Breast milk selenocystine as a biomarker for selenium intake in lactating women at differential geographical deficiency risk in China

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Running title: Breast milk selenocystine is a good biomarker

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ABSTRACT

Background and Objectives: A reliable biomarker for optimal selenium (Se) intake in lactating women is not currently available. **Methods and Study Design:** Daily dietary Se intake in lactating women was calculated from a 24-hour meal record survey for over 3 days. Se levels in plasma and breast milk were measured through inductively coupled plasma mass spectrometry. Plasma selenoprotein P 1 levels and glutathione peroxidase 3 activity were measured using an enzyme-linked immunosorbent assay. Ultra-performance liquid chromatography-tandem mass spectrometry was used to analyze proteinaceous Se species in enzymatically digested breast milk. **Results:** Dietary Se intakes of lactating women from Liangshan, Beijing, and Enshi were 41.57±21.19 ng/d, 51.06±22.60 ng/d, and 614.84±177.80 ng/d, respectively (p<0.05). The Se levels in the blood and breast milk were significantly associated with the dietary Se intake (p<0.05). The proteinaceous Se species in breast milk were SeMet and SeCys2. The levels of SeMet in the lactating women from Liangshan, Beijing, and Enshi were 3.31±2.44 ng Se/mL , 7.34±3.70 ng Se/mL, and 8.99±9.64 ng Se/mL, while that of SeCys2 were 13.74±12.01 ng Se/mL, 35.61±20.85 ng Se/mL, and 57.35±13.20 ng Se/mL, respectively. Notably, the concentration of SeCys2, the metabolite of unstable SeCys, reached a saturation platform, whereas no similar phenomenon were found for the total Se SeMet from Se-containing proteins. **Conclusions:** SeCys2 in breast milk is a potential biomarker for determining the optimal Se intake in lactating women.

Key Words: selenium speciation, human milk, dietary Se intake, Se-adequate lactating woman, selenocystine

INTRODUCTION

Selenium (Se) is an essential trace element and is necessary for healthy metabolism in humans.[1] Se deficiency has been associated with Keshan disease and Kashin-Beck disease. Keshan disease is an endemic cardiomyopathy. Repeated outbreaks of Keshan disease have been reported in a broad diagonal (northeast to southwest) belt in China. However, it has rarely been observed since the end of the 20th century. Kashin-Beck disease[2] is an endemic chronic osteoarthritis, which occurs even today in a few areas in Russia, Nepal, North Korea, and China.[3] Insufficient Se intake may have several short- and long-term medical implications, including male infertility, neurological disorders, impaired immune response, abnormal thyroid hormone metabolism, and oxidative stress.[4] However, excessive Se intake is also associated with health disorders. For example, acute or chronic Se toxicity may cause skin,

nail, and hair abnormalities as well as gastrointestinal or neurological symptoms.[5,6] Se functions through 25 selenoproteins in the human body, and the difference between the optimal and toxic levels of Se is relatively small . Moreover, Se supplements (tablets or capsules) are often used to prevent tumorigenesis in humans; however, an increased risk of type 2 diabetes (T2D) has been reported in individuals with high baseline Se levels.[7] Therefore, a reliable biomarker for monitoring the levels of Se in pregnant women, lactating women, and their infants is urgently required. Se levels are often assessed for three major purposes, determining the risk of Se-deficiency diseases; estimating the anticancer potential of Se at supernutritional levels; and monitoring the risk of Se toxicity at very high levels of Se.

Accordingly, dietary reference intakes (DRIs) for Se were developed in China in 2000 and modified in 2013 to provide standard reference values to establish dietary guidelines for Chinese residents. According to the scientific opinions for policy makers in health administration, the reference nutrient intake (RNI) is 78 μg Se/d and the tolerable upper intake level (UL) is 400 μg Se/d for lactating women.[8] However, monitoring the daily intake of Se in lactating women in China, particularly in some Se-deficient or Se-toxic areas is difficult because detailed information regarding the Se levels in staple foods, vegetables, and fruits is not available. Moreover, the Se levels in a single type of food may vary according to the geographic locations (among regions as well as among fields) in which it is available.

In addition to daily intake, several biomarkers can be used to assess the levels of Se in humans. These biochemical indicators can be classified as invasive and noninvasive indicators.[9] The invasive biomarkers usually are Se levels in whole blood, plasma or serum, erythrocyte, and platelet samples as well as the activity of plasma glutathione peroxidase (GPx) and concentration of plasma selenoprotein P (SEPP1). Plasma GPx is the most sensitive biomarker, and GPx, which is a selenoprotein or selenoenzyme, is commonly measured previously to estimate the bioactivity of Se. Plasma GPx has now replaced by another plasma selenoprotein, SEPP1, which is predominantly expressed in the liver and secreted into the plasma (accounting for 50% of the Se content of human plasma) SEPP1 has multiple functions in the human body; it is an extracellular antioxidant and a transporter of Se to the brain and other extrahepatic organs and tissues along with apolipoprotein E receptor-2 (apoER2).[10] However, the use of invasive biomarkers present in blood is not feasible for repeatedly biomonitoring the level of Se in lactating women and their infants. The use of relatively less invasive biomarkers, such as those indicating Se levels in urine, hair, and nails may be more practical. In particular, estimating Se levels in breast milk is the most feasible

option for simultaneously biomonitoring dietary Se in lactating mothers and their exclusively breastfed infants.[11,12]

The content of Se in human breast milk is considerably higher than that in cow milk, and the major Se components in breast milk are usually organic species, such as 8–12 selenoproteins and free selenoamino acids.[11,13] The order of abundance of these major Se components in breast milk is generally GPx (4%–32% of total Se) > selenocystamine > selenocystine (SeCys2) > selenomethionine (SeMet).[14]

In this study, the data of daily Se intake in lactating Chinese women living in three regions of China were collected using 24-h recall surveys conducted for 3 days. Furthermore, samples of blood and breast milk were also collected from the women. The levels of SeCys2 and SeMet in enzymatically hydrolyzed breast milk were measured through UPLC-MS/MS for the first time in this study. The effect of dietary Se levels on the levels of SeCys2 and SeMet in the breast milk samples obtained from the lactating women was analyzed.

MATERIALS AND METHODS

Study population

A stratified random sampling method was used to select 20 lactating women per site in a low-Se region in Liangshan Autonomous Prefecture in Sichuan Province, an adequate-Se region in Xicheng District in Beijing City, and a high-Se region in Enshi City in Hubei Province. This study is a subset of a larger study conducted for examining the current status of Se in lactating Chinese women and collecting reliable evidence for modification of AI values for infants in China.

The present study was conducted according to guidelines stated in the Declaration of Helsinki, and all procedures involving human participants were approved by the Ethics Committee of the National Institute for Nutrition and Health, Chinese Center for Disease Control and Prevention. Written informed consent was obtained from all the participants.

Dietary surveys

In the study, we selected a sample of 60 lactating mothers who participated in 24-hour dietary recall surveys conducted for 3 days staring from approximately postpartum day 42 (including 1 day during a weekend). The daily dietary Se intake was calculated using the levels of Se in local foods in the three regions (including low-Se, adequate-Se, and high-Se).

Milk and blood sample collection

All samples were collected in 2014. On postpartum day 42, with assistance from local nurses, 5 mL of blood and 20 mL of mature breast milk were collected from the recruited lactating women. Each blood sample was divided into two parts. A 3-mL aliquot was centrifuged at 10000×g at 4°C for 10 min to separate plasma for determining the levels of SePP1 and activity of GPx3. The remaining 2 mL of the whole blood sample was used to measure the total Se content. A mechanical pump was used to collect 20 mL of breast milk. All the samples were frozen immediately at -20°C after collection and stored below 0°C during air transport. The samples were stored at -80°C in a laboratory at CDC, Beijing.

Analytical method

The total Se content in the whole blood samples was determined through inductively coupled plasma mass spectrometry (ICP-MS).[15] GPx3 activity and SePP1 levels in plasma were measured according to manufacturer instructions by using a commercial enzyme-linked immunosorbent assay kit (Andy Gene). Enzyme activity was expressed as units per liter (U/L) of plasma. The intra-assay coefficient of variation for different materials (10-20 analyses of each sample) was <3%.

An ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) method was used for the quantification of SeMet and SeCys2 in samples of human breast milk and cow milk.[16,17] The analyte was separated using an Acquity UPLC Amide column with gradient elution (Table 1). In the positive electrospray ionization mode, multiple reaction monitoring of the precursor-product ion transitions of m/z 198.0→181.1 (SeMet) and m/z 337.0→248.0 (SeCys2) was used for the quantification of SeMet and SeCys2. Acetonitrile precipitation was used as a pretreatment of free Se speciation analysis, and enzyme hydrolysis was used as pretreatment of free and protein-bound Se speciation analysis.[17-19]

Statistical analysis

Data were analyzed using SAS 9.4. All values are reported as means ± standard deviation. Analysis of variance was used to analyze the significance of differences among the three geographic locations (Liangshan, Beijing, and Enshi). Significance was determined at the 95% two-sided confidence level ($p < 0.05$). Relationships between the appropriate variables were evaluated using Pearson correlation coefficient and polynomial curve fitting.

RESULTS

Dietary intakes of selenium and other nutrients

From Table 2, the daily intakes of all nutrients except Se exhibited trends identical to those of the intakes of total energy, protein, and fat in the lactating women living in the three sampled regions. The Se intake in Beijing was lower than that in Liangshan and Enshi. However, the average dietary Se intakes in the lactating mothers from Enshi, Beijing, and Liangshan were as follows: 614.84 μg/d, 51.06 μg/d, and 41.57 μg/d; thus, the Se intake in Liangshan was lower than the intakes in Beijing and Enshi.

Biomarkers for Se level in the blood or plasma

The results of Se biomarker in blood are shown in Table 3. Significant differences (p<0.0001) were observed in the total levels of Se in the whole blood samples from the three regions (Enshi, Beijing, and Liangshan). However, no significant differences were observed in the SePP1 levels and GPx activities in the plasma among the three regions (p>0.05). Correlation analysis showed that the daily dietary intake of Se in the lactating mothers was associated with only Se levels in the blood samples (y = 0.826 x + 43.839, R2 = 0.7104, p<0.05) and not with the activity of GPx3 or level of SEPP1 in the plasma (Figure 1a and 1b).

Proteinaceous Se species and total Se percentage in human breast milk

The total Se levels in the breast milk samples obtained from lactating mothers in the three regions are shown in Table 4. The average levels of Se in the breast milk samples obtained from the lactating mothers in Liangshan, Beijing, and Enshi were 10.33 ng/mL, 25.62 ng/mL, and 38.13 ng/mL, respectively (p=0.002).

Two proteinaceous Se species (SeMet and SeCys) in the enzymatically digested breast milk samples from the lactating mothers were analyzed through UPLC-MS/MS. The average concentrations of SeMet and SeCys in the enzymatically digested breast milk were significantly different among the three regions (p=0.002 or p<0.001, shown in Table 4).

Effect of the daily dietary intake of Se on the total content of Se and two proteinaceous Se species in human breast milk

Correlation analysis showed, in Figure 1a-c, that dietary Se intake was positively correlated with the total content of Se as well as the concentrations of the two proteinaceous Se species in breast milk. However, only SeCys2, the metabolite of unstable SeCys in breast milk, was found to achieve a saturation platform (Figure 1c).

DISCUSSION

Although human infants are born with Se reserves, they rely on the Se supplied by human breast milk. Breast milk is the primary source of optimum levels of dietary Se, and it is the only source of dietary Se in exclusively breastfed infants. Se is present in breast milk in the form of organic compounds such as selenoproteins, Se-GSH, selenocystamine, SeCys2, and SeMet.[20] Among the nine selenoproteins present in human breast milk, GPXs alone accounts for 15%–30% of the total Se.[21] Both maternal physical conditions and environmental factors can affect the metabolism and secretion of Se in the breasts.[22]

In this study, several biomarkers of Se level (including daily dietary Se intake and Se levels in the whole blood) differed significantly among lactating Chinese women from the three regions (Tables 2 and 3). Therefore, geographical and geological factors affect the Se intake in lactating mothers because the Se content of local foods varies according to geographical factors. The average dietary Se intake in lactating mothers in Liangshan (41.57μg/day) was nearly half of the RNI value (78 μg/day) recommended by the revised DRIs for lactating Chinese women (2013 version), while that in Enshi (614.84 μg/day) was 1.5-fold of the recommended UL value (400 μg/day).

In low-Se areas, many Se-fortified foods or Se-biofortified crops have been developed under research projects and nutritional policies in China for increasing the daily Se intake of residents, including local lactating women. Several reliable biomarkers for monitoring the improvement in Se nutrition, such as the activity of GPx3 and the concentration of SEPP1 in plasma have been used. Each of these biomarkers can achieve a saturation platform when the Se intake is sufficient in the human body[23] or when the Se intake of a lactating woman and her exclusively breastfed infant is adequate.

However, the maximum value of the dietary Se intake was recently reported in the two high-Se areas of Enshi (2144 μg/day) and Ziyang (1067 μg/day).[24,25] In this study, the maximum dietary intake of Se in lactating Chinese women was 800 μg/day, which exceeds the toxic dose reported in China. Moreover, a growing body of evidence obtained from Se-supplementation cohort studies and cross-sectional epidemiological studies suggests that the risks of insulin resistance (IR) and T2D are relatively high at supranutritonal levels of dietary Se many in countries including China.[7,24,26,27] Thus, this potential risk in several population groups warrants attention, including women during pregnancy and lactation and their infants (during childhood). Thus far, changes in nail color, nail deformation, and hair loss have been used to indicate Se toxicity. Currently, no biomarker of Se can be used as a cutoff value to reliably determine a safe maximum value of dietary Se intake to reduce the potential risk of

IR or T2D caused by excessive Se intake and safeguard the health of infants during the first 1000 days of life.

In this study, neither the level of SePP1 nor the activity of GPx3 in plasma exhibited a linear correlation with the daily dietary Se intake in the 60 lactating Chinese women (Figures 1a and 1b). The SePP1 level and activity of GPx3 in the plasma were not sensitive (biomarkers) to the levels of daily dietary Se intake in the lactating mothers. Hence, the optimal biomarker was breast milk. Based on the measurement of Se levels in the breast milk samples and correlation analysis between the level Se in the breast milk samples and daily dietary intake of Se (Table 4) the content of Se in human milk is sensitive to the dietary intake of Se, but it does not have a cutoff value (Figure 2a). SeCys2, one of the two proteinaceous Se species (SeCys2 and SeMet), was found to achieve a saturation platform following an increase in the daily dietary Se intake in 55 lactating Chinese women; the cutoff value was approximately 300 μg Se/day (Figure 2b and 2c). This saturation platform was similar to that of the activity of GPx3 or SEPP1 in plasma reported previously in adults. Thus, we believe that SeCys2 is also a potential biomarker for determining the optimal Se level in lactating women and is useable to determine permitted safe levels and the adequate Se levels for infants in high-Se regions because it reaches a saturation platform completely digested by enzymes. The saturation platform is similar to that of the activity of GPx3 or concentration of SEPP1 in the plasma, which is used in low-Se areas.

To our knowledge, this study is the first of its type; however, it has some limitations. First, we did not determine the Se levels in the local foods available in the three regions by collecting meal samples for 3 days corresponding to each participant. Second, we did not measure the activity of GPXs or the levels of other selenoproteins in the breast milk samples. Third, the direct relationship between each proteinaceous Se species and selenoproteins (one at a time) remains unclear. In the future, additional studies with Se supplementation in Se-deficient or Se-marginal lactating women are needed to verify the saturation platform of SeCys-2 levels in completely enzymatically digested breast milk.

In conclusion, geographical and geological factors may affect the current levels of Se in lactating Chinese women, daily dietary intake of Se, Se level in whole blood, and Se levels in breast milk. The activities of GPx3 and SEPP1 level in plasma were not sensitive to the current dietary intake of Se daily. SeCys2, one of two proteinaceous Se species in breast milk, is a new potential biomarker for determining the optimal Se level in lactating women.

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CONFLICT OF INTREST AND FUNDING DISCLOSURE

All authors declare no conflict of interest. The present study was supported by the National Natural Science Foundation of China (Grant No. 81273073).

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Table 1. Gradient elution of mobile phase

Time (min)	Flow rate (mL/min)	0.1% formic acid solution	0.1% formic acid acetonitrile solution
0	0.4	10	90
0.7	0.4	10	90
1.0	0.4	50	50
3.0	0.4	50	50
3.1	0.4	10	90
4.0	0.4	10	90

Table 2. Dietary energy and nutrients intake in Liangshan, Beijing and Enshi ($\bar{x}$±SD)

Nutrients	Enshi (n=20)	Beijing (n=20)	Liangshan (n=20)	*p*
Energy (kcal/d)	2004.18±371.16^{a}	1656.50±527.46^{b}	2144.82±713.52^{a}	<0.0001
Fat (g/d)	63.93±26.00^{b}	52.21±26.43^{b}	77.58±35.48^{a}	0.0002
Protein (g/d)	112.01±27.04^{b}	84.55±33.77^{c}	142.83±49.97^{a}	<0.0001
Se (μg/d)	614.84±177.80^{a}	51.06±22.60^{b}	41.57±21.19^{b}	<0.0001
Fe (mg/d)	19.43±5.50^{b}	15.86±5.63^{c}	23.08±7.72^{a}	<0.0001
Zn (mg/d)	17.52±13.26^{a}	10.19±3.39^{b}	17.34±5.16^{a}	<0.0001
VA (μg/d)	397.25±227.58^{b}	362.99±190.51^{b}	795.47±456.16^{a}	<0.0001
VE (mg/d)	16.05±18.76^{a}	9.98±4.66^{b}	13.54±6.88^{b}	0.0391

abc indicated that there was significant difference among these groups in the same row.

Table 3. The comparison of Se content in whole blood among three regions ($\bar{x}$±SD)

	Enshi (n=20)	Beijing (n=20)	Liangshan (n=20)	*p*
Total Se in blood (μg/L)	152.01±121.33^{a}	41.58±10.48^{b}	31.18±13.28^{b}	<0.0001
Plasma SePP1 (μg/L)	31.38±7.85	29.79±7.09	27.86±5.80	0.297
Plasma GPx3 (U/L)	194.59±47.49	177.54±53.23	178.80±47.95	0.488

abc indicated that there was significant difference among these groups in the same row.

Table 4. The comparison of selenium biomarker in human breast milk among three different Se-level regions

	Total	Enshi	Beijing	Liangshan	F	*p*
n	55	17	19	19		
Total milk Se (ng/mL)	16.29±8.78	38.13±39.56^{a}	25.62±6.27^{a,b}	10.33±4.14^{b}	6.75	0.002
SeMet (ng/mL)	16.59±15.97	22.77±23.44^{a}	18.29±9.04^{a}	8.30±6.12^{b}	4.39	0.017
SeCys2 (ng/mL)	70.75±50.80	105.61±48.53^{c}	75.27±43.39^{b}	29.30±25.62	33.63	<0.001

abc indicated that there was significant difference among these groups in the same row.

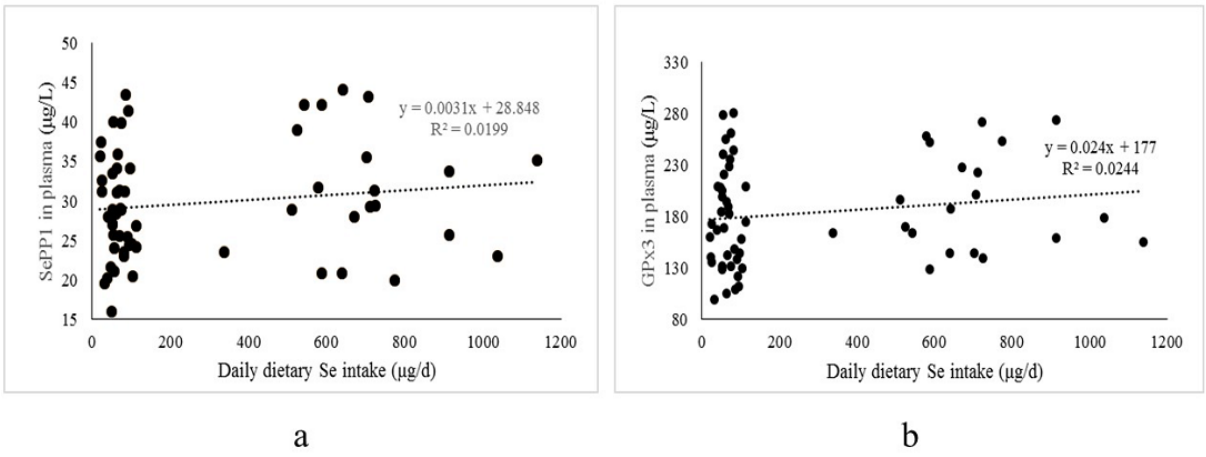

Figure 1. The scatter plot of daily dietary Se intake and SePP1 and Gpx3 in plasma. (a) The scatter plot of daily dietary Se intake and SePP1 in plasma (b) The scatter plot of daily dietary Se intake and Gpx3 in plasma

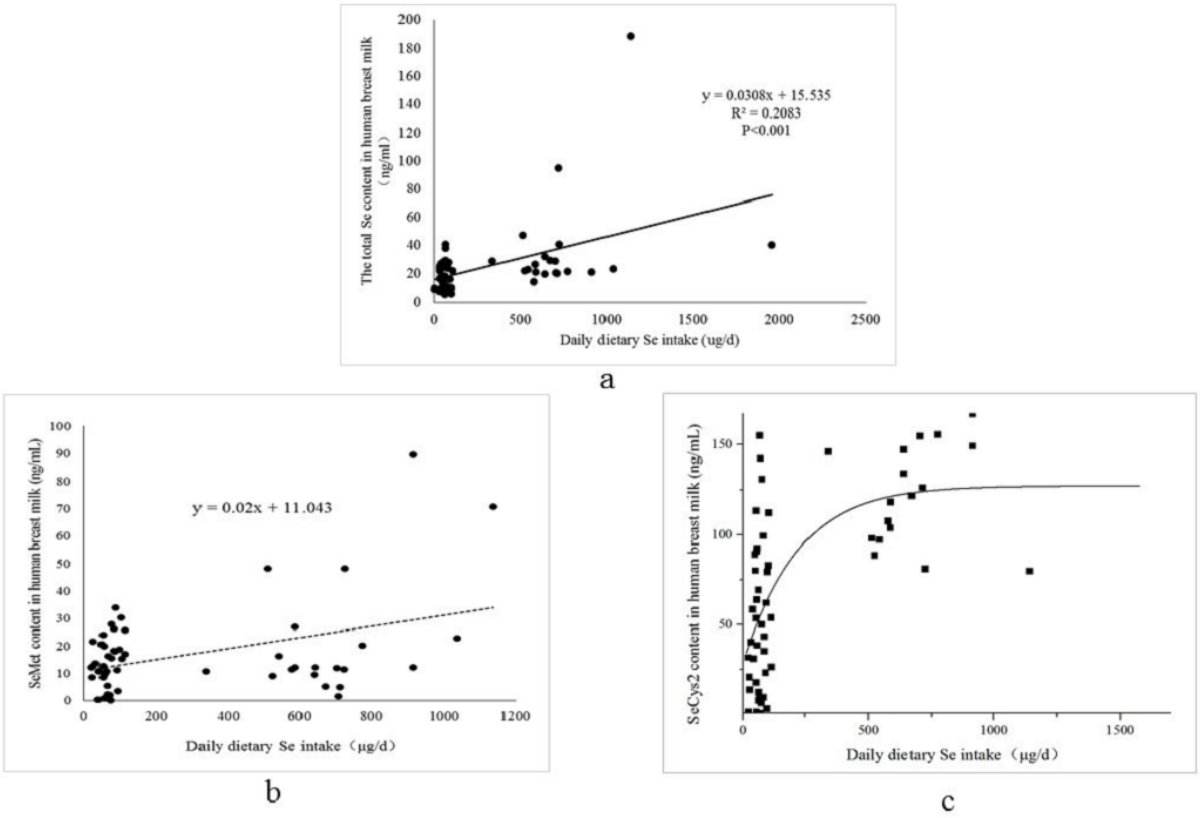

Figure 2. The scatter plot of daily dietary Se intake and selenium biomarker in human breast milk. (a) The scatter plot of daily dietary Se intake and total Se content in human breast milk (b) The scatter plot of daily dietary Se intake and SeMet content in human breast (c) The scatter plot of daily dietary Se intake and SeCys2 content in human breast