high reliability, especially when isotopic dilution analysis is used. However, the methodology may be adapted to each particular sample for the absolute quantification of the present individual selenospecies (*e.g.* serum, biopsy, bronchoalveolar lavage fluid, urine, etc), but also to the disorder under study. For this purpose, an especial effort should be performed first on the correct identification of the main selenospecies involved in the disease and the used biosample. For example, the main selenospecies in human serum are SELENOP>SelenoAlb>GPx>inorganic selenium [104]. Therefore, studies related with the absolute quantification of this selenoproteins in human serum from cancer or neurodegenerative disorder patients are a very interesting challenge. In this sense, selenospecies could be used as biomarkers in medicine.

On the other hand, the essentiality or toxicity action of selenium strongly depends on both, a narrow range of concentration and the chemical specie involved. Therefore, the relation between selenium intake and status is not linear, but is a U-shape with adverse effects found at low and high Se intake. For this reason, the supplementation using functional foods enriched in selenium or nutraceuticals should be carried out in view of these facts and considering also the bioavailability and bioactivity of species. From our point of view, supplementation with selenium (*e.g.* formula milk for infants, pills for nutritional purposes or used during pregnancy, etc) should be carried out with very special care, regarding the doses and the chemical species used.

Therefore, studies related with the biological function and bioaccessibility/bioavailability/bioactivity of selenoproteins and selenometabolites in different functional foods enriched in selenium and nutraceuticals are highly recommended. Then, experiments using model organisms (mammals) or cell cultures supplemented with selenospecies is a key goal. In this last case, it is important to consider that selenium is a well known antagonist of several xenobiotics, for example against mercury toxicity, which was first reported in 1967 following an experiment with rats exposed to mercury chloride and selenite. Humans, and in general living organisms incorporate elements and their species together from the environment and diet and numerous antagonistic and synergistic interactions take place as have been described [55]. Therefore, the metabolism of trace elements, and in particular for selenium, cannot be considered in isolation and multielemental determinations are strongly recommended.

On the other hand, in the particular case of cancer research and carcinogenesis, there are two different points of view to explain the role of metals in the organism, considering them as a cause or as an effect. The first one, is supported by the fact that the natural chemical form and concentration of elements are disturbed during cancer onset and progression. The second one considers that the carcinogenic process is a consequence of their high exposure. If selenium acts as an antagonist against the toxic action of metals or the cancer onset by altering the selenoproteome expression, then selenoproteins and selenometabolites could be potential biomarkers of cancer and in turn of other diseases. For this reason, studies about the levels and chemical forms of selenium in human biofluids and tissues from patients at different stages are recommended.

Recent studies demonstrate that the brain-gut microbiota axis is thought to be on the basis of several disorders (allergies, intestinal inflammatory problems, diabetes, obesity and also neurological and cognitive disorders). Furthermore, as described previously, the influence of gut microbiota on the status of selenium and the expression of selenoproteins has been demonstrated. However, these studies focus on determinations of the enzymatic activity, but the absolute quantification of low molecular mass selenium species (inorganic selenium, selenomethionine, selenocystine, etc) and selenoproteins, their distribution and concentration have not been studied in connection with the gut microbiota. Moreover, although there are studies that establish a relation between the oral exposure of metals and gut microbiota in mice [89], and that the later acts as a barrier against chronic exposure to heavy metals [118] there are not previous studies with "chemical cocktails" of elements which includes selenium to deep insight into the global goal in the microbiota nor the antagonisms of selenium through the later.

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Figure 1. Selenium metabolism in human body after intake.

Selenoproteins	Disease	Organism and sample	Analytical Method	Reference
Cancer				
GPx1	Colorectal Cancer	Human tissue	Immunohistochemistry	[119]
	Lung Cancer	Carcinoma Cells	PCR assay	[46]
	Lung Cancer	Human Blood	PCR assay	[47]
	Laryngeal Cancer	Human Blood	PCR assay	[47]
GPx2	Colorectal Cancer	Human tissue	Immunohistochemistry	[119]
	Renal Cancer	Carcinoma Cells	PCR assay	[46]
GPx3	Colorectal Cancer	Human tissue	Immunohistochemistry	[119]
	Endometrial carcinoma	Rat endometrial carcinoma cell	PCR assay	[43]
	Endometrial carcinoma	Human endometrial carcinoma cell	PCR assay	[43]
	Prostate Cancer	Human prostate cancer cells	Immunohistochemistry	[120]

	Melanoma	Murine tumor cells	PCR assay	[45]
GPx4	Fibrosarcoma	Murine tumor cells	PCR assay	[45]
	Lung Cancer	Human Blood	PCR assay	[47]
	Laryngeal Cancer	Human Blood	PCR assay	[47]
SELENOP	Prostate Cancer	Human prostate tissue	Hapmap databse	[121]
	Lung Cancer	Human Blood	PCR assay	[47]
	Laryngeal Cancer	Human Blood	PCR assay	[47]
	Lung Cancer	Human Blood	ELISA	[122]
	Hepatocellular carcinome	Human serum	ELISA	[56,123]
	Gastric adenocaarcinome	Human Gastric adenocarcinoma tissue	Immunohistochemistry	[124]

	Colorectal Cancer	Human colon mucosa	PCR assay	[125]
	Prostate Cancer	Murine prostate cancer cells	ELISA	[126]
	Colorectal Cancer	Human tissue	Immunohistochemistry	[119]
TRx	Colorectal Cancer	Human tissue	Immunohistochemistry	[119]
TXNRD2	Lung Cancer	Human Blood	PCR assay	[47]
	Laryngeal Cancer	Human Blood	PCR assay	[47]
Neurodegenerative disease				
GPX3	Alzheimer	Human Celebrospinal fluid	ICP-DRC-MS	[127]
	MCI	Human Celebrospinal fluid	ICP-DRC-MS	[127]
SeAlb	Alzheimer	Human Celebrospinal fluid	ICP-DRC-MS	[127]
	MCI	Human Celebrospinal fluid	ICP-DRC-MS	[127]
SELENOK	Alzheimer	Mouse BV2 microglial cells	PCR assay	[78]

	Parkinson	Mouse BV2 microglial cells	PCR assay	[78]
SELENOP	Alzheimer	Human Celebrospinal fluid	ICP-DRC-MS	[127]
	MCI	Human Celebrospinal fluid	ICP-DRC-MS	[127]
SELENOR	Alzheimer	Human Celebrospinal fluid	FRET analysis	[128]
	MCI	Human Celebrospinal fluid	FRET analysis	[128]
TXNRD	Alzheimer	Human Celebrospinal fluid	ICP-DRC-MS	[127]
	MCI	Human Celebrospinal fluid	ICP-DRC-MS	[127]

Table 1. Selenoproteins determined in biofluids and tissues from cancer and neurodegenerative disease patients and model organisms or cells.