UNIVERSITY OF SÃO PAULO
Faculty Of Pharmaceutical Sciences
Post Graduation Program in Food Sciences
Area of Experimental Nutrition

Influence of different genotypes in the pattern of selenoprotein expression in response to Brazil nut supplementation

Janaina Lombello Santos Donadio

São Paulo
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Thesis presented for the degree of
DOCTOR OF PHILOSOPHY

Supervisor:
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DEDICATION

To my lovely nieces, Helena and Letícia, who fill my heart with the most pure and sincere love I can ever had imagined to fell...

To my mother and my father, Silvia and Pedro, who have always supported me in every possible way. Thank you. I love you.

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"And do not choose to be conformed to this age, but instead choose to be reformed in the newness of your mind, so that you may demonstrate what is the will of God: what is good, and what is well-pleasing, and what is perfect."

Romans12:2

"Be the change you want to see in the world."

Mahatma Gandhi

"Faith is the confirmation of things we don't see and the conviction of their reality, perceiving as real fact what is not revealed to the senses"

Unknown...

RESUMO

DONADIO, Janaina L S. **Influência de diferentes genótipos no perfil de expressão de selenoproteínas em resposta à suplementação com castanha-do-brasil.** São Paulo, 2016. Tese de Doutorado – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo.

O micronutriente selênio é essencial para a fisiologia humana, inserido nas selenoproteínas na forma do aminoácido selenocisteína. As selenoproteínas são importantes para a função antioxidante, controle do metabolismo dos hormônios tireoidianos, melhora do sistema imune, função cerebral, fertilidade e reprodução. O estado nutricional de selênio deficiente ou marginal está associado com aumento do risco de doenças crônicas, como câncer, diabetes e doença cardiovascular. Sendo assim, diversos estudos procuraram investigar se a suplementação com selênio poderia reduzir o risco dessas doenças. Entretanto, as variações genéticas podem afetar a resposta dos indivíduos a uma intervenção dietética. Portanto, esse estudo foi conduzido para investigar a influência de variações genéticas em genes de selenoproteínas na resposta à suplementação com castanha-do-brasil, melhor fonte de selênio da natureza. Participaram do estudo 130 adultos de ambos os gêneros, com idade de 20 a 60 anos, selecionados na Universidade de São Paulo. Os indivíduos receberam castanhas suficientes para 8 semanas, ingerindo uma unidade por dia e, após o período de suplementação realizaram um período de *washout* também por 8 semanas. Todos realizaram cinco coletas de material biológico a cada quatro semanas. Foram realizadas medidas antropométricas, perfil lipídico, malondialdeído (MDA), concentração de selênio e selenoproteína P no plasma, eritrócitos, atividade da GPx eritrocitária e plasmática, expressão gênica da *GPX1, SEPP1, SELS e SEP15*. Além disso, os participantes foram genotipados para os SNPs rs1050450, rs3811699, rs1800699, rs713041, rs3877899, rs7579, rs34713741 e rs5845. Cada unidade de castanha forneceu em média 350μg de selênio. Todos os 130

voluntários concluíram o estudo. As concentrações de glicose e colesterol total diminuíram após 8 semanas de suplementação. Além disso, as concentrações de HDL-c foram influenciadas pelo SNP rs713041 no gene da GPX4, sendo os valores mais altos encontrados para os indivíduos com o alelo variante T (CT+TT). As frequências dos genótipos variantes foram 5,4% para rs1050450, rs3811699 e rs1088668, 10% para rs3877899, 19,2% para rs713041 e rs7579, 11,5% para rs5845 e 8,5% para rs34713741. Os níveis dos cinco biomarcadores aumentaram significativamente após a suplementação. Além disso, a atividade da GPx eritrocitária foi influenciada pelos rs1050450, rs713041 e rs5845, o selênio eritrocitário foi influenciado pelo rs5845 e o selênio plasmático pelo rs3877899. A expressão dos genes GPX1 e SEPP foram maiores após a suplementação. Tendo em vista esses resultados, conclui-se que a suplementação com uma unidade de castanha-do-brasil durante 8 semanas foi suficiente para reduzir as concentrações de colesterol e de glicose, e elevar as concentrações dos principais biomarcadores do estado nutricional de selênio. Além disso, observou-se que o polimorfismo rs713041 parece influenciar as concentrações de HDL-c e atividade da GPx1, o polimorfismo rs1050450 parece influenciar a atividade da GPx1, o polimorfismo rs5845 parece influenciar a atividade da GPx1 e o selênio eritrocitário e o polimorfismo rs3877899 parece influenciar a o selênio plasmático. Portanto, sugere-se considerar o perfil genético dos indivíduos em futuros estudos avaliando a resposta à suplementação com castanha-do-brasil no estado nutricional de selênio da população.

Palavras-chave: castanha-do-brasil. Selênio. Polimorfismos. Nutrigenética.

ABSTRACT

DONADIO, J.L.S. **Influence of differents genotypes on the pattern of selenoprotein expression in response to supplementation with Brazil nuts.** São Paulo, 2016. PhD Thesis – Faculty of Pharmaceutical Sciences, University of São Paulo.

The micronutrient selenium is essential to human physiology. As the amino acid selenocysteine, it is inserted into selenoproteins with a wide range of functions including antioxidant capacity, thyroid hormone metabolism, improvement of immune system, brain function, fertility and reproduction. Low selenium status has been associated with increased risk for chronic diseases, such as cancer, type-2 diabetes and cardiovascular disease. In this context, several studies have been conducted in order to investigate if selenium supplementation could reduce the risk of such diseases. However, genetic variations may interfere in the response of individuals to a dietary intervention and must be considered as a important source of inter-individual variation. Therefore, this study was conducted was conducted to investigate the influence of genetic variations in selenoproteins genes on the response to an intervention with Brazil nuts, the richest source of selenium known in nature. The study included 130 healthy volunteers with both genders, aged 20 to 60 years old selected in University of São Paulo. They received nuts for 8 weeks, eating one nut a day, and did a washout period for more 8 weeks. All volunteers had a blood sampling collection every 4 weeks during 4 months, in a total of 5. The following analysis were done: anthropometric measurements, lipid profile, plasma malondialdehyde, plasma and erythrocyte Se, selenoprotein P, plasma and erythrocyte GPx activity, gene expression of *GPX1, SEPP1, SELS* and *SEP15*. The volunteers were also genotyped for SNPs rs1050450, rs3811699, rs1800699, rs713041, rs3877899, rs7579, rs34713741 and rs5845. Each unit of Brazil nut provided an average of 300 μg of selenium. All 130 volunteers completed the protocol. The

concentrations of total cholesterol and glucose decreased after 8 weeks of supplementation. Moreover, HDL concentrations were higher for carriers of the variant T allele for *GPX4*_rs713041. The frequencies of the variant genotypes were 5,4% for rs1050450, rs3811699 e rs1088668, 10% for rs3877899, 19,2% for rs713041 e rs7579, 11,5% for rs5845 and 8,5% for rs34713741. The levels of the five biomarkers increased significantly after supplementation. In addition, erythrocyte GPx activity was influenced by rs1050450, rs713041 and rs5845; erythrocyte selenium was influenced by rs5845 and plasma selenium by rs3877899. Gene expression of *GPX1*, *SEPP1* and *SEP15* were higher after supplementation. The SNP rs1050450 influenced GPX1 mRNA expression and rs7579 influenced SEPP1 mRNA expression. Therefore, it can be concluded that the supplementation with one of Brazil nut for 8 weeks was efficient to reduce total cholesterol and glucose levels and to increase the concentrations of the main biomarkers of selenium status in healthy adults. Furthermore, our results suggest that *GPX4*_rs713041 might interfere on HDL concentrations and GPx1 activity, *GPX1*_rs1050450 might interfere on GPx1 activity, *SEP15*_rs5845 might interfere on GPx1 activity and erythrocyte selenium and *SEPP1*_3877899 might interfere on plasma Se levels. Therefore, the effect of genetic variations should be considered in future nutritional interventions evaluating the response to Brazil nut supplementation.

Key words: Brazil nuts. Selenium. Polymorphisms. Nutrigenetics.

ABREVIATIONS

BMI	Body mass index
CH_3SeH	Methyl-selenol
CVD	Cardiovascular Disease
DIO	Iodothyronine Deiodinase
DIO	Deiodinase
DMBA	7,12-dimethylbenzoanthracene
DNMT	DNA methyl transferase
DNPH	dinitrophenilhydrazine
EAR	Estimated Average Requirement
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assays
GPx	Glutathione peroxidase
GPX1	Glutathione Peroxidase 1
GPX2	Glutathione Peroxidase 2
GPX3	Glutathione Peroxidase 3
GPX4	Glutathione Peroxidase 4
GSEA	Gene Set Enrichment Analysis
H_2O_2	hydrogen peroxides
H_2Se	Selenide
HDL-c	High density lipoprotein
HGFAAS	hydride generation flame atomic absorption spectrometry
HPFS	Health Professionals Follow-up Study
HPLC	High Performance Liquid Chromatography
$HSePO_3^{2-}$	selenophosphate
IPP	isopentenyl pyrophosphate
LDL-c	Low density lipoprotein
MDA	Malondialdehyde
MSigDB	Molecular Signature Database
MUFA	monounsaturated fatty acids
NHANES	National Health and Nutrition Examination Survey
NHS	Nurse's Health Study
PUFA	polyunsaturated fatty acids
qPCR	real-time quantitative polymerase chain reaction
RIN	RNA integrity number

RNAt-Sec	Selenocysteine RNA transporter
RT-PCR	real-time polymerase chain reaction
Se	Selenium
Sec	Selenocysteine
SELH	Selenoprotein H
SELW	Selenoprotein W
SeMet	Selenomethione
SEP15	Selenoprotein 15
SePP	Selenoprotein P
SEPP1	Selenoprotein P
SEPS	Selenoprotein S
SNPs	single nucleotide polymorphisms
SPS2	selenophosphate synthetase 2
TAG	triacylglycerol
TC	Total cholesterol
TR	Thioredoxin Reductase
TR	Tioredoxin Reductase
VLDL-c	Very low density lipoprotein

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SUMMARY

CHAPTER 1. Literature review

Importance of Selenium to Human Biology

Selenium (Se) is a trace element with a essential role in human biology. Unlikely other minerals that interact with proteins as cofactors, Se is inserted into the mammalian selenoproteins as the amino acid selenocystein (Sec). Generally, Sec is located in the active site of the enzymes by a mechanism involving the recoding of the stop codon UGA during the translation process (KRYUKOV et al., 2003; PAPP et al., 2007; RAYMAN, 2008). The functions attributed to Se are directly related to the functions of selenoproteins and subproducts of its metabolism. One of the most known functions of selenoproteins is the redox activity, reducing hydrogen peroxides (H_2O_2) and phospholipids peroxides, which can reduce the overall oxidative damage to cell membranes, biomolecules and DNA, therefore, decreasing the risk of cancer and atherosclerosis (RAYMAN, 2000).

Se was reported to be essential to humans after the discovery that increasing Se intake could prevent Keshan Disease, a cardiomyopathy, and Kashin-Beck Disease, an osteoarthritis, observed only in selenium-deficient zones of China, Mongolia, Tibet and North Korea (GE; YANG, 1993; HUANG et al., 2013). Low Se status have been associated with the incidence of the juvenil cardiomyopathy Keshan Disease, characterized by myocardial necrosis and calcification. The infection by coxsackie virus was also implicated in the development of this myocarditis (BENSTOEM et al., 2015; CHEN, 2012). The osteoarthropathy Keshin-Beck Disease is characterized by deformalities of bones, joints and cartilages mostly of ankles, knees, elbows and wrists. It is also a juvenil disease, occuring in children of 5 to 15 years old. The risk factors are Se and iodine deficiency and infection by mycotoxins (HUANG et al., 2013).

Besides the antioxidant function, the mineral is related to immune function (BELLINGER et al., 2009; MATTMILLER; CARLSON; SORDILLO, 2013), protection against toxic heavy metals, (RAYMAN, 2012), thyroid function (BECKETT; ARTHUR, 2005), neurological function (SANTOS et al., 2014; STEINBRENNER; SIES, 2013), male fertility and reproduction (FORESTA et al., 2002), reduction of the risk of cardiovascular disease (TANGUY et al., 2012), cancer (BERA et al., 2013; ZENG; COMBS, 2008) and diabetes mellitus type 2 (RAYMAN; STRANGES, 2013; STEINBRENNER, 2013). In addition, low molecular weight methylated metabolites of Se, such as acid methyl-seleninic and methyl-selenocystein, could have anticarcinogenic function, as demonstrated in *in vivo* and *in vitro* studies (FINLEY, 2006; RAYMAN, 2012). Moreover, there are evidences that selenomethionine (SeMet) supplementation could improve genomic stability, maintaining DNA integrity by reducing the formation of DNA adducts induced by the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) (EL-BAYOUMY, 2001; FERGUSON et al., 2012; XIANG et al., 2008).

Dietary sources, bioavalability and requirements

The concentration of Se in foods are related to the concentrations in the soils, which depends basically on the geological formation. In the soils there are inorganic forms, such as selenates and selenites, with different bioavailability to plants. Great inhibitors are high content of organic matter, and Fe hydroxydes, that can bind Se. Selenite is also less bioavailable to plants than selenate (RAYMAN, 2008; WINKEL et al., 2015). Plants can be stratified by their ability to accumulate Se in three categories: non-accumulators, secondary accumulators and accumulators. Some examples of secondary accumulators are the *Brassica* species (brocoli and cabbage) and *Allium* species (onions, garlic and leek). The only accumulator plant used as a source of Se to humans is the Brazil nut tree (*Bertholetthia*

excelsa, *Lecitidae* family) (RAYMAN, 2008). Accumulator plants store Se as methyl-selenocysteine mainly in young leaves, pollen, ovules and seeds. Possible physiological advantages of Se in plants are upregulation of antioxidant responses, protection from pathogens and herbivores, decreased competition from other species of plants and enhanced growth (WINKEL et al., 2015).

Once absorbed by plants, Se can be found in several chemical forms, such as selenates, SeMet, Sec, Se-methyl-selenocysteine and γ-glutamyl-Se-methyl-selenocysteine. There is a combination chemical forms of Se in different foods. For instance, while in Brazil nuts, the richest Se food source, Se is presented mostly as SeMet, in garlic, onion and brocoli the main forms are Se-methyl-selenocysteine and γ-glutamyl-Se-methyl-selenocysteine (RAYMAN; INFANTE; SARGENT, 2008). In animals, the most predominant forms are SeMet and Sec. Seafood and fish have SeMet and selenates, but apparently SeMet from fish is less bioavailable than SeMet from vegetable sources (RAYMAN; INFANTE; SARGENT, 2008).

Among the Se food sources, such as fish, seafood, meat, grains, eggs and cereals Brazil nuts (*Bertholetthia excelsa*, *Lecitidae* family) have the highest concentration of Se known in nature presented mainly as SeMet, which contributes to 74% of total Se and has greater bioavailability to humans compared to Sec (DA SILVA; MATAVELI; ZEZZI ARRUDA, 2013). Se concentration in Brazil nuts varies from 8 to 83μg/g, however the values can vary even more (THOMSON et al., 2008). Few intervention studies have used Brazil nuts as a Se food source in human trials in order to observe its bioavailability by increasing functional biomarkers such as GPx activity and plasma Se. Organic forms (SeMet and Sec) have greater bioavailability to humans than inorganic forms (selenites and selenates) because they are more efficient in increasing blood Se levels (RAYMAN; INFANTE; SARGENT, 2008). Thomson (2008) found that after 12 weeks of supplementation with

SeMet or 2 units of Brazil nuts, both SeMet and Brazil nuts were efficient in raising plasma Se . Furthermore, Brazil nut was more efficient than SeMet in raising glutathione peroxidase (GPx) activity, which could indicate a better Se bioavailability to selenoprotein synthesis (THOMSON et al., 2008). Two other studies have also found higher levels of plasma Se and GPx activity after Brazil nut supplementation than baseline (CARDOSO et al., 2015b; COMINETTI et al., 2012). The higher bioavailability of SeMet is probably due to its incorporation in replacement of methionine in muscles, erythrocytes and plasma albumin, where it serves as a Se store. It was observed that SeMet is retained 2.5 times more than inorganic forms and the selenoenzymes activities are mantained for longer periods after Se depletion when using SeMet as a supplement (RAYMAN; INFANTE; SARGENT, 2008).

Current dietary Se requirements in most countries are based on the intake necessary to maximize plasma selenoproteins, such as glutathione peroxidase 3 (GPx3) and selenoprotein P (SePP). The most recent recommendations proposed by the Institute of Medicine of the USA and Canada in 2000 were based on the intake needed to maximize GPx3. The experts used the average of two different studies to choose the EAR of 45μg/d (Estimated Average Requirement). The convertion of this value to the RDA (Recommended Dietary Allowance) was further calculated to be 55 μg/d (NÈVE, 2000). In Brazil, the reference used is the same proposed by USA and Canada, namely 55 μg/d for adults. However, different organizations around the world have tried to establish their recommendations, as summarized recently (HURST et al., 2013). Furthermore, new studies have demonstrated that SePP may be a better biomarker for Se intake, as it responds to different forms of Se and could be used in populations with low to adequate Se status (HURST et al., 2013; KIPP et al., 2015). Recently, the European Food Safety Authority (EFSA) have revised the dietary reference values for Se. It was concluded that the habitual intake of 50 – 60 μg/d was not sufficient to

reach the plateau for SePP in a Finish study. Therefore, they concluded that the reference intake for Se should be set at 70 μg/d (EFSA, 2014).

Metabolism

The metabolism of organic and inorganic forms of Se is different Figure 1. Food sources such as meat, fish and Brazil nuts, have most Se compounds in the form of SeMet, which could be incorporated into proteins *en lieu* of methionine, and few compounds in the form of SeCys. Both are metabolized to selenide (H_2Se). SeMet can form methyl-selenol (CH_3SeH) by the action of γ-lyase. Food sources such as garlic and onions have most Se in the methylated form of γ-glutamyl-CH_3SeCys, which will be converted to CH_3SeH by the enzyme β-lyase. Inorganic supplements and some vegetal sources have selenate and selenite, which will be reduced to H_2Se. It's important to highlight that H_2Se can enter the pathway of selenoprotein synthesis by being transformed into selenophosphate ($HSePO_3^{2-}$) by the enzyme selenophosphate synthetase 2 (SPS2), reacting with residues of RNAt-seryl, forming RNAt-Sec, the specific Sec RNA transporter, which will incorporate Sec into the selenoproteins by recoding the stop codon UGA (HATFIELD; GLADYSHEV, 2002). The selenoproteins formed will exert its various functions in human physiology, and depending on the genetic variation in their genes, can have impaired biological function or contribute to increase or reduce the risk of diseases (RAYMAN; INFANTE; SARGENT, 2008).

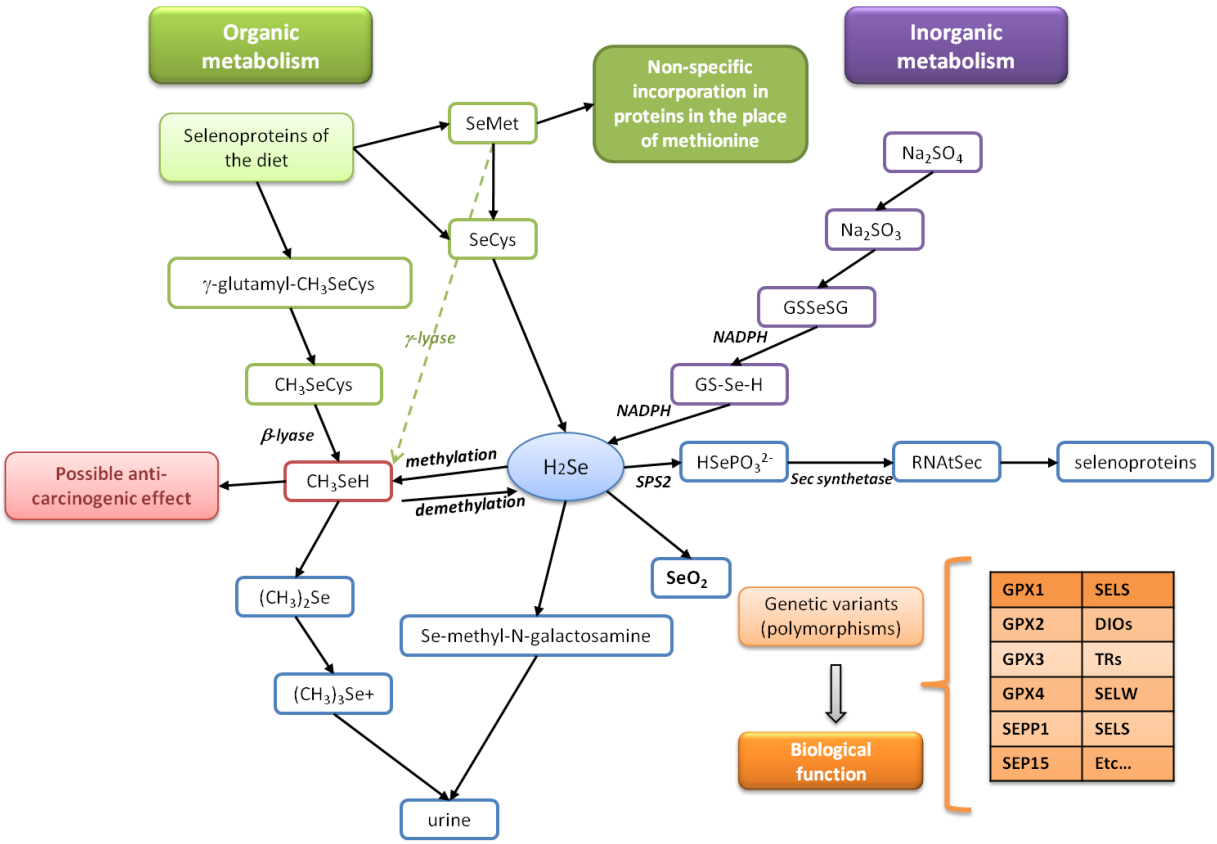


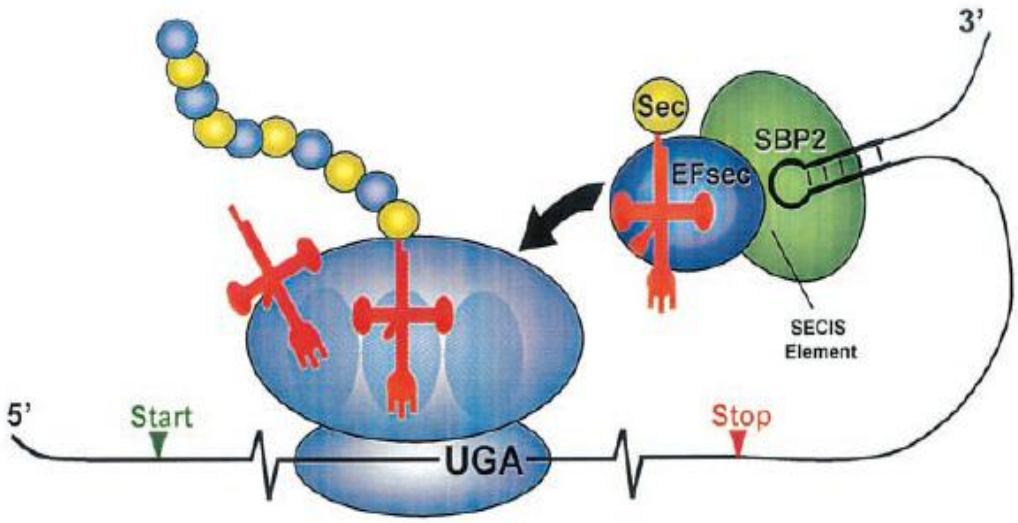
Figure 1. Metabolism of organic and inorganic forms of Se.

Incorporation of Sec into proteins

Se can be incorporated into proteins by two different forms. In the first one is a nonspecifically incorporation, when Se substitutes sulfur in the synthesis of the amino acids cysteine and methionine (HATFIELD; GLADYSHEV, 2002). In the second one, the amino acid Sec is incorporated into proteins during translation, which requires the recoding of the stop codon UGA (LOW; BERRY, 1996; LOW et al., 2000). The discovery of this recoding mechanism has transformed the understanding of the genetic code, which needed to be expanded to include the 21st amino acid Sec.

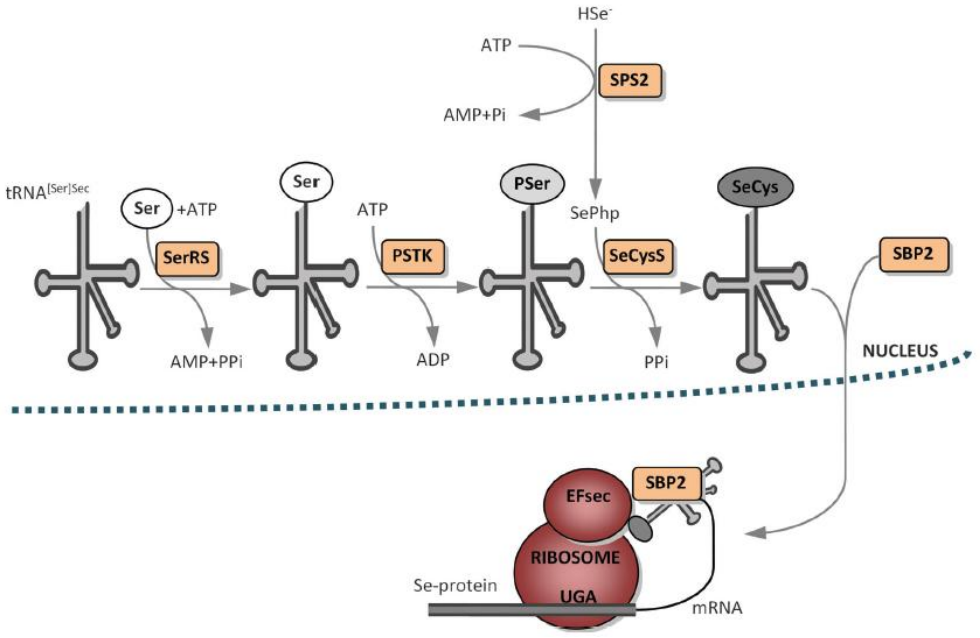
This recoding requires the presence of a specific sequence in the mRNA, a stem loop structure called SECIS (Sec Insertion Sequence) in the 3'UTR of the mRNA Figure 2. The key component of this insertion is the $tRNA^{[Ser]Sec}$, the tRNA specific for Sec. Its synthesis is

related to the amount of Sec available, which is dependent on the Se status. Essentially, the synthesis involves three steps (Figure 3). First, the $tRNA^{[Ser]Sec}$ is charged with serine. Then, it is phosphorylated by a kinase (O-phosphoseryl-$tRNA^{[Ser]Sec}$ kinase). Finally, the phosphoserine is converted to selenocysteine by a synthetase (selenocysteine synthetase), which uses monoselenophosphate as a substrate (BURK; HILL, 2015). It was discovered that there are two isoforms of $tRNA^{[Ser]Sec}$, the only difference being a methylation in the structure when Se is available. After its synthesis, the $tRNA^{[Ser]Sec}$ undergoes four base modifications, which transform this tRNA into its active form (BURK; HILL, 2015). There are two classes of SECIS elements in eukaryotes, named Type I and II (KROL, 2002). Other proteins are needed to bring about the recoding mechanism, such as SECIS Binding Protein two (SBP2) and a specific elongation factor (EFsec). SBP2 is responsible for creating a tight complex with SECIS and for recruiting EFsec ,which is different from EF1 used for the other 20 amino acids (HATFIELD; GLADYSHEV, 2002). The strength of the binding of SBP2 to the different SECIS elements is an important determinant to the hierarchy of selenoprotein expression (LOW et al., 2000).



(HATFIELD; GLADYSHEV, 2002)

Figure 2. Details of Sec insertion into proteins.



(ROMAN; JITARU; BARBANTE, 2014)

Figure 3. Synthesis of $tRNA^{[Ser]Sec}$.

Selenoproteins and their functions

The Glutathione Peroxidases (GPx) are enzymes that can reduce hydrogen peroxides, lipids peroxides using glutathione as a reductant. In mammals there are five GPx (GPx 1, GPx 2, GPx 3, GPx 4, GPx 6) which have selenocysteine in their structure (LABUNSKYY; HATFIELD; GLADYSHEV, 2014; PAPP et al., 2007), but at least eight GPx have been described (BRIGELIUS-FLOHÉ; MAIORINO, 2013). The five enzymes have similar, but not identical, antioxidant proprieties, but different subcellular location (Chart 1). GPx1 was first discovered as an erythrocyte enzyme (LEI; CHENG; MCCLUNG, 2007) and is one of the most studied glutathione peroxidases enzymes, mainly due to its capacity of regulating H_2O_2 concentrations in the human body; $H_2O_2$is a signalling molecule triggering processes

such as stress response, apoptosis, cell proliferation and mitochondrial functions (LABUNSKYY; HATFIELD; GLADYSHEV, 2014).

GPx2 is located in the gastrointestinal system, expressed mainly in the epithelium of the oesophagus. It was first characterized as a specific gastrointestinal protein acting as a barrier against hydroperoxides generated from food. Within the gastrointestinal system, GPx2 is highly expressed in colonic crypt bases, where occurs the growth and differentiation of intestinal stem cells. This process is regulated by the Wnt pathway, which also targets GPx2. GPx2 is also regulated by the transcription factor Nrf2, a upregulator of the cellular defense system (BRIGELIUS-FLOHÉ; MAIORINO, 2013). It is positioned high in the hierarchy of selenoprotein expression, based on studies in mice (BANNING et al., 2008) due to its high mRNA stability under Se deficient conditions (BANNING et al., 2005).

GPx3 is a glycoprotein synthetized in the kidneys with extracellular antioxidant function (PAPP et al., 2007). Initially, the studies were focused on the erythrocyte GPx (GPx1), however, with the discover that GPx3 could use other substrates in the place of glutathione, such as thioredoxin and glutaredoxin, more studies were conducted with this enzyme (BRIGELIUS-FLOHÉ, 1999). GPx3 can also reduce hydrogen peroxide, fatty acid and phospholipid hydroperoxide (PAPP et al., 2007). It accounts for 20% of total plasma Se, based on HPLC-ICP-MS experiments (JACOBSON et al., 2006). . In obese individuals, not only GPx3 concentration but also its enzyme activity were reduced. Another important discovery was the association of lower GPx3 activity with increased risk of stroke (KENET et al., 1999). A role for the enzyme in the inflammatory process has been proposed due to its high activity in children with inflammatory bowel disease (HOFFENBERG et al., 1997). Moreover, GPx3 gene expression is reduced in some types of cancer (BRIGELIUS-FLOHÉ; MAIORINO, 2013; WANG et al., 2010), such as prostate, thyroid and gastric, while the increased gene expression inhibit tumor growth and metastasis (WANG et al., 2010).

However, less is known about the mechanisms underlying its transcriptional regulation (BRIGELIUS-FLOHÉ; MAIORINO, 2013).

GPx4 has three isoforms, one in the nucleus, one in the mitochondria and one in the cytosol. This enzyme is different from the others glutathione peroxidases for several reasons. Its smaller size facilitates the interaction with lipids in the cell membrane and it can use not only glutathione as a substrate, but also hydroperoxides of phospholipids. The deletion of the entire gene is lethal, however mice with the deletion of the mitochondrial isoform were viable. The spermatozoa showed structure abnormalities causing males to be completely infertile (BRIGELIUS-FLOHÉ; MAIORINO, 2013). GPx4 is related to male fertility due to the high expression of the mitochondrial form in testis, where is crucial for the formation of sperm capsules (BRIGELIUS-FLOHÉ, 1999; DIAMOND, 2015). The cytosolic GPx4 is not only essential for embryogenesis, as knockout models of this isoform are embryonically lethal, but also for adults. It was observed a downregulation of GPX4 in the brain of mouse models of Alzheimer's disease. As GPx4 is an important regulator of lipid peroxidation, this decrease could indicate a protective role in the neurons (BRIGELIUS-FLOHÉ; MAIORINO, 2013). GPx4 is also involved in the regulation of leukotrienes biosynthesis: the synthesis of leukotrienes requires the activation of the enzyme 5-lypoxigenase, which is activated by hydroperoxides of lipids in the cell membrane. Therefore, GPx4 is critical to the amount of hydroperoxides available to activate the enzyme 5- lipoxygenase (VILLETTE et al., 2002).

Chart 1 - Chromosome location and function of selenoproteins.

Selenoprotein	Gene location	Function	Tissue	Subcellular location
GPX 1	3p21.3	Antioxidant	All tissues	Cytoplasm
GPX 2	14q23.3	Antioxidant	Gastrointestinal	Cytoplasm
GPX 3	5q33.1	Antioxidant	Plasma	
GPX 4	19p13.3	Antioxidant	All tissues (testis)	Mitochondria, Cell Membrane, nucleus
TR1	12q23.3	Antioxidant	All tissues	Cytoplasm, Mitochondria
TR 2	22q11.21	Antioxidant	Liver, kidney, heart	Mitochondria
TR 3	3q21.2	Antioxidant	Testis	Cytoplasm
DIO 1	1p32.3	Thyroid hormones metabolism	Thyroid	Plasma membrane
DIO 2	14q31.1	Thyroid hormones metabolism	Thyroid	ER membrane
DIO 3	14q32	Thyroid hormones metabolism	Brain, muscle, placenta	Plasma membrane
SPS2	16p11.2	Synthesis of selenoproteins	All tissues	Cytoplasm
SEP15	1p22.3	Quality control of protein folding in ER	All tissues	ER
SEPP1	5p12	Se tranport	Liver, Plasma	Extracellular
SelS	15q26.3	ER-associated degradation, protein folding	Unknown	ER
SelK	3p21.31	ER-associated degradation, protein folding	Unknown	Plasma membrane
SelH	11q22.1	Antioxidant	Unknown	Cytoplasm
SelM	22q12.2			
SelN	1p36.11	Muscle development, Ca homeostasis	Wide	ER membrane
SelW	19q13.32	Antioxidant	Muscle	Cytoplasm
SelI	2p22.3			
SelO	22q13.33			
SelR	16p13.3			
SelV	19q13.13			

Adapted from Labunskyy, 2014; Kryukov, 2003; Bellinger, 2009. Selenoproteins with unknown functions: SelI, SelO, SelR, SelV

Selenoprotein P (SePP) is the most abundant selenoprotein in plasma, constituting almost 50% of plasma Se (ASHTON et al., 2009). Differently from other selenoproteins, SePP has 10 residues of selenocysteine, being expressed mostly in the liver, but it is also found in the other tissues such as brain, gut, heart and kidneys (BURK; HILL, 2005). In Se-deficient conditions, it is preferentially directed to the brain and testis, which implies in the presence of SePP receptors APOER2 in these two tissues (BURK; HILL, 2005). SePP functions and its role in the maintenance of Se homeostasis were discovered after studies conducted in knockout mice for this gene (*Sepp*-/-). Such deletion caused a sharp decrease in

Se concentration in the brain and testis, no changes in the liver and moderate decrease in kidneys and other tissues. It was observed irreversible neurological damages when the animals received a poor Se diet, proving the essentiality of SePP in supply Se to the brain. Moreover, while males *Sepp-/-* were completely sterile, even when fed with a rich Se diet, females *Sepp-/-* were fertile again (BURK; HILL, 2005).

The Thioredoxin Reductase (TR) enzymes are part of the thioredoxin system, the major cellular oxidoreductase system in mammals. This system, which has three components, thioredoxin, thioredoxin reductase and NADPH, is important to maintain cellular redox homeostasis in different biological processes, such as cell growth, apoptosis, regulation of transcription factors, DNA synthesis and repair. Moreover, the tumor suppressor gene p53 is activated by TR1 (LABUNSKYY; HATFIELD; GLADYSHEV, 2014; LU; BERNDT; HOLMGREN, 2009; PAPP et al., 2007). The Iodothyronine deiodinases (DIOs) are enzymes responsible for the activation and inactivation of thyroid hormones that are essential to various biologic processes in human physiology, such as thermogenesis and growth (LABUNSKYY; HATFIELD; GLADYSHEV, 2014; PAPP et al., 2007). Under severe Se deficiency, the DIOs can maintain their expression in thyroid gland, fact that ranks these enzymes high in the hierarchy of selenoprotein expression (BECKETT; ARTHUR, 2005).

Selenoprotein 15 (Sep15) is a thioredoxin-like enzyme located in the endoplasmatic reticulum (ER) of the cells. It is expressed in several tissues, mostly in the brain, liver, testis, lung, kidney and thyroid, being regulated by dietary Se (LABUNSKYY; HATFIELD; GLADYSHEV, 2014). It has been proposed that Sep15 is involved in the quality control of post-translational protein folding in the ER (PAPP et al., 2007). Knockout mice, lacking 2 exons of the the gene, have been developed and they were characterized by oxidative stress in the liver and cataract development (LABUNSKYY; HATFIELD; GLADYSHEV, 2014).

Selenoprotein S (SELS) is located in the endoplasmatic reticulum membrane and it is associated with the transport of proteins from the ER to the cytosol for further degradation by the ubiquitin/proteasome system (LABUNSKYY; HATFIELD; GLADYSHEV, 2014). It has been proposed that this selenoprotein has an important role in inflammation by protecting the ER stability. When the ER is not able to function properly due to misfolded proteins, the expression of pro-inflammatory cytokines is activated through stimulation of the transcription factor NF-κB (RAYMAN, 2009). A genetic variation located in the promoter region of SELS gene was associated with three pro-inflammatory cytokines: interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) (CURRAN et al., 2005)

Regulation of selenoproteins expression

It has been established that dietary Se modulates the expression of some selenoproteins in both protein and mRNA levels (HESKETH, 2008). There are two principles of regulation of selenoprotein expression: the tissue level and the selenoprotein level (SCHOMBURG; SCHWEIZER, 2009). The first principle describes the tissue hierarchy. It states that not all tissues respond equally when the Se supply is scarce. In a severe deficiency condition, the Se concentration in the brain and endocrine tissues is maintained, whereas the quantity in the kidneys and plasma is reduced dramatically. In deficient rats, there is a sharply decrease in GPx1 activity and mRNA in liver. Furthermore, only males became infertile under Se deficiency (HESKETH, 2008; SCHOMBURG; SCHWEIZER, 2009).

The second principle describes the selenoprotein hierarchy. Some proteins have the preference in transcription during Se depletion. It was demonstrated that selenoprotein transcripts compete for the supply of Sec during translation. The expression of DIO1 was maintained *in vivo* in comparison to GPx1; GPx4 and GPx2 transcripts were preferentially synthetized during Se deficiency whereas GPx1 transcripts were degradated (SCHOMBURG;

SCHWEIZER, 2009). Selenoprotein W and H also had a decrease in mRNA expression under Se deficiency (BURK; HILL, 2015). The selenoprotein mRNAs that are sensitive to dietary Se are ranked low in the hierarchy of selenoprotein expression; the ones whose mRNA expression is stable under deficiency are ranked high in the hierarchy. One important aspect of this second principle is how Se supply regulates selenoprotein expression. It has been demonstrated that the regulation is mainly post-transcriptional (BERMANO et al., 1995). Under Se deficiency, the synthesis of $tRNA^{[Ser}Sec$ is reduced. During translation, if there is no $tRNA^{[Ser}Sec$ available, the machinery reads the UGA as a stop codon in the ORF. The remaining mRNA with premature termination codon (PTC) is then eliminated by NMD (Nonsense-Mediated Decay) (DUBEY; COPELAND, 2015). However, the elimination by NMD was demonstrated only for GPX1 mRNA; not all selenoprotein transcripts are degradated by this mechanism (BURK; HILL, 2015).

The regulation of selenoprotein transcripts is very complex and not well understood. In mouse models, Se-deficiency alters only a subset of selenoprotein transcripts in the liver and kidney (SUNDE et al., 2009). Limited Se intake reduced *GPX1* mRNA expression in the liver and kidney (SUNDE et al., 2009). It is established that the regulation of *GPX1* expression is not transcriptional, but due to a mechanism called Nonsense Mediate Decay (NMD), which degrades mRNAs with a UGA stop codon positioned at 55 nucleotides upstream of the exon-intron region of splicing (SUNDE; RAINES, 2011). Therefore, it seems reasonable to conclude that, *GPX1* mRNA expression could be a potential molecular biomarker to alterations of Se status due to the sensibility of its mRNA to degradation by NMD.

Se and diseases

Cancer

There are a large amount of epidemiological evidences linking Se intake and reduced risk of cancer mortality (MÉPLAN; HESKETH, 2012, 2014; PETERS; TAKATA, 2008; WHANGER, 2004). One of the strongest pieces of evidence was shown in 1996, when the supplementation of healthy Americans with 200 μg/d of selenized yeast reduced the incidence of colon, lung and prostate cancers by 58, 46 and 64%, mainly in individuals with plasma Se < 106 μg/L (CLARK, 1996). However, the same results were not achieved in another study, the SELECT trial (Selenium and Vitamin E Cancer Prevention Trial), in which the supplementation of selenomethione and vitamin E were studied combined or alone to prevent prostate cancer. The trial was discontinued after 7 years of supplementation with no positive results of the study agents (LIPPMAN et al., 2009). These opposite results from both studies shed the light into possible explanations with regard to the form of Se used, the baseline Se status of participants (136μg/L) and the genetic profile not being considered in either trial (HATFIELD; GLADYSHEV, 2009; RAYMAN, 2012).

Possible mechanisms by which Se interferes with cancer progression are through antioxidant proprieties of selenoproteins, such as GPx, TR, SePP, Sep15; the monomethylated intermediates of its metabolism, such as methylselenol, that can stimulate tumor cells apoptosis; regulation of programmed cell death; maintaince of DNA integrity, the modulation of immune system and inflammation (BARRERA et al., 2012; HATFIELD et al., 2009; WHANGER, 2004). Usually, the chemopreventive dose of Se is supranutritional and enough to maximize selenoproteins activity. Thus, it was proposed that its chemical proprieties and metabolites have a more important role in cancer prevention, especially in the early stages (BARRERA et al., 2012; WHANGER, 2004).

Low Se status measured by serum, plasma and toenail Se concentrations has been associated with cancer risk. For prostate cancer, a recent meta-analysis demonstrated a reduced risk over the narrow range of plasma Se between 120 to 170 μg/L (HURST et al., 2012). Plasma Se concentration above 80 μg/L were associated with a reduced risk for lung and laryngeal cancers (JAWORSKA et al., 2013). Lower concentrations of serum Se and SePP were observed in patients with prostate cancer in Germany, however the authors questioned if this was a predisposition or a consequence of the disease (MEYER et al., 2009).

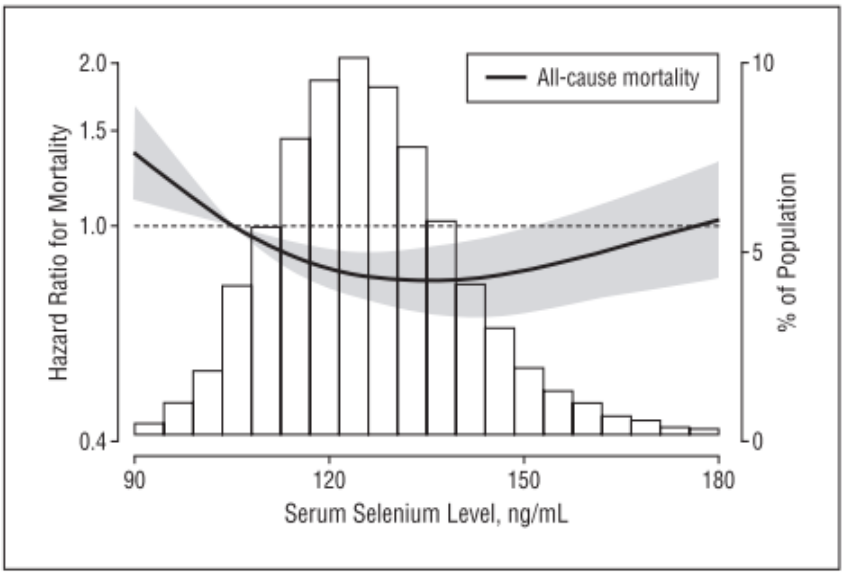
The molecular pathways that are influenced by Se associated with cancer are: the induction of the tumor supressor gene p53, alteration in the pattern of DNA methylation, improvement of immune function, inactivation of the signalling receptor PKC (protein kinase C) inhibiting tumor cell growth, induction of caspases enzymes that estimulates apotosis of cancer cells (PETERS, 2008; RAYMAN, 2005). Other possible mechanism by which Se influences cancer progression involves the pattern of global methylation of the DNA, an epigenetic mechanism that changes the pattern of gene expression without altering the nucleotyde sequence of the DNA. DNA methylation is controled by the enzyme DNA methyl transferase (DNMT) which was found to be downregulated by dietary Se (BARRERA et al., 2012; XIANG et al., 2008). In cancer cells the is a different pattern of DNA methylation, with hypermethylation in the promoter region causing gene silencing and hypomethylation of genomic DNA causing chromosomal instability (BARRERA et al., 2012).

Cardiovascular Disease

Studies investigating the relation between Se and reduced risk of cardiovascular disease (CVD) have inconsistent findings (RAYMAN, 2000, 2012; TANGUY et al., 2012). In the analysis of data from the Third Nutrition and Health Examination survey (NHANES III) conducted in the USA, Se supplementation did not have an effect on cardiovascular

disease endpoints. Currently, randomized human trials do not support the hypothesis that Se supplementation may prevent CVD, unless the population is already at risk for the disease or with low Se status (STRANGES et al., 2010). In this context, it was proposed a "U" dose-shape curve for serum Se levels and mortality from CVD (Figure 4) (BLEYS; NAVAS-ACIEN; GUALLAR, 2008). In individuals with low (below 90 μg/L) and very high Se status (above 150μg/L), there is a high risk of mortality from CVD, demonstrating that there is a narrow window for the beneficial effects of Se supplementation in reducing the risk for CDV.

In the context of cardiometabolic health, the selenoproteins that have special attention are the GPx family because of its capacity to protect membrane lipids from oxidation (RAYMAN, 2012). Since LDL oxidation is the main cause of the development of the atherosclerotic plate, plasma selenoproteins such as GPx and SePP may prevent its formation (BENSTOEM et al., 2015). However, other selenoproteins K and R have been studied recently due to their involvement in the antioxidant defence in cardiomyocytes (TANGUY et al., 2012). There is some evidence that Se supplementation in patients with cardiomyopathy is effective in restore cardiac function (JOSEPH; LOSCALZO, 2013). Within the optimal Se levels, the cardioprotective effect of Se may be explained by some mechanisms. Apart from the already mentioned plasma antioxidant capacity, Se may also interfere in the progression of the disease by modulating the inflammatory system (BENSTOEM et al., 2015). Other possibility is by generating an anti-apoptotic signal which reduces ischemia and reperfusion (TANGUY et al., 2012).



(BLEYS; NAVAS-ACIEN; GUALLAR, 2008)

Figure 4. The proposed U-shape dose response of serum Se levels and all cause mortality

Diabetes Mellitus type II

The association between selenium status and insulin resistance is not completely understood. Epidemiological studies have shown a association of high Se status and lower risk of type 2 diabetes. Experiments *in vitro* and *in vivo* demonstrated that treatment with supraphisiological doses of purified SePP from human plasma (10μg/mL) impaired insulin signaling and dysregulates glucose metabolism (MISU et al., 2010). Evidence from clinical studies demonstrate that both low and high Se status increase risk for type-2 diabetes (RAYMAN; STRANGES, 2013).

On the one hand, high Se status could interfere in insulin metabolism by increasing GPx1activity which reduces the concentration of hydrogen peroxide, a second messenger in insulin signalling.On the other hand, low Se status could increase the oxidative stress in pancreatic β cells (RAYMAN; STRANGES, 2013). In the NPC trial, a post-hoc analysis revealed that the supplementation of 200μg of Se increased the risk of type-2 diabetes in

participants in the highest tertile of plasma Se on baseline (RAYMAN et al., 2012). The post-hoc analysis of NHANES trial corroborated these findings (STRANGES et al., 2010). The pilot UK PRECISE (Prevention of Cancer by Intervention with Selenium) study carried out with 500 elderly adults showed no effect of Se supplementation as selenium-enriched yeast on plasma adiponectin, a marker of lower risk of type-2 diabetes (RAYMAN et al., 2012).

The findings of these trials have to be considered carefully because some of them were conducted in very specific groups of the population. The NPC trial, for instance, were carried out only in men with history of skin melanoma, and the pilot UK-PRECISE study used only elderly adults. Besides, none of these trials considered either the genetic profile or the baseline Se status of the participants. Therefore, better designed trials are urgently needed to help the scientific community understand the effect of different doses of Se in reducing the risk of type-2 diabetes (RAYMAN; STRANGES, 2013).

Biomarkers of Se status

Se nutritional status is determined by the quantification of different biomarkers. The most currently used are plasma Se, erythrocyte Se, whole blood Se, urinary Se, plasma GPx activity, erythrocyte GPx activity, whole blood GPx activity, plasma SePP and (COMBS et al., 2011). The most sensitive biomarker to changes in Se intake is plasma Se. In individuals with marginal to adequate Se levels, plasma Se is considered an excellent biomarker; for those with low Se levels, erythrocyte GPx activity is a good choice. Its activity reaches a plateau when plasma Se is 100μg/L (ASHTON et al., 2009) and when erythrocyte Se reaches 140 μg/L (NÈVE, 2000). Another good biomarker that can be used in individuals with low Se levels is plasma SePP. In Brazilian studies the most used biomarkers are plasma Se, erythrocyte Se, erythrocyte GPx activity and whole blood GPx activity. Nonetheless, it is

imperative to also quantify SePP concentrations in plasma and GPx1, GPx3 and GPx4 activities separately. Recent studies have shown that the measurement of different selenoproteins is crucial to the assessment of Se nutritional status (COMBS et al., 2011).

The great advantage in using different biomarkers to assess Se status is the possibility of assessing Se intake (COMBS, 2015). This approach is adequate in case of populations with marginal Se status, in which the increase in Se blood levels is proportional to the amount of Se supplemented (COMBS, 2015; XIA et al., 2005). In populations with adequate Se status, the increase in Se biomarkers is dependent of the form of Se in the diet. It is known that sodium selenite has lower bioavailability compared to SeMet (FAIRWEATHER-TAIT; COLLINGS; HURST, 2010), and increases plasma Se in 20% in those individuals with basal concentration of 70μg/L (BURK et al., 2006; DUFFIELD et al., 1999). On the other hand, SeMet is capable of raising plasma Se and SePP concentrations even in those individuals with adequate Se status (BURK et al., 2006; COMBS, 2015; COMBS et al., 2012; HURST et al., 2010). In those individuals with adequate Se status consuming Se as SeMet, the Se intake can be estimated by plasma Se (COMBS, 2015; COMBS et al., 2012).

SePP concentration is sensitive to dietary Se, therefore is an excellent biomarker of Se intake, mainly in individuals with low to marginal Se status (HURST et al., 2010).Previous work has shown that supplementation with 200μg/d of SeMet for four weeks is enough to change plasma SePP concentration (ASHTON et al., 2009). Plasma SePP concentration has being used in many international studies as a biomarker of Se status. There are still no reference values for this biomarker, however, and its measurement could help to establish a possible reference for plasma Se (DUFFIELD et al., 1999)Even without a reference value for SePP concentrations, some studies have tried to identify the plasma Se concentration needed to reach a plateau of SePP concentration in plasma. In a Chinese study, it was noted that an intake of 37μg/d of SeMet and 66 μg/d of selenite was enough to maximize plasma GPx,

however it was not enough to maximize SePP. Therefore, to optimize SePP concentrations it is necessary to have a higher Se intake or a longer period of intervention (XIA et al., 2005). In a study of healthy adults in UK Se supplementation with 50μg/d of SeMet was sufficient SePP concentration in plasma to reach a plateau (HURST et al., 2010).

The problem of evaluating Se intake using the traditional methods, such as 24h Food Recall, 3-day nonconsecutive dietary food record and Food Frequency Questionnaires, is the real Se concentration of foods in Brazilians Food Databases. It is established that a huge variation of Se concentrations in foods exists as a result of the variation of Se content in the soil of different countries (COMBS, 2001; OLDFIELD, 2002). This variation is reflected in different plasma Se concentration worldwide. Another important point is the lack of information regarding Se concentration in Brazilian foods and the insertion of this data into computer software used to calculate dietary intake. Few works have evaluated Se concentration in food consumed in Brazil (FERREIRA et al., 2002; LEMIRE et al., 2010). Therefore, because of the problems in evaluate Se intake, it is preferable to quantify different biomarkers in plasma in order to obtain a real Se status of individuals, and still be able to infer Se intake with more precision.

Genomic Studies

After the sequencing of the human genome, the nutritional field was revolutionized. New genomic approaches were established to answer traditional questions, such as how a nutrient modulates the risk of chronic diseases by interacting with the environment and the genome. In this context, the nutritional genomics field emerged, with the goals of understand how a specific nutrient alters the pattern of gene expression and how the genetic background interferes in the response to nutrients modulating the risk of diseases (KAUWELL, 2005; MÉPLAN; DONADIO; HESKETH, 2015).

As a consequence of the great effort made by the Human Genome Project, genetic variations or polymorphisms were discovered and received special attention. There are different types of polymorphisms in the human genome. By definition, a polymorphic locus in a chromosome is that in which the most common variant occurs in less than 99% of the population, or in terms of the variant allele, the rarer allele must occurs in more than 1% of the population (KAUWELL, 2005; SCHORK; FALLIN; LANCHBURY, 2000). Polymorphisms are originated from mutations that were fixed in the population. Therefore, different types of polymorphisms originated different types of mutations. The substitution of a single nucleotide in the DNA sequence is the simplest and most common type of polymorphism, termed single nucleotide polymorphism or SNP. Other polymorphisms are consequence of insertions or deletions (INDELS) of parts of the DNA. These INDELS could be of hundred of base pairs, called variable number of tandem repeats (VNTRs or minisatellites), or just two, three or four bases, named simple tandem repeats (STRs or microsatellites) (SCHORK; FALLIN; LANCHBURY, 2000). STRs are often used as markers of ancestry in population genetics (SANTOS et al., 2009).

One important concept in population genetics is the linkage disequilibrium and haplotype blocks. By definition, a haplotype is a group of alleles in a specific locus in the chromosome; and the linkage disequilibrium is the association among these alleles. Alleles in one specific region that are physically close to each other tend to be inherited together. Therefore, if one of these alleles is a marker for a disease and if they are in linkage disequilibrium, the identification of only one allele in the haplotype is sufficient to predict the presence of the other ones (BROOKES, 1999; SCHORK; FALLIN; LANCHBURY, 2000).

In the nutritional field, the most important genetic variations are the SNPs. It is noteworthy that most SNPs do not have an effect on phenotype for several reasons: they could be in a intronic region not close to the splicing juntion, they could be in exons and do

not change the amino acid because the genetic code is degenerated or even if they change the amino acid, this modification is not esential for the structure, stability and function of the protein. Thus, the great current challenge is to discover SNPs that alone or interacting with other SNPs or nutritional components have functional consequences (HESKETH, 2008). There is a huge amount of data associating the frequency of genotypes for different SNPs and the risk of chronic diseases. With regard to selenium biology, there is a consensus for the functionality of SNPs in at least ten selenoprotein genes. Detailed information about each of them can be seen in **Chart 2**.

Chart 2. Functional SNPs in selenoprotein genes.

Gene symbol	Chromossomal location	SNPs	Location/ modification	base change	Functional consequences	Associated diseases or	References
SEP15	1p22.3	rs5845 rs5859 rs540049	3'UTR 3'UTR 3'UTR	G>A G>A or C>T C>T	Sec insertion Sec insertion enzyme activity(GPX3)	colorectal , prostate cancer colorectal, prostate, lung cancer prostate cancer	(JABLONSKA et al., 2008; KARUNASINGHE et al., 2012a; MÉPLAN et al., 2010; STEINBRECHER et al., 2010; SUTHERLAND et al., 2010)
GPX1	3p21.3	rs1050450 rs8179169 rs1800668 rs3811699	Pro198Leu Arg5Pro promoter promoter	C>T G>C C>T G>A	enzyme activity enzyme activity? gene expression gene expression	breast, lung, prostate and bladder cancer no diseases breast cancer diabetes	(MÉPLAN et al., 2013; PELLATT et al., 2013; PETERS; TAKATA, 2008; RAVN-HAREN et al., 2006; STEINBRECHER et al., 2010; ZHAO et al., 2005)
GPX2	14q23.3	rs4902347	tagSNP	C>T	?	rectal cancer	(HAUG et al., 2012)
GPX3	5q33.1	rs8177447 rs3828599 rs736775	tagSNP tagSNP tagSNP	C>T C>T C>T	? ? ?	rectal cancer rectal cancer rectal cancer	(HAUG et al., 2012; PETERS et al., 2008)
GPX4	19p13.3	rs713041	3'UTR	C>T	Sec insertion	colorectal, breast and lung cancer	(JAWORSKA et al., 2013; MÉPLAN et al., 2008, 2013; STEINBRECHER et al., 2010; VILLETTE et al., 2002)
SELK	3p21.31	rs9880056	taggingSNP/ promoter	T>C	?	prostate cancer	(MÉPLAN et al., 2012)
SEPP1	5p12	rs3877899 rs7579 rs12055266 rs3797310 rs2972994 rs230813 rs230819	Ala234Thr 3'UTR tagSNP tagSNP tagSNP tagSNP (3'UTR) intron	G>A G>A A>G G>A C>T C>G C>A	enzyme activity Sec insertion ? ? ? ? ?	breast cancer colorectal and prostate colorectal cancer colorectal cancer colorectal cancer MDA levels MDA levels	(MÉPLAN et al., 2007, 2013; PETERS et al., 2008; STEINBRECHER et al., 2010; TAKATA et al., 2012)
SELS	15q26.3	rs34713741 rs28665122	promoter promoter	C>T C>T	gene expression? gene expression	colorectal cancer gastric cancer	(CURRAN et al., 2005; MÉPLAN et al., 2010; PELLATT et al., 2013; SHIBATA et al., 2009; SUTHERLAND et al., 2010)
TXNRD1	12q23.3	rs7310505	intron	C>A	?	prostate cancer	(MÉPLAN et al., 2012)
TXNRD2	22q11.21	rs9605030 rs9605031	intron intron	C>T C>T	?	prostate cancer colorectal cancer	(MÉPLAN et al., 2010, 2012)

One of the most studied SNPs in selenoprotein genes is the *GPX1*_rs1050450. It is C>T substitution located in the coding region of the gene which changes the amino acid proline to leucine in the position 198 of the protein. The rare allele T was found to be more frequent in breast cancer cells, with loss of heterozygosity in *GPX1* locus 3p21 observed in 36% of breast tumors (HU; DIAMOND, 2003) and was associated with decreased GPx1 activity and higher risk of breast cancer in a Danish study (RAVN-HAREN et al., 2006). This association was confirmed latter in a more recent study demonstrating that the allele T increased the risk for non-ductal tumors (MÉPLAN et al., 2013). The rare allele T was also associated with higher risk for prostate cancer in only one study (KARUNASINGHE et al., 2012a) and with modulation of the disease risk by interaction with Se status, decreasing the risk with increasing serum Se levels (STEINBRECHER et al., 2010). The risk for colorectal cancer was increased by an interaction of *GPX1*_rs1050450 and *SELS*_rs34713741 (MÉPLAN et al., 2010). Other studies demonstrated that the T allele increased risk for lung cancer (RATNASINGHE et al., 2000), bladder cancer (ZHAO et al., 2005), metabolic syndrome (KUZUYA et al., 2008) and cardiovascular disease (HAMANISHI et al., 2004). In healthy subjects, it was demonstrated that individuals with the TT genotype had lower plasma Se at baseline compared to CC (COMBS et al., 2012).

Two SNPs were discovered in *SEPP1* gene with functional consequences: one is a G>A substitution located in the 3´UTR (rs7579) and the other one is also a G>A substitution located in the coding region, predicted to change the amino acid alanine to threonine (rs3877899) (MÉPLAN et al., 2007). After intervention in healthy adults in the UK with 100µg/d of sodium selenite for 6 weeks, it was observed that both SNPs affected the Se availability to selenoprotein synthesis, as SePP is the most important Se transporter

in plasma (MÉPLAN et al., 2007). In Danish adults, the rare allele (A) for rs3877899 decreased the risk for breast cancer, however, the interaction with *GPX1*_rs1050450 increased the risk (RASMUSSEN et al., 2009). In adults from Czech Republic, the interaction of *SEPP1*_rs3877899 and *GPX4*_rs713041 decreased the risk of colorectal cancer (MÉPLAN et al., 2010). In American adults, plasma SePP concentrations were lower in GA individuals compared to GG for *SEPP1*_rs3877899 (COMBS et al., 2011).

Considering the SNP located in the 3´UTR (rs7579), it was noticed that the AA genotype increased the risk of colorectal cancer in adults from Czech Republic, but the interaction with *SEP15*_rs5859 decreased the risk (MÉPLAN et al., 2010). In a German study, men with AA genotype had an increased risk of developing prostate cancer. Moreover, higher plasma SePP concentrations were found in individuals carriers of the A allele (GA+AA) (STEINBRECHER et al., 2010).

There is a C>T substitution (rs713041) in the GPX4 gene in the region corresponding to the 3'UTR, the region of the mRNA important for Sec insertion. Experimental evidence, both *in vitro* and *in vivo,* have demonstrated that this SNP has functional consequences. Levels of lymphocyte lypoxigenase products were higher in individuals with the CC genotype and the C allele were associated with higher risk for colorectal cancer (VILLETTE et al., 2002). It was demonstrated *in vitro* that *GPX4* transcripts containing the C allele showed a better ability to bind proteins, competing strongly with the transcripts containing the T allele and with the *GPX1* transcripts (MÉPLAN et al., 2008). In human endothelial cells, the T allele was associated with higher levels of lipid hydroperoxides and was also more sensitive to oxidative challenge (CROSLEY et al., 2013). The rare T allele was associated with higher risk for breast cancer in a study conducted in the UK (UDLER et al., 2007) and with colorectal cancer in adults

from Czech Republic, in interaction with SOD2_rs4880 and TR2_rs9605031 (MÉPLAN et al., 2010). In healthy men from New Zealand, the TT genotype was associated with reduction of DNA damage with increasing serum Se (KARUNASINGHE et al., 2012b). Moreover, this SNP affected the response after supplementation with 100μg of sodium selenite in healthy adults from UK. The GPx4 activity measured in the lymphocytes were dramatically decreased in TT subjects during the washout period (MÉPLAN et al., 2008).

OBJECTIVES

In the context of selenium biology and its effects in human health, it is important to understand how individuals may respond to the intake of a high Se content food, such as Brazil nuts. It is also fundamental to investigate the consequences of this intake on both biochemical and molecular levels in order to choose the best biomarker for a specific group of the population. Based on the literature presented, no studies have investigated the effect of Brazil nut supplementation in healthy individuals at the molecular level considering the genetic profile of the participants. Therefore, this work was conducted to investigate the influence of genetic variants in selenoprotein genes on the response to supplementation with Brazil nuts at the biochemical and molecular levels.

Specific objectives

- Determine the influence of genetic variants on blood lipid profile and oxidative stress after Brazil nut supplementation
- Quantify two new biomarkers of Se status in plasma: SePP concentrations and GPx3 activity
- Determine the influence of SNPs in selenoproteins genes on five biomarkers of Se status after Brazil nut supplementation
- Determine the influence of genetic variants on the pattern of selenoprotein expression
- Determine the influence of the SNP rs713041 in *GPX4* gene on the global gene expression after Brazil nut supplementation

METHODS

Study population and supplementation protocol

One hundred and thirty unrelated healthy adults (males and females) aged 20 to 60 years old were selected in Sao Paulo University, Brazil. Volunteers taking multivitamins and mineral supplements, anti-inflammatory drugs, those with excessive alcohol consumption, those athletes, those with chronic diseases such as cancer, diabetes and cardiovascular disease were excluded from the study. The participants were invited by electronic correspondence and personal communication when the protocol was explained. They were asked to give a 20mL blood sample initially (baseline) and take a daily supplement of Brazil nuts for 4 weeks, eating one nut a day. After four weeks, they returned to another 20mL blood sampling and received nuts for more four weeks. After eight weeks of supplementation with Brazil nuts, another 20mL blood sample was taken, and then two more blood samples were taken at four week intervals during eight weeks of washout period (Figure **5**). The compliance with Brazil nut supplementation was estimated by checking a control calendar given in the first blood sampling. The adhesion to the supplementation was checked by high reported compliance. For all participants, weight and height were measured and the body mass index (BMI) was calculated. Written inform consent were signed by 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 Declaration of Helsinski.

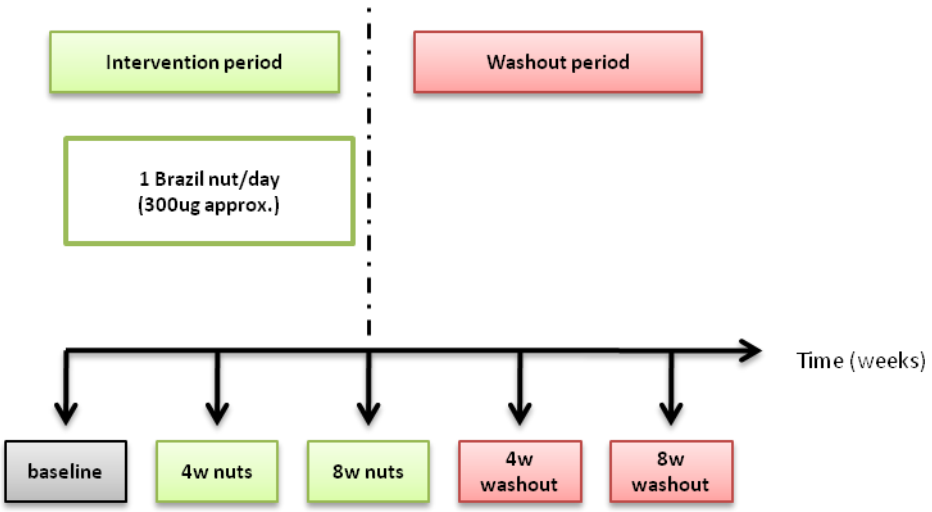


Figure 5. Brazil nut supplementation protocol.

Centesimal Composition and Se content of Brazil nuts

The centesimal composition of a random sample of Brazil nuts representative of the eight batches used in the study was analyzed according to the methods proposed by the A.O.A.C. (AOAC, 1990). Se content of Brazil nuts was done using hydride generation flame atomic absorption spectrometry (HGFAAS). Brazil nuts were prepared for analysis by digestion with 68% nitric acid (Merck, Darmstadt, Germany) and heating at 150^{o}C. After volatilization of the organic material, solutions were reduced from SeVI to SeIV by the addition of 1.2N hydrochloric acid and heating at 100^{o}C for 2 h. The samples were diluted to 50mL volume in ultrapure water. A calibration curve was prepared with Titrisol 1000 mg/mL (Merck) diluted in 1% nitric acid at the following concentrations: 0, 0.1, 0.3, 0.5, 1.0, 3.0, 5.0, 15.0, and 30.0 mg/L. Wheat Flour Standard Reference Material 1567a from the National Institute of Standards & Technology (Gaithersburg, MD, USA) with a certified Se concentration was used as an external control.

Blood sampling

Fasting blood samples (15 mL) were drawn by venipuncture into three 5 mL EDTA tubes. An aliquot of 1.5 mL of EDTA whole blood was used for DNA extraction and subsequent genotyping. Another 5mL of blood were collected in a tube without anticoagulant to obtain serum. 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.

Anthropometry

Body weight (kg) was measured using a digital scale and height (m) was self-reported by the volunteers. Body Mass index (BMI), in kilograms per square meter, was calculated and compared to the guidelines proposed by World Health Organization.

Serum lipid profile

Fasting glucose and lipid profile - total cholesterol (TC), HDL-c, LDL-c and triacylglycerol (TAG) - were measured in serum using commercial kits (Labtest, Minas Gerais, Brazil) adapted to a biochemical analyzer (LabMax 240, Labtest, Minas Gerais, Brazil).

Evaluation of plasma levels of malondyaldehyde (MDA)

To assess the oxidative stress, plasma malondyaldehyde (MDA) concentrations were determined before and after Brazil nut supplementation by High Performance Liquid Chromatography (HPLC) (Shimadzu, Kyoto, Japan) , quantifying the reaction of MDA and dinitrophenylhydrazine (DNPH) using an absorbance of 306nm.

Plasma was previously centrifuged at 5000 *rpm* for 10min. In a microtube, it was added 100μL of plasma, 10 μL of NaOH 4M and 10 μL of butylated hydroxytoluene (BHT) 10%, left 30min at 60^{o}C and 5 min on ice. It was added 150μL of sulphuric acid (H_2SO_4, 1%) on each tube, mixed and centrifuged for 10min at 14000 *rpm.* 200 μL of the supernatant was added into another identified microtube. 25 μL of DNPH 0,001g/mL diluted on HCl 2M was added, mixed and left 30min at 40^{o}C. The tubes were dry on speedvac (miVac Duo Concentrator, GeneVac, England) for 1h. The solution was ressuspended on 70 μL of a solution with 985 μL of acetocholonitrile and 15 μL of amonium hydroxyde and centrifuged for 10min at 14000 *rpm.* 60 μL were transferred into a vial and 50 μL were injected into the chromatographer. A standard curve with the points 0.25, 0.5, 1.0, 2.0, 4.0, 6.0 and 12.0 was prepared and run with the samples on each day of analysis. The column used in the equipment was a Luna C18 column (250 mm x 4,6 mm, Phenomenex, Torrance, CA) with a pre-column C18 (4,0 x 3,0 mm, Phenomenex, Torrance, CA). The temperature of the sampler was kept at 4 ^{o}C and the column at 30 ^{o}C. The mobile fase used was composed by a acetonitrile solution with a solution of acetic acid 0.2% v/v with a flux of 1ml/min. The detection used an absorbance of 306nm.

Plasma Se concentrations

Plasma Se were determined by inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer DRC II) according to the method described previously (BATISTA et al., 2009), at the Laboratório de Toxicologia e Essencialidade de Metais, Universidade de São Paulo, Ribeirão Preto, SP, Brazil. Samples were diluted 1:50 into a 15mL polypropylene tube with a solution containing 0.01% (v/v) Triton X-100, 0.5% (v/v) nitric acid and 10 g/L of each one of the internal standards.

Erythrocyte GPx activity

Erythrocyte GPx activity was done for all time points using commercial kit (Randox, Labtest, Minas Gerais, Brazil) according to manufacturer's instructions. Briefly, 50µL of erythrocytes were mixed with 1mL of the diluting solution, followed by 5 min of incubation and by the addition of 1mL of 2x colour reagent. 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 in order to express eGPx activity in U/g Hb.

Plasma GPx3 activity

GPx3 activity was done in plasma, as proposed by Paglia e Valentine (PAGLIA; VALENTINE, 1967), modifying to the specific enzyme. This method is based on the reduction of hydrogen peroxide catalyzed by GPx, using glutathione as a reductor agent. The glutathione concentration is maintained by the addition of glutathione reductase and NADPH (PAGLIA; VALENTINE, 1967). First, a reaction mix for 50R was done using

5mg of $NADPH_2$, 46mg of glutathione reduced, 3mL of destiladed water, 24mL of phosphate buffer, 1mL of Na Azide and 20U of glutathione reductase. In a cuvette, it was added 915μL of the mix, 35μL of hydrogen peroxide and 50 μL of plasma. The absorbance was read at 340nm in a 8cell spectrophotometer (Ultrospec 4000, UV/visible spectrophotometer Pharmacia Biotech Inc, NJ, USA).

Plasma SePP concentrations

SePP concentration was determined in plasma by enzyme-linked immunosorbent assays (ELISA) in a 96-well microplate reader (FLUOstar Omega microplate reader, BMG Labtech, Ortenberg, Germany). The customized antibody anti-SePP was incubated in a 96-well plate for 2h, 37°C. The solution with the antibody was rinsed and blocked overnight with 0.05% caesin solution at room temperature. Plasma samples were diluted and combined with selenoprotein P monoclonal antibody (Selenoprotein P antibody, clone 37A1, LifeSpan Biosciences) overnight, 4°C on another 96-well plate. Standard curves were constructed using human purified SePP using the points 16, 8, 4, 2, 1 and 0. The first plate was washed three times with 300μL of PBS-tween. 100μL of diluted samples from the second plate were transferred to the washed plate and incubated at 37°C for 2h. After 2h the plate was washed with PBS-tween. A secondary antibody (antimouse IgG, HRP-linked, horseradish peroxidase- linked, Cell Signalling Technology) was added to the plate which was incubated at 37°C for 1h. The plate was washed again three times with 300μL of PBS-tween. 100 μL of TMB was added to the plate and left at room temperature for 5 min until the colour was developed. The reaction was stopped by the addition of 100 μL of H_2SO_4 and the absorbance was read at 450nm.

Genotyping

Total DNA was extracted from 200μL of whole blood using Purelink Genomic DNA Minikit (Invitrogen, Life Technologies, California, USA), the final concentration measured in a NanoDrop ND 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and adjusted for further analysis. The SNPs selected were chosen based on the literature evidence for functional consequences and relation to risk of chronic diseases. Using these criteria, the SNPs selected are shown in Table 1.The SNPs were determined by real-time polymerase chain reaction (RT-PCR) using Taqman SNP Genotyping Assays (Life Technologies, California, USA). Each RT-PCR reaction contained 20ng of adjusted genomic DNA, 0,5μL of 20X SNP Genotyping Assay (Life Technologies), 5,0μL of TaqMan Genotyping Master Mix (Life Technologies) in a 10μL final reaction volume. Samples were assayed along with no-template and internal controls for each genotype and run in the StepOne Plus Real Time PCR system under the following conditions: enzyme activation at 95 ^{o}C for 10min, followed by 40 cycles at 92 ^{o}C for 15s and 60 ^{o}C for 1 min for annealing and extension. The allelic discrimination was obtained by performing an endpoint read. Hardy-Weinberg equilibrium was tested by the chi-squared test. The haplotype distribution and linkage disequilibrium were done in the software Haploview 4.2. SNPs were considered in linkage disequilibrium when D'>0,5.

Chart 3. Information about the SNPs used in the study

Genes	Chromossome	rs (dbSNP)	SNP region	Base change	Codon change	aminoacid change
GPX1	3	rs1050450	Coding region	C679T	C**C**C / C**T**C	Pro198Leu
		rs1800668	promoter	C>T	*	*
		rs8179169	Codin region	G94C	C**G**G / C**C**G	Arg5Pro
		rs3811699	promoter	G-602A	*	*
GPX4	19	rs713041	3'UTR	C718T	*	*
SEPP1	5	rs3877899	Coding region	G215A	**G**CT - **A**CT	Ala234Thr
		rs7579	3'UTR	G25191A	*	*
SELS	15	rs34713741	promoter	C>T	*	*
SEP15	1	rs5845	3'UTR	G>A	*	*

*: no change.

Gene expression

The gene expression of *GPX1, SEPP1, SELS* and *SEP15* was analysed before and after Brazil nut supplementation on previously genotyped participants. RNA was extracted from whole blood using Ribopure Blood Kit (Ambion, Life Technologies) and treated with DNase I to prevent DNA contamination. The final concentration was measured in a NanoDrop ND 1000 spectrophotometer (Thermo Scientific). RNA was considered of good quality when the absorbance ratios between 260 and 280 nm wavelengths were between 1,9 and 2,1. RNA was adjusted to 20ng/μL in a final volume of 20μL. High Capacity Reverse Transcriptase kit (Life Technolgies) was used to synthetise the cDNA using 10μL of adjusted RNA. cDNA was further diluted 1:5 in DNA/RNA free water to perform the real time PCR reactions.

Analysis of selenoproteins gene expression was performed by real-time quantitative PCR (qPCR) in the QuantStudio 12K Real Time PCR System (Life Technologies) using specific probes for each gene (Taqman Gene expression Assays, Life Technologies). The qPCR reactions were performed in a 384-well plate in triplicate for the target gene and

duplicate for the reference gene GAPDH. Each qPCR reaction contained 2μL of diluted cDNA, 0,5μL of 20X Taqman Gene Expression Assays (Life Technologies), 5,0μL of TaqMan Gene Expression Master Mix (Life Technologies), 2,5μL of RNase free water in a 10μL final reaction volume. GAPDH was used as a reference gene as its expression was stable among samples. A non template reaction without cDNA was included in each plate as a negative control. The cycling conditions were 2 min at 50 ^{o}C for denaturation, followed by 45 cycles 95 ^{o}C for 15s and 1 min at 60 ^{o}C for hybridisation and extension.

Differential gene expression of the selenoprotein genes was evaluated considering the previously genotyped SNPs in each gene. We used the dominant genetic model to choose the samples of the two genotypic groups for each SNP: homozygous dominant x heterozygous + homozygous variant. Each group had 18 samples. The method used to quantify the relative gene expression was based on the $2^{-\Delta\Delta\ Cq}$ method (LIVAK; SCHMITTGEN, 2001). The Δ Cq was calculated normalizing the values by the internal control gene GAPDH (Δ Cq target gene - Δ Cq reference gene). The ΔΔ Cq was obtained by subtracting the Δ Cq of the samples by the average of Δ Cq of each genotype group before supplementation.

Microarray analysis

To investigate the significance of C>T SNP located on the 3'UTR of *GPX4* the pattern of gene expression was analysed using microarray analysis using RNA from six volunteers who were CC and six volunteers who were TT and using samples before and after supplementation (**Figure 1**). Total RNA was extracted from whole blood using Purelink Blood MiniKit (Ambion). The integrity of these samples and more 12 spare

samples was checked using Tape Station 2000 (Agilent, Santa Clara, USA) with the Agilent RNA Nano kit. Samples with a RNA integrity number (RIN) of above seven were used for whole genome microarray using the Illumina HumanHT-12 v4 BeadChip. These RNA quality control measurements were also carried out by Service XS using Agilent Bioanalyzer, as can be seen in **Figure 2**. RNA labeling, amplification, and hybridization were performed by Service XS. Raw microarray scan files were exported using the Illumina Beadstudio program and loaded into R for downstream analysis using the BioConductor and specific packages for each step of the bioinformatics analysis (RITCHIE et al., 2011). The data were transformed and normalized using VST (variance stabilizing transform) and RSN (robust spline normalization) from the Lumi BioConductor package. Probes with signals that fullfilled the criteria of Illumina probe detection *P* value of 0.05 were considered different. The bioinformatic analysis was performed by the Newcastle Bioinformatics Support Unit at the Faculty of Medical Sciences in Newcastle University, UK.

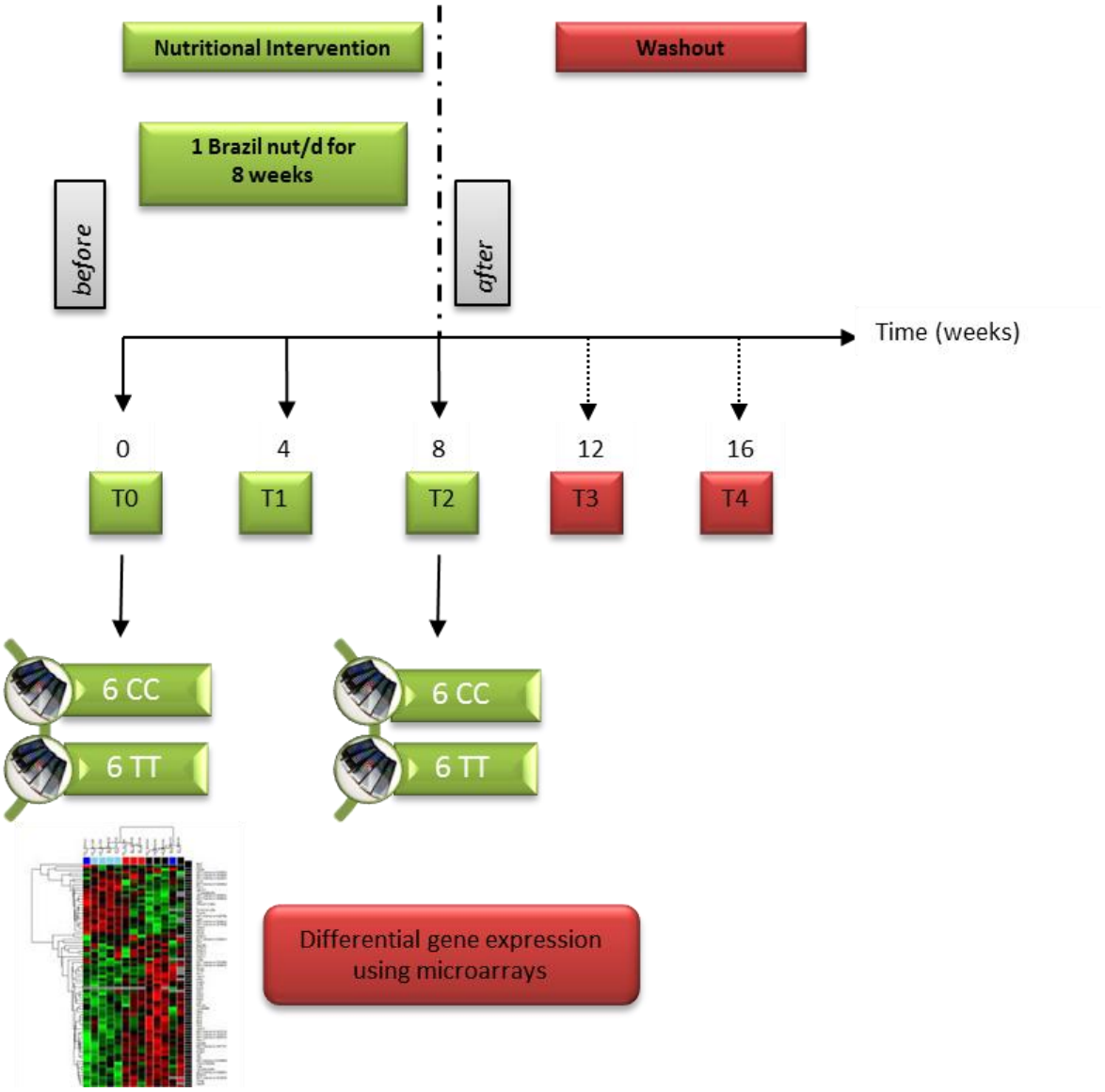


Figure 6. Nutritional intervention protocol indicating time of biological sample collection (T0 – T4).

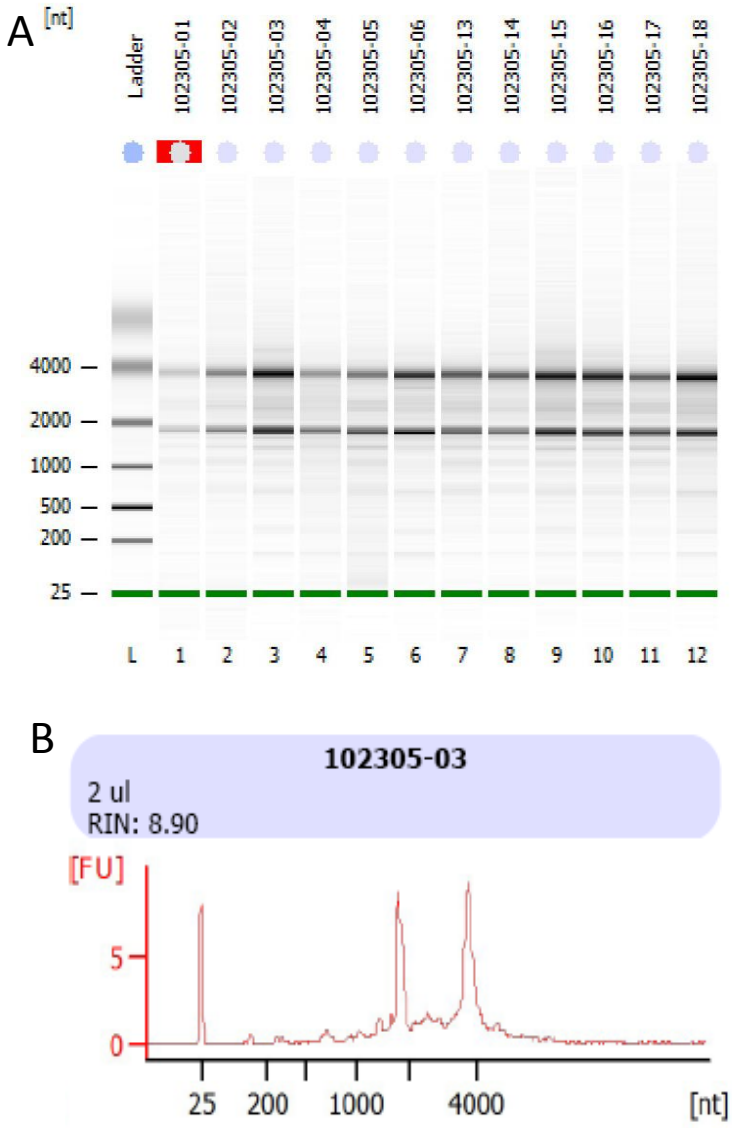


Figure 7. RNA integrity used for microarray analysis.

A.Gel-like eletropherogram image generated from Bioanalyser for 12 samples showing two clear bands. B. Bioanalyser electropherogram output of one sample showing 2 clear peaks indicative of good quality RNA. The RIN number stands to RNA Integrity Number and have to be greater than 7.0 to be of sufficient quality.

Real time confirmation

Real time PCR on specific transcripts in order to conform the microarray data was carried out using a Roche LightCycler 480 and SYBER green I Master Mix (Roche) with 10µL reaction in a white 96-well plate with 0.5mM of primers in the Institute for Cell and Molecular Biosciences in Newcastle University, UK. Primers specific for each gene were designed using NCBI Primer Blast and the amplicon was further submitted to fold in an online software available on mfold.rna.albany.edu, using the DNA Folding Form and the following conditions: 60^{o}C, Na^{+} 0,1 and Mg^{2+} 0,005. The folding structures are available as requested. To select the genes for confirmation, the criterion was the highest or lowest fold change and the genes that were overlapping in different metabolic pathways in Ingenuity Pathways Analysis. The genes selected were: ALAS2, NFkBIA and HaD3.

Chart 4. List of primers used for real time confirmation

primer	sequence	Tm	%GC	Yield (nmol)
NFKBIA_F	TGCTCAAGGAGCCCTGTAATG	59,4	55	18,6
NFKBIA_R	GACATCAGCCCACACTTCA	59,4	55	20,9
DEFA_F	AGCATCCAGGCTCAAGGAAA	57,3	50	7,2
DEFA_R	ATGCAGGTTCCATAGCGACG	59,4	55	15,3
ALAS2_F	TGCCAGGTCCTAACCCAAGT	59,4	55	20,2
ALAS2_R	AACCTAAAGTCCTGTTGCCCT	57,9	47,6	24,1

Gene Set Enrichment Analysis (GSEA)

The transcriptome data were analysed by gene set enrichment analysis (GSEA), which ranks the genes in a list by their differential expression and tests for coordinated differences in a set of genes in response to a specific situation, rather than individual genes with increased or decreased expression in two conditions. One of the great advantages of this integrated approach is the facility of interpreting a large amount of data by identifying biological pathways and processes. The other one is that GSEA considers the entire list of genes of the experiment, rather than the ones that passed a fold-change cut-toff (SUBRAMANIAN et al., 2005). The GSEA application from the Broad Institute was used in the analysis, available online at http://software.broadinstitute.org/gsea/index.jsp. Three files were created (dataset file.gct, phenotype file.cls and gene sets file.gmt) and loaded into the software. The dataset file contained the normalised microarray data, in our case with 19835 probes and 23 arrays. The phenotype file contained the information about the experimental conditions, which have to be numbered. In our experiment we used the genotypes for GPX4_rs713041 and the supplementation. Therefore, we ended up with four conditions: 0 = CC_before, 1 = CC_after, 2 = TT_before and 3 = TT_after. The gene sets file was downloaded from the Molecular Signature Database v5.1 (MSigDB), a online collection of gene sets from different databases available for free to use with the GSEA application. The MSigDB has 8 different collections. We used only 2 that were applicable to our conditions: C2, curated gene sets from online pathways databases and C5, Gene Ontology gene sets.

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CHAPTER 2. Influence of *GPX4* rs713041 on blood lipid profile and oxidative stress in response to Brazil nut supplementation

Introduction

The consumption of tree nuts (peanuts, almonds, hazelnuts, walnuts, Brazil nuts, pistachios, cashew, macadamia) has been associated with decreased risk of cardiovascular disease in human trials (JIANG et al., 2006; MUKUDDEM-PETERSEN; OOSTHUIZEN; JERLING, 2005; O'NEIL; FULGONI; NICKLAS, 2015; SABATÉ; ANG, 2009). The benefits of nut consumption are probably associated with their nutritional composition. Nuts are rich in monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA), magnesium, copper, selenium, vitamin E, folic acid and other bioactive compounds such as phytosterols and phenolic acids (O'NEIL; FULGONI; NICKLAS, 2015; SEGURA et al., 2006; WELNA; KLIMPEL; ZYRNICKI, 2008). The analysis of two trials, the Nurse's Health Study (NHS) conducted with female nurses and The Health Professionals Follow-up Study (HPFS) conducted with males, both in the USA, revealed that the intake of tree nuts was associated with lower risk for cardiovascular disease (BAO et al., 2013). Comparable results were found in the analysis of the National Health and Nutrition Examination Survey (NHANES) from 2005 to 2010 (O'NEIL et al., 2015).

There are some mechanisms that could explain this positive effect of nuts on cardiovascular health. The most accepted is the lipid-lowering effect (MUKUDDEM-PETERSEN; OOSTHUIZEN; JERLING, 2005). Nuts have a high content of PUFA that could explain the reduction of total cholesterol and LDL-c, observed for almonds, peanuts, walnuts and pecan nuts (MUKUDDEM-PETERSEN; OOSTHUIZEN; JERLING, 2005). It

is established that PUFA stimulate the release of cholesterol particles from its hepatic receptor, reducing LDL production (ROS; MATAIX, 2006).

The lipid-lowering effect of Brazil nuts has been investigated in several studies. The supplementation of one unit of Brazil nuts for 18 weeks in obese females reduced cardiovascular risk by increasing HDL-c concentrations (COMINETTI et al., 2012). The increase in HDL-c was observed in healthy adults (COLPO et al., 2013) and a decrease in total cholesterol and LDL-c was observed in obese female adolescents (MARANHÃO et al., 2011). Brazil nut supplementation did not affect HDL-c and LDL-c concentrations in another study with healthy adults, however it did affect the transfer of cholesterol esters by HDL, which can be important to reverse cholesterol transport (STRUNZ et al., 2008).

Brazil nuts are not only a source of PUFA and bioactive compounds, but also the richest source of selenium known in nature (THOMSON et al., 2008). As the amino acid selenocysteine (Sec), selenium is inserted into selenoproteins which have important functions in the antioxidant system, lipid peroxidation, immune function, cardiovascular disease risk, among others (RAYMAN, 2012). The insertion of Sec into selenoproteins occurs during translation and it requires the presence of a specific structure in the 3'untranslated region (3'UTR) of the mRNA, a specific RNA for Sec ($tRNA^{[Ser]Sec}$), and other structures (HATFIELD; GLADYSHEV, 2002). Under selenium deficiency, some selenoproteins have preference for synthesis, a phenomenon called selenoprotein hierarchy (SCHOMBURG; SCHWEIZER, 2009). Differences in the interaction between the 3'UTR mRNA and the structure required for Sec incorporation may explain this hierarchy (HESKETH, 2008; SMALL-HOWARD et al., 2006).

Glutathione Peroxidase 4 (GPx4) is a selenoprotein that can reduce hydroperoxide of phospholipids in cell membranes and it is ranked high in the selenoprotein hierarchy

(SCHOMBURG; SCHWEIZER, 2009). Previous studies have demonstrated that a genetic variation in the *GPX4* gene, a C>T variation located in the 3'UTR region important to Sec insertion (rs713041), has functional consequences (BERMANO et al., 2007; CROSLEY et al., 2013; MÉPLAN et al., 2008; VILLETTE et al., 2002). In addition, it was demonstrated that fatty acids could modulate *GPX4* gene expression (CROSLEY et al., 2013; SNEDDON et al., 2003). Until now no study has investigated the influence of this genetic variation in *GPX4* gene on blood lipids after a supplementation with nuts. Therefore, the aim of the present work was to verify the influence of *GPX4*_rs713041 on blood lipids concentrations after supplementation with Brazil nuts in healthy adults.

Methods

Study population and supplementation protocol

One hundred and thirty unrelated healthy adults (males and females) aged 20 to 60 years old were selected in Sao Paulo University, Brazil. Volunteers taking multivitamins and mineral supplements, anti-inflammatory drugs, those with excessive alcohol consumption, those athletes, those with chronic diseases such as cancer, diabetes and cardiovascular disease were excluded from the study. The participants were invited by electronic correspondence and personal communication when the protocol was explained.
They were asked to give a 20mL blood sample initially (baseline) and take a daily supplement of Brazil nuts for 4 weeks, eating one nut a day. After four weeks, they returned to another 20mL blood sampling and received nuts for more four weeks. After eight weeks of supplementation with Brazil nuts, another 20mL blood sample was taken,

and then two more blood samples were taken at four week intervals during eight weeks of washout period. The compliance with Brazil nut supplementation was estimated by checking a control calendar given in the first blood sampling. The adhesion to the supplementation was checked by high reported compliance. For all participants, weight and height were measured and the body mass index (BMI) was calculated. Written inform consent were signed by 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 Declaration of Helsinski.

Centesimal Composition and Se content of Brazil nuts

The centesimal composition of a random sample of Brazil nuts representative of the eight batches used in the study was analyzed according to the methods proposed by the A.O.A.C. (AOAC, 1990). Se content of Brazil nuts was done using hydride generation flame atomic absorption spectrometry (HGFAAS). Brazil nuts were prepared for analysis by digestion with 68% nitric acid (Merck, Darmstadt, Germany) and heating at 150^{o}C. After volatilization of the organic material, solutions were reduced from SeVI to SeIV by the addition of 1.2N hydrochloric acid and heating at 100^{o}C for 2 h. The samples were diluted to 50mL volume in ultrapure water. A calibration curve was prepared with Titrisol 1000 mg/mL (Merck) diluted in 1% nitric acid at the following concentrations: 0, 0.1, 0.3, 0.5, 1.0, 3.0, 5.0, 15.0, and 30.0 mg/L. Wheat Flour Standard Reference Material 1567a from the National Institute of Standards & Technology (Gaithersburg, MD, USA) with a certified Se concentration was used as an external control.

Blood sampling

Fasting blood samples (15 mL) were drawn by venipuncture into three 5 mL EDTA tubes. An aliquot of 1.5 mL of EDTA whole blood was used for DNA extraction and subsequent genotyping. Another 5mL of blood were collected in a tube without anticoagulant to obtain serum. 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, serum, plasma and erythrocytes were frozen at -80 °C in sterile, demineralized tubes until the analyses were performed.

Biochemical parameters

Fasting glucose and lipid profile - total cholesterol (TC), HDL-c, LDL-c and triacylglycerol (TAG) - were measured in serum using commercial kits (Labtest, Minas Gerais, Brazil) adapted to a biochemical analyzer (LabMax 240, Labtest, Minas Gerais, Brazil). Malondyaldehyde (MDA) was measured by high performance liquid chromatography (HPLC) on a Shimadzu (Kyoto, Japan) instrument with a Phenomenex (Torrance, CA, USA) reverse phase C18 column. The instrument was calibrated with a MDA standard stock solution in the following concentrations: 0.0, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0 and 12.0 μM.

Genotyping

Total DNA was extracted from 200μL of whole blood using Purelink Genomic DNA Minikit (Invitrogen, Life Technologies, California, USA), the final concentration measured in a NanoDrop ND 1000 spectrophotometer (Thermo Scientific, Wilmington,

DE, USA) and adjusted for further analysis. *GPX4* polymorphism (rs713041, Assay ID C___2561693_20) was determined by real-time polymerase chain reaction (RT-PCR) using Taqman SNP Genotyping Assays (Life Technologies, California, USA). Each RT-PCR reaction contained 20ng of adjusted genomic DNA, 0,5μL of 20X SNP Genotyping Assay (Life Technologies), 5,0μL of TaqMan Genotyping Master Mix (Life Technologies) in a 10μL final reaction volume. Samples were assayed along with no-template and internal controls for each genotype and run in the StepOne Plus Real Time PCR system under the following conditions: enzyme activation at 95 ^{o}C for 10min, followed by 40 cycles at 92 ^{o}C for 15s and 60 ^{o}C for 1 min for annealing and extension. The allelic discrimination was obtained by performing an endpoint read.

Statistical Analysis

Continuous variables were tested for Normality using the Kolmogorov-Smirnov test and presented as mean ± standard deviation (sd). Mean values were compared in the different time points using ANOVA repeated measures or Friedman's test. A genetic dominant model was used in order to observe differences in the presence of the variant allele T for *GPX4*_rs713041. In this model, individuals with the variant allele T (CT+TT) were combined together in one category, leaving the dominant genotype (CC) in another category. Mean values were compared in the different time points for each category using ANOVA repeated measures or Friedman's test. For MDA, paired t-test was used. The percentage variation for each biochemical parameter was calculated, subtracting the values of each time point (delta).

Results

A total of 130 healthy volunteers completed the entire study protocol. Mean age was 29.8 ± 9.2 with mean body weight of 63,9 ± 12,1 and BMI of 23.3 ± 3.3. Females constituted 75% of the group and 72% self-reported as being Caucasian. Family history of chronic diseases, such as cancer, diabetes mellitus and cardiovascular disease, were reported by 87% of the volunteers.

The Se content of Brazil nuts and centesimal composition are shown in Table 1. 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. Considering the average weight of the nuts from 3g to 4g , each nut provided approximately from 300 to 400 μg of Se.

Table 1. Centesimal composition and selenium content in Brazil nuts used during the protocol

Nutrient	Mean ± sd
Energy (kcal)	732,9 ± 2,8
Carbohydrates (g)	15,0 ± 0,6
Proteins (g)	13,1 ± 0,2
Lipids (g)	69,0 ± 0,6
Ash (%)	3,1 ± 0,9
Humidity (%)	4,7 ± 0,7
Selenium (μg/g)	100,4 ± 5,3

Lipid profile and MDA levels are reported in Table 2. Fasting Glucose concentrations decreased after 4 weeks and 8 weeks of daily consumption of Brazil nuts. During the washout period, the concentrations were still lower than baseline. Total

cholesterol concentrations were also decreased after 8 weeks of supplementation and after 8 weeks of washout period the concentrations increased again. No significant differences were observed for triglycerides, HDL-c, LDL-c and MDA concentrations.

Table 2 Biochemical parameters during supplementation with Brazil nuts

Variables	Time points				
	baseline	4w nuts	8w nuts	4w washout	8w washout
GLU (mg/dL)	84,4 ± 11,0	81,7 ± 10,3 [b]	77,5 ± 10,4 [b]	80,4 ± 11,0 [b]	80,0 ± 11,9 [b]
TC (mg/dL)	195,4 ± 39,3[a]	190,5 ± 41,4	186,0 ± 37,4	190,1 ± 44,5	195,4 ± 42,8[a]
TAG (mg/dL)	98,0 ± 50,4	100,1 ± 54,6	99,3 ± 51,9	94,6 ± 49,2	101,0 ± 51,6
HDL-c (mg/dL)	57,6 ± 15,6	57,5 ± 15,1	57,5 ± 16,7	59,2 ± 16,7	58,9 ± 17,2
LDL-c (mg/dL)	108,1 ± 27,9	110,9 ± 35,1	111,5 ± 33,0	110,6 ± 34,2	109,2 ± 29,6
MDA (μmol/L)	0,49 ± 0,33	nd	0,56 ± 0,33	nd	nd

Values are mean ± SD. ANOVA repeated measures with pos hoc Tukey for TC, HDL and LDL. Friedman test with pos hoc Dunn for TAG and GLU. Wilcoxon Test for MDA.

[a]: different from 8w nuts (ANOVA, repeated measures $p<0,05$)

[b]: different from baseline (Friedman test $p<0,05$)

nd: not determined

The frequency of the genotypes and alleles for *GPX4* SNP was CC 38,4% (n=50), CT 42,3% (n=55) and TT 19,2% (n=25). The respective allele frequencies were 0,60 for the common allele C and 0,40 for the rare allele T.

Biochemical parameters stratified by genotypes using the genetic dominant model for *GPX4*_rs713041 are shown in Table 3. Differences were only observed in glucose and HDL concentrations. In individuals with the dominant genotype CC, glucose concentrations decreased after 8 weeks of supplementation with Brazil nuts. In carriers of the variant allele T (CT+TT), the reduction on glucose levels was observed after 4 weeks of nuts and it remained lower than baseline during the washout period . No differences were observed for HDL-c, LDL-c, triglycerides and MDA stratified by genotype during supplementation. Comparing the different genotype groups, HDL-c concentrations in

carriers of the variant allele (CT+TT) were higher than individuals with the wild-type genotype in baseline and after 4 weeks of nuts.

Table 3 Biochemical parameters during supplementation with Brazil nuts stratified by genotypes for GPX4_rs713041.

	Genotypes	N	baseline	4w nuts	8w nuts	4w washout	8w washout
Fasting Glucose							
(mg/dL)	CC	50	82,3 ± 11,8	81,9 ± 10,7	77,5 ± 11,6 [b]	81,3 ± 10,5	79,8 ± 11,7
	CT + TT	80	85,6 ± 10,1	81,6 ± 10,1 [b]	77,6 ± 9,7 [b]	79,9 ± 11,4 [b]	80,2 ± 12,1 [b]
	Δ% CC			0,5	-5,4	4,9	-1,8
	Δ% CT+TT			-4,7	-4,9	3,0	0,4
Total Cholesterol							
(mg/dL)	CC	50	191,4 ± 32,7	189,7 ± 36,8	185,1 ± 36,8	190,6 ± 42,5	198,2 ± 33,9
	CT + TT	80	198,0 ± 42,9	191,1 ± 44,2	186,7 ± 38,1	192,2 ± 43,4	193,7 ± 47,6
	Δ% CC			-0,9	-2,4	3,0	4,0
	Δ% CT+TT			-3,5	-2,3	2,9	0,8
HDL-c							
(mg/dL)	CC	50	53,9 ± 14,7	53,6 ± 13,3	55,1 ± 14,2	57,0 ± 14,6	57,4 ± 18,4
	CT + TT	80	59,9 ± 15,7*	58,9 ± 15,7*	58,9 ± 18,1	60,6 ± 17,8	59,9 ± 16,5
	Δ% CC			-0,6	2,8	3,4	0,7
	Δ% CT+TT			0	-1,7	2,9	-1,2
LDL-c							
(mg/dL)	CC	50	109,1 ± 27,8	114,7 ± 37,0	112,3 ± 31,0	114,9 ± 37,0	110,1 ± 25,9
	CT + TT	80	107,6 ± 28,2	108,6 ± 33,9	111,2 ± 34,4	106,5 ± 32,1	108,8 ± 31,9
	Δ% CC			5,1	-2,1	2,3	-4,2
	Δ% CT+TT			0,9	2,4	-4,2	2,2
Triglycerides							
(mg/dL)	CC	50	102,0 ± 59,6	106,9 ± 61,8	103,7 ± 56,4	97,3 ± 53,0	102,4 ± 51,4
	CT + TT	80	95,6 ± 44,1	95,8 ± 49,5	96,5 ± 49,0	91,6 ± 45,1	100,2 ± 52,1
	Δ% CC			4,8	-3,0	-6,2	5,2
	Δ% CT+TT			0,2	0,7	-5,1	9,4
MDA							
(mmol/L)	CC	50	0,49 ± 0,34	nd	0,54 ± 0,30	nd	nd
	CT + TT	80	0,49 ± 0,32	nd	0,57 ± 0,35	nd	nd
	Δ% CC				10,2		
	Δ% CT+TT				16,3		

Values are mean ± SD. Nd: not determined. Differences in time points accessed by ANOVA repeated measures with pos hoc Tukey for GPx1 and GPx3 activities or Friedman test with pos hoc Dunn for SePP and Plasma Se. Differences between genotype groups measured by t-Test for GPx1 and GPx3 activities or Mann-Whitney Test for SePP and Plasma Se.

[b]: different from baseline ($P < 0.05$); *: different from wild-type($P < 0.05$); nd: not determined. Δ%: variation between each time point

Discussion

Previous studies have demonstrated that the intake of Brazil nuts improves blood lipid profile in adults (CARVALHO et al., 2015; COLPO et al., 2013; COMINETTI et al., 2012; MARANHÃO et al., 2011). The present results support and extend these observations by showing that supplementation with one unit of Brazil nut for eight weeks decreases total cholesterol and glucose in healthy adults. In addition, a genetic variation in *GPX4* gene (rs713041) significantly affected HDL-c concentrations both at baseline and after four weeks of supplementation with Brazil nuts. This work is the first to observe the association between circulating HDL-c concentrations and the *GPX4*_rs713041 polymorphism after Brazil nuts intake.

Recent reports have demonstrated that the intake of Brazil nuts could increase HDL-c in obese females (COMINETTI et al., 2012) and healthy adults (COLPO et al., 2013), decrease total cholesterol and LDL-c in obese females adolescents (MARANHÃO et al., 2011) and decrease total cholesterol in dislipidemic adults (CARVALHO et al., 2015). In the present work, we found that the daily intake of one unit of Brazil nut for eight weeks decreased total cholesterol. However, another work conducted with healthy adults did not find an improvement in lipid profile after the consumption of ten units of Brazil nuts for two weeks (STRUNZ et al., 2008). Conflicting findings may be explained by differences in study population, duration of the intervention and amount of nuts ingested. Although the decrease in total cholesterol can be considered of limited clinical application because these volunteers were not dislipidemic, this reduction after 8 weeks of ingestion and the following increase after washout suggest that Brazil nuts may interfere in cholesterol metabolism.

Possible mechanisms that might explain this improvement in blood lipid profile are the nutritional composition of Brazil nuts (RYAN et al., 2006) and the connection between selenium metabolism and mevalonate pathway (MOOSMANN; BEHL, 2004). Although Brazil nuts have higher saturated fatty acids (SFA) concentrations compared to other nuts, the MUFA and PUFA contents are sufficient to exert its cholesterol lowering effect (MUKUDDEM-PETERSEN; OOSTHUIZEN; JERLING, 2005). Furthermore, Brazil nuts are also source of dietary phytosterols that can interfere in the cholesterol absorption, even though the concentration in one unit, around 5mg/g, may not be sufficient to exerts this effect (SEGURA et al., 2006).

Another explanation for the decrease observed in total cholesterol concentration is the relation between cholesterol and selenoprotein biosynthesis. The two metabolisms are connected through the mevalonate pathway (MOOSMANN; BEHL, 2004). In order to be functional, selenoproteins need to have the amino acid Sec inserted on their structure during translation. This requires the synthesis and activation of the $tRNA^{[Ser]Sec}$. The activation of $tRNA^{[Ser]Sec}$ depend on four bases modifications. One of these modifications is the isopentenylation of adenosine 37, which requires isopentenyl pyrophosphate (IPP), a direct metabolite of mevalonate during cholesterol biosynthesis (MOOSMANN; BEHL, 2004). As a result, during the supplementation with a high Se content nut, the synthesis of selenoproteins might be stimulated, which could be directing the IPP for the isopentenylation of adenosine 37 of the $tRNA^{[Ser]Sec}$, to the detriment of cholesterol synthesis. Selenoproteins that are ranked high in the hierarchy of selenoprotein expression, such as GPx4, could be preferentially synthesized under such conditions (SCHOMBURG; SCHWEIZER, 2009).

Experimental evidence has demonstrated that the *GPX4*_rs713041 has functional consequences (BERMANO et al., 2007; CROSLEY et al., 2013; MÉPLAN et al., 2008). Our results confirm these earlier observations indicating that individuals with the variant allele T have higher concentrations of HDL-c at baseline and after four weeks of supplementation with Brazil nuts than individuals with the C allele. This difference disappeared after eight weeks of supplementation and during the washout period. The small sample size of the groups could have masked real differences between genotypes. It is known that fatty acids can modulate *GPX4* expression (SNEDDON et al., 2003) and the T-variant is more susceptible to oxidative stress (CROSLEY et al., 2013). However, the mechanism by which this genetic variation could influence HDL-c concentrations is not known.

Apart from the improvement on blood lipid profile, we observed a decrease on fasting glucose concentrations after supplementation with Brazil nuts. This glucose lowering effect in healthy adults after a daily supplementation with one unit of Brazil nut for eight weeks was not observed before. Moreover, the glucose concentrations remained lower than baseline during the washout period. A recent meta-analysis of randomized controlled trials investigating the influence of nuts consumption on glycemic control in diabetes demonstrated that diets with tree nuts significantly reduced fasting glucose in individuals with type-2 diabetes (VIGUILIOUK et al., 2014).

One of the limitations of this study is the small sample size, fact that can interfere with the results stratified by genotypes. However, this pilot study can be used to drive future nutritional interventions or mechanistic studies in cell cultures in order to understand the relation between this genetic variation and the lipid metabolism. It can also be used to reanalyze human clinical trials considering the genetic background.

In conclusion, this study indicates that the supplementation with one unit of Brazil nuts for eight weeks can improve lipid profile in healthy adults and this response could be influenced by the *GPX4*_rs713041. In addition, we also demonstrated that the supplementation reduces fasting glucose. These results could be important to direct future nutritional interventions considering the genetic background of the participants with the ultimate goal of preventing chronic diseases, such as cardiovascular disease and type-2 diabetes.

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CHAPTER 3. Influence of genetic profile on biomarkers of Se status after Brazil nut supplementation in healthy Brazilians

Introduction

The micronutrient selenium (Se) has crucial roles in human biology as a constituent of various selenoproteins, whose functions are related to antioxidant and redox reaction, immune function, thyroid hormone metabolism, reproduction and fertility (RAYMAN, 2012). Se is inserted into the structure of proteins as the amino acid selenocysteine (Sec) during translation. This requires the recoding of the stop codon UGA, a specific tRNA for Sec and a stem-loop structure located in the 3'-untranslated region (3'UTR) called SECIS, Sec Insertion Sequence, and other molecular components (LABUNSKYY; HATFIELD; GLADYSHEV, 2014). Se status can modulate the function of this molecular insertion complex, regulating selenoprotein expression (LOW et al., 2000).

Low Se status has been associated with increased risk for several diseases, such as cancer, cardiovascular disease, viral infections, male infertility and inflammatory disorders (RAYMAN, 2012). One factor that could modulate this risk is the genetic component which could interfere in the response to Se supplementation (MÉPLAN et al., 2007). Several studies have demonstrated that polymorphisms in selenoprotein genes have functional consequences (HESKETH, 2008).

Glutathione peroxidase 1 (GPx1) is a antioxidant enzyme that reduces hydrogen peroxides and is expressed ubiquitously in all tissues (LEI; CHENG; MCCLUNG, 2007). Its enzyme activity is sensitive to alterations in Se status mainly in individuals with low to moderate intake (ASHTON et al., 2009). There is a single nucleotide polymorphism (SNP)

in the coding region of this gene, changing the amino acid Proline to Leucine in the position 198 of the protein (*GPX1*_rs1050450)(MOSCOW et al., 1994). This variation has been associated with increased risk for lung, breast, prostate and bladder cancers (KARUNASINGHE et al., 2012a; RATNASINGHE et al., 2000; RAVN-HAREN et al., 2006; ZHAO et al., 2005) and has been found to modulate the response to Se supplementation (COMBS et al., 2012). Glutathione peroxidase 4 (GPx4) is the only GPx that can reduce phospholipid hydroperoxides in the cell membrane (BELLINGER et al., 2009). There is a C>T substitution located in the 3'UTR (*GPX4*_rs713041), important for Sec insertion, and the C allele was reported to be more frequent in patients with colorectal cancer and more efficient at incorporating Se (BERMANO et al., 2007). This SNP has also been associated with increased mortality of breast cancer and it has been reported to affect GPx1 activity (MÉPLAN et al., 2013). Furthermore, this SNP had an effect on the response to selenium supplementation in healthy adults (MÉPLAN et al., 2008).

Selenoprotein P (SePP) is the most important Se transporter in the human body and is the only selenoprotein with ten Sec residues per molecule. It has two domains: the N-terminal with one Sec seems to have enzyme antioxidant properties and the C-terminal with the other nine Sec residues containing the Se in transport (BURK; HILL, 2009). SePP can also have an antioxidant role in plasma, protecting LDL from oxidation (TRAULSEN et al., 2004) and is important for brain function and fertility (BURK; HILL, 2009). Two SNPs were discovered in *SEPP1* gene. One in the coding region, changing the amino acid Alanine to Threonine in position 234 (*SEPP1*_rs3877899), the other is located in the 3'UTR (*SEPP1*_rs7579), both a G>A substitution. It was demonstrated that both genetic variations modulate the response to Se supplementation in healthy adults (MÉPLAN et al., 2007). Selenoprotein S (SelS) is a endoplasmatic reticulum (ER) selenoprotein involved in

protecting ER from stress caused by misfolded proteins (BOS et al., 2009) and it is associated with the control of inflammatory response (CURRAN et al., 2005). The C>T substitution located in the promoter region of the gene (*SELS*_rs34713741) was associated with increased risk for rectal cancer (SUTHERLAND et al., 2010). Selenoprotein 15 (Sep15) is an another selenoprotein involved in maintaining ER integrity (FERGUSON et al., 2006). Genetic variation in the 3'UTR of this gene, a G>A substitution in position 1125 (*SEP15*_rs5845) was associated with increased risk for rectal cancer (SUTHERLAND et al., 2010) and lung cancer (JABLONSKA et al., 2008).

Most of the studies investigating the effect of Se supplementation on biomarkers of Se status are conducted using inorganic supplement such as sodium selenite (MÉPLAN et al., 2007, 2008) or the organic form selenomethionine (COMBS et al., 2012). Few studies were conducted using a natural Se source like Brazil nuts (*Bertholetia excelsa*, family Lecythidaceae). The supplementation with Brazil nuts has been found to be effective in increasing concentrations of Se biomarkers in several studies. However, only three biomarkers were commonly used (GPx1 activity, Plasma Se and Erythrocyte Se) and only one genetic variation in *GPX1* gene, rs1050450, is mostly considered (COMINETTI et al., 2011, 2012; THOMSON et al., 2008).

The aim of this work was to investigate if the efficacy of Brazil nuts in increasing Se status, as assessed by the quantification of five biomarkers of Se status (GPx1 activity, Erythrocyte Se concentrations, GPx3 activity, Plasma Se and SePP concentrations), was modulated by the six previously mentioned functional genetic polymorphisms in selenoprotein genes in healthy Brazilians.

Methods

Study population and supplementation protocol

One hundred and thirty unrelated healthy adults (males and females) aged 20 to 60 years old were selected in Sao Paulo University, Brazil. Volunteers taking multivitamins and mineral supplements, anti-inflammatory drugs, those with excessive alcohol consumption, those athletes, those with chronic diseases such as cancer, diabetes and cardiovascular disease were excluded from the study. The participants were invited by electronic correspondence and personal communication when the protocol was explained. They were asked to give a 20mL blood sample initially (baseline) and take a daily supplement of Brazil nuts for 4 weeks, eating one nut a day. After four weeks, they returned to another 20mL blood sampling and received nuts for more four weeks. After eight weeks of supplementation with Brazil nuts, another 20mL blood sample was taken, and then two more blood samples were taken at four week intervals during eight weeks of washout period. The compliance with Brazil nut supplementation was estimated by checking a control calendar given in the first blood sampling. The adhesion to the supplementation was checked by high reported compliance. For all participants, weight and height were measured and the body mass index (BMI) was calculated. Written inform consent were signed by 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 Declaration of Helsinski.

Centesimal Composition and Se content of Brazil nuts

The centesimal composition of a random sample of Brazil nuts representative of the eight batches used in the study was analyzed according to the methods proposed by the A.O.A.C. (AOAC, 1990). Se content of Brazil nuts was done using hydride generation flame atomic absorption spectrometry (HGFAAS). Brazil nuts were prepared for analysis by digestion with 68% nitric acid (Merck, Darmstadt, Germany) and heating at 150^{o}C. After volatilization of the organic material, solutions were reduced from SeVI to SeIV by the addition of 1.2N hydrochloric acid and heating at 100^{o}C for 2 h. The samples were diluted to 50mL volume in ultrapure water. A calibration curve was prepared with Titrisol 1000 mg/mL (Merck) diluted in 1% nitric acid at the following concentrations: 0, 0.1, 0.3, 0.5, 1.0, 3.0, 5.0, 15.0, and 30.0 mg/L. Wheat Flour Standard Reference Material 1567a from the National Institute of Standards & Technology (Gaithersburg, MD, USA) with a certified Se concentration was used as an external control.

Blood sampling collection

Fasting blood samples (15 mL) were drawn by venipuncture into three 5 mL EDTA tubes. An aliquot of 1.5 mL of EDTA whole blood was used for DNA extraction and subsequent genotyping. Another 5mL of blood were collected in a tube without anticoagulant to obtain serum. 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 were determined by inductively coupled plasma mass spectrometry (ICP-MS, Perkin Elmer DRC II) according to the method proposed by Batista et al. (2009) (BATISTA et al., 2009), at the Laboratório de Toxicologia e Essencialidade de Metais, Universidade de São Paulo, Ribeirão Preto, SP, Brazil. Samples were diluted 1:50 into a 15mL polypropylene tube with a solution containing 0.01% (v/v) Triton X-100, 0.5% (v/v) nitric acid and 10μg/L of each one of the internal standards.

Erythrocyte GPx activity was determined using commercial kit (Randox, Labtest, Minas Gerais, Brazil) according to manufacturer's instructions. Briefly, 50μL of erythrocytes were mixed with 1mL of the diluting solution, followed by 5 min of incubation and by the addition of 1mL of 2x colour reagent. 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 in order to express eGPx activity in U/g Hb.

GPx3 activity was done in plasma, as proposed by Paglia e Valentine (PAGLIA; VALENTINE, 1967), modifying to the specific enzyme. This method is based on the reduction of hydrogen peroxide catalyzed by GPx, using glutathione as a reductor agent. The glutathione concentration is maintained by the addition of glutathione reductase and NADPH (PAGLIA; VALENTINE, 1967). First, a reaction mix for 50 reactions was done using 5mg of $NADPH_2$, 46mg of glutathione reduced, 3mL of destiladed water, 24mL of phosphate buffer, 1mL of Na Azide and 20U of glutathione reductase. In a cuvette, it was added 915μL of the mix, 35μL of hydrogen peroxide and 50 μL of plasma. The absorbance

was read at 340nm in a spectrophotometer (Ultrospec 4000, UV/visible spectrophotometer Pharmacia Biotech Inc, NJ, USA).

SePP concentration was determined in plasma by enzyme-linked immunosorbent assays (ELISA) in 96-well microplate reader (FLUOstar Omega microplate reader, BMG Labtech, Ortenberg, Germany). The customized antibody anti-SePP was incubated in a 96-well plate for 2h, 37°C. The solution with the antibody was rinsed and blocked overnight with 0.05% caesin solution at room temperature. Plasma samples were diluted and combined with selenoprotein P monoclonal antibody (Selenoprotein P antibody, clone 37A1, LifeSpan Biosciences) overnight, 4°C. Standard curves were constructed using human purified SePP. The first plate was washed three times with 300μL of PBS-tween. 100μL of diluted samples were transferred to the washed plate and incubated at 37°C for 2h. After 2h the plate was washed with PBS-tween. A secondary antibody (antimouse IgG, HRP-linked, horseradish peroxidase- linked, Cell Signalling Technology) was added to the plate which was incubated at 37°C for 1h. The plate was washed again with PBS. 100μL of TMB was added to the plate and left at room temperature for 5 min until the colour was developed. The reaction was stopped by the addition of 100 μL of H_2SO_4 and the absorbance was read at 450nm.

Genotyping

Total DNA was extracted from 200μL of whole blood using Purelink Genomic DNA Minikit (Invitrogen, Life Technologies, California, USA), the final concentration measured in a NanoDrop ND 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and adjusted for further analysis. The SNPs selected were chosen based on the

literature evidence for functional consequences and relation to risk of chronic diseases. Using these criteria, the SNPs selected are shown in Chart 5. The SNPs were determined by real-time polymerase chain reaction (RT-PCR) using Taqman SNP Genotyping Assays (Life Technologies, California, USA). Each RT-PCR reaction contained 20ng of adjusted genomic DNA, 0,5μL of 20X SNP Genotyping Assay (Life Technologies), 5,0μL of TaqMan Genotyping Master Mix (Life Technologies) in a 10μL final reaction volume. Samples were assayed along with no-template and internal controls for each genotype and run in the StepOne Plus Real Time PCR system under the following conditions: enzyme activation at 95 ^{o}C for 10min, followed by 40 cycles at 92 ^{o}C for 15s and 60 ^{o}C for 1 min for annealing and extension. The allelic discrimination was obtained by performing an endpoint read. Hardy-Weinberg equilibrium was tested by the chi-squared test. The haplotype distribution and linkage disequilibrium were done in the software Haploview 4.2. SNPs were considered in linkage disequilibrium when D'>0,5.

Chart 5. Information about the SNPs used in the study

Genes	Chromossome location	rs (dbSNP)	SNP region	Base change	Codon change	aminoacid change
GPX1	3p21.3	rs1050450	Coding region	C679T	C**C**C / C**T**C	Pro198Leu
		rs1800668	promoter	C>T	*	*
		rs3811699	promoter	G-602A	*	*
GPX4	19p13.3	rs713041	3'UTR	C718T	*	*
SEPP1	5p12	rs3877899	Coding region	G24731A	**G**CT - **A**CT	Ala234Thr
		rs7579	3'UTR	G25191A	*	*
SELS	15q26.3	rs34713741	promoter	C>T	*	*
SEP15	1p31	rs5845	3'UTR	G1125A	*	*

*: no change. 3'UTR: 3'untranslated region.

Results

The characteristics of the volunteers are summarized in Table 4. A total of 130 volunteers completed the study protocol. Females constituted 75% of the group, 80% were non smokers and 73% self reported being Caucasian. Baseline plasma Se was 96.7 ± 29.6 μg/L and after 8 weeks of supplementation with Brazil nuts the concentration was 292.8 ± 95.4 μg/L.

Table 4. Characteristics of the study volunteers

Parameters	Total (n= 130)
Age (y) *m±sd*	29,8 ± 9,2
Gender *n(%)*	
male	32 (24,6)
female	98 (75,4)
BMI (kg/m^2)	23,3 ± 3,3
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)
Chronic diseases historical *n(%)*	
yes	113 (86,9)
no	17 (13,1)
Ethnicity *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

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

The Se content of Brazil nuts and centesimal composition are shown in Table 5. Four different batches, with the most similar Se content, were used during the supplementation. The mean ± standard deviation for Se content of these four batches was 100,4 ± 5,3 μg/g. Considering the average weight of the nuts from 3g to 4g, each nut provided approximately from 300 to 400 μg of Se.

Table 5. Centesimal composition and selenium content in Brazil nuts used during the protocol

Nutrient	Mean ± sd
Energy (kcal)	732,9 ± 2,8
Carbohydrates (g)	15,0 ± 0,6
Proteins (g)	13,1 ± 0,2
Lipids (g)	69,0 ± 0,6
Ash (%)	3,1 ± 0,9
Humidity (%)	4,7 ± 0,7
Selenium (μg/g)	100,4 ± 5,3

The biomarkers of Se status measured during the supplementation with Brazil nuts are shown in Table 9 and Figure 8 . Two biomarkers were measured in the erythrocytes (GPx1 activity and Erythrocyte Se) and three were measured in plasma (GPx3 activity, SePP concentration and Plasma Se concentration). The biomarkers were measured before, during the supplementation and during the washout period, except for SePP concentrations which were evaluated only before, after the supplementation and at the end of the washout period. GPx1 activity decreased significantly after 4 weeks of supplementation, increased again until the first 4 weeks of Brazil nuts withdrawal and decreased after 8 weeks of washout (Figure 8A). Erythrocyte Se concentrations during the supplementation and after

Brazil nuts withdrawal were higher than baseline. The supplementation increased erythrocyte Se concentrations gradually until the first four weeks of washout. The values decreased again in the end of the washout period (Figure 8B) .

The supplementation with Brazil nuts directly affected plasma biomarkers GPx3 activity, plasma Se and plasma SePP concentrations. There was a significant increase in GPx3 activity after 4 weeks of supplementation. Moreover, the activity during the washout period was significantly lower than the activity during the supplementation (Figure 8C). Similarly, plasma Se concentrations increased significantly after 4 and 8 weeks of supplementation. During the washout period, there was a sharp decrease in plasma Se concentrations compared to baseline and 8 weeks of nuts (Figure 8D). The concentrations of plasma SePP were also increased after the supplementation and reduced after nuts withdrawal; however, the concentrations at the end of the washout period remained significantly higher than at baseline (Figure 8E).

Table 6. Concentrations of biomarkers of Se status during supplementation with Brazil nuts.

	Time Points				
Biomarkers	baseline	4w nuts	8w nuts	4w washout	8w washout
GPx 1 activity (U/g Hb)	64,5 ± 18,3 [c]	56,5 ± 15,2	64,8 ± 19,9 [c]	71,2 ± 19,1 [ac]	67,2 ± 24,1 [c]
Erythrocyte Se (μg/L)	184,3 ± 87,4	376,4±120,0 [ab]	467,0± 176,0 [b]	519,3 ± 188,0 [bc]	367,8 ± 170,2 [ab]
GPx 3 activity (U/L)	538,5± 105,7 [c]	585,7 ± 116,3	558,7 ± 129,8	520,3 ± 110,9 [ac]	498,6 ± 123,2 [ac]
Plasma Se (μg/L)	96,7 ± 29,6	218,6 ± 67,3[b]	292,8 ± 95,4 [bc]	202,2 ± 54,5 [ab]	162,2 ± 38,0 [abc]
SePP (mg/L)	3,52 ± 0,94 [a]	nd	4,00 ± 0,99	nd	3,66 ± 0,92 [ab]

Values are mean ± sd. ANOVA repeated measures with pos hoc Tukey for GPx 1 and GPx 3 activities. Friedman test with pos hoc Dunn for Erythrocyte Se, Plasma Se and SePP.
[a]: different from 8w nuts ($P < 0.05$)
[b]: different from baseline ($P < 0.05$)
[c]: different from 4w nuts ($P < 0.05$)
nd: not determined

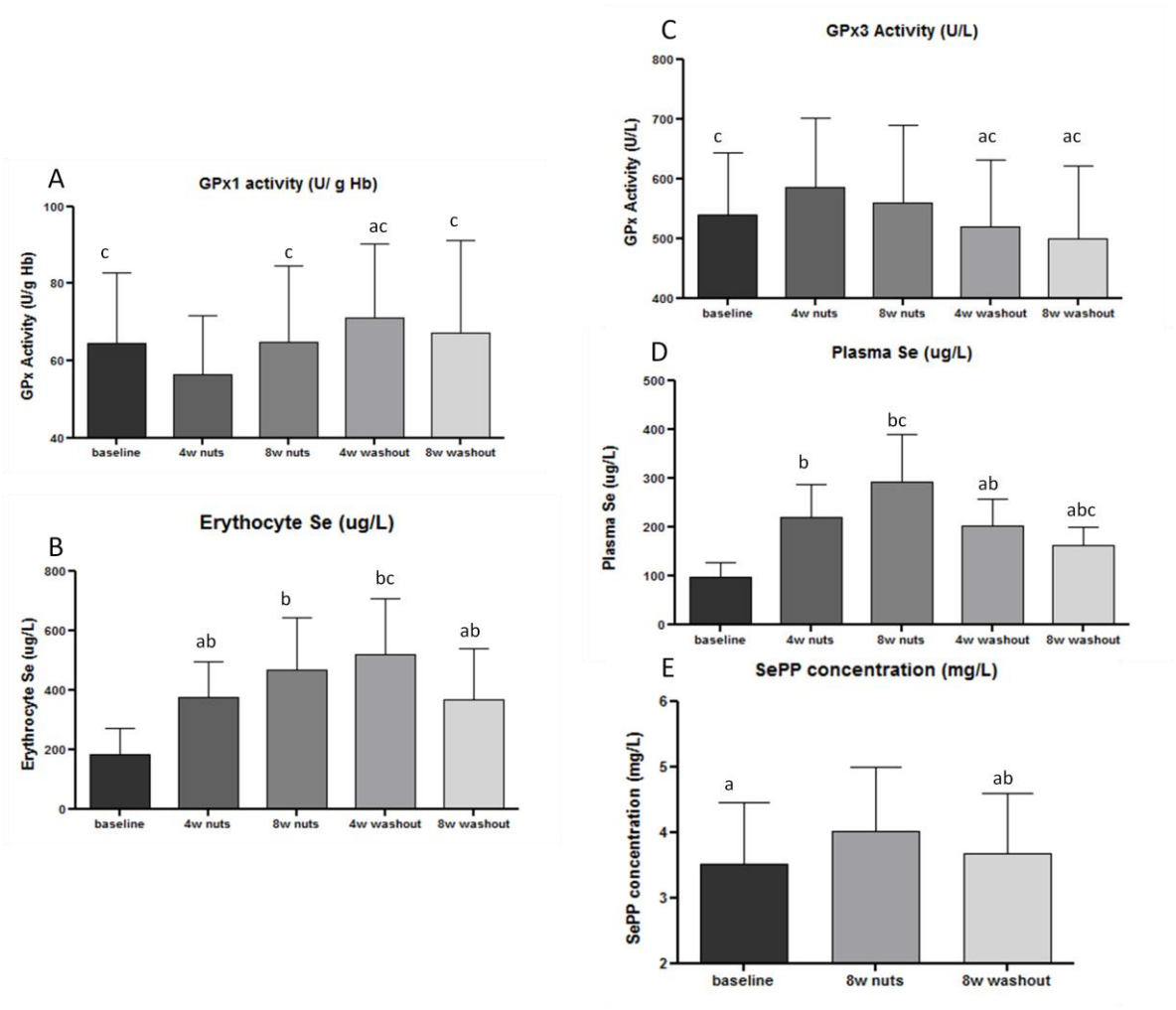

Figure 8. Effect of Brazil nut supplementation on biomarkers of Se status.

A) GPx1 activity, B) erythrocyte Se, C) GPx3 activity, D) Plasma Se and E) SePP concentrations.

Values are mean ± SD. ANOVA repeated measures with post hoc Tukey for GPx1 and GPx3 activities. Friedman test with pos hoc Dunn for Erythrocyte Se, Plasma Se and SePP.

[a]: different from 8w nuts ($p<0.05$)

[b]: different from baseline ($p<0.05$)

[c]: different from 4w nuts ($p<0.05$)

Genotype and allele frequencies of SNPs in selenoprotein genes are shown inTable 7. All SNPs were in Hardy-Weinberg Equilibrium. Haplotype analyses, using Haploview software, showed evidence of linkage disequilibrium for SNPs in *GPX1* gene: rs1050450 x

rs1800699 (D'= 1,0 e r^2= 0,98) and rs1050450 x rs3811699 (D'= 1,0 e r^2= 1,0). Only two haplotypes were observed (Table 8). Linkage disequilibrium were also evident for SNPs in *SEPP1* gene (D'= 1,0 e r^2= 0,15) with three haplotypes observed (Table 8)

Table 7. Genotype and allele frequency of SNPs in selenoprotein genes

SNPs	N	%	SNPs	N	%
*GPX1*_rs1050450			*GPX4*_rs713041		
CC	70	53,85	CC	50	38,46
CT	53	40,77	CT	55	42,31
TT	7	5,38	TT	25	19,23
C		0,75	C		0,60
T		0,25	T		0,40
*GPX1*_rs3811699			*SEP15*_rs5845		
GG	70	53,85	GG	55	42,31
GA	53	40,77	GA	60	46,15
AA	7	5,38	AA	15	11,54
G		0,75	G		0,65
A		0,25	A		0,35
*GPX1*_rs1800668			*SELS*_rs34713741		
CC	70	53,85	CC	70	53,85
CT	53	40,77	CT	49	37,69
TT	7	5,38	TT	11	8,46
C		0,75	C		0,73
T		0,25	T		0,27
*SEPP1*_rs3877899			*SEPP1*_rs7579		
GG	70	53,85	GG	50	38,46
GA	47	36,15	GA	55	42,31
AA	13	10,00	AA	25	19,23
G		0,72	G		0,60
A		0,28	A		0,40

GPX1: Glutathione Peroxidase 1; SEPP1: Selenoprotein P; GPX4: Glutathione Peroxidase 4; SEP15: Selenoprotein 15; SELS: Selenoprotein S. A: adenine; T: timine; C: citosine; G: guanine.

Table 8. Haplotypes observed in *GPX1* and *SEPP1*genes.

Haplotypes	**SNPs**			**Frequency**
GPX1	rs1050450	rs1800668	rs3811699	
haplotype a (common)	C	C	G	0.742
haplotype b	T	T	A	0.254
SEPP1	rs7579	rs3877899		
haplotype a (common)	G	G		0.442
haplotype b	A	G		0.281
haplotype c	G	A		0.277

The biomarkers of Se status stratified by genotypes for the six SNPs in selenoprotein genes are shown in Table 9. The genotype for three SNPs affected GPx1 activity. Volunteers who were carriers of the variant allele for *GPX1*_rs1050450 had lower GPx1 activity than volunteers with the common genotype after 8 weeks of washout (Figure 9A). GPx1 activity was also lower for individuals GA+AA for *SEP15*_rs5845 compared to GG after 4 weeks of supplementation and in the end of the washout period (Figure 9C). On contrary, GPx1 activity was higher in carriers of the variant allele for *GPX4*_rs713041 after 8 weeks of washout (Figure 9B). In addition, Plasma Se was affected by genotype for *SEPP1*_3877899, with lower concentrations found in carriers of the variant allele A in the end of the washout period (Figure 9D). Finally, erythrocyte Se concentrations were higher in volunteers carriers of the variant allele A for *SEP15*_rs5845 compared to GG after 4 weeks of supplementation (Figure 9E). No differences were observed for GPx3 activity and SePP concentrations.

Table 9. Biomarkers of Se status stratified by genotypes for SNPs in selenoprotein genes

	Genotypes	N	baseline	4w nuts	8w nuts	4w washout	8w washout
GPx1 activity (U/g Hb)							
*GPX1*_rs1050450	CC	70	66,1 ± 17,5 [c]	58,8 ± 14,8	68,7 ± 18,7[c]	72,0 ± 18,0[c]	68,6 ± 25,2[c]
	CT+TT	60	62,6 ± 19,2 [c]	53,7 ± 15,4	60,1 ± 20,5 *	70,2 ± 20,3 [c]	65,4 ± 22,7[c]
*GPX4*_ rs713041	CC	50	61,1 ± 15,3	54,9 ± 13,5	63,5 ± 19,0 [c]	70,1 ± 18,5[bc]	61,6 ± 22,5
	CT + TT	80	66,6 ± 19,8 [c]	57,4 ± 16,2	65,5 ± 20,6 [c]	71,9 ± 19,5 [c]	70,7 ± 24,4 [c]*
*SEPP1*_rs3877899	GG	70	63,0 ± 20,7 [c]	54,4 ± 12,7	64,4 ± 19,3 [c]	71,7 ± 18,0 [c]	68,2 ± 25,7 [c]
	GA+AA	60	66,2 ± 14,9 [c]	58,8 ± 17,5	65,1 ± 20,8	70,6 ± 20,3 [c]	66,0 ± 22,1 [c]
*SEPP1*_rs7579	GG	70	66,7 ± 18,1[c]	56,5 ± 17,1	63,6 ± 18,8 [c]	69,9 ± 19,9 [c]	65,1 ± 23,8 [c]
	GA+AA	60	61,8 ± 18,3	56,3 ± 12,7	66,1 ± 21,3 [c]	72,6 ± 18,1 [c]	69,6 ± 24,2 [c]
*SELS*_rs34713741	CC	55	64,1 ± 17,6	58,2 ± 16,6	63,1 ± 17,8	69,2 ± 19,4 [c]	64,3 ± 24,3
	CT+TT	75	64,7 ± 18,9 [c]	55,1 ± 14,0	65,9 ± 21,4 [c]	72,6 ± 18,8 [c]	69,2 ± 23,7 [c]
*SEP15*_rs5845	GG	77	64,6 ± 18,7	58,7 ± 16,5	65,4 ± 21,3	72,7 ± 19,3 [abc]	70,6 ± 24,7 [c]
	GA+AA	53	64,3 ± 17,9 [c]	53,2 ± 12,5 *	63,8 ± 17,9 [c]	68,9 ± 18,6 [c]	62,1 ± 22,2 [c] *
Erythrocyte Se (µg/L)							
*GPX1*_rs1050450	CC	70	183,6 ± 78,9	373,9 ± 104,5	457,2 ± 167,1	516,5 ± 201,2	371,1 ± 183,4
	CT+TT	60	185,1 ± 97,2	379,3 ± 136,7	478,5 ± 186,6	522,6 ± 173,1	364,0 ± 154,9
*GPX4*_ rs713041	CC	50	170,6 ± 68,1	367,4 ± 102,8	474,2 ± 164,7	522,5 ± 193,1	379,3 ± 161,4
	CT + TT	80	192,8 ± 97,0	382,1 ± 129,9	462,6 ± 183,6	517,2 ± 186,0	360,6 ± 176,1
*SEPP1*_rs3877899	GG	70	168,9 ± 60,2	357,3 ± 98,7	467,0 ± 152,9	518,2 ± 169,5	364,2 ± 146,0
	GA+AA	60	202,2 ± 109,0	398,7 ± 138,4	467,1 ± 200,9	520,0 ± 209,0	372,0 ± 196,0
*SEPP1*_rs7579	GG	70	188,2 ± 79,3	375,5 ± 100,6	463,7 ± 166,0	499,6 ± 163,9	363,7 ± 166,6
	GA+AA	60	179,7 ± 96,6	377,5 ± 140,2	470,9 ± 188,3	542,2 ± 211,9	372,6 ± 175,7
*SELS*_rs34713741	CC	55	193,2 ± 107,2	386,3 ± 152,2	464,6 ± 186,2	506,5 ± 211,8	375,2 ± 171,6
	CT+TT	75	177,7 ± 69,6	369,2 ± 89,8	468,8 ± 169,4	528,6 ± 169,4	362,4 ± 170,2
*SEP15*_rs5845	GG	77	181,5 ± 71,8	357,8 ± 98,6	454,8 ± 175,2	503,8 ± 178,8	356,5 ± 152,0
	GA+AA	53	188,3 ± 106,8	403,6 ± 142,3*	484,8 ± 177,2	541,8 ± 200,4	384,2 ± 194,1
Plasma Se, µg/L							
*GPX1*_rs1050450	CC	70	92,6 ± 26,2	215,6 ± 72,8[ab]	293,5 ± 96,0[b]	205,3 ± 58,5[ab]	165,8 ± 41,5[ab]
	CT+TT	60	99,8 ± 30,9	218,9 ± 65,0 [ab]	282,3 ± 88,7[b]	198,7 ± 50,5[ab]	160,8 ± 35,8 [ab]
*GPX4*_ rs713041	CC	50	96,9 ± 26,2	222,6 ± 71,8 [ab]	293,0 ± 95,3 [b]	202,5 ± 48,4 [ab]	162,6 ± 36,8 [ab]
	CT + TT	80	95,3 ± 30,1	213,7 ± 67,5 [ab]	285,4 ± 91,2 [b]	202,1 ± 58,8 [ab]	164,1 ± 40,4 [ab]
*SEPP1*_rs3877899	GG	70	93,9 ± 25,1	223,8 ± 62,9 [ab]	296,9 ± 93,1 [b]	199,6 ± 56,1 [ab]	170,4 ± 41,2 [ab]
	GA+AA	60	98,3 ± 32,2	209,3 ± 75,4 [ab]	278,3 ± 91,6 [b]	205,4 ± 53,7 [ab]	155,5 ± 34,7 [ab*]
*SEPP1*_rs7579	GG	70	94,5 ± 23,6	221,6 ± 70,1 [ab]	284,6 ± 96,0 [b]	206,5 ± 55,6 [ab]	160,7 ± 40,7 [ab]
	GA+AA	60	97,5 ± 33,6	211,9 ± 68,0 [ab]	292,7 ± 88,9 [b]	197,2 ± 54,0 [ab]	166,7 ± 36,8 [ab]
*SELS*_rs34713741	CC	55	97,4 ± 31,7	210,8 ± 68,2 [ab]	287,7 ± 88,3 [b]	202,0 ± 56,3 [ab]	162,1 ± 41,1 [ab]
	CT+TT	75	94,8 ± 26,2	221,8 ± 69,8 [ab]	288,8 ± 96,1 [b]	202,4 ± 54,2 [ab]	164,6 ± 37,5 [ab]
*SEP15*_rs5845	GG	77	94,1 ± 25,5	214,9 ± 71,8 [ab]	281,8 ± 93,9 [b]	200,8 ± 56,0 [ab]	161,6 ± 36,1 [ab]
	GA+AA	53	98,5 ± 32,5	220,4 ± 65,3 [ab]	297,8 ± 90,4 [b]	204,4 ± 53,7 [ab]	166,3 ± 42,8 [ab]

Values are mean ± SD. ANOVA repeated measures with pos hoc Tukey for GPx 1 and GPx 3 activities. Friedman test with pos hoc Dunn for Erythrocyte Se, Plasma Se and SePP. [a]: different from 8w nuts ($p<0.05$), [b]: different from baseline ($p<0.05$), [c]: different from 4w nuts ($p<0.05$), *: different from wildtype ($p<0.05$).

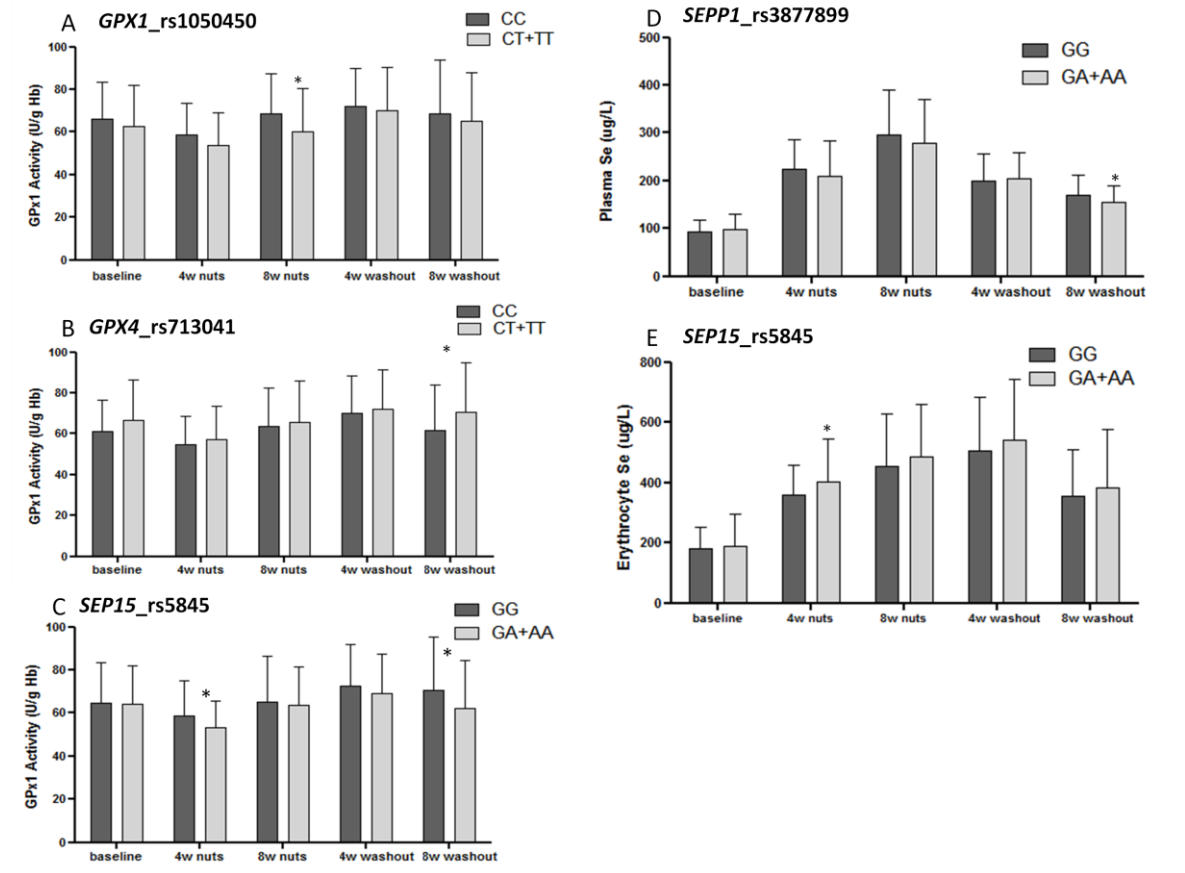

Figure 9. Biomarkers of Se status stratified by SNPs in selenoprotein genes.

GPx1 activity was stratified by *GPX1*_rs1050450 (A), *GPX4*_rs713041 (B) and *SEP15*_rs5845 (C). Plasma Se was stratified by *SEPP1*_rs3877899 (D) and Erythrocyte Se by *SEP15*_rs5845 (E). Values are mean ± SD. Differences between genotypes tested by Mann-Whitney test. *: different from wildtype ($P<0.05$)

Discussion

The influence of genetic variants on the response to Se supplementation have been proposed before (COMBS et al., 2012; MÉPLAN et al., 2007, 2008). Our results not only confirm these earlier observations by demonstrating that erythrocyte GPx1 activity is influenced by *GPX1*_rs1050450 and *GPX4*_rs713041 and that plasma Se is influenced by *SEPP1*_rs3877899, but also extend them by demonstrating that the *SEP15*_rs5845

influence erythrocyte GPx1 activity and Se concentrations. This work is the first to analyze the association between *SEP15*_rs5845 and these two biomarkers of Se status after supplementation with Brazil nuts.

In our work we quantified five biomarkers of Se status, two in erythrocytes and three in plasma. GPx1 activity and erythrocyte Se concentrations have been used in several studies as biomarkers of Se status that respond to Se supplementation *via* Brazil nuts (CARDOSO et al., 2015a; COMINETTI et al., 2012; THOMSON et al., 2008). Erythrocyte Se correlates strongly with plasma Se. Therefore, it can be used as a useful biomarker of Se status in human studies (ASHTON et al., 2009; STEFANOWICZ et al., 2013). We observed a delayed response of these two erythrocyte biomarkers 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 to appear in the mature red cell population (ROBBERECHT; CAUWENBERGH; HERMANS, 2012; THOMSON et al., 2008). Plasma biomarkers used were Se, SePP concentrations and GPx3 activity. This is the first study to quantify plasma SePP concentrations in Brazilians. The baseline plasma Se concentration of 96,7 μg/L (1,22 μmol/L) would be expected to be sufficient to maximize plasma GPx activity, but not SePP concentration since earlier work has shown that the plasma Se concentration needed to maximize GPx3 activity is 90 μg/L (DUFFIELD et al., 1999) and to maximize plasma SePP it is 120 μg/L (HURST et al., 2010). As a result, the present population can be considered to have a moderate to adequate Se status which could respond with an increase in plasma biomarkers after Se supplementation. Indeed, plasma SePP concentrations and GPx3 activity increased

significantly after supplementation with Brazil nuts and decreased during the washout period. Accordingly, plasma Se concentrations had a direct response to the supplementation, probably due to Se incorporation into SePP and GPx3. These results are consistent with the Se in Brazil nuts, mostly as selenomethionine, being bioavailable for the synthesis of selenoproteins in humans (DA SILVA; MATAVELI; ZEZZI ARRUDA, 2013; THOMSON et al., 2008).

In our study, GPx1 activity was lower in carriers of the variant allele T for SNP rs1050450. Previous studies corroborate our results (BASTAKI et al., 2006; HAMANISHI et al., 2004; HANSEN et al., 2009; RAVN-HAREN et al., 2006). Bastaki et al. found an interaction between gender and the genotype for this variation: TT males had lower GPx1 activity than males with the other genotypes. Hamanishi et al. observed in *in vitro* studies that the presence of the variant allele T decreases 40% the GPx1 activity. Finally, in a Danish study, the presence of the allele T was associated with lower GPx1 activity. One possible explanation for this lower activity is that the change of the amino acid Proline to Leucine alters the secondary structure of the protein, which can have profound effects on its activity and stability (RATNASINGHE et al., 2000). *In vitro* studies demonstrated that Se supplementation reduced enzyme thermostability for the Leu-variant (ZHUO et al., 2009).

After the supplementation with Brazil nuts, GPx1 activity was significantly higher in individuals with at least one variant allele T for *GPX4*_rs713041. A similar effect of this genetic variation on GPx1 activity was reported in earlier studies both *in vivo* and *in vitro* (BERMANO et al., 2007; CROSLEY et al., 2013; GONG et al., 2012; MÉPLAN et al., 2008). At first sight it surprising that GPx1 activity was affected by one SNP in another gene, however, the hierarchy of selenoprotein expression could explain this result. RNA-protein binding assays *in vitro* demonstrated that the C-variant for *GPX4* transcripts bind

protein more strongly than the T-variant and *GPX1* transcripts (MÉPLAN et al., 2008). In addition, GPx4 protein is ranked high in hierarchy of selenoprotein expression, which means that its transcript has a preference for protein synthesis during translation (SCHOMBURG; SCHWEIZER, 2009). Such evidence suggests that in individuals with the T allele, the synthesis of GPx1 protein is higher because during translation, the C transcript for *GPX4* can compete more strongly against the GPx1 transcript for Se incorporation into GPx4 protein (MEPLAN et al., 2008; CROSLEY et al., 2013).

There are two genetic variations in *SEPP1* gene that influence Se metabolism. Both SNPs are G>A substitutions: one is located in the coding region (Ala234Thr, rs3877899) and the other is located in the 3'UTR (rs7579), close to SECIS element which is important to Sec insertion. It is proposed that the first one regulates the SePP stability or uptake by cells, and the second one affects SePP synthesis (MÉPLAN et al., 2007). In our study, plasma Se concentrations were significantly lower in GA+AA individuals after 8 weeks of washout. This result confirms earlier work which demonstrated the influence of rs3877899 on plasma Se concentrations: GA individuals had lower plasma Se levels compared to GG individuals post supplementation and 2 weeks after the washout period (MÉPLAN et al., 2007). SePP has two isoforms in plasma, the 50kDa and the 60kDa, that are influenced by the genotype for both SNPs in *SEPP1* gene. Individuals with the GA genotype for rs3877899 had lower proportion of the SePP 60kD, with the Sec-rich domain (MÉPLAN et al., 2009). This difference in the proportion of SePP isoforms may affect Se availability in plasma for selenoprotein synthesis in different tissues.

Two biomarkers of Se status were influenced by *SEP15* _rs5845: erythrocyte Se concentrations and GPx1 activity. We found that individuals with at least one variant allele A (GA+AA) had lower GPx1 activity after 4weeks of supplementation with Brazil nuts and

in the end of the washout period and higher erythrocyte Se concentrations. To our knowledge, this is the first time that rs5845 is associated with these erythrocyte biomarkers. The functionality of rs5845 was indicated previously (HU et al., 2001). This SNP is located in the 3'UTR which is important for Sec insertion. It was demonstrated that the SECIS (Sec Insertion Sequence) element containing the A variant was less responsive to Se and may influence the translation of Sep15 (HU et al., 2001). The association of this genetic variation with lung cancer was revealed in a Polish population. There was a higher risk for individuals with low Se status and lower risk for those with plasma Se above 80μg/L (JABLONSKA et al., 2008). However, the exact mechanism by which erythrocyte biomarkers of Se status may be influenced by rs5845 in *SEP15* gene requires further investigation.

This work has some limitations. The first one is the small sample size of the group, which could have masked significant difference between genotypes. Therefore, it may be considered a pilot study. The second one is the absence of quantification of protein expression, since the main effects of Se are during translation.

In summary, the results of this pilot study suggest that genetic variants in selenoprotein genes influence the response of plasma and erythrocyte biomarkers of Se status to a supplementation with Brazil nuts in healthy Brazilians. This could have an impact on future nutritional recommendations of selenium for specific groups of the population, with the ultimate goal of preventing and reducing the risk of chronic diseases.

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CHAPTER 4. Influence of genetic variations in selenoprotein genes on the pattern of gene expression after supplementation with Brazil nuts

INTRODUCTION

The adequate intake of vitamins and minerals is essential for maintenance of health and longevity in humans. The regular intake of fruits, vegetables and nuts decrease risk for chronic diseases, such as cancer, cardiovascular disease and type-2 diabetes (BLOMHOFF et al., 2006; DONALDSON, 2004; GROSSO et al., 2015; TAPSELL; PROBST, 2008). This beneficial effect is due to their high concentration of vitamins, micronutrients and bioactive compounds. One of the micronutrients that have an important role in human biology is the trace element selenium (Se). There are 25 genes encoding selenoproteins with a wide range of functions, including antioxidant defense, redox function, thyroid hormone metabolism, immune function, reproduction and fertility (KRYUKOV et al., 2003; RAYMAN, 2012). Se is unique among the micronutrients because of its mechanism of insertion into proteins. Unlikely most minerals that interact as cofactors in the active site of enzymes, Se is inserted as the amino acid selenocysteine (Sec) during translation. This process requires the presence of a stem-loop structure in the mRNA of selenoproteins called SECIS (Sec Insertion Sequence), and other molecular components, such as the specific tRNA of Sec ($tRNA^{[Ser]Sec}$) to recode the stop codon UGA and insert Sec (LABUNSKYY; HATFIELD; GLADYSHEV, 2014).

Some examples of selenoproteins are the antioxidant enzyme family Glutathione Peroxidases (GPx 1 – 4), the deiodinases (DIO 1 – 3) related to thyroid hormone metabolism, Selenoprotein P (SePP) which has a role as the principal Se transporter and

also has antioxidant function, the Thioredoxin Reductase enzyme family (TR 1 – 3) important for redox regulation in cells, selenoprotein 15 (Sep15) which regulates misfolded proteins in the endoplasmatic reticullum (ER) and selenoprotein S (SelS) is also essential for maintaining the integrity of the ER and may have an important role in inflammation (LABUNSKYY; HATFIELD; GLADYSHEV, 2014).

There is a principle that explains the regulation of selenoprotein expression by Se supply, namely the selenoprotein hierarchy. This controls which selenoproteins will be synthesized under a specific condition so that how the available Se is used for selenoprotein synthesis depends on Se status, the specific tissue and the specific selenoprotein. Under Se-deficient conditions, Se is directed to brain and endocrine tissues rather than to liver and kidneys (SCHOMBURG; SCHWEIZER, 2009). Even in the same tissue, not all selenoproteins have the same regulation. Some proteins have preference for synthesis when the Se supply is limiting. It was observed in mouse models that under conditions of Se deficiency the mRNAs of *GPX1, DIO1* and *SEP15* were reduced in liver. On contrary, no decrease was observed for *GPX4* transcript. Upon repletion, *SEPP1* transcript was the most abundant in liver (SUNDE et al., 2009). This difference in regulation of selenoprotein expression demonstrates that some selenoproteins are more important than others, and during deficiency states, the selenoproteins ranked high in the hierarchy, such as *GPX4*, have preference for synthesis (SCHOMBURG; SCHWEIZER, 2009).

The concept of hierarchy in regulation of selenoprotein expression shed light into the possible use of molecular biomarkers of Se status in supplementation studies using humans and rodents. In mouse models, nine selenoproteins genes had reduced mRNA expression in liver under Se-deficiency, including *GPX1, SELH, SELW, TR1, TR2, DIO1*

and *SEP15*. This reduction ranked them low in the hierarchy and made them a possible target for use as molecular biomarkers in rodents (SUNDE et al., 2009). Nevertheless, human studies have failed to demonstrate an association of Se status and selenoproteins transcripts (RAVN-HAREN et al., 2008a, 2008b; SUNDE et al., 2008). Only two studies have observed a positive relationship between Se supplementation and increased selenoprotein expression (CARDOSO et al., 2015a; PAGMANTIDIS et al., 2008).

Genetic polymorphisms are an important source of inter-individual variation in response to nutritional supplementation (HESKETH; MÉPLAN, 2011). Several single nucleotide polymorphisms (SNPs) in selenoproteins genes were proved to affect the response to Se supplementation by changing the concentration of biomarkers of Se status (COMBS et al., 2012; JABLONSKA et al., 2009; MÉPLAN et al., 2007, 2008). However, none of the studies investigating the effect of Se supplementation on the pattern of gene expression have considered the genetic profile of the participants. Therefore, the aim of our work was to investigate the influence of genetic variations in selenoprotein genes on the pattern of gene expression in response to Brazil nut supplementation in healthy Brazilians.

METHODS

Brazil nut supplementation and blood sampling

The present study involved 130 unrelated healthy volunteers who took part of the Supplementation with Brazil Nuts study (SUBRANUT) described previously. Volunteers taking multivitamins and mineral supplements, anti-inflammatory drugs, those with

excessive alcohol consumption, those athletes, those with chronic diseases such as cancer, diabetes and cardiovascular disease were excluded. At the beginning of the study, 20mL of blood sample were drawn (baseline) and the volunteers took a daily supplement of Brazil nuts for 8 weeks, eating one nut a day. At the end of eight weeks of supplementation with Brazil nuts, another 20mL blood sample was taken, and then two more blood samples were taken at four week intervals during eight weeks of washout period. The compliance with Brazil nut supplementation was estimated by checking a control calendar given in the first blood sampling. The adhesion to the supplementation was checked by high reported compliance. Written inform consent were signed by 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 Declaration of Helsinski.

RNA was isolated from whole blood of previously genotyped volunteers before and after Brazil nut supplementation. To analyze the expression of *GPX1*, *SEPP1*, *SELS* and *SEP15* we used previously genotyped samples. We chose one SNP in each gene, except for *SEPP1* which we used two SNPs, and used a dominant genetic model. In this model, the homozygous dominant genotype was separated from the carriers of the variant allele, heterozygous and homozygous variant genotypes together. Specifically for microarray analysis, the C>T SNP located on the 3'UTR of *GPX4* gene was chosen. Six volunteers who were CC and six volunteers who were TT were selected and analyzed before and after supplementation (Figure 10).

Genotyping

Total DNA was extracted from 200μL of whole blood using Purelink Genomic DNA Minikit (Invitrogen, Life Technologies, California, USA), the final concentration measured in a NanoDrop ND 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and adjusted for further analysis. The SNPs selected were chosen based on the literature evidence for functional consequences and relation to risk of chronic diseases. Details of the SNPs selected are shown in Chart 6. Genotyping was done by real-time polymerase chain reaction (RT-PCR) using Taqman SNP Genotyping Assays (Life Technologies, California, USA). Each RT-PCR reaction contained 20ng of adjusted genomic DNA, 0,5μL of 20X SNP Genotyping Assay (Life Technologies), 5,0μL of TaqMan Genotyping Master Mix (Life Technologies) and 2,5 μL of DNase free water in a 10μL final reaction volume. Samples were assayed along with no-template and internal controls for each genotype and run in a 96 well plate in the StepOne Plus Real Time PCR system under the following conditions: enzyme activation at 95 ^{o}C for 10min, followed by 40 cycles at 92 ^{o}C for 15s and 60 ^{o}C for 1 min for annealing and extension. The allelic discrimination was obtained by performing an endpoint read. Hardy-Weinberg equilibrium was tested by the chi-squared test.

Chart 6. Information about the SNPs analysed in the study

Genes	Chromossome location	rs (dbSNP)	SNP region	Base change	Codon change	aminoacid change
GPX1	3p21.3	rs1050450	Coding region	C679T	C**C**C / C**T**C	Pro198Leu
GPX4	19p13.3	rs713041	3'UTR	C718T	*	*
SEPP1	5p12	rs3877899	Coding region	G24731A	**G**CT - **A**CT	Ala234Thr
		rs7579	3'UTR	G25191A	*	*
SELS	15q26.3	rs34713741	promoter	C>T	*	*
SEP15	1p31	rs5845	3'UTR	G1125A	*	*

*: no change. 3'UTR: 3'untranslated region.

Selenoprotein gene expression

The gene expression of *GPX1*, *SEPP1*, *SELS* and *SEP15* was analysed before and after Brazil nut supplementation on previously genotyped participants. RNA was extracted from whole blood using Ribopure Blood Kit (Ambion, Life Technologies) and treated with DNase I to prevent DNA contamination. The final concentration was measured in a NanoDrop ND 1000 spectrophotometer (Thermo Scientific). RNA was considered of good quality when the absorbance ratios between 260 and 280 nm wavelengths were between 1,9 and 2,1. RNA was adjusted to 20ng/μL in a final volume of 20μL. High Capacity Reverse Transcriptase kit (Life Technolgies) was used to synthetise the cDNA using 10μL of adjusted RNA. cDNA was further diluted 1:5 in DNA/RNA free water to perform the real time PCR reactions.

Analysis of selenoproteins gene expression was performed by real-time quantitative polymerase chain reaction (qPCR) in the QuantStudio 12K Real Time PCR System (Life Technologies) using specific probes for each gene (Taqman Gene expression Assays, Life Technologies). The qPCR reactions were performed in a 384-well plate in triplicate for the

target gene and duplicate for the reference gene GAPDH. Each qPCR reaction contained 2μL of diluted cDNA, 0,5μL of 20X Taqman Gene Expression Assays (Life Technologies), 5,0μL of TaqMan Gene Expression Master Mix (Life Technologies), 2,5μL of RNase free water in a 10μL final reaction volume. GAPDH was used as a reference gene as its expression was stable among samples. A non template reaction without cDNA was included in each plate as a negative control. The cycling conditions were 2 min at 50 ^{o}C for denaturation, followed by 45 cycles 95 ^{o}C for 15s and 1 min at 60 ^{o}C for hybridisation and extension.

Differential gene expression of the selenoprotein genes was evaluated considering the previously genotyped SNPs in each gene. We used the dominant genetic model to choose the samples of the two genotypic groups for each SNP: homozygous dominant x heterozygous + homozygous variant. Each group had 18 samples. The method used to quantify the relative gene expression was based on the $2^{-\Delta\Delta\ Cq}$ method (LIVAK; SCHMITTGEN, 2001). The Δ Cq was calculated normalizing the values by the internal control gene GAPDH (Δ Cq target gene - Δ Cq reference gene). The ΔΔ Cq was obtained by subtracting the Δ Cq of the samples by the average of Δ Cq of each genotype group before supplementation.

Microarray analysis

The microarray analysis was done in order to investigate the influence of the C>T SNP located on the 3'UTR of *GPX4* in the pattern of gene expression. mRNA was extracted from 6 CC and 6 TT volunteers before and after Brazil nut supplementation (Figure 10). Total RNA was extracted from whole blood using Purelink Blood MiniKit (Ambion). The integrity of these samples and more 12 spare samples was checked using

Tape Station 2000 (Agilent, Santa Clara, USA) with the Agilent RNA Nano kit. Samples with a RNA integrity number (RIN) of above seven were used for whole genome microarray using the Illumina HumanHT-12 v4 BeadChip. These RNA quality control measurements were also carried out by Service XS using Agilent Bioanalyzer, as can be seen in Figure 11. RNA labeling, amplification, and hybridization were performed by Service XS. Raw microarray scan files were exported using the Illumina Beadstudio program and loaded into R for downstream analysis using the BioConductor and specific packages for each step of the bioinformatics analysis (RITCHIE et al., 2011). The data were transformed and normalized using VST (variance stabilizing transform) and RSN (robust spline normalization) from the Lumi BioConductor package. Probes with signals that fullfilled the criteria of Illumina probe detection *P* value of 0.05 were considered different. The bioinformatic analysis was performed by the Newcastle Bioinformatics Support Unit at the Faculty of Medical Sciences in Newcastle University, UK.

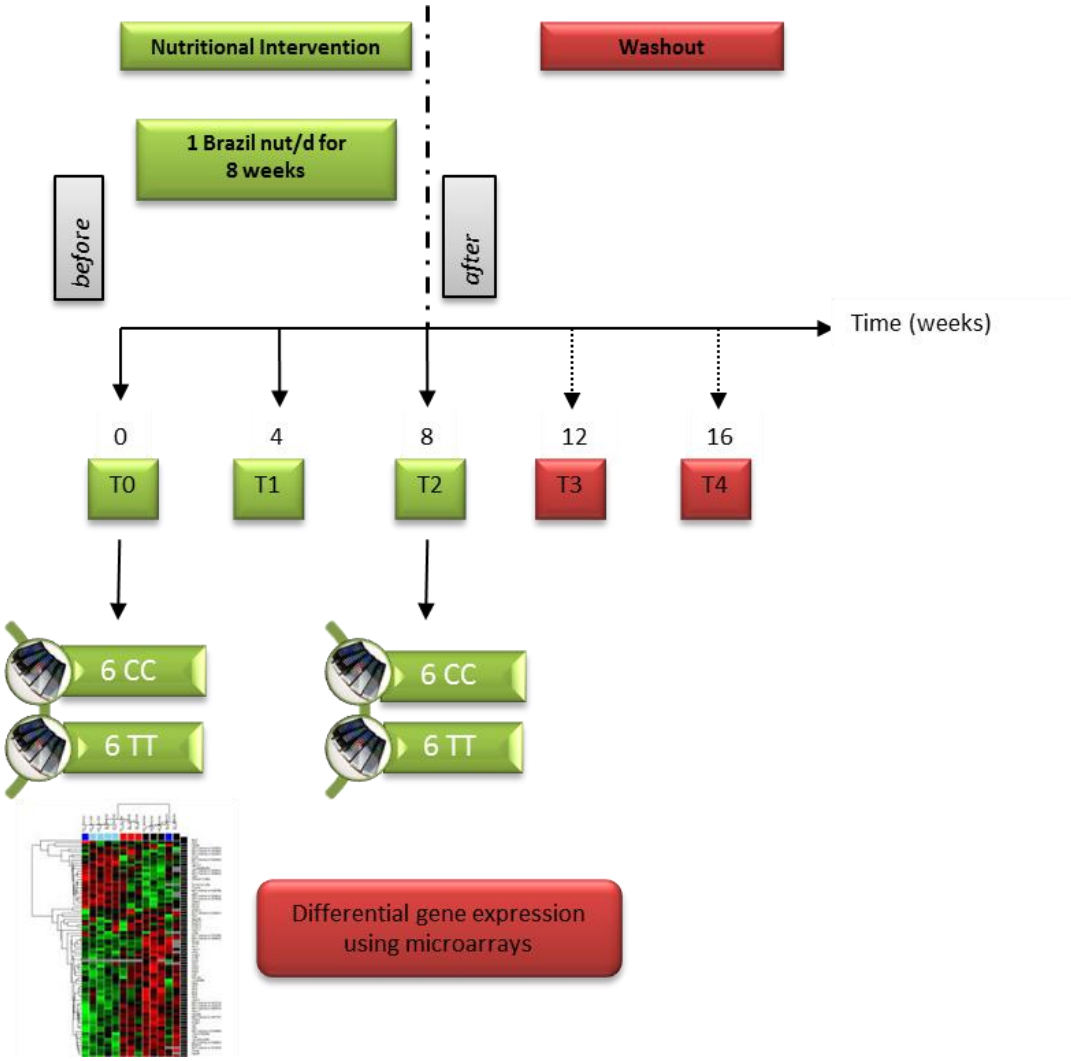

Figure 10. Nutritional intervention protocol indicating time of biological sample collection (T0 – T4).

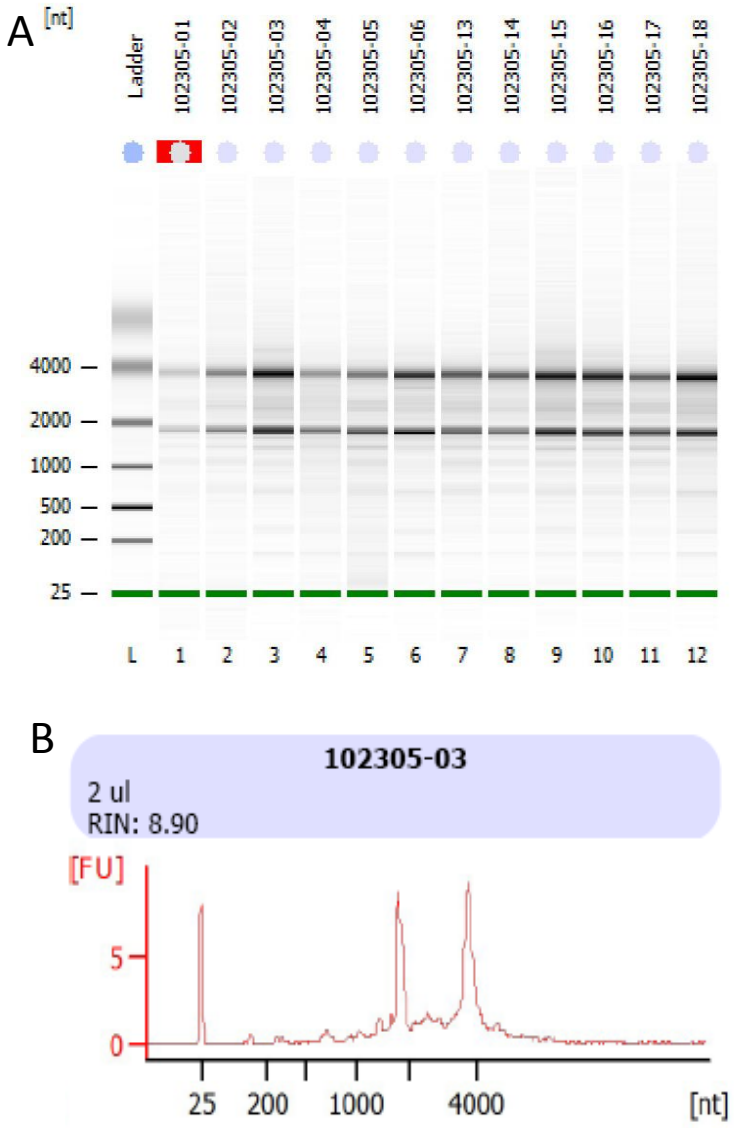

Figure 11. RNA integrity used for microarray analysis.

A.Gel-like eletropherogram image generated from Bioanalyser for 12 samples showing two clear bands. B. Bioanalyser electropherogram output of one sample showing 2 clear peaks indicative of good quality RNA. The RIN number stands to RNA Integrity Number and have to be greater than 7.0 to be of sufficient quality.

Real time confirmation

Real time PCR on specific transcripts in order to confirm the microarray data was carried out using a Roche LightCycler 480 and SYBER green I Master Mix (Roche) with 10μL reaction in a white 96-well plate with 0.5mM of primers in the Institute for Cell and Molecular Biosciences in Newcastle University, UK. Primers specific for each gene were designed using NCBI Primer Blast and can be seen in Chart 7. The amplicon was further submitted to fold in an online software available on mfold.rna.albany.edu, using the DNA Folding Form and the following conditions: 60^{o}C, Na^{+} 0,1 and Mg^{2+} 0,005. The folding structures are available as requested. To select the genes for confirmation, the criterion was the highest or lowest fold change and the genes that were overlapping in different metabolic pathways using Ingenuity Pathways Analysis, used to perfom a canonical pathway analysis (Ingenuity Systems, Redwood City, California). The genes selected were: ALAS2, NFkBIA and HaD3.

Chart 7. List of primers used for real time confirmation

primer	sequence	Tm	%GC	Yield (nmol)
NFKBIA_F	TGCTCAAGGAGCCCTGTAATG	59,4	55	18,6
NFKBIA_R	GACATCAGCCCACACTTCA	59,4	55	20,9
DEFA_F	AGCATCCAGGCTCAAGGAAA	57,3	50	7,2
DEFA_R	ATGCAGGTTCCATAGCGACG	59,4	55	15,3
ALAS2_F	TGCCAGGTCCTAACCCAAGT	59,4	55	20,2
ALAS2_R	AACCTAAAGTCCTGTTGCCCT	57,9	47,6	24,1

Gene set enrichment analysis (GSEA)

The transcriptome data were analysed by gene set enrichment analysis (GSEA), which ranks the genes in a list by their differential expression and tests for coordinated

differences in a set of genes in response to a specific situation, rather than individual genes with increased or decreased expression in two conditions. One of the great advantages of this integrated approach is the facility of interpreting a large amount of data by identifying biological pathways and processes. The other one is that GSEA considers the entire list of genes of the experiment, rather than the ones that passed a fold-change cut-toff (SUBRAMANIAN et al., 2005). The GSEA application from the Broad Institute was used in the analysis, available online at http://software.broadinstitute.org/gsea/index.jsp. Three files were created (dataset file.gct, phenotype file.cls and gene sets file.gmt) and loaded into the software. The dataset file contained the normalised microarray data, in our case with 19835 probes and 23 arrays. The phenotype file contained the information about the experimental conditions, which have to be numbered. In our experiment we used the genotypes for GPX4_rs713041 and the supplementation. Therefore, we ended up with four conditions: 0 = CC_before, 1 = CC_after, 2 = TT_before and 3 = TT_after. The gene sets file was downloaded from the Molecular Signature Database v5.1 (MSigDB), a online collection of gene sets from different databases available for free to use with the GSEA application. The MSigDB has 8 different collections. We used only 2 that were applicable to our conditions: C2, curated gene sets from online pathways databases and C5, Gene Ontology gene sets.

Statistical analysis

For all statistical analysis, individuals who were homozygous and heterozygous for the variant alleles were combined together in one group. Volunteers were selected based on their genotype for each of the five SNPs analysed and a total of 18 individuals were selected in each group. mRNA was extracted from whole blood of these 36 individuals

before and after nuts. Relative gene expression of each gene was normalized by GAPDH reference gene using the $2^{-\Delta\Delta Cq}$ method. The final fold-change was used for statistical comparisons and submitted to Normality tests using the Shapiro-Wilk test. The genotype effect before and after nuts was assessed by the Mann-Whitney test. The supplementation effect in each genotype was assessed by the Wilcoxon Test. Differences were considered significant if $P < 0.05$.

RESULTS

The Supplemention with Brazil Nut study (SUBRANUT) was carried out to investigate the influence of genetic variations on the response to Brazil nut intake in different biomarkers. The study was conducted with 130 healthy adults, of these, 66 were selected according to the genotype to analyse the expression of four selenoproteins genes and 12 were selected to the microarray analysis. The supplementation for 8 weeks was effective on increasing plasma Se concentrations from 96.7 ± 29.6 to 292.8 ± 95.4 μg/L ($P < 0.05$). Moreover, Brazil nut supplementation led to greater plasma SePP concentrations.

Gene expression of four selenoprotein genes (*GPX1*, *SEPP1*, *SELS* and *SEP15*) was analysed in previously genotyped volunteers before and after Brazil nut supplementation. The results are shown in Figure 12. The *GPX1*_rs1050450 had an effect on *GPX1* mRNA expression. In CC individuals, there was an increase in *GPX1* expression after supplementation ($P = 0.026$, Wilcoxon test). In addition, after Brazil nut consumption, the *GPX1* expression was lower in T-carriers compared to CC group (Figure 12A). The genotype for *SEPP1*_rs7579 affected *SEPP* expression before and after nut supplementation. In carriers of the variant allele A, *SEPP1* mRNA expression was higher

than GG individuals either before or after supplementation (Figure 12C). After supplementation, *SEPP1*_rs3877899 apparently had an effect on *SEPP1* mRNA expression, being higher in A-carriers, however it did not reach statistical significance (Figure 12B). No significant differences were observed for *SELS* and *SEP15* genes (Figure 12D and E).

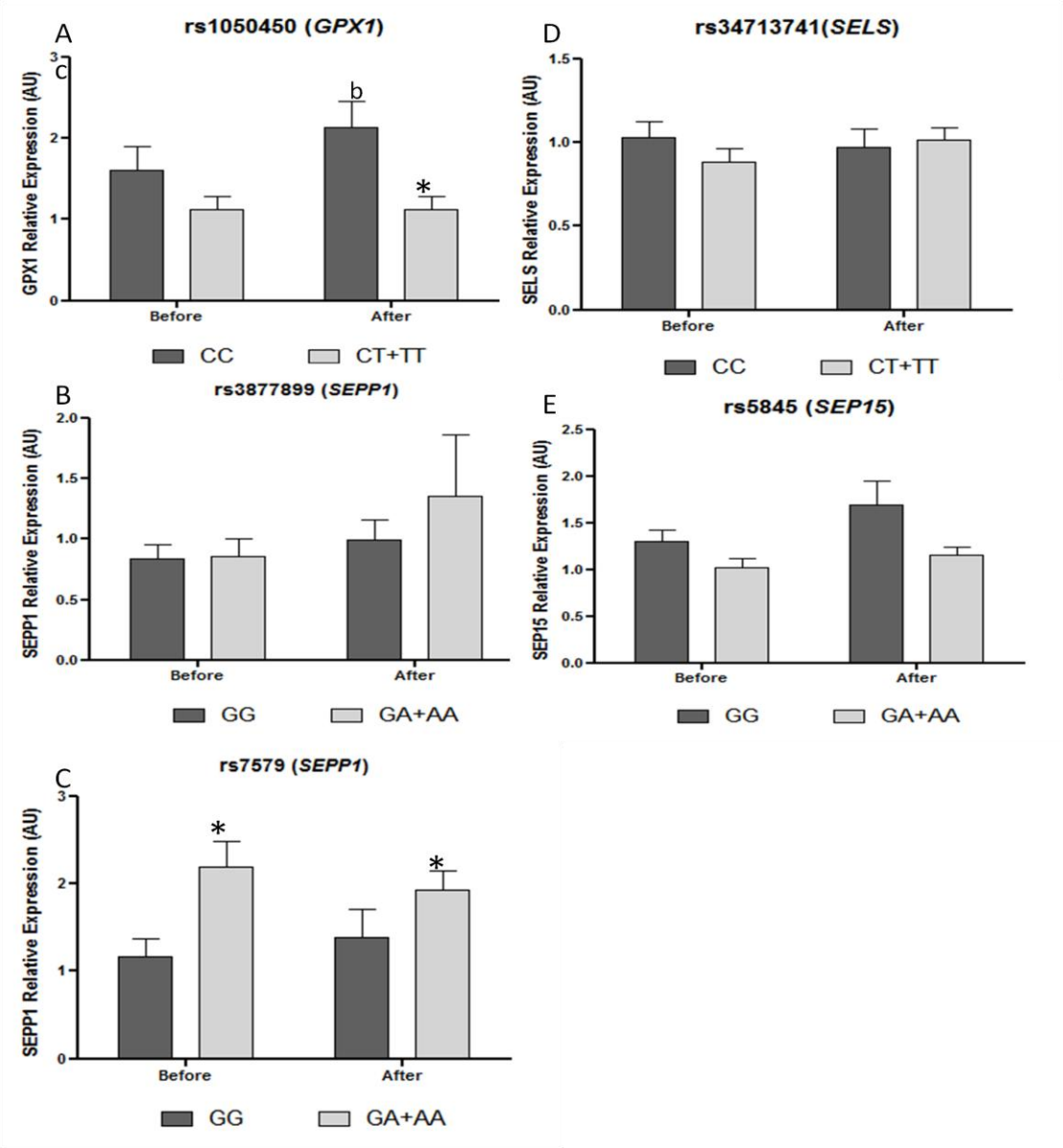

Figure 12. Pattern of selenoprotein gene expression in response to Brazil nut supplementation on previously genotyped volunteers.

A. GPX1_rs1050450, B. SEPP1_rs3877899, C. SEPP1_rs7579, D. SELS_rs34713741 and E. SEP15_rs5845. * $P < 0.05$, Mann-Whitney test.

The microarray experiment was carried out using RNA from 12 individuals (before and after Brazil nuts)previously genotyped for *GPX4*_ rs713041 using Illumina HumanHT-12 v4 BeadChip. Figure 13 shows the overall pattern of differential gene expression in four groups tested; 2 genotypes and 2 experimental conditions. No genes were detected as being significantly altered in expression before supplementation in either CC or TT genotype (Figure 13A). On contrary, after supplementation there was some evidence of genes altered in expression between CC and TT genotypes, using a fold change of 1.0 and *P* value of 0.05 (Figure 13B). One gene of this list was used for assessment of real time confirmation (HaD3, Human alpha Defensin 3). Figure 13C shows the pattern of differential expressed genes before and after supplementation for CC individuals. Two genes of this minor list were selected for real time confirmation (NFkBIA, nuclear factor of kappa light polypeptide inhibitor alpha, and ALAS2, aminolevulinate delta synthetase-2). Another type of visualization of differential expressed genes is the heatmap. Such visualization of the gene sets after the supplementation comparing the genotypes CC x TT showed a different pattern of response of individuals with different genotypes (Figure 14). In fact, the response was opposite. Genes that were down regulated in TT individuals after nuts (top left blue square) were up regulated in CC individuals (top right red square).

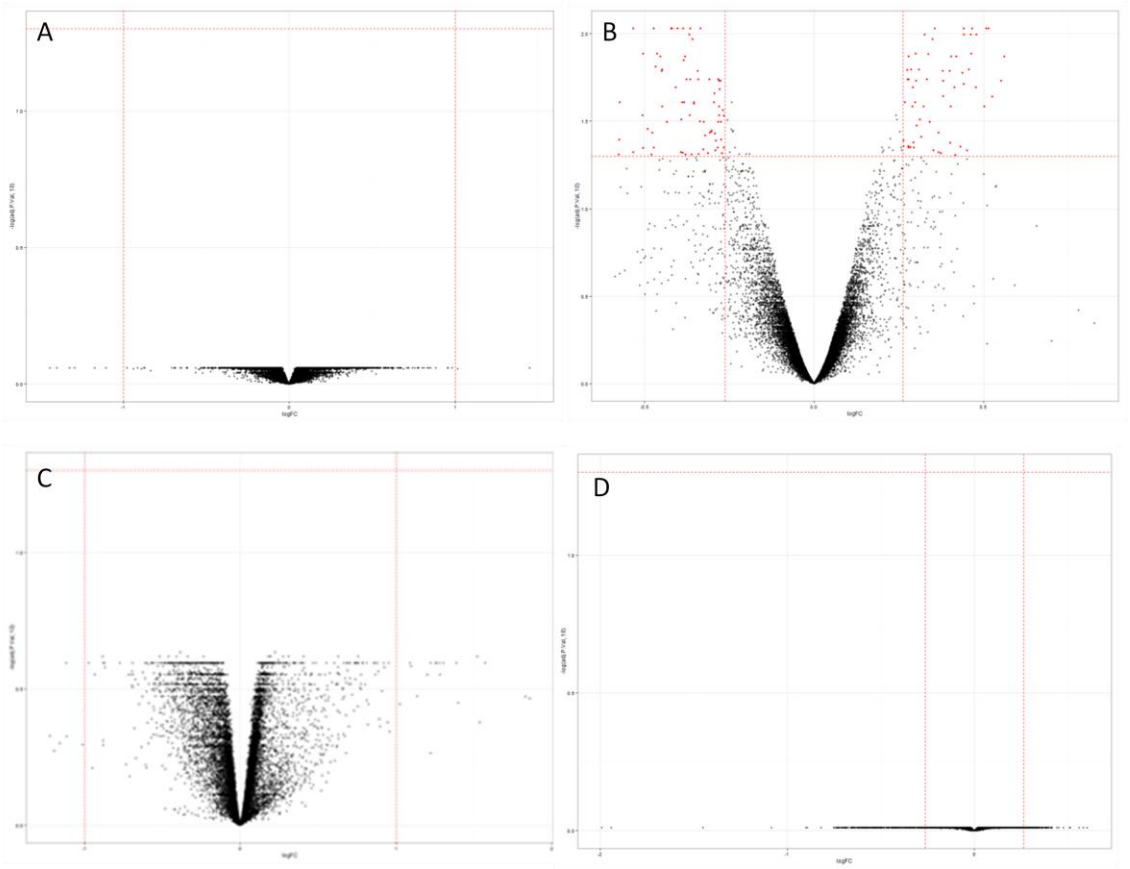

Figure 13. Volcano plots for the four conditions investigated.

A. testing the genotypes before supplemetantation (before: CC x TT), B. testing genotypes after supplementation (after: CC x TT), C. testing the supplementation in CC genotype (CC: before x after), D. testing the supplementation in TT genotype (TT: before x after).

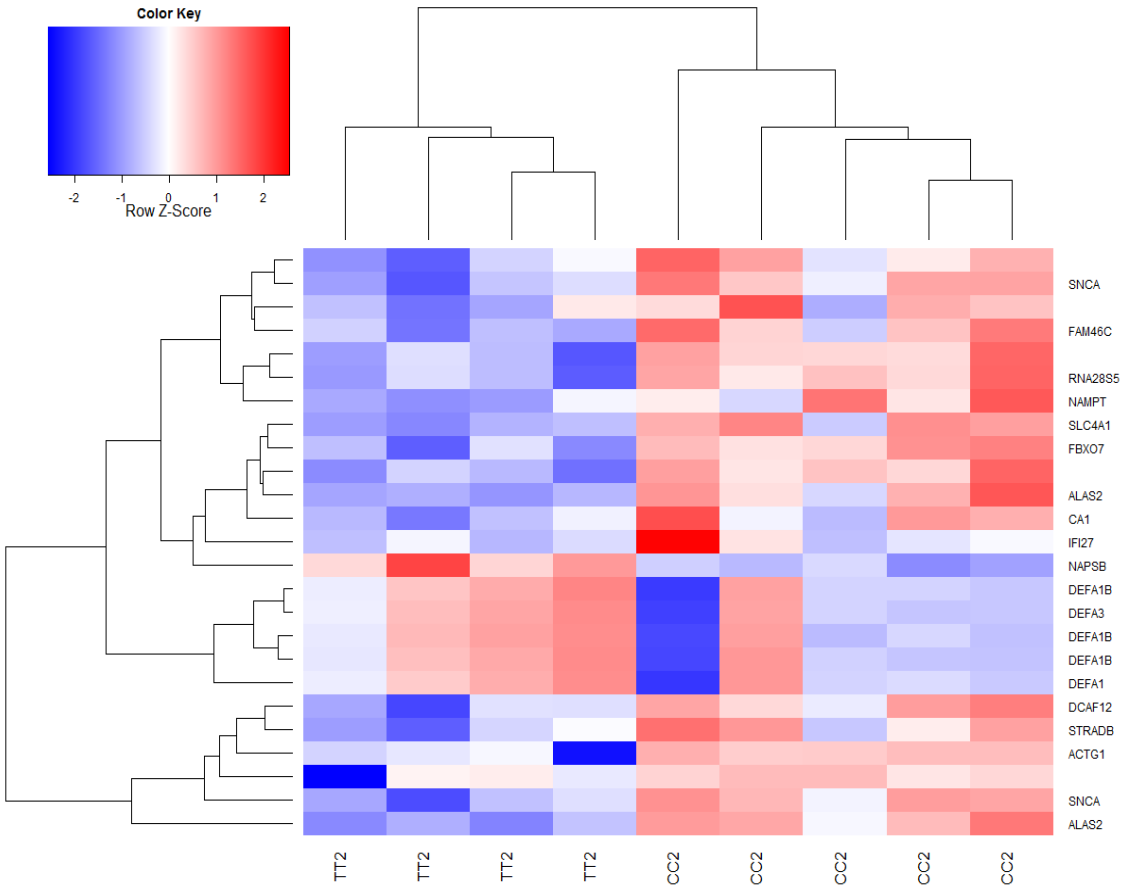

Figure 14. Heatmap showing patterns of differential expression in TT and CC genotype after Brazil nuts supplementation.

Red indicates genes with higher expression levels and blue genes with lower expression levels.

Real time confirmation of the microarray data was performed using three genes. Two were up regulated in the microarray after supplementation in CC individuals (NFkBIA and ALAS2) and one was down regulated (HaD3). The confirmation of the expression of these three genes by real time PCR corroborates the transcriptomic analysis, although only one gene reached statistical significance (Figure 15).

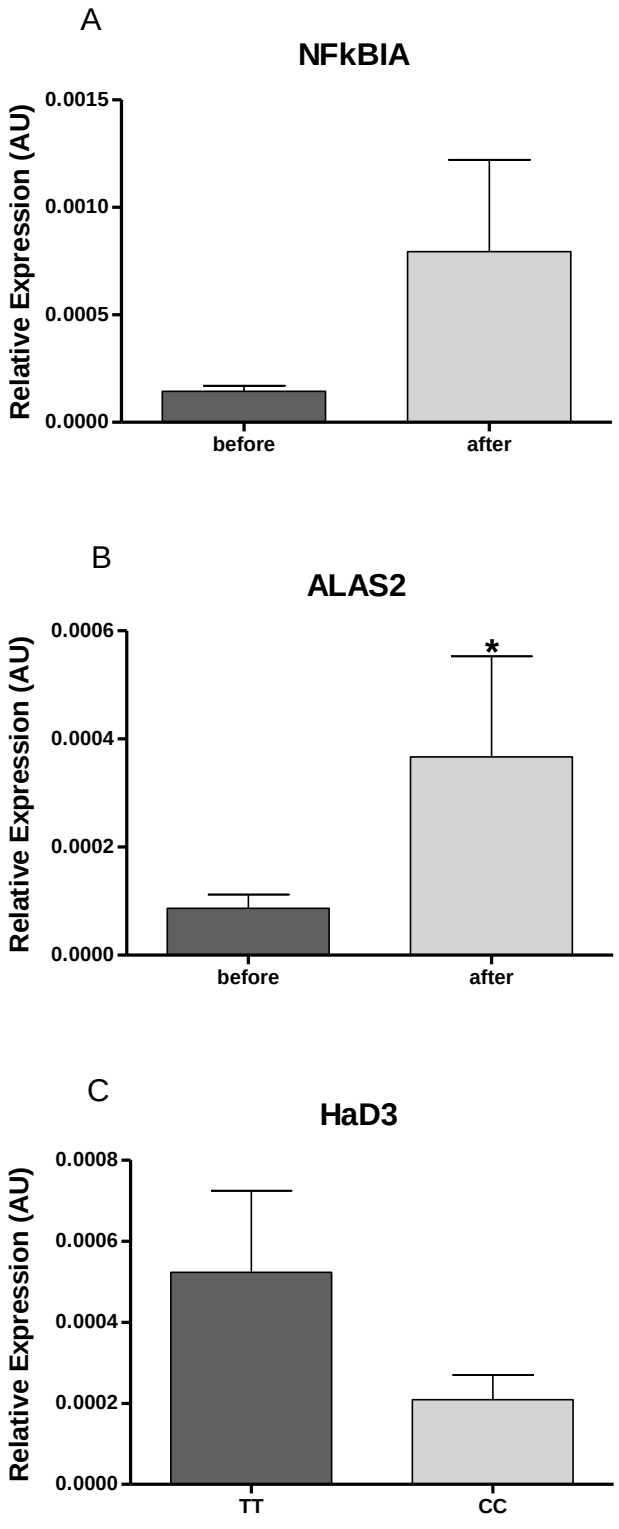

Figure 15. Relative gene expression for confirmation of transcriptomic data by real time PCR.
A. NF-kBIA, B. ALAS2, C. HaD3. *: different from before nuts ($P < 0,05$, Wilcoxon test)

Gene set enrichment analysis was carried out using 19835 probes and 23 arrays. We used both genotypes and the supplementation as conditions for the comparisons. The collection of gene sets available in MSigDB as C2 and C5 were tested. No gene sets from C2 pathways were enriched either in CC individuals before and after nuts or in both genotypes CC and TT after nuts. However, 13 gene sets related to ribosomes, ER and Golgi compartmets and mitochondria were found to be enriched in TT individuals after nuts when the Cellular Component list from Gene Ontology gene sets (C5:CC) were used (Table 10).

Table 10. Enriched gene sets from Gene Ontology (C5) in TT individuals for GPX4_rs713041 after supplementation with Brazil nuts compared with CC individuals. Gene sets were considered to be enriched at an FDR cut off of 25%.

Name	Size	ES	NES	NOM *p*-value	FDR *q*-value
Cellular component					
Organellar ribosome	22	-0.69	-1.56	0.035	1.000
Mitochondrial ribosome	22	-0.69	-1.56	0.035	0.571
Early endosome	15	-0.74	-1.53	0.012	0.582
ER Golgi Intermediate compartment	20	-0.52	-1.52	0.004	0.496
Microtubule cytoskeleton	101	-0.41	-1.51	0.025	0.397
Ribosomal subunit	20	-0.66	-1.51	0.076	0.332
Intrinsic to endoplasmic reticulum membrane	23	-0.58	-1.50	0.035	0.320
Integral to endoplasmic reticulum membrane	23	-0.58	-1.50	0.035	0.280
Mitochondrial matrix	44	-0.55	-1.49	0.066	0.289
Mitochondrial lumen	44	-0.55	-1.49	0.066	0.260
Replication fork	16	-0.61	-1.48	0.014	0.242
Nuclear chromosome part	25	-0.55	-1.48	0.036	0.223
Golgi apparatus	166	-0.37	-1.45	0.008	0.280

ES = Enrichment score, NES = normalised enrichment score, FDR = false discovery rate.

DISCUSSION

Previous works have tried to associate Se supplementation with molecular biomarkers of Se status in human studies, mainly transcripts of selenoproteins in white blood cells (PAGMANTIDIS et al., 2008; RAVN-HAREN et al., 2008a, 2008b; SUNDE et al., 2008). These studies were not able to find an association between plasma Se biomarkers and selenoprotein expression after Se supplementation, except for one study conducted with healthy adults in the UK, which could observe the up-regulation of some selenoprotein genes after Se supplementation (PAGMANTIDIS et al., 2008). The present work extends these earlier observations by investigating whether the response in pattern of blood cell gene expression to a natural source of Se *via* Brazil nuts is different depending upon the genetic background of individuals. To do this we used a transcriptomics approach and the present work is the first to use such an approach to investigate the response to Brazil nut supplementation based on genetic profile. This work demonstrated that three genetic variants in selenoprotein genes (*GPX1*_rs1050450, *SEPP1*_rs7579 and *GPX4*_rs713041) affect the response to supplementation with Brazil nuts at the transcriptional level. In addition, the effect of *GPX4*_ rs713041 on the response in global gene expression profile after Brazil nut supplementation has not been observed before.

It was observed in the present work that the supplementation with one unit of Brazil nut for 8 weeks was sufficient to significantly increase *GPX1* mRNA expression in whole blood. This result was different from other three human studies which have tried to investigate if Se supplementation would have an effect on selenoprotein transcripts. A small study conducted in Denmark found no association of Se supplementation as Se-

enriched milk, yeast and selenate for 1 week and *GPX1* mRNA expression (RAVN-HAREN et al., 2008a). Accordingly, the 5-years PRECISE Danish study and a longitudinal study conducted in the UK also found no association (RAVN-HAREN et al., 2008b; SUNDE et al., 2008). Nevertheless, two studies are in agreement with our results. One study conducted with healthy adults in the UK observed that the supplementation with 100μg/d with sodium selenite for 6 weeks increased the expression of selenoprotein K (*SELK*) and selenoprotein 15 (*SEP15*), showing that these selenoproteins are sensible to alterations of Se status (PAGMANTIDIS et al., 2008). The other study conducted with Alzheimer's patients also found an increase in *GPX1* mRNA expression after supplementation with 1 unit of Brazil nut for 6 months (CARDOSO et al., 2015a). A possible explanation for this variation in response to Se supplementation is the presence of genetic variants, which most of the aforementioned works have not considered. Other factors that could contribute to this variation are the chemical form of Se used in the supplements, the duration of the intervention and the baseline plasma Se levels of the participants.

Interestingly, the increase in *GPX1* mRNA expression observed in our study was different based on the presence of genetic polymorphisms. The increase on *GPX1* transcript was significant only for individuals with the common genotype CC. No difference was observed in T-carriers. This genetic variation was associated with increased *GPX1* mRNA expression in other Brazilian work conducted with Alzheimer's patients, but the authors found the increase in T-carriers instead of CC (CARDOSO et al., 2015a). Although some studies have investigated the association of this variation with differences on GPx1 activity (HAMANISHI et al., 2004; HANSEN et al., 2009; JABLONSKA et al., 2009), few studies

have associated this variation with *GPX1* gene expression. The only study available in humans was conducted with a different population from ours and maybe this could be influencing the opposite results. In our group of healthy individuals, there was strong linkage disequilibrium among rs1050450, rs3811699 and rs1800668 (see chapter 3). This indicates that these SNPs were linked, meaning that the presence of the variant allele for one SNP implicates in the presence of the variant allele of the other two polymorphisms. This linkage was also observed in a Japanese study conducted with type-2 diabetic patients, in which the combination of rs3811699 and rs1800668 were associated with a reduction of 25% in *GPX1* transcription (HAMANISHI et al., 2004). The T-carriers of rs1050450 were not responsive to the supplementation at the molecular level in our study. One possible explanation is that the presence of these three genetic variations is affecting the transcriptional process.

The presence of genetic variants also influenced *SEPP1* expression. Although the supplementation did not affect significantly *SEPP1* mRNA levels, the presence of rs7579 in the *SEPP1* gene affected transcript levels at baseline and after supplementation: A-carriers had higher *SEPP1* mRNA levels than GG (Figure 3C). Previous work with humans have not found an association between Se supplementation and *SEPP1* mRNA expression in white blood cells (RAVN-HAREN et al., 2008a; SUNDE et al., 2008). In rodents, *SEPP1* mRNA in liver was not affected by Se-deficient diet, however, upon repletion *SEPP1* transcript was the most abundant in liver (SUNDE et al., 2009). The lack of sensitivity of *SEPP1* to Se deficiency may be explained in part by the position of this protein into the hierarchy of selenoprotein expression, apparently in the middle. Therefore, under deficiency, the selenoproteins which are ranked low in hierarchy have their mRNA decreased, such as *GPX1*,

Our work also had the goal of determining if genetic variants would influence the pattern of gene expression in response to a natural source of Se, such as Brazil nuts. We have chosen the *GPX4*_rs713041 to test this hypothesis, as there is evidence that this SNP is functional (BERMANO et al., 2007; CROSLEY et al., 2013; MÉPLAN et al., 2008). The pattern of gene expression was obtained by using microarrays in samples previously genotyped for this SNP before and after supplementation with Brazil nuts. We observed that although not statistically significant, the heatmap and volcano plots suggest opposite response to Brazil nut supplementation based on genotype. To our knowledge, the association of *GPX4*_rs713041 genotype with the profile of global gene expression has not been observed previously. Moreover, gene set enrichment analysis showed that the biological processes and cellular compartments altered by the supplementation were related to protein synthesis, mitochondria and endoplasmatic reticulum. This supports the findings of previous humans studies which used microarrays to investigate the effect of Se supplementation (HAWKES; RICHTER; ALKAN, 2013; PAGMANTIDIS et al., 2008). These studies observed that processes related to protein biosynthesis were up-regulated after Se supplementation. This could be explained by the molecular biosynthesis of selenoproteins, which needs the synthesis of a specific $tRNA^{[Ser]Sec}$ for the aminoacid selenocysteine, inserted in the proteins during translation.

In conclusion, the present study has shown that supplementation with Brazil nuts alters the gene expression of some selenoproteins depending upon the presence of genetic polymorphisms. In addition, it has demonstrated that the use of microarrays to investigate the pattern of global gene expression in response to a nutritional intervention with nuts is feasible, and the genetic profile is an important source of inter-individual variation. This could be relevant to direct future nutritional interventions to the use of molecular and

biochemical biomarkers considering the interaction with the genetic variations. These three factors could help to elucidate the complex interaction of nutritional status and risk for chronic diseases.

Table S1. List of genes significantly different before and after supplementation in CC volunteers.

symbol	description	logFC	adj.P.Val
IL10	interleukin 10	-0,57556	0,0491
MCM8	minichromosome maintenance complex component 8	-0,57277	0,0247
PNPT1	polyribonucleotide nucleotidyltransferase 1	-0,53279	0,0476
AOC4P	amine oxidase, copper containing 4, pseudogene	-0,53273	0,0093
BLZF1	basic leucine zipper nuclear factor 1	-0,50503	0,0293
NLRP8	NLR family, pyrin domain containing 8	-0,50329	0,0451
DMC1	DNA meiotic recombinase 1	-0,49029	0,0350
ALPP	alkaline phosphatase, placental	-0,47602	0,0369
OCIAD1	OCIA domain containing 1	-0,47264	0,0093
FLJ44124	uncharacterized LOC641737	-0,4528	0,0135
LRRC37BP1	leucine rich repeat containing 37B pseudogene 1	-0,44844	0,0164
ZNF682	zinc finger protein 682	-0,44794	0,0262
SPTLC1	serine palmitoyltransferase, long chain base subunit 1	-0,44791	0,0160
MCMDC2	minichromosome maintenance domain containing 2	-0,44679	0,0262
DTWD2	DTW domain containing 2	-0,4332	0,0320
FKBP14	FK506 binding protein 14, 22 kDa	-0,42125	0,0093
KIAA1751	KIAA1751	-0,41819	0,0093
CATSPER2	cation channel, sperm associated 2	-0,40203	0,0093
DUXAP3	double homeobox A pseudogene 3	-0,39173	0,0476
LOC642947	uncharacterized LOC642947	-0,39115	0,0309
C2orf69	chromosome 2 open reading frame 69	-0,38795	0,0247
ZNF430	zinc finger protein 430	-0,38697	0,0480
CRCP	CGRP receptor component	-0,38437	0,0142
FAM175A	family with sequence similarity 175, member A	-0,37988	0,0135
PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)	-0,37879	0,0491
KCNH6	potassium voltage-gated channel, subfamily H (eag-related), member 6	-0,37532	0,0182
ITPK1-AS1	ITPK1 antisense RNA 1	-0,36787	0,0101
C8orf37	chromosome 8 open reading frame 37	-0,36656	0,0293
YRDC	yrdC N(6)-threonylcarbamoyltransferase domain containing	-0,3648	0,0182
DENR	density-regulated protein	-0,36069	0,0491
ZNF14	zinc finger protein 14	-0,35848	0,0107
CDKN2AIPNL	CDKN2A interacting protein N-terminal like	-0,35447	0,0247
LOC100128288	uncharacterized LOC100128288	-0,34254	0,0164
DDX51	DEAD (Asp-Glu-Ala-Asp) box polypeptide 51	-0,34074	0,0489
SULT1A1	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	-0,32622	0,0458
LOC399900	uncharacterized LOC399900	-0,30826	0,0182
C21orf58	chromosome 21 open reading frame 58	-0,30643	0,0369
XRCC2	X-ray repair complementing defective repair in Chinese hamster	-0,30183	0,0359

	cells 2		
TMEM17	transmembrane protein 17	-0,29464	0,0247
HNRNPA2B1	heterogeneous nuclear ribonucleoprotein A2/B1	-0,29352	0,0220
BIRC3	baculoviral IAP repeat containing 3	-0,29167	0,0372
RPS26P15	ribosomal protein S26 pseudogene 15	-0,291	0,0464
ZRANB2	zinc finger, RAN-binding domain containing 2	-0,28891	0,0409
BRD2	bromodomain containing 2	-0,28358	0,0449
EXO5	exonuclease 5	-0,28214	0,0182
EID2B	EP300 interacting inhibitor of differentiation 2B	-0,28175	0,0319
SF3B1	splicing factor 3b, subunit 1, 155kDa	-0,27945	0,0262
ZNF549	zinc finger protein 549	-0,27897	0,0186
FLI1	Fli-1 proto-oncogene, ETS transcription factor	-0,2741	0,0319
EIF4G2	eukaryotic translation initiation factor 4 gamma, 2	-0,27113	0,0483
CCBE1	collagen and calcium binding EGF domains 1	-0,26877	0,0274
NBPF11	neuroblastoma breakpoint family, member 11	-0,26615	0,0295
ANXA1	annexin A1	-0,26584	0,0448
TP53BP2	tumor protein p53 binding protein, 2	0,263575	0,0409
ROPN1B	rhophilin associated tail protein 1B	0,274406	0,0161
FTL	ferritin, light polypeptide	0,276674	0,0182
COX6B1	cytochrome c oxidase subunit VIb polypeptide 1 (ubiquitous)	0,277768	0,0441
HBB	hemoglobin, beta	0,279192	0,0448
FTL	ferritin, light polypeptide	0,279251	0,0182
AP2S1	adaptor-related protein complex 2, sigma 1 subunit	0,279503	0,0135
HBG2	hemoglobin, gamma G	0,285092	0,0446
RPS27	ribosomal protein S27	0,285828	0,0160
HBA1	hemoglobin, alpha 1	0,291537	0,0203
DPYSL5	dihydropyrimidinase-like 5	0,292535	0,0419
BABAM1	BRISC and BRCA1 A complex member 1	0,294191	0,0449
RPLP2	ribosomal protein, large, P2	0,298868	0,0247
UBB	ubiquitin B	0,299241	0,0130
HBA2	hemoglobin, alpha 2	0,301329	0,0186
HBA2	hemoglobin, alpha 2	0,303268	0,0335
GYPC	glycophorin C (Gerbich blood group)	0,309872	0,0160
DENND6B	DENN/MADD domain containing 6B	0,311742	0,0309
PSMF1	proteasome (prosome, macropain) inhibitor subunit 1 (PI31)	0,316729	0,0388
SPRYD3	SPRY domain containing 3	0,333429	0,0182
RBM38	RNA binding motif protein 38	0,337281	0,0131
SEMA6B	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6B	0,340984	0,0320
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	0,349092	0,0107
MPP1	membrane protein, palmitoylated 1, 55kDa	0,35203	0,0466
GUK1	guanylate kinase 1	0,356251	0,0093
ALAS2	aminolevulinate, delta-, synthase 2	0,366856	0,0369

SLC6A10P	solute carrier family 6 (neurotransmitter transporter), member 10, pseudogene	0,367952	0,0476
GPX1	glutathione peroxidase 1	0,373893	0,0483
CLUAP1	clusterin associated protein 1	0,380028	0,0164
MYL4	myosin, light chain 4, alkali; atrial, embryonic	0,380605	0,0186
MUC6	mucin 6, oligomeric mucus/gel-forming	0,381097	0,0228
AHSP	alpha hemoglobin stabilizing protein	0,400277	0,0164
FIS1	fission 1 (mitochondrial outer membrane) homolog (S. cerevisiae)	0,401752	0,0422
GSPT1	G1 to S phase transition 1	0,403064	0,0262
DMTN	dematin actin binding protein	0,404564	0,0135
GUCD1	guanylyl cyclase domain containing 1	0,413719	0,0203
GMPR	guanosine monophosphate reductase	0,416145	0,0495
HBM	hemoglobin, mu	0,420055	0,0135
FOXO4	forkhead box O4	0,431478	0,0444
SLC25A39	solute carrier family 25, member 39	0,437888	0,0168
HBD	hemoglobin, delta	0,440986	0,0194
TESC	tescalcin	0,441821	0,0101
SERF2	small EDRK-rich factor 2	0,442123	0,0093
GLRX5	glutaredoxin 5	0,451635	0,0466
LYL1	lymphoblastic leukemia associated hematopoiesis regulator 1	0,45735	0,0160
GYPC	glycophorin C (Gerbich blood group)	0,461942	0,0101
VWCE	von Willebrand factor C and EGF domains	0,463549	0,0130
HBQ1	hemoglobin, theta 1	0,466787	0,0093
TSPAN5	tetraspanin 5	0,477096	0,0203
BSG	basigin (Ok blood group)	0,478764	0,0101
HAGH	hydroxyacylglutathione hydrolase	0,502051	0,0262
ALAS2	aminolevulinate, delta-, synthase 2	0,508163	0,0093
RBM38	RNA binding motif protein 38	0,514278	0,0093
CARM1	coactivator-associated arginine methyltransferase 1	0,525426	0,0229
UBXN6	UBX domain protein 6	0,552289	0,0186
ASCC2	activating signal cointegrator 1 complex subunit 2	0,561756	0,0135

Table S2. List of genes significantly different after supplementation between CC and TT volunteers.

symbol	description	logFC	adj.P.Val
ASAP1	ArfGAP with SH3 domain, ankyrin repeat and PH domain 1	0,5082	0,0456
BOLA2	bolA family member 2	0,2795	0,0277
COL18A1	collagen, type XVIII, alpha 1	-0,3679	0,0357
CDK13	cyclin-dependent kinase 13	0,4788	0,0488
FAM78A	family with sequence similarity 78, member A	-0,4020	0,0277
IARS	isoleucyl-tRNA synthetase	-0,2821	0,0467
MMGT1	membrane magnesium transporter 1	0,3563	0,0269
MRPL46	mitochondrial ribosomal protein L46	0,4668	0,0456
MOB1A	MOB kinase activator 1A	0,4619	0,0467
MOB1B	MOB kinase activator 1B	0,3099	0,0456
RALY	RALY heterogeneous nuclear ribonucleoprotein	-0,2750	0,0456
TOM1L2	target of myb1-like 2 (chicken)	-0,4182	0,0357

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