Genetic variants in selenoprotein genes modulate biomarkers of selenium status in 1 response to Brazil nut supplementation (the SU.BRA.NUT study) 2

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Abstract

Background: The beneficial effects of selenium (Se) to human health are exerted by selenoproteins, which can be quantified in blood and used as biomarkers of Se status. Different responses of Se biomarkers after supplementation with selenomethionine and sodium selenite have been observed and some of them could be due to genetic polymorphisms, mainly single nucleotide polymorphisms (SNPs). Brazil nuts are known to be the richest natural source of Se. **Objective:** Investigate how genetic variations in selenoprotein genes modulate biomarkers of Se status in response to Brazil nut supplementation. **Methods**: The SU.BRA.NUT study was a four month interventional trial which involved healthy volunteers of both genders, selected in University of Sao Paulo. The supplementation was done with one Brazil nut a day for 8 weeks, followed by 8 weeks of washout. Blood samples were collected at 5 time points: baseline, 4 and 8 weeks of supplementation and 4 and 8 weeks of washout for analysis of five biomarkers of Se status – erythrocyte GPx1 (Glutathione Peroxidase 1) activity, plasma GPx3 activity, plasma Se, erythrocyte Se, and plasma selenoprotein P. The gene expression of *GPX1*, *SELENOP*, *SELENOF* and *SELENOS* was done before and after 8 weeks of supplementation. The volunteers were genotyped for SNPs in *GPX1 (*rs1050450, rs3811699 and rs1800699), *GPX4 (*rs713041), *SELENOP (*rs3877899 and rs7579), *SELENOF (*rs5845) and *SELENOS (*rs34713741). **Results:** A total of 130 volunteers finished the protocol. The concentrations of four biomarkers of Se status increased significantly after 4 and 8 weeks of supplementation, being modulated by gender. In addition, erythrocyte GPx1 activity was associated with rs1050450, rs713041 and rs5845. Plasma Se was associated with rs7579 and selenoprotein P with plasma Se at baseline. Nut supplementation significantly increased *GPX1* mRNA expression only in subjects with CC genotype at rs1050450. *SELENOP* mRNA expression was

significantly lower in subjects with GG genotype at rs7579 before and after supplementation. **Conclusion**: Genetic variations in *GPX1* and *SELENOP* genes are associated with different responses of molecular and biochemical biomarkers of Se status after Brazil nut supplementation in healthy Brazilians. The SU.BRA.NUT study was registred at www.clinicaltrials.gov as NCT 03111355.

Keywords: Glutathione Peroxidase, SNPs, selenium, polymorphisms, nutrigenetics

Abbreviations

Se, Selenium; Sec, selenocysteine; SNP, single nucleotide polymorphisms; GPx, glutathione peroxidase enzyme; *GPX1*, cytosolic glutathione peroxidase gene or erythrocyte glutathione peroxidase gene ; *GPX3*, plasma glutathione peroxidase gene ; *GPX4*, phospholipid glutathione peroxidase gene; *SELENOP*, Selenoprotein P gene; *SELENOS*, Selenoprotein S gene; *SELENOF*, Selenoprotein 15 gene; SePP, Selenoprotein P protein; SU.BRA.NUT, Supplementation with Brazil Nuts study.

Funding source

This work was supported by Brazilian grants from São Paulo Research Foundation to JLSD (Fundação de Amparo à Pesquisa do Estado de São Paulo -FAPESP processes: 2011/17720-0 and 2015/10146-8). Funding source had no involvement in study design, collection, analysis and interpretation of data from the present research.

INTRODUCTION

The regular intake of nuts has been proposed to decrease risk for chronic diseases such as cancer, cardiovascular disease and type 2 diabetes [1–4]. Brazil nuts (*Bertholletia excelsa*, family Lecythidaceae) are known to be the richest source of selenium (Se), an essential micronutrient for human health. The main form of Se in Brazil nuts are selenomethionine [5]. Se is incorporated as the amino acid selenocysteine (Sec) during translation into 25 selenoproteins encoded by the human genome, many of which show a wide range of functions, including antioxidant defense, redox function, thyroid hormone metabolism, immune function, reproduction and fertility [6,7].

Low Se status has been associated with increased risk for several diseases, such as cancer, cardiovascular disease, viral infections, male infertility and inflammatory disorders [7]. Potentially, genetic variations could modulate this risk by affecting responses to Se intake [8]. Several studies have demonstrated that polymorphisms in genes encoding selenoproteins have functional consequences [9]. For instance, the rs1050450 (Pro198Leu) in *GPX1* (Glutathione Peroxidase 1) gene was associated with lower erythrocyte GPx1 activity [10,11] and lower plasma Se in humans [12]. Although the regulation of selenoproteins expression is mainly during translation, the mRNA expression of selenoproteins, such as SELENOF (Selenoprotein 15), SELENOK and SEPHS1 can be altered by Se status, as shown previously [13]. Nevertheless, human studies have failed to demonstrate an association between Se status and selenoprotein transcripts [14–16]. Only three studies have observed a positive association between Se supplementation and increased selenoprotein expression in humans (SELENOF, SELENOK, GPX1 and SELENOP) [13,17,18].

Glutathione peroxidase 1 (GPx1) activity is sensitive to alterations in Se status in individuals with low to moderate intake [19]. The *GPX1* gene contains a single nucleotide

polymorphism (SNP) in the coding region, which causes a Proline to Leucine amino acid change at position 198 (rs1050450) [20]. This variation has been associated with increased risk for lung, breast, prostate and bladder cancers [11,21–23] and has been found to modulate the response to Se supplementation in healthy subjects [12,24]. Carriers of the minor allele T had lower plasma Se at baseline and after one year of supplementation with selenomethionine they had increased urinary Se excretion [12,24]. Glutathione peroxidase 4 (GPx4) is the only GPx that can reduce phospholipid hydroperoxides in cell membranes [25]. There is a C>T substitution located in the 3'UTR of the *GPX4* gene (rs713041) and this variant affects Se incorporation in cell culture models [26] and the response to Se supplementation in healthy adults [27]. It was demonstrated that subjects with the TT genotype had lower GPx3 activity after 6 weeks of supplementation with sodium selenite and lower GPx4 activity during the washout period. Also, females with the TT genotype had lower GPx1 and TR1 concentration during the supplementation and the washout period [27].

Selenoprotein P (*SELENOP*) is the major component of blood Se and the key Se transporter in the body [28]. Two SNPs with functional consequences are present in this gene; one G>A substitution in the coding region causes an amino acid substitution Alanine to Threonine at position 234 of the protein (rs3877899), and the other G>A substitution is located in the 3'UTR, important for Sec insertion (rs7579). Both SNPs modulate the response to Se supplementation in healthy adults [8]. It was demonstrated that carriers of the minor allele A for both SNPs had higher SePP concentrations after supplementation. Also, males with the AA genotype for rs7579 had lower GPx3 activity after supplementation and during the washout period compared with males with the GG genotype [8]. Selenoprotein S (*SELENOS*) is an endoplasmic reticulum (ER) selenoprotein involved in protecting ER from stress caused by misfolded proteins [29]. A

C>T substitution located in the promoter region of the gene (rs34713741) has been associated with increased risk for rectal cancer [30]. Selenoprotein 15 (*SELENOF*) is another selenoprotein involved in maintaining ER integrity [31]. A SNP in the 3'UTR of this gene, a G>A substitution in position 1125 (rs5845), has been associated with increased risk for rectal cancer [30] and lung cancer [32].

Most of the studies investigating the effect of Se supplementation on biomarkers of Se status were conducted using different chemical forms of selenium in different concentrations [8,12,27,33,34]. The studies with Brazil nut supplementation were conducted only in specific groups of the population and considered mainly three SNPS in selenoproteins (rs1050450, rs3877899 and rs7579) genes with just three biomarkers evaluated before and after supplementation. No study was conducted in healthy Brazilians using other functional SNPs in selenoproteins, other plasma biomarkers and evaluated the washout period to investigate how the biomarkers return after supplementation withdraw. Therefore, this study was conducted to evaluate if the six functional polymorphisms in selenoprotein genes modulate the response of biomarkers of Se status, on both molecular and biochemical levels, during supplementation with Brazil nuts and the washout period in healthy Brazilians.

SUBJECT AND METHODS

Study population and supplementation protocol

The present study involved 130 unrelated healthy volunteers selected at University of Sao Paulo who took part of the Supplementation with Brazil Nuts study (SU.BRA.NUT) described previously [35]. Subjects were excluded if they were pregnant, younger than 20y and older than 60y taking multivitamins and mineral supplements, anti-inflammatory drugs, with excessive alcohol consumption, athletes, with chronic

diseases such as cancer, diabetes, and cardiovascular disease and obese (BMI > 35). At the beginning of the study (baseline), 20 mL venous blood samples were drawn and the volunteers received. plastic bottles with nuts enough for four weeks. They were oriented to take a daily supplement of one Brazil nut a day with a meal. At the end of four weeks of supplementation, they returned and received another plastic bottle with nuts for the last four weeks. At the end of four (4-week-intervention) and eight weeks (8-week intervention) of supplementation, another 20 mL blood sample was taken, and then two more blood samples were taken after a further four (4-week washout) and eight weeks without intervention (8-week washout) . Volunteers were asked to complete a control calendar and mark with an "x" when they consumed each nut throughout the intervention period. Written informed consent was obtained from all volunteers before blood sampling. The protocol was approved by Faculty of Pharmaceutical Sciences Ethical Committee (CAE: 00961112.3.0000.0067) and was conducted according to the Helsinki Declaration. The SU.BRA.NUT study was registred at clinicaltrials.gov as NCT 03111355.

Composition and Se content of Brazil nuts

The Se content of a random sample of Brazil nuts representative of the four batches used in the study was analyzed using hydride generation flame atomic absorption spectrometry as described previously [36] and the centesimal composition was done as proposed by the Association of Official Analytical Chemists [37].

Sample collection

Fasting blood samples (20 mL) were drawn by venipuncture into four 5 mL EDTA tubes for quantification of the five biomarkers of Se status. An aliquot of 1.5 mL of whole blood from one EDTA tube was stored into 1,5mL sterile plastic tubes used

for DNA extraction and subsequent genotyping, and an aliquot of 500 µL of whole blood from the same EDTA tube was stored into 1,5mL sterile plastic tubes used for RNA extraction and subsequent gene expression. Another 5mL of blood were collected in a tube without anticoagulant to obtain serum for determination of the lipid profile which was described previously [35]. The total volume of blood samples collected was 25mL. Plasma was separated by centrifugation at 3,000 *rpm* for 15 min at 4 °C. The erythrocyte pellet was washed three times with 5 mL sterile 9 g/L NaCl solution, slowly mixed by inversion, and centrifuged at 10,000 *rpm* for 10 min (Eppendorf, C5408) at 4 °C, and the supernatant fluid was discarded. Aliquots of whole blood, plasma and erythrocytes were frozen at -80 °C in sterile, demineralized tubes until the analyses were performed.

Biomarkers of Se status

Plasma Se and erythrocyte Se concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer DRC II) as described previously [38]. Samples were diluted 1:50 into a 15 mL polypropylene tube with a solution containing 0.01% (v/v) Triton X-100, 0.5% (v/v) nitric acid and 10 µg/L of each one of the internal standards. The certified reference material Seronorm Trace Elements Serum (SERO AS, Billingstad, Norway) was used for the quality control assessment. Erythrocyte GPx activity was determined using commercial kit (Randox, Labtest, Minas Gerais, Brazil) according to manufacturer's instructions. The enzyme activity was evaluated spectrophotometrically at 37 °C at 340 nm using an automated biochemical analyzer Labmax 240 (Labtest, Minas Gerais, Brazil). Hemoglobin (Hb) concentration was also determined spectrophotometrically in order to express erythrocyte GPx activity in U/g Hb. Plasma GPx (GPx3) activity was calculated by the method of Paglia and Valentine (1967), as modified previously [39], using hydrogen

peroxide as a substrate. One unit of GPx3 activity is defined as that which oxidizes 1 μmol NADPH/min. SePP concentration was determined in plasma using an in-house SePP ELISA (Enzyme Liked Immunosorbent Assay) assays as described previously [40] using a 96-well microplate reader (FLUOstar Omega microplate reader, BMG Labtech, Ortenberg, Germany). Each sample was analyzed in duplicate with aliquots of purified SePP incubated alongside (range 0.25–16 ng/well) acting as the standard curve. A pooled plasma from a previous study conducted at The Rowett Institute was used as a quality control.

Genotyping

Total genomic DNA was extracted from whole blood using the Purelink Genomic DNA Minikit (Invitrogen, Thermo Scientific, CA, USA) and the final concentration and purity were measured by spectrophotometry at 260 and 280 nm (NanoDrop ND 1000 Thermo Scientific, Wilmington, DE, USA). Genotyping was carried out by real-time PCR using the StepOne Plus Real-Time system with Taqman SNP Genotyping Assays (Applied Biosystems, Thermo Scientific, Foster City, CA, USA). The allelic discrimination was obtained by performing an endpoint read. The SNPs selected were located in *GPX1* gene (rs1050450, rs3811699 and rs1800668), *GPX4* gene (rs713041), *SELENOP* gene (rs3877899 and rs7579), *SELENOS* gene (rs34713741) and *SELENOF* gene *(*rs5845).

Selenoprotein gene expression

Total RNA was extracted from whole blood using the Ribopure Blood Kit (Ambion, Thermo Scientific, Austin, TX, USA) and final concentration was measured in a NanoDrop ND 1000 spectrophotometer (NanoDrop ND 1000, Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized by reverse transcription PCR using the High Capacity Reverse Transcriptase kit (Applied Biosystems, Thermo Scientific,

Foster City, CA, USA). Analysis of gene expression was performed by real-time quantitative PCR (qPCR) in the QuantStudio 12K Real Time PCR System using Taqman Gene expression Assays for *GPX1*, *SELENOP*, *SELENOS* and *SELENOF* (Applied Biosystems, Thermo Scientific, Foster City, CA, USA). GPX4 mRNA expression was not evaluated because the literature states that this protein is ranked high in the hierarchy of selenoprotein expression with no predictable changes in gene expression after supplementation. Glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA expression was used as a reference gene. Relative gene expression was calculated based on the $2^{-\Delta\Delta Cq}$ method [41].

Statistical Analysis

Continuous variables were tested for normality using the Kolmogorov–Smirnov test. The data were presented as geometric means (CI 95%). Concentrations of the five biomarkers were compared in the different time points using ANOVA repeated measures or Friedman's test. The percentage of variation for each biomarker was calculated considering the time point immediate before, for example, the percentage of variation for GPx1 activity at 4weeks of nuts was calculated using the formula:

$$\frac{(\text{GPx1 activity 4w}) - (\text{GPx1 activity baseline}) * 100}{(\text{GPx1 activity baseline})}$$

The variables used for multiple linear regressions in table 5 were done considering only 3 time points: baseline, 8 weeks of nuts and 8 weeks of washout. Therefore, change 1 was referring to the entire supplementation period (baseline until 8 weeks of nuts) and change 2 to the washout period (8 weeks of nuts until 8 weeks of washout. For example the variable "Change1_GPx1" was calculated using the formula:

$$\frac{(\text{GPx1 activity 8w}) - (\text{GPx1 activity baseline}) * 100}{(\text{GPx1 activity baseline})}$$

A genetic dominant model was used to evaluate differences in the presence of the rare allele. In this model, individuals with the rare allele were combined together in one category, leaving the common genotype in another category. Multiple linear regression models were created using the biomarkers at each time of intervention as response variables. Age, body fat percentage, gender, plasma Se, erythrocyte Se and six SNPs were included as predictors. Only six SNPs were used because the three SNPs in *GPX1* gene were in linkage disequilibrium with an r = 1 and D'> 0,5. Repeated measures analysis of covariance (ANCOVA) was performed to investigate the effect of the genotypes for SNPs appointed in the linear regression models. The Chi-square test with continuity correction was used to determine whether genotype frequencies followed the Hardy-Weinberg Equilibrium. The haplotype distribution and linkage disequilibrium analysis were done in the software Haploview 4.2. SNPs were considered in linkage disequilibrium when D'> 0,5. Differences were considered significant at $P < 0.05$. The analyses were performed using the Statistical Package for the Social Sciences software version 17.0 for Windows (SPSS, Chicago, IL, USA) and GraphPad Prism (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA).

RESULTS

A total of 135 adults were enrolled in the study during 2013 and started the supplementation. Of these, 2 females stopped the supplementation complaining of side effects (ex. sickness), leaving 133 subjects that finished the 8 weeks of intervention. Of these 133 volunteers, 2 males withdraw during the first month of washout and one male during the second month, leaving a total of 130 volunteers that finished the entire protocol.

Characteristics of the volunteers and nut composition

The characteristics of the volunteers are summarized in **Table 1**. Mean age was 29.8 ± 9.2 y and mean BMI was 23.3 ± 3.3 kg/m^2. There was a 100% adherence to the supplementation confirmed by the control calendar given at baseline, the empty plastic bottles at the end of supplementation and the increase of three times in plasma Se after 8 weeks of supplementation with Brazil nuts. The Se content and centesimal composition of Brazil nuts are shown in **Table S1**. Four different batches were used during the supplementation. The mean ± standard deviation for Se content of these four batches was 100.4 ± 5.3 µg/g. The average weight of the nuts ranged from 3g to 4g, therefore each nut provided from 300 µg of Se to 400 µg of Se, which is approximately six times higher than the RDA (Recommended Dietary Allowances) for adults (55µg/d).

Effect of Brazil nut supplementation on five biomarkers of Se status

The concentrations of the five biomarkers of Se status measured during nut supplementation and washout period are shown in **Table 2**. GPx1 activity decreased

significantly after 4 weeks of supplementation, increased by 8 weeks of supplementation ($P < 0.001$) and remained high during the first 4 weeks of Brazil nut withdrawal; it finally decreased after 8 weeks washout ($P = 0.004$). Erythrocyte Se concentrations increased after 4 and 8 weeks intervention and decreased after 8 weeks washout ($P < 0.001$).There was a significant increase in GPx3 activity after 4 weeks of supplementation ($P = 0.004$). Similarly, plasma Se concentrations increased significantly after 4 and 8 weeks of supplementation ($P < 0.001$). During the washout period, there was a sharp decrease in plasma Se compared to 8 weeks of Brazil nut intake ($P < 0.001$). The concentrations of plasma SePP were also increased after the supplementation and reduced after nut withdrawal ($P = 0.001$). The percentage of the variation for each biomarker during the supplementation and the washout period is shown in **Figure 1**.

Genotypes and haplotypes in selenoprotein genes

Genotype and allele frequencies of SNPs in selenoprotein genes are shown in **Table 3**. All SNPs were in Hardy-Weinberg Equilibrium. Haplotype analyses showed evidence of linkage disequilibrium for SNPs in *GPX1* gene: rs1050450 x rs1800668 (D'= 1.0 and r^2= 0.98) and rs1050450 x rs3811699 (D'= 1.0 and r^2= 1.0). In fact, in Table 3 the genotypes frequencies of the three SNPs are exactly the same. Only two haplotypes were observed, the common haplotype CCG for rs1050450, 1800668 and rs3811699 with a frequency of 74% and the rare haplotype TTA with 25%. Therefore, only rs1050450 was used for further analysis. For the SNPs in the *GPX4*, *SELENOF* and *SELENOS* genes, the frequency of the rare genotypes was between 19% and 8%. No haplotype analysis was performed in those genes because only one SNP was genotyped in each gen**e.Variables influencing biomarkers of Se status during Brazil nut supplementation and washout period**

Multiple linear regression models for the five biomarkers of Se status in each time point of the protocol are shown in **Table 4**. GPx1 activity was associated with rs1050450 after 4 (P = 0.037) and 8 weeks (P = 0.017) of intervention and with rs5845 after 4 weeks (P = 0.003) of intervention with both SNPs reducing GPx1 activity. After 8 weeks of washout, GPx1 activity was associated with rs5845 in *SELENOF*, which was lower in the presence of the rare allele A (P = 0.049) and with rs713041 in *GPX4*, which increased GPx1 activity in individuals carrying the rare allele T (P = 0.036). The SNP rs713041 in *GPX4* gene was associated with Erythrocyte Se concentrations at baseline, reducing its concentrations in the presence of the rare allele T (P = 0.038).

Plasma Se concentration was associated with rs7579 in *SELENOP* (P = 0.034) and rs34713741 in *SELENOS* (P = 0.038) after 4 weeks of supplementation, which was lower in the presence of the rare allele for both SNPs. Plasma SePP concentrations were positively associated with plasma Se at baseline and GPx3 activity was positively associated with plasma Se after 4 (P = 0.050) and 8 weeks (P = 0.025) of supplementation, as expected due to the same blood compartment location of both biomarkers (Table 4). A complete table of the biomarkers stratified by all functional SNPs can be seen at Supplementary table 2 (Table S2).

Erythrocyte GPx1 activity was stratified by rs1050450, as appointed in the multivariate regression analysis (**Figure 2**). During the intervention, GPx1 activity was lower in carriers of the rare allele T (CT+TT), almost reaching statistical significance at 4 weeks of supplementation (P = 0.057, Figure 2a). SePP concentrations were stratified by rs7579 (Figure 2b). At baseline, SePP concentrations were higher for carriers of the rare allele A, when the interaction with gender was considered (P = 0.048).

The percentage of variation on the concentrations of biomarkers of Se status is shown in **Table 5**. The difference in GPx1 activity comparing baseline and 8 weeks of

supplementation (Change1_GPx1) was associated with rs7579 in *SELENOP* ($P = 0.044$) and with rs5845 ($P < 0.001$), in which the presence of the rare allele increased this difference, meaning that the values were higher for carriers of the rare allele A after 8 weeks of supplementation. The difference in Erythrocyte Se concentrations during the supplementation (Change1_ Erythrocyte Se) was associated with rs34713741 in *SELENOS* ($P = 0.010$), in which the presence of the rare allele T also increased this difference. BMI and gender had a negative effect on the variation in Erythrocyte Se, in which individuals with higher BMI and females had higher values at baseline. The change in GPx3 activity in response to the supplementation (Change1_ GPx3 activity) was negatively associated with rs5845 in *SELENOF* ($P = 0.014$), in which carriers of the rare allele A had higher GPx3 activity at baseline. However, interaction with BMI increased the variation ($P = 0.011$). The only two variables affecting the variation in Plasma Se were gender and BMI, both having a negative effect. The difference in GPx1 activity comparing 8 weeks of washout and 8 weeks of supplementation (Change2_GPx1) was negatively associated with gender, in which females had higher GPx1 activity after 8 weeks of supplementation. However, the interaction with rs713041 had the opposite effect: females carriers of the variant allele T had higher GPx1 activity at 8 weeks of washout. Finally, the difference in SePP concentrations comparing 8 weeks of washout and 8 weeks of supplementation (Change2_SePP) was negatively associated with rs5845 in *SELENOF* gene, in which carriers of the rare allele A had higher SePP concentrations after supplementation (Table 5).

Effect of Brazil nut supplementation on mRNA expression of selenoprotein genes

Gene expression of four selenoprotein genes (*GPX1, SELENOP, SELENOS* and *SELENOF*) was analyzed on previously genotyped volunteers before and after 8 weeks of Brazil nut supplementation. The results are shown in **Figure 3**. *GPX1* mRNA

expression increased after supplementation in individuals with the CC genotype for rs1050450 ($P = 0.026$) while it did not change for carriers of the T allele (Figure 3a). Consequently, after Brazil nut supplementation, *GPX1* mRNA expression was lower in individuals with the CT and TT genotypes compared to the CC group ($P < 0.05$). *SELENOP* mRNA expression was higher in carriers of the rare allele A for rs7579 either before or after supplementation (Figure 3b, $P < 0.05$), and nut supplementation did not significantly changed SELENOP mRNA expression whatever the genotype group. No effect of genotypes on SELENOF and SELENOS mRNA expression was observed either before or after the supplementation (Figure 3c and 3d).

DISCUSSION

The influence of genetic variants on the response to Se supplementation have been proposed before [8,12,27,34]. Our results not only confirm these earlier observations, but also extend them by demonstrating that the rs5845 in *SELENOF* gene modulated erythrocyte GPx1 activity, the variation of GPx1, GPx3 activity after supplementation and the variation on SePP after nut withdraw. This study is the first to report this unexpected association between rs5845 in *SELENOF* and Se biomarkers after supplementation with Brazil nuts, the first to use SePP concentrations as a biomarker of Se status in Brazilians and the first to measure all five biomarkers during the washout period. Moreover, this study demonstrated that Brazil nut supplementation was effective in increasing mRNA expression of *GPX1* and *SELENOP* and that this effect was modulated by functional polymorphisms on those genes.

It was observed a delayed response of the two erythrocyte biomarkers of Se status to Brazil nut supplementation, with values increasing up to the first four weeks of the washout period. This apparently slow response could be explained by erythrocytes having a lifespan of 120 days so that it takes time for changes in selenoprotein synthesis

382 to appear in the mature red cell population [42,43]. This is the first study to use plasma
383 SePP concentrations as a biomarker of Se status in healthy Brazilians. The baseline
384 plasma Se concentration of 96.7 µg/L (1.22 µmol/L) would be expected to maximize
385 plasma GPx activity, but not SePP concentration since earlier work has shown that the
386 plasma Se concentration needed to maximize GPx3 activity is about 90 µg/L [44] and to
387 maximize plasma SePP concentration it is approximately 120 µg/L [45]. As a result, the
388 studied population could be considered to have a moderate to adequate Se status which
389 was able to respond to Se supplementation with an increase in concentrations of plasma
390 Se and SePP [43,46]. We suggest that the threshold for maximize GPx3 activity be
391 reviewed, as our work demonstrated that this population with a baseline plasma Se of
392 90µL/L had a significant increase in GPx3 activity after 4 weeks of supplementation.
393 In our study subjects with the rare allele T for rs1050450 in *GPX1* gene had
394 lower GPx1 activity. This observation is consistent with previous studies [10,11,47,48]. It is
395 hypothesized that the change of the amino acid Proline to Leucine alters the secondary
396 structure of the protein, which can have profound effects on its activity and stability [21].
397 This was confirmed by *in vitro* studies where Se supplementation reduced enzyme
398 thermostability for the Leu-variant [49]. It was observed that the SNPs rs1050450,
399 rs3811699 and rs1800668 were in linkage disequilibrium. This linkage was also
400 observed in a Japanese study conducted with type 2 diabetic patients [10]. One possible
401 explanation for the reduced GPx1 activity is that the presence of these three genetic
402 variations affected the transcriptional process and, as a result, the final enzyme activity
403 was lower. Moreover, in our work, the Brazil nut supplementation was effective in
404 increase *GPX1* mRNA expression in whole blood, only in individuals with the CC
405 genotype at rs1050450. Previous studies did not find a positive association of Se
406 supplementation and selenoprotein gene expression [14–16], however, three studies confirm

our results [13,17,18] . Differences may be explained by the lack of the genotype analysis on previous studies, maybe to observe this effect of Se supplementation on mRNA levels of *GPX1* is necessary to stratify by genotypes.

The presence of rs7579 in the *SELENOP* gene influenced not only SePP plasma concentrations but also *SELENOP* mRNA expression. SePP protein concentrations were higher at baseline in carriers of the variant allele A for this 3'UTR SNP. Furthermore, A-carriers had higher *SELENOP* mRNA levels than GG at baseline and after supplementation. Previous work with humans have not found an association between Se supplementation and *SELENOP* mRNA expression in white blood cells [14,16]. It should be noted that the present work used whole blood for the mRNA expression. Only one study found a positive association by showing that rs7579 influenced *SELENOP* mRNA expression [17]. The SePP protein has two isoforms in plasma, the 50kDa and the 60kDa, that are influenced by the genotype for both SNPs in *SELENOP* gene. Individuals with the GA genotype for rs3877899 had a lower proportion of the SePP 60kD, with the Sec-rich domain [50]. This difference in the proportion of SePP isoforms may affect Se availability in plasma for selenoprotein synthesis in different tissues.

The 3'UTR region of *SELENOF* gene has two SNPs which are in the same haplotype. The first one is a located a C>T substitution at position 811 (rs5859) and the other is a G>A substitution at position 1125 (rs5845). The two possible haplotypes are 811C/1125G or 811T/1125A [51]. Previous work has demonstrated that the SECIS element containing the A variant is less responsive to Se supplementation and may influence the translation of Selenoprotein 15 protein [52]. Two biomarkers of Se status were associated with rs5845 in *SELENOF* gene in our study: erythrocyte Se concentrations and GPx1 activity. We also observed that this SNP modulated the percentage of variation of GPx1 and GPx3 activities after supplementation and the

variation in SePP during washout period. To our knowledge, this is the first time that rs5845 is associated with these biomarkers. These were unexpected associations, since the SNP is located in the SELENOF gene and the biological effect was observed in other selenoproteins. However, the biological function of the Selenoprotein 15 is still unknown and maybe the hierarchy of selenoprotein expression may help explain these associations with other selenoproteins.

This study has several limitations. The first one is the small sample size, which could have masked significant associations of the genotypes with the biomarkers. The second one is the absence of a control group, however, since the main goal of this before-after interventional trial was to investigate the effect of the genotypes on the response to the dietary intervention, we decided that each person before supplementation would be a better control. The other limitation is the small number of males in the study group which could have biased the gender effect in the statistical modeling.

In summary, the results of this study suggest that genetic variants in selenoprotein genes and gender influence the response of plasma and erythrocyte biomarkers of Se status to a daily supplementation with one unit of Brazil nut in healthy adults in Brazil. Furthermore, the genotypes for rs1050450 and rs7579 affected the gene expression of *GPX1* and *SELENOP*. Future nutritional interventions with Brazil nuts should consider the genetic background of the volunteers when evaluating the biomarkers of Se status used in this study.

Acknowledgments

The authors are very grateful to all volunteers who took part in this study. The authors' responsibilities were as follows – JLSD, MMR, SMFC: designed research;

457 JLSD: conducted research; FB Jr: was responsible for quantification of Plasma and
458 Erythrocyte Selenium; AS: provided essential reagents for Selenoprotein P
459 concentrations and Glutathione Peroxidase 3 activity; JLSD, EMGS, CD, PB:
460 performed data analysis and statistics; JLSD, MMR, JH, PB: wrote the manuscript. All
461 authors read and approved the final version of the manuscript for submission. The
462 authors declare no conflict of interest.

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TABLE 1

Characteristics of the study volunteers[1]

Parameters	Total (n= 130)
Gender, *n (%)*	
male	32 (24.6)
female	98 (75.4)
Age (y), *n (%)*	
20 – 30y	92 (70.8)
31 – 40y	20 (15.4)
41 – 50y	9 (6.9)
51 – 60y	9 (6.9)
BMI (kg/m^2), *n (%)*	
< 25	93 (71.5)
25 – 30	31 (23.8)
> 30	6 (4.6)
Smoking status, *n (%)*	
Never	104 (80.0)
ex-smoker	20 (5.4)
current	6 (4.6)
Physical activity, *n (%)*	
yes	94 (72.3)
no	36 (27.7)
History of chronic diseases, *n (%)*	
yes	113 (86.9)
no	17 (13.1)
Race, *n (%)*	
caucasian	94 (72.3)
black	26 (20.0)
asian/indigen	10 (7.7)
Plasma Se (μg/L)	
before supplementation	96.7 ± 29.6
after supplementation	292.8 ± 95.4

[1] Numerical variables are presented as mean ± standard deviation (sd). Categorical variables are presented as n (%).

TABLE 2

Concentrations of biomarkers[1] of Se status during supplementation with Brazil nuts and washout period in healthy adults (n = 130)

Biomarker	Supplementation period			
	baseline	4 weeks	8 weeks	*P* value[2]
GPx 1 activity (U/g Hb)	61.8 (58.8 – 65.1)[a]	54.4 (52.0 – 57.1)[b]	61.3 (57.7 – 65.1)[a]	< **0.001**
Erythrocyte Se (µg/L)	169.8 (158.8 – 181.4)[a]	360.2 (342.5 – 378.9)[b]	438.8 (406.6 – 464.8)[c]	< **0.001**
GPx 3 activity (U/L)	528.1 (510.2 – 546.6)[a]	574.1 (554.4 – 594.5)[b]	544.4 (523.5 – 566.2)[ab]	**0.004**
Plasma Se (µg/L)	90.7 (86.4 – 95.2)[a]	219.5 (208.4 – 231.2)[b]	267.0 (252.8 – 282.0)[c]	< **0.001**
SePP (mg/L)	3.4 (3.2 – 3.5)[a]	na	3.9 (3.7 – 4.1)[b]	< **0.001**
Biomarker	Washout period			
	8 weeks	4 weeks	8 weeks	*P* value
GPx 1 activity (U/g Hb)	61.3 (57.7 – 65.1)[a]	68.5 (65.3 – 71.9)[b]	62.8 (58.9 – 67.0)[ab]	**0.005**
Erythrocyte Se (µg/L)	438.8 (406.6 – 464.8)[a]	484.1 (452.7 – 517.5)[b]	332.1 (307.0 – 359.3)[c]	< **0.001**
GPx 3 activity (U/L)	544.4 (523.5 – 566.2)[a]	507.1 (486.9 – 528.2)[ab]	480.9 (457.7 – 505.2)[b]	0.021
Plasma Se (µg/L)	267.0 (252.8 – 282.0)[a]	195.3 (186.8 – 204.2)[b]	160.2 (154.3 – 166.2)[c]	< **0.001**
SePP (mg/L)	3.9 (3.7 – 4.1)[a]	na[3]	3.5 (3.4 – 3.7)[b]	**0.001**

[1] Values are geometric means (CI 95%).
[2] ANOVA repeated measures with post hoc Tukey for GPx1 activity; Friedman test with post hoc Dunn for Erythrocyte Se, GPx3 activity and Plasma Se; Wilcoxon test for SePP concentrations. Different letters in the row mean statistical difference in the time points. GPx1 activity and Erythrocyte Se measured in erythrocytes and GPx3 activity, Plasma Se and SePP measured in Plasma. GPx, Glutathione Peroxidase; SePP, Selenoprotein P.
[3] na: not analyzed

TABLE 3

Genotype and allele frequency of SNPs in selenoprotein genes

Gene	SNP	N	CC/GG	CT/GA	TT/AA	alleles	MAF	HWE (*p* value)
GPX1	rs1050450	130	53.85	40.77	5.38	C > T	T: 0.25	0.560
	rs3811699	130	53.85	40.77	5.38	C > T	T: 0.25	0.560
	rs1800668	130	53.85	40.77	5.38	C > T	T: 0.25	0.560
GPX4	rs713041	130	38.46	42.31	19.23	C > T	T: 0.40	1.914
SELENOP	rs3877899	130	53.85	36.15	10.00	G > A	T: 0.28	1.428
	rs7579	130	38.46	42.31	19.23	G >A	T: 0.40	1.914
SELENOS	rs34713741	130	53.85	37.69	8.46	C > T	T: 0.27	0.332
SELENOF	rs5845	130	42.31	46.15	11.54	C > T	T: 0.35	0.050

GPX1: Glutathione Peroxidase 1, GPX4: Glutathione Peroxidase 4, SELENOP: Selenoprotein P; SELENOF: Selenoprotein 15; SELENOS: Selenoprotein S.

TABLE 4

Multiple Linear Regression models for biomarkers of Se status during daily supplementation with Brazil nuts and washout period in healthy subjects

Dependent Variables	Predictors	β coefficient	Standard error	*P* value
GPx1 activity (U/g Hb)				
Baseline	Erythrocyte Se	0.050	0.019	0.011
4 weeks nuts	Erythrocyte Se	0.029	0.012	0.014
	rs1050450 (*GPX1*)	-5.385	2.546	0.037
	rs5845 (*SELENOF*)	-7.959	2.605	0.003
8 weeks nuts	rs1050450	-8.300	3.424	0.017
8 weeks washout	rs5845	-8.333	4.189	0.049
	rs713041	8.955	4.231	0.036
Erythrocyte Se concentration (µg/L)				
Baseline	Plasma Se	1.570	0.215	< 0.001
	rs713041 (*GPX4)*	-26.698	12.752	0.038
4 weeks nuts	Plasma Se	0.405	0.150	0.008
8 weeks nuts	Plasma Se	0.692	0.158	< 0.001
Plasma Se concentration (µg/L)				
4 weeks nuts	rs7579 (*SELENOP)*	-0.115	0.054	0.034
	rs34713741 (*SELENOS)*	-0.110	0.053	0.038
SePP concentration (mg/dL)				
Baseline	Plasma Se	0.007	0.003	0.015
GPx3 activity (U/L)				
4 weeks nuts	Plasma Se	0.323	0.165	0.050
8 weeks nuts	Plasma Se	0.304	0.134	0.025

[1] Multivariate linear regression models were done separately in different time points for each biomarker using SPSS. Only significant p values (< 0.05) are shown in the table. GPx1 activity and Erythrocyte Se measured in erythrocytes and GPx3 activity, Plasma Se and SePP measured in Plasma. GPx, Glutathione Peroxidase; SePP, Selenoprotein P. All the models were adjusted for age, gender and body fat percentage.

TABLE 5

Multiple Linear Regression models for the change[1] on biomarkers concentrations during daily supplementation with Brazil nuts

Dependent Variables	Predictors	β coefficient	Standard error	*P* value
Change1_GPx1	rs7579	7.372	3.624	**0.044**
	rs5845	54.582	11.334	< **0.001**
Change1_ Erythrocyte Se	rs34713741	262.445	100.909	**0.010**
	BMI	-11.849	4.348	**0.007**
	gender	-107.745	32.802	**0.001**
Change1_GPx3	rs5845	-397.353	158.625	**0.014**
	BMI*rs5845	17.412	6.769	**0.011**
Change1_ Plasma Se	gender	-57.110	18.159	**0.002**
	BMI	-8.429	2.440	**0.001**
Change2_GPx1	gender	-40.057	18.162	**0.029**
	gender*rs713041	26.269	10.728	**0.016**
Change2_SePP	rs5845	-3.110	1.521	**0.043**

[1]: Change 1 = (8 weeks nuts) – (baseline) and Change 2 = (8 weeks washout) – (8 weeks nuts)
All the models were adjusted for age, gender and body fat percentage. GPx, Glutathione Peroxidase; SePP, Selenoprotein P.

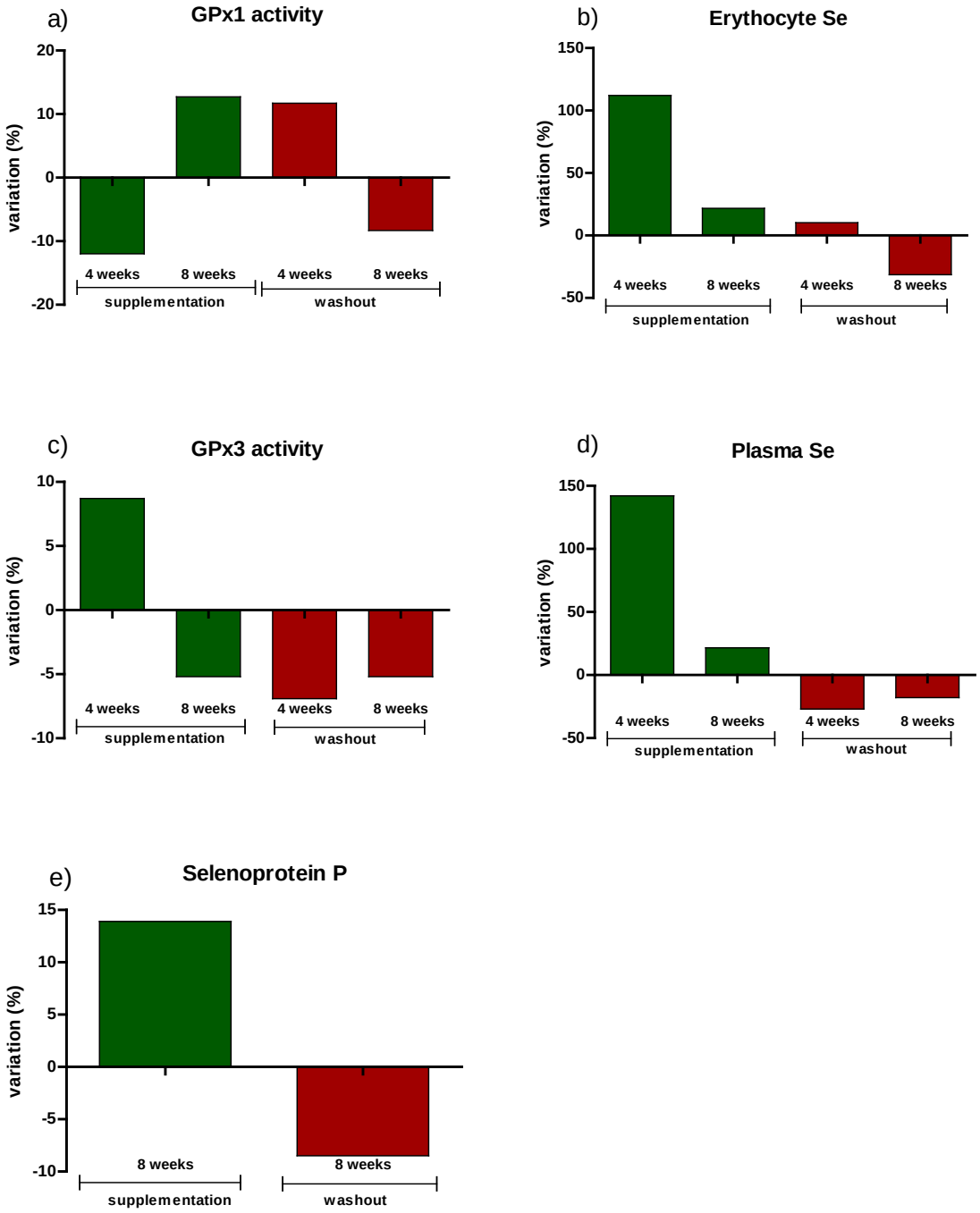

FIGURE 1. Percentage of variation[1] in five biomarkers of Se status during and after daily supplementation with one unit of Brazil nuts in healthy adults

[1] The variation was calculated comparing with the time point immediate before: 4 weeks nuts compared to baseline, 8 weeks nuts compared to 4 weeks nuts, 4 weeks washout compared with 8 weeks nuts and 8 weeks washout compared to 4 weeks washout. a) percentage of variation for GPx1 activity, b) percentage of variation for Erythrocyte Se, c) percentage of variation for GPx3 activity, d) percentage of variation for Plasma Se, e) percentage of variation for SePP.

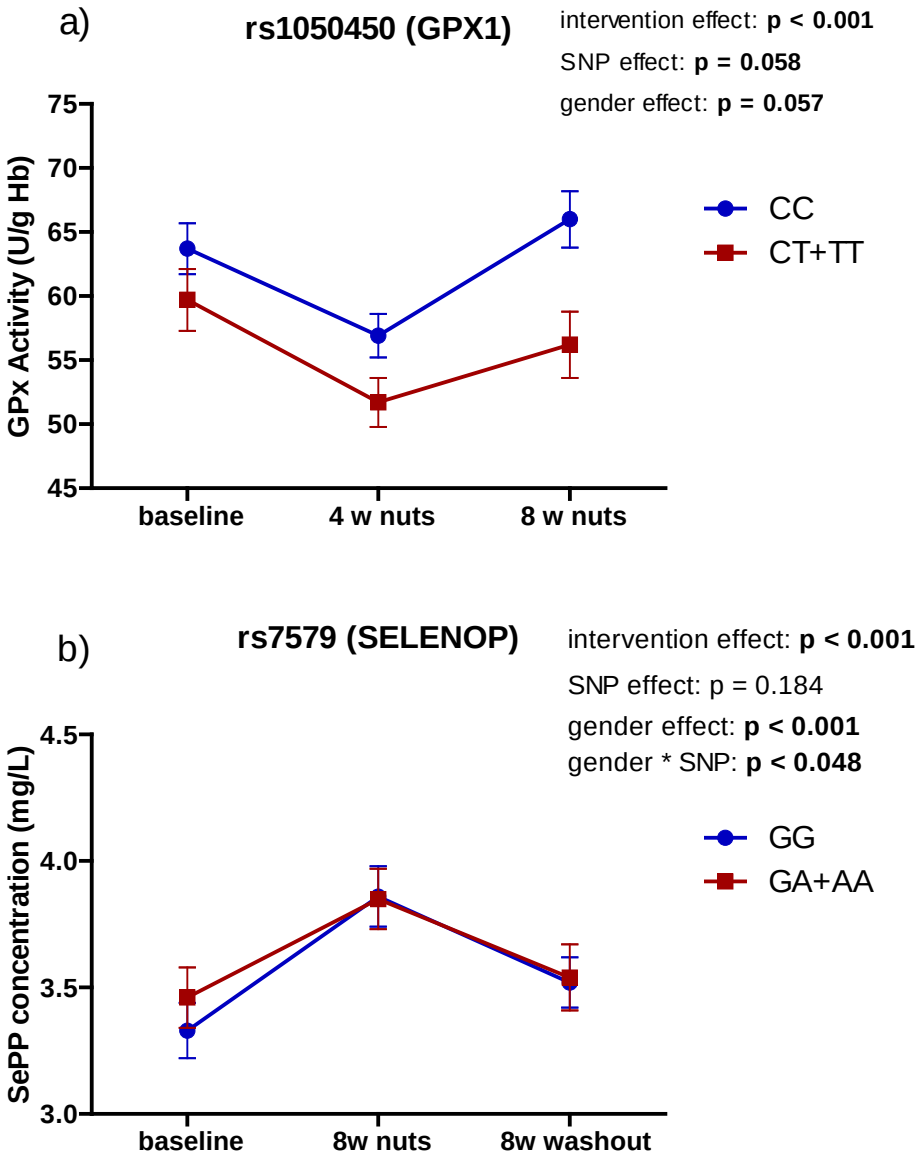

FIGURE 2. Biomarkers of Se status stratified by SNPs in *GPX1* and *SELENOP* genes[1].

[1] Values are geometric means ± standard errors. Two way ANOVA repeated measures adjusted for multiple comparisons with Bonferroni test. a) Erythrocyte GPx1 activity stratified by rs1050450 genotypes, b) Plasma SePP concentration stratified by rs7579 genotypes.

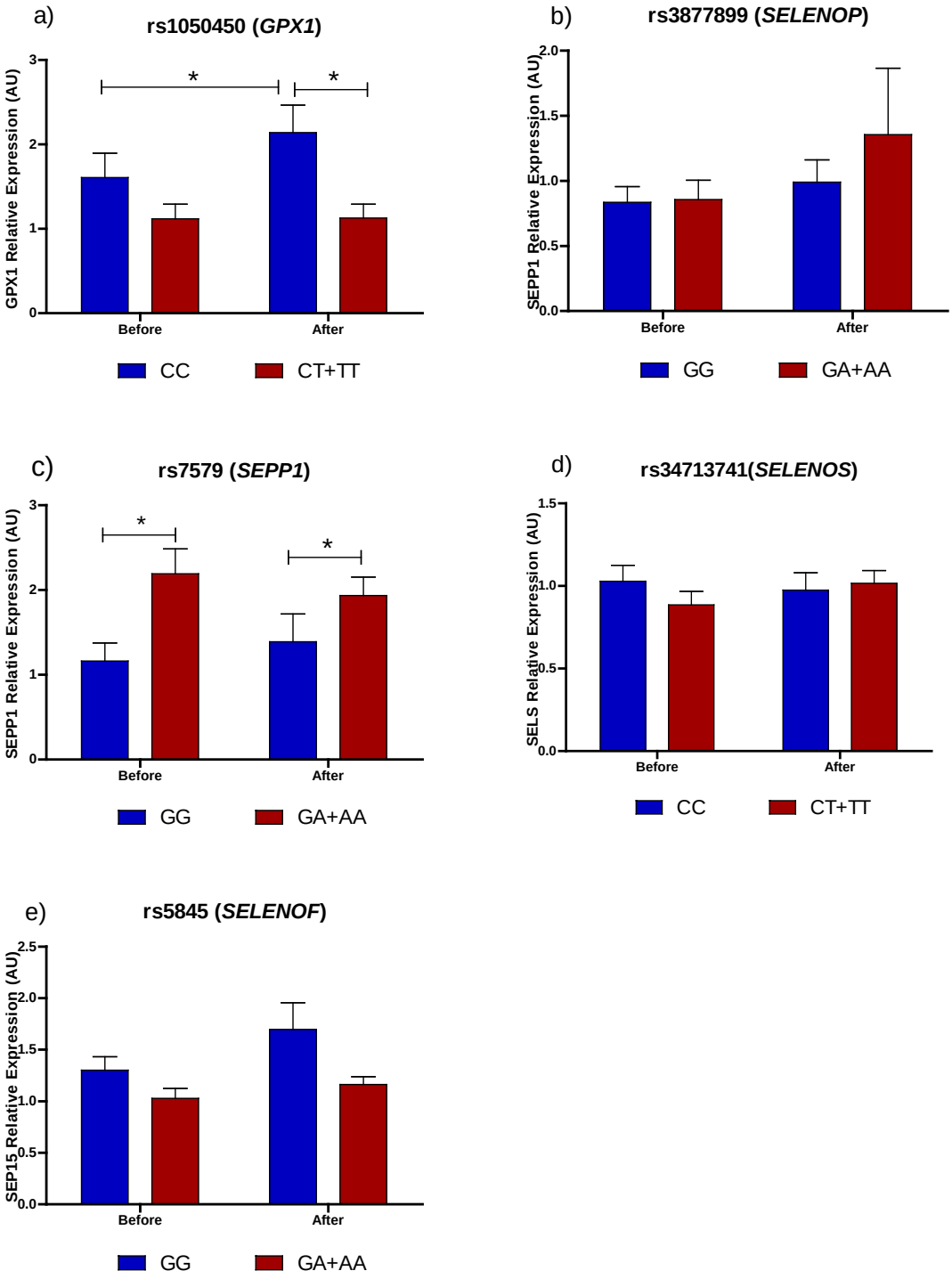

FIGURE 3. Pattern of selenoprotein gene expression in response to Brazil nut supplementation on previously genotyped volunteers[1]

[1] Gene expression relative to GAPDH a) GPx1 mRNA expression separated by rs1050450 genotypes, b) SELENOP mRNA expression separated by rs3877899 genotypes, c) SELENOP mRNA expression separated by rs7579 genotypes, d) SELENOS mRNA expression separated by rs34713741 genotypes, e) SELENOF mRNA expression separated by rs5845 genotypes.
* $P < 0.05$, Mann-Whitney test, ** $P < 0.05$, Wilcoxon test