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HAL Authorization

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

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25 Abstract

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

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

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

56 **Abbreviations**

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

63

64 **Funding source**

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68 collection, analysis and interpretation of data from the present research.

69

70 INTRODUCTION

71 The regular intake of nuts has been proposed to decrease risk for chronic diseases
72 such as cancer, cardiovascular disease and type 2 diabetes¹⁻⁴. Brazil nuts (*Bertholletia*
73 *excelsa*, family Lecythidaceae) are known to be the richest source of selenium (Se), an
74 essential micronutrient for human health. The main form of Se in Brazil nuts are
75 selenomethionine⁵. Se is incorporated as the amino acid selenocysteine (Sec) during
76 translation into 25 selenoproteins encoded by the human genome, many of which show
77 a wide range of functions, including antioxidant defense, redox function, thyroid
78 hormone metabolism, immune function, reproduction and fertility^{6,7}.

79 Low Se status has been associated with increased risk for several diseases, such
80 as cancer, cardiovascular disease, viral infections, male infertility and inflammatory
81 disorders⁷. Potentially, genetic variations could modulate this risk by affecting
82 responses to Se intake⁸. Several studies have demonstrated that polymorphisms in
83 genes encoding selenoproteins have functional consequences⁹. For instance, the
84 rs1050450 (Pro198Leu) in *GPX1* (Glutathione Peroxidase 1) gene was associated with
85 lower erythrocyte GPx1 activity^{10,11} and lower plasma Se in humans¹². Although the
86 regulation of selenoproteins expression is mainly during translation, the mRNA
87 expression of selenoproteins, such as SELENOF (Selenoprotein 15), SELENOK and
88 SEPHS1 can be altered by Se status, as shown previously¹³. Nevertheless, human
89 studies have failed to demonstrate an association between Se status and selenoprotein
90 transcripts¹⁴⁻¹⁶. Only three studies have observed a positive association between Se
91 supplementation and increased selenoprotein expression in humans (SELENOF,
92 SELENOK, GPX1 and SELENOP)^{13,17,18}.

93 Glutathione peroxidase 1 (GPx1) activity is sensitive to alterations in Se status in
94 individuals with low to moderate intake¹⁹. The *GPX1* gene contains a single nucleotide

95 polymorphism (SNP) in the coding region, which causes a Proline to Leucine amino
96 acid change at position 198 (rs1050450)²⁰. This variation has been associated with
97 increased risk for lung, breast, prostate and bladder cancers^{11,21–23} and has been found
98 to modulate the response to Se supplementation in healthy subjects^{12,24}. Carriers of the
99 minor allele T had lower plasma Se at baseline and after one year of supplementation
100 with selenomethionine they had increased urinary Se excretion^{12,24}. Glutathione
101 peroxidase 4 (GPx4) is the only GPx that can reduce phospholipid hydroperoxides in
102 cell membranes²⁵. There is a C>T substitution located in the 3'UTR of the *GPX4* gene
103 (rs713041) and this variant affects Se incorporation in cell culture models²⁶ and the
104 response to Se supplementation in healthy adults²⁷. It was demonstrated that subjects
105 with the TT genotype had lower GPx3 activity after 6 weeks of supplementation with
106 sodium selenite and lower GPx4 activity during the washout period. Also, females with
107 the TT genotype had lower GPx1 and TR1 concentration during the supplementation
108 and the washout period²⁷.

109 Selenoprotein P (*SELENOP*) is the major component of blood Se and the key Se
110 transporter in the body²⁸. Two SNPs with functional consequences are present in this
111 gene; one G>A substitution in the coding region causes an amino acid substitution
112 Alanine to Threonine at position 234 of the protein (rs3877899), and the other G>A
113 substitution is located in the 3'UTR, important for Sec insertion (rs7579). Both SNPs
114 modulate the response to Se supplementation in healthy adults⁸. It was demonstrated
115 that carriers of the minor allele A for both SNPs had higher SePP concentrations after
116 supplementation. Also, males with the AA genotype for rs7579 had lower GPx3 activity
117 after supplementation and during the washout period compared with males with the GG
118 genotype⁸. Selenoprotein S (*SELENOS*) is an endoplasmic reticulum (ER)
119 selenoprotein involved in protecting ER from stress caused by misfolded proteins²⁹. A

120 C>T substitution located in the promoter region of the gene (rs34713741) has been
121 associated with increased risk for rectal cancer³⁰. Selenoprotein 15 (*SELENOP*) is
122 another selenoprotein involved in maintaining ER integrity³¹. A SNP in the 3'UTR of
123 this gene, a G>A substitution in position 1125 (rs5845), has been associated with
124 increased risk for rectal cancer³⁰ and lung cancer³².

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

137

138 **SUBJECT AND METHODS**

139 **Study population and supplementation protocol**

140 The present study involved 130 unrelated healthy volunteers selected at
141 University of Sao Paulo who took part of the Supplementation with Brazil Nuts study
142 (SU.BRA.NUT) described previously³⁵. Subjects were excluded if they were pregnant,
143 younger than 20y and older than 60y taking multivitamins and mineral supplements,
144 anti-inflammatory drugs, with excessive alcohol consumption, athletes, with chronic

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

160 **Composition and Se content of Brazil nuts**

161 The Se content of a random sample of Brazil nuts representative of the four
162 batches used in the study was analyzed using hydride generation flame atomic
163 absorption spectrometry as described previously³⁶ and the centesimal composition was
164 done as proposed by the Association of Official Analytical Chemists³⁷.

165

166 **Sample collection**

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

170 for DNA extraction and subsequent genotyping, and an aliquot of 500 μ L of whole
171 blood from the same EDTA tube was stored into 1,5mL sterile plastic tubes used for
172 RNA extraction and subsequent gene expression. Another 5mL of blood were collected
173 in a tube without anticoagulant to obtain serum for determination of the lipid profile
174 which was described previously³⁵. The total volume of blood samples collected was
175 25mL. Plasma was separated by centrifugation at 3,000 *rpm* for 15 min at 4 °C. The
176 erythrocyte pellet was washed three times with 5 mL sterile 9 g/L NaCl solution, slowly
177 mixed by inversion, and centrifuged at 10,000 *rpm* for 10 min (Eppendorf, C5408) at 4
178 °C, and the supernatant fluid was discarded. Aliquots of whole blood, plasma and
179 erythrocytes were frozen at -80 °C in sterile, demineralized tubes until the analyses
180 were performed.

181 **Biomarkers of Se status**

182 Plasma Se and erythrocyte Se concentrations were determined by inductively
183 coupled plasma mass spectrometry (ICP-MS, Perkin Elmer DRC II) as described
184 previously³⁸. Samples were diluted 1:50 into a 15 mL polypropylene tube with a
185 solution containing 0.01% (v/v) Triton X-100, 0.5% (v/v) nitric acid and 10 μ g/L of
186 each one of the internal standards. The certified reference material Seronorm Trace
187 Elements Serum (SERO AS, Billingstad, Norway) was used for the quality control
188 assessment. Erythrocyte GPx activity was determined using commercial kit (Randox,
189 Labtest, Minas Gerais, Brazil) according to manufacturer's instructions. The enzyme
190 activity was evaluated spectrophotometrically at 37 °C at 340 nm using an automated
191 biochemical analyzer Labmax 240 (Labtest, Minas Gerais, Brazil). Hemoglobin (Hb)
192 concentration was also determined spectrophotometrically in order to express
193 erythrocyte GPx activity in U/g Hb. Plasma GPx (GPx3) activity was calculated by the
194 method of Paglia and Valentine (1967), as modified previously³⁹, using hydrogen

195 peroxide as a substrate. One unit of GPx3 activity is defined as that which oxidizes 1
196 μmol NADPH/min. SePP concentration was determined in plasma using an in-house
197 SePP ELISA (Enzyme Liked Immunosorbent Assay) assays as described previously⁴⁰
198 using a 96-well microplate reader (FLUOstar Omega microplate reader, BMG Labtech,
199 Ortenberg, Germany). Each sample was analyzed in duplicate with aliquots of purified
200 SePP incubated alongside (range 0.25–16 ng/well) acting as the standard curve. A
201 pooled plasma from a previous study conducted at The Rowett Institute was used as a
202 quality control.

203 **Genotyping**

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

214 **Selenoprotein gene expression**

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

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

229

230 **Statistical Analysis**

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

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

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

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

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

259 **RESULTS**

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

266

267 **Characteristics of the volunteers and nut composition**

268 The characteristics of the volunteers are summarized in **Table 1**. Mean age was
269 29.8 ± 9.2 y and mean BMI was 23.3 ± 3.3 kg/m². There was a 100% adherence to the
270 supplementation confirmed by the control calendar given at baseline, the empty plastic
271 bottles at the end of supplementation and the increase of three times in plasma Se after 8
272 weeks of supplementation with Brazil nuts. The Se content and centesimal composition
273 of Brazil nuts are shown in **Table S1**. Four different batches were used during the
274 supplementation. The mean \pm standard deviation for Se content of these four batches
275 was 100.4 ± 5.3 μ g/g. The average weight of the nuts ranged from 3g to 4g, therefore
276 each nut provided from 300 μ g of Se to 400 μ g of Se, which is approximately six times
277 higher than the RDA (Recommended Dietary Allowances) for adults (55 μ g/d).

278

279 **Effect of Brazil nut supplementation on five biomarkers of Se status**

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

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

294 **Genotypes and haplotypes in selenoprotein genes**

295 Genotype and allele frequencies of SNPs in selenoprotein genes are shown in **Table 3**.
296 All SNPs were in Hardy-Weinberg Equilibrium. Haplotype analyses showed evidence
297 of linkage disequilibrium for SNPs in *GPX1* gene: rs1050450 x rs1800668 ($D' = 1.0$ and
298 $r^2 = 0.98$) and rs1050450 x rs3811699 ($D' = 1.0$ and $r^2 = 1.0$). In fact, in Table 3 the
299 genotypes frequencies of the three SNPs are exactly the same. Only two haplotypes
300 were observed, the common haplotype CCG for rs1050450, 1800668 and rs3811699
301 with a frequency of 74% and the rare haplotype TTA with 25%. Therefore, only
302 rs1050450 was used for further analysis. For the SNPs in the *GPX4*, *SELENOF* and
303 *SELENOS* genes, the frequency of the rare genotypes was between 19% and 8%. No
304 haplotype analysis was performed in those genes because only one SNP was genotyped
305 in each gene. **Variables influencing biomarkers of Se status during Brazil nut**
306 **supplementation and washout period**

307 Multiple linear regression models for the five biomarkers of Se status in each time
308 point of the protocol are shown in **Table 4**. GPx1 activity was associated with
309 rs1050450 after 4 ($P = 0.037$) and 8 weeks ($P = 0.017$) of intervention and with rs5845
310 after 4 weeks ($P = 0.003$) of intervention with both SNPs reducing GPx1 activity. After
311 8 weeks of washout, GPx1 activity was associated with rs5845 in *SELENOF*, which was
312 lower in the presence of the rare allele A ($P = 0.049$) and with rs713041 in *GPX4*,
313 which increased GPx1 activity in individuals carrying the rare allele T ($P = 0.036$). The
314 SNP rs713041 in *GPX4* gene was associated with Erythrocyte Se concentrations at
315 baseline, reducing its concentrations in the presence of the rare allele T ($P = 0.038$).

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

324 Erythrocyte GPx1 activity was stratified by rs1050450, as appointed in the
325 multivariate regression analysis (**Figure 2**). During the intervention, GPx1 activity was
326 lower in carriers of the rare allele T (CT+TT), almost reaching statistical significance at
327 4 weeks of supplementation ($P = 0.057$, Figure 2a). SePP concentrations were stratified
328 by rs7579 (Figure 2b). At baseline, SePP concentrations were higher for carriers of the
329 rare allele A, when the interaction with gender was considered ($P = 0.048$).

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

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

353 **Effect of Brazil nut supplementation on mRNA expression of selenoprotein genes**

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

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

366 **DISCUSSION**

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

378 It was observed a delayed response of the two erythrocyte biomarkers of Se
379 status to Brazil nut supplementation, with values increasing up to the first four weeks of
380 the washout period. This apparently slow response could be explained by erythrocytes
381 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/L⁴⁴ and to
387 maximize plasma SePP concentration it is approximately 120 µg/L⁴⁵. 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 stability²¹.
397 This was confirmed by *in vitro* studies where Se supplementation reduced enzyme
398 thermostability for the Leu-variant⁴⁹. 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 patients¹⁰. 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 expression¹⁴⁻¹⁶, however, three studies confirm

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

410 The presence of rs7579 in the *SELENOP* gene influenced not only SePP plasma
411 concentrations but also *SELENOP* mRNA expression. SePP protein concentrations were
412 higher at baseline in carriers of the variant allele A for this 3'UTR SNP. Furthermore,
413 A-carriers had higher *SELENOP* mRNA levels than GG at baseline and after
414 supplementation. Previous work with humans have not found an association between Se
415 supplementation and *SELENOP* mRNA expression in white blood cells^{14,16}. It should
416 be noted that the present work used whole blood for the mRNA expression. Only one
417 study found a positive association by showing that rs7579 influenced *SELENOP* mRNA
418 expression¹⁷. The SePP protein has two isoforms in plasma, the 50kDa and the 60kDa,
419 that are influenced by the genotype for both SNPs in *SELENOP* gene. Individuals with
420 the GA genotype for rs3877899 had a lower proportion of the SePP 60kD, with the Sec-
421 rich domain⁵⁰. This difference in the proportion of SePP isoforms may affect Se
422 availability in plasma for selenoprotein synthesis in different tissues.

423 The 3'UTR region of *SELENOP* gene has two SNPs which are in the same
424 haplotype. The first one is a located a C>T substitution at position 811 (rs5859) and the
425 other is a G>A substitution at position 1125 (rs5845). The two possible haplotypes are
426 811C/1125G or 811T/1125A⁵¹. Previous work has demonstrated that the SECIS
427 element containing the A variant is less responsive to Se supplementation and may
428 influence the translation of Selenoprotein 15 protein⁵². Two biomarkers of Se status
429 were associated with rs5845 in *SELENOP* gene in our study: erythrocyte Se
430 concentrations and GPx1 activity. We also observed that this SNP modulated the
431 percentage of variation of GPx1 and GPx3 activities after supplementation and the

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

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

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

453

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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 volunteers¹

Parameters	Total (n= 130)
Gender, <i>n</i> (%)	
male	32 (24.6)
female	98 (75.4)
Age (y), <i>n</i> (%)	
20 – 30y	92 (70.8)
31 – 40y	20 (15.4)
41 – 50y	9 (6.9)
51 – 60y	9 (6.9)
BMI (kg/m ²), <i>n</i> (%)	
< 25	93 (71.5)
25 – 30	31 (23.8)
> 30	6 (4.6)
Smoking status, <i>n</i> (%)	
Never	104 (80.0)
ex-smoker	20 (5.4)
current	6 (4.6)
Physical activity, <i>n</i> (%)	
yes	94 (72.3)
no	36 (27.7)
History of chronic diseases, <i>n</i> (%)	
yes	113 (86.9)
no	17 (13.1)
Race, <i>n</i> (%)	
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 biomarkers¹ of Se status during supplementation with Brazil nuts and washout period in healthy adults ($n = 130$)

Biomarker	Supplementation period			<i>P</i> value ²
	baseline	4 weeks	8 weeks	
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			<i>P</i> value
	8 weeks	4 weeks	8 weeks	
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 (<i>p</i> 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	<i>P</i> 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 (<i>GPXI</i>)	-5.385	2.546	0.037
	rs5845 (<i>SELENOF</i>)	-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 ($\mu\text{g/L}$)				
Baseline	Plasma Se	1.570	0.215	< 0.001
	rs713041 (<i>GPX4</i>)	-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 ($\mu\text{g/L}$)				
4 weeks nuts	rs7579 (<i>SELENOF</i>)	-0.115	0.054	0.034
	rs34713741	-0.110	0.053	0.038
	(<i>SELENOF</i>)			
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 change¹ on biomarkers concentrations during daily supplementation with Brazil nuts

Dependent Variables	Predictors	β coefficient	Standard error	<i>P</i> 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.

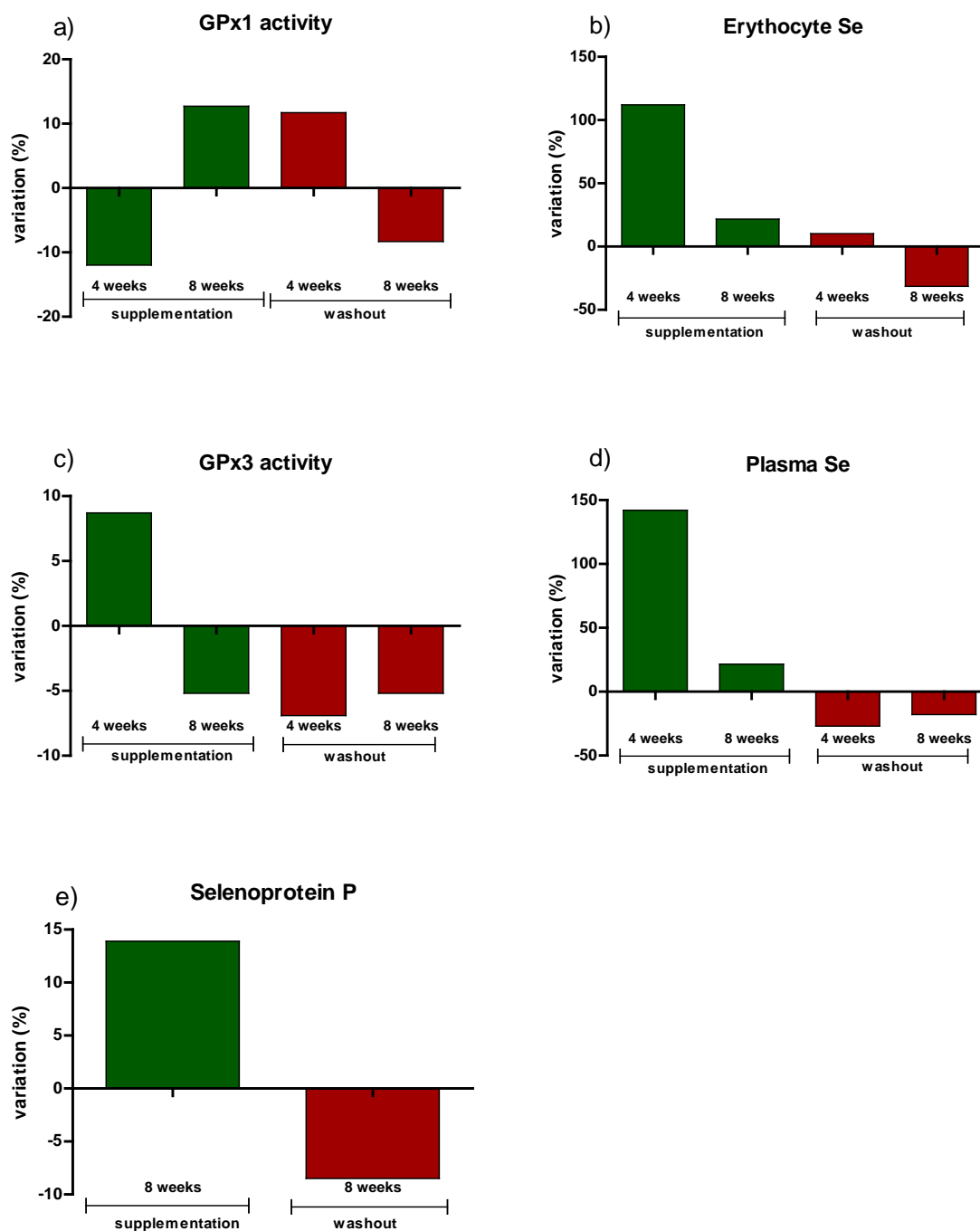


FIGURE 1. Percentage of variation¹ in five biomarkers of Se status during and after daily supplementation with one unit of Brazil nuts in healthy adults

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.

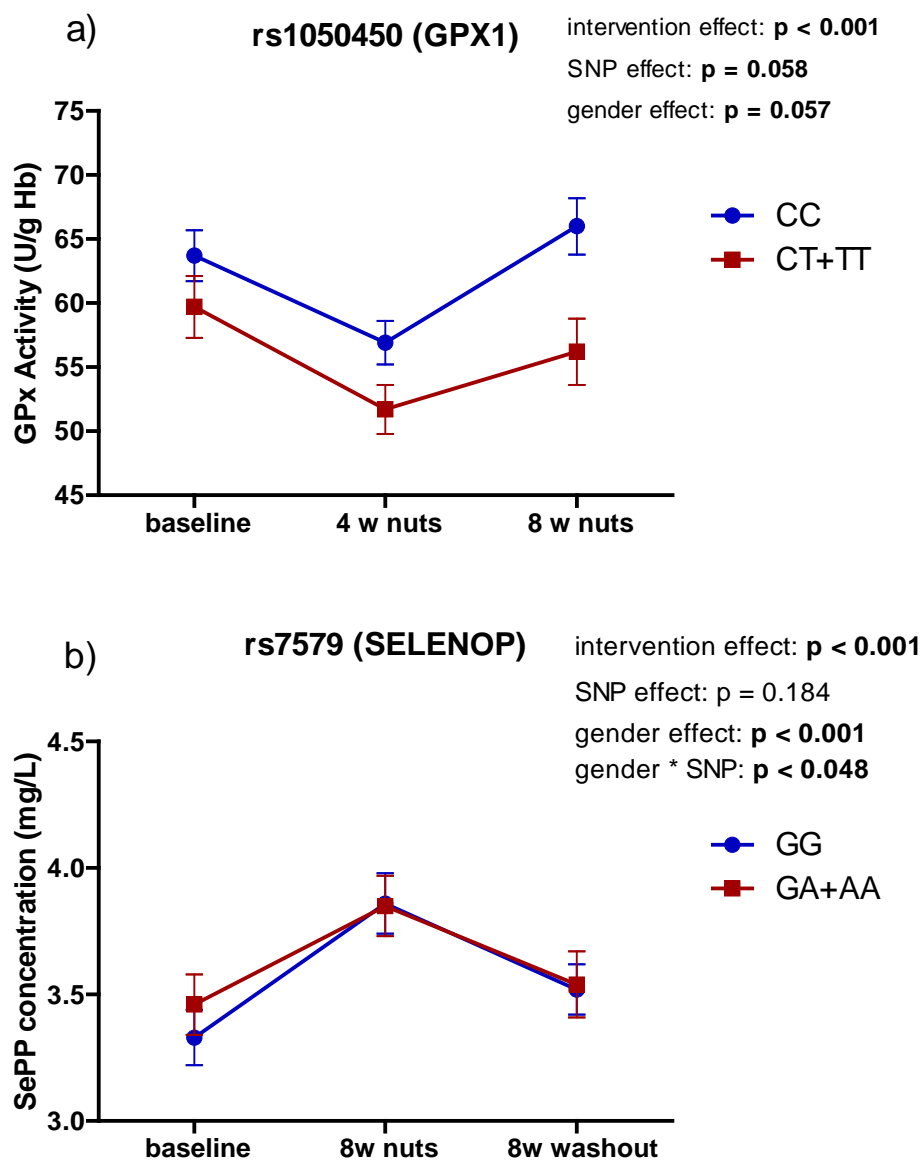


FIGURE 2. Biomarkers of Se status stratified by SNPs in *GPX1* and *SELENOP* genes¹.

¹ Values are geometric means \pm 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.

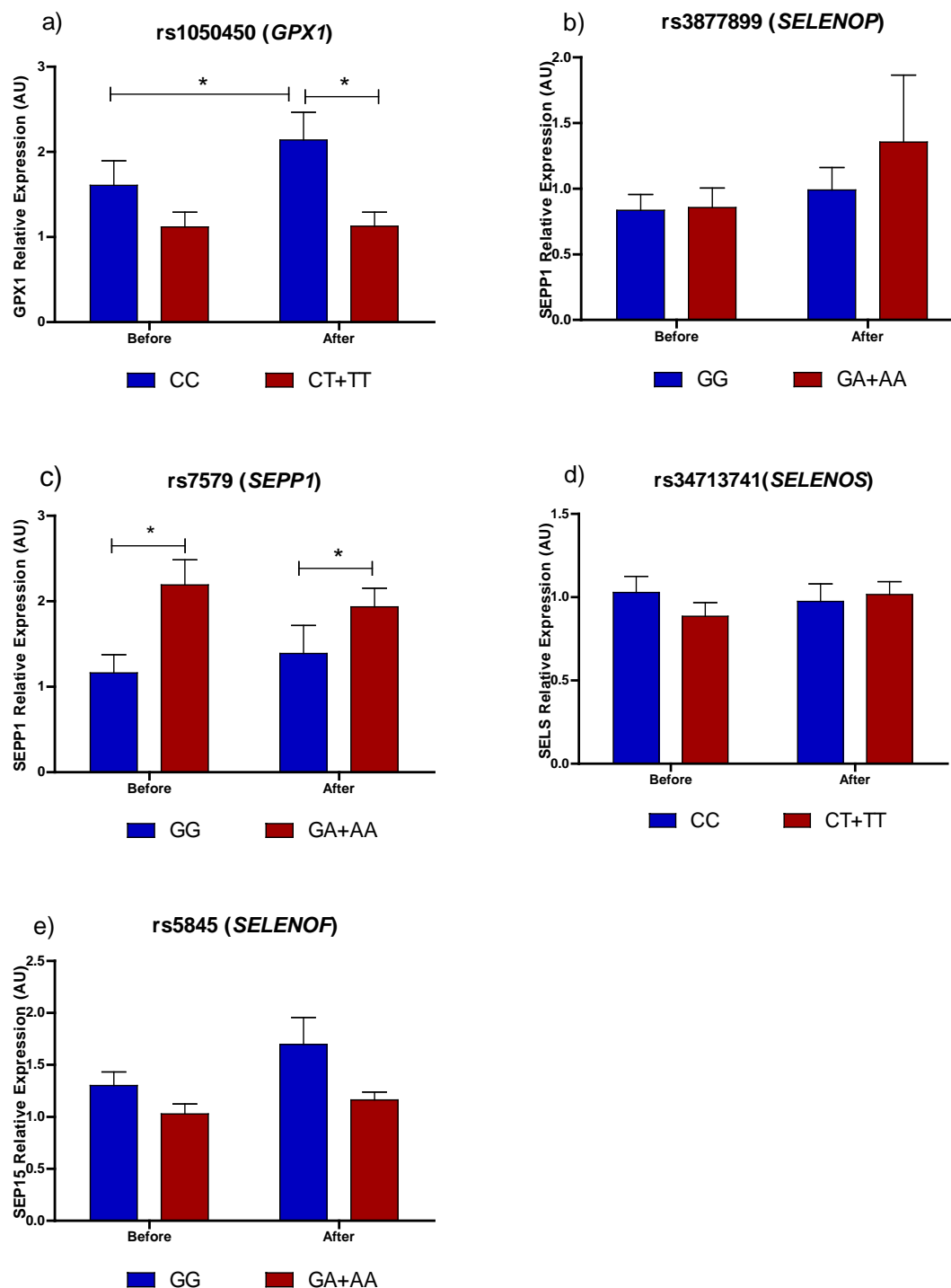


FIGURE 3. Pattern of selenoprotein gene expression in response to Brazil nut supplementation on previously genotyped volunteers¹

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