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Efeito do consumo da castanha-do-brasil (Bertholetia excelsa H.B.K.) sobre selenocompostos no uso de estatinas

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Área de concentração: Investigação biomédica

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Epígrafe

"*A mente que se abre a uma nova ideia jamais voltará ao tamanho inicial".*

Albert Einstein

RESUMO

WATANABE, L.M**. Efeito do consumo da castanha-do-brasil (Bertholetia excelsa H.B.K.) sobre selenocompostos no uso de estatinas**. 2020. 138 f. Tese (Doutorado) – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2020.

Introdução e objetivos: Embora os mecanismos responsáveis pelos distúrbios musculares promovidos pelo uso de estatina permaneçam indeterminados, a suplementação com antioxidantes provenientes de alimentos parece mitigar os efeitos colaterais do medicamento. Nos seres humanos, a variação individual na resposta à suplementação de selênio (Se), não relacionada ao estado basal de Se, pode indicar a ocorrência de variantes genéticas. Vários polimorfismos de nucleotídeo único (SNPs) foram reconhecidos como fontes importantes de variações interindividuais no metabolismo de Se em resposta à suplementação, dentre eles SNPs em genes que codificam para as selenoproteínas glutationa peroxidase 1 (*GPX1*) e selenoproteína P (*SELENOP*). Este estudo teve como objetivo investigar se a suplementação de Se pelo consumo de castanhas-do-brasil um alimento rico em Se, poderia modular os biomarcadores do status Se, como exemplo as concentrações de Se no sangue, atividade da enzima GPX e expressão gênica da selenoproteína P (SELENOP), selenoproteína N (SELENON) e GPX, além de influenciar no controle da atividade sérica da creatina quinase (CK), um marcador indireto de dano muscular, de pacientes em uso regular de estatinas. Além disso, foi inferido se as variações genéticas em selenoproteínas modulam a resposta ao consumo das castanhas-do-brasil na mesma população do estudo. **Métodos:** O estudo foi realizado no Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto. Trinta e dois pacientes em uso regular de estatinas receberam uma unidade de castanha-do-brasil diariamente por 3 meses. A composição corporal, as concentrações de Se no sangue, a atividade da GPX dos eritrócitos, os parâmetros de estresse oxidativo e a atividade da CK foram avaliados antes e após a suplementação. No primeiro momento, para análise inicial, os indivíduos foram alocados de acordo com os níveis de atividade da CK (G1: aumentado ou G2: normal) e para a análise das variantes gênicas das selenoproteínas, os voluntários foram previamente genotipados e alocados de acordo com os SNPs nos genótipos GPX1 (rs1050450) e SELENOP (rs3877899 e rs7579). **Resultados:** A suplementação com uma castanha-do-brasil diariamente por 3 meses contribuiu para diminuir a atividade da CK no soro, alterando de maneira positiva as concentrações plasmáticas e de eritrócitos Se ($p < 0,0001$) e com aumento dos níveis de atividade da GPX. Entre os parâmetros relacionados ao estresse oxidativo, observamos níveis reduzidos de

malondialdeído (MDA) e superóxido dismutase (SOD) nos dois grupos após a suplementação. Também encontramos uma associação moderadamente negativa entre a atividade da CK e da GPX ($r = -41$; $p < 0,02$). Não detectamos alterações na expressão das selenoproteínas GPX1, SELENOP e selenoproteína N (SELENON) após a suplementação com a castanha-do-brasil. A presença de alelos variantes para SELENOP rs3877899 e SELENOP rs7579 modulou a resposta da suplementação de Se para atividade da GPX e níveis de CK. Além disso, a suplementação com a castanha-do-brasil aumentou significativamente a expressão de RNAm de GPX1 apenas em indivíduos com genótipo CC em rs1050450. A expressão do RNAm do SELENOP foi significativamente menor nos indivíduos com genótipo GG no rs7579, tanto antes quanto após a suplementação de Se. **Conclusão:** O consumo de castanha-do-brasil aumentou o controle da atividade da CK, melhorando os biomarcadores do estresse oxidativo em pacientes em uso de estatinas, mas não modulou a expressão do RNAm das selenoproteínas. Além disso, nossos resultados indicaram que as variações gênicas em selenoproteínas modulam a resposta da atividade GPX e dos níveis de CK à suplementação de Se via castanha-do-brasil. Também demonstramos que os polimorfismos em selenoproteínas levam a diferentes respostas na expressão do RNAm das selenoproteínas após a suplementação de Se.

Palavras-chave: estatinas, castanha-do-brasil, selenoproteínas, polimorfismo de nucleotídeo único, estresse oxidativo

ABSTRACT

WATANABE, L.M. **Effect of Brazil nut (Bertholetia excelsa H.B.K.) consumption on selenocompouds in chronic use of statins.** 2020. 138 f. Tese (Doutorado) – Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, 2020.

Background and aims: Although the mechanisms by which statins promote muscle disorders remain unclear, supplementation with dietary antioxidants may mitigate statins' side effects. In humans, the individual variation in response to selenium (Se) supplementation may indicate the occurrence of gene variants. Several single nucleotide polymorphisms (SNPs) have been recognized as important sources of inter-individual variations in Se metabolism in response to Se supplementation. In particular, SNPs in *GPX1* (encoding GPX1) and *SELENOP* (encoding SELENOP) have been shown to affect blood selenium or selenoprotein levels in response to supplementation. This study aimed to investigate whether the supplementation with Brazil nuts, a source of dietary Se, could modulate the biomarkers of Se status such as blood Se and GPX activity, and mRNA expression of selenoprotein P (SELENOP), selenoprotein N (SELENON), and GPX. We also verified the influence of Brazil nut supplementation on the control of serum creatine kinase (CK) activity, an indirect marker of muscle damage, of patients in regular use of statins. Moreover, we investigated if nucleotide variations in selenoprotein genes could modulate the response to Brazil nuts in the same study population. **Methods**: The study was performed in the Ribeirão Preto Medical School University Hospital. Thirty-two patients in regular use of statins received one unit of Brazil nut daily for 3 months. Body composition, blood selenium (Se) concentrations, erythrocyte glutathione peroxidase (GPX) activity, oxidative stress parameters, and CK activity were evaluated before and after Brazil nut supplementation. At first, individuals were divided in groups according to CK activity levels (G1: increased or G2: normal). For the analysis of selenoproteins gene variants, participants were previously genotyped and allocated according to SNPs in GPX1 (rs1050450) and SELENOP (rs3877899 and rs7579) genotypes. **Results:** Supplementation with one Brazil nut daily for 3 months decreased levels of CK activity in serum, with positive changes in plasma and erythrocyte Se concentrations ($p<0.0001$), while increasing levels of GPX activity. Regarding oxidative stress, we observed reduced levels of malondialdehyde (MDA) and superoxide dismutase (SOD) in both groups after supplementation. We also found a moderately negative association between CK and GPX activity ($r=-41$; $p<0.02$). We did not detect changes in the expression of selenoproteins *GPX1*, *SELENOP*, and *SELENON* after Brazil nut

supplementation. The presence of variant alleles rs3877899 and rs7579 for *SELENOP* modulates response to Se supplementation for blood GPX activity and CK levels. Brazil nut supplementation significantly increased GPX1 mRNA expression only in subjects with CC genotype at rs1050450 in the *GPX1* gene. SELENOP mRNA expression was significantly lower in subjects with GG genotype of rs7579, both before and after supplementation. **Conclusion:** Brazil nut consumption improved the control of CK activity, as well as oxidative stress biomarkers in patients using statins but did not modulate the mRNA expression of selenoproteins. Furthermore, our findings indicated that selenoprotein gene variations modulate the response of GPX activity and CK levels to Brazil nut supplementation. We also demonstrated that selenoprotein polymorphisms lead to different responses in mRNA expression of selenoproteins following Se supplementation with Brazil nut.

Keywords: Statin, Brazil nut, selenoproteins, single nucleotide polymorphisms, oxidative stress

LIST OF DIAGRAMS, FIGURES, AND TABLES

ACRONYM LIST

A	Adenine
ACC	The American College of Cardiology
AHA	American Heart Association
Ala	Alanine
BMI	Body mass index
CK	Creatine Kinase
CWG	Canadian Working Group
CYP2C9	Cytochrome P450 2C9
CYP3A4	Cytochrome P450 3A4
EFSec	Sec-Specific elongation factor
DIO	Iodothyronine deiodinases
DMSe	Dimethyl selenide
G	Guanine
G1	Group 1
G2	Group 2
GSH	Glutathione
GR	Glutathione reductase
GSSeH	Glutathione selenopersulfide
GSSeSG	Selenodiglutathione
HMG-COA	Hydroxy-methyl-glutaryl-coenzyme-A
i6A	Isopentenyl adenosine
Met	Methionine
mRNA	Messenger RNA

MSRB1	Methionine sulfoxide reductase B1
MDA	Malondiadehyde
NADPH	Nicotinamide adenine dinucleotide phosphate
NLA	National Lipid Association
RBR	Brazilian Clinical Trial Registry
RDA	Recommended Dietary Allowances
RDI	Recommended Dietary Intake
RYR	Ryanodine receptors
SAMS	Statin-adverse muscle symptoms
SBP2	SECIS-binding protein 2
Se	Selenium
Sec/SeCys	Selenocysteine
SeMet	Selenomethionine
SECIS	SeCys Insertion Sequence
SELENOF	15-kDa selenoprotein
SELENOH	Selenoprotein H
SELENOI	Selenoprotein I
SELENOK	Selenoprotein K
SELENOM	Selenoprotein M
SELENON	Selenoprotein N
SELENOO	Selenoprotein O
SELENOP	Selenoprotein P
SELENOS	Selenoprotein S

SELENOT	Selenoprotein T
SELENOV	Selenoprotein V
SELENOW	Selenoprotein W
SEM	Standard error of the mean
SEPHS2	Selenophosphate synthetase
SD	Standard deviation
SNP	Single nucleotide polymorphisms
SOD	Superoxide dismutase
SUS	Brazilian Unified Health System
Thr	Threonine
TXNRD	Thioredoxin reductases
UPC	University Hospital Clinical Research Unit
USA	United States of America
UTR	Untranslated region
WHO	World Health Organization

SUMMARY

1 Introduction

1.1 Interaction between Statin-associated muscle symptoms (SAMS), selenoproteins and genetic variations

Statins are pharmacological drugs that inhibit the hydroxy-methyl-glutaryl-coenzyme-A (HMG-CoA) reductase enzyme, and are the most prescribed and effective pharmacological therapy for the treatment of hypercholesterolemia and prevention of cardiovascular events [1–3]. Statins are specific and potent inhibitors of the rate-limiting step of cholesterol biosynthesis, namely the reduction of HMG-CoA to mevalonic acid by the enzyme HMG-CoA reductase [4]. Despite their favorable overall safety profile [1,5,6], statins evoke distinct side effects whose molecular origins have remained unsettled [7].

Statin-associated muscle symptoms (SAMS) are the most commonly reported adverse effect of statin, present in 10% to 25%-29% of patients receiving statin therapy in the clinical practice and according to observational studies [2,6,8]. Varying definitions and terms are used to define the SAMS and often vary by guidelines [8]. The American College of Cardiology (ACC) and the American Heart Association (AHA), a Canadian Working Group (CWG), and the National Lipid Association (NLA) have proposed definitions for SAMS [2]. The definitions range from myalgia to marked creatine kinase (CK) elevations and/or clinical rhabdomyolysis [2,6]. CK is an enzyme found in both cytosol and mitochondria of tissues where energy demands are high, most notably skeletal muscle. Elevated serum CK activity can indicate muscular tissue damage and are observed in several pathological conditions, including SAMS [2,9,10]. However, the presence of SAMS can occur even with normal or slightly elevated levels of serum CK [1,2,11]. Confirmation of SAMS remains a challenge in the absence of validated tests and a specific, sensitive biomarker [4]. Thus, the diagnostic of SAMS still is subjective and based on clinical manifestations such as myalgia, cramps, and weakness [2,6,8].

Symptoms of statin-related myopathy usually occur soon after initiation of statin therapy. However, symptoms may also appear after years of treatment [12] and usually dissipate after cessation of therapy, although it may take several months to totally resolve [2]. Several factors can increase the risk of SAMS, including high doses, the interaction

of statins with other drugs that inhibit statin catabolism, hypothyroidism, reduced muscle mass and increased physical activity. Moreover, advanced age, female sex, and alcohol consumption can increase the risk of SAMS [2,8].

With the exception of pravastatin, statins are metabolized by the cytochrome P450 pathway, which transforms lipophilic compounds into hydrophobic compounds in the liver for excretion [1,2]. Atorvastatin, lovastatin and simvastatin are metabolized primarily by cytochrome P450 3A4 (CYP3A4) [2,8,13]. Medications that are also metabolized by CYP3A4, including cyclosporine, erythromycin and other macrolide antibiotics, azole antifungals, diltiazem and other nondihydropyridine calcium channel blockers, ritonavir and other protease inhibitors, nefazodone, and grapefruit juice [14], can interact with statins, increasing the risk of SAMS [2,8,14]. Other statins, such as fluvastatin, pitavastatin and rosuvastatin, are minimally metabolized by CYP2C9 and have fewer statin–drug interactions [2,8,12]. SAMS are most commonly observed with simvastatin therapy than other available statins [2], due to its high lipophilicity that displays greater levels of passive diffusion across cellular membranes, increasing the distribution of the medication [15].

The mechanisms of SAMS development are unclear [3], but several hypotheses have been proposed, including a possible mitochondrial disfunction and cellular energy utilization related to the depletion of coenzyme Q10, oxidative stress, decreased intracellular concentrations of cholesterol, impairment of calcium homeostasis, immune response, up-regulation of atrogin-1 gene, and aberrant expression of ryanodine receptors (RyR) [6,16]. Other potential mechanisms include the decrease of downstream intermediaries, such as mevalonate isoprenoids, dolichol, and side chain of coenzyme Q [6,12].

One of the products derived from mevalonate, isopentenyl pyrophosphate, is responsible for isopentenylation of adenosine at position 37 whereupon creates isopentenyl adenosine (i6A). i6A is required for efficient translation of selenocysteine (Sec) codons by $tRNA^{[Ser]Sec}$ which decodes specific UGA codons and inserts selenocysteine into designated proteins defined as selenoproteins [7,17–21]. When this pathway is inhibited, diminished availability of selenoproteins may occur [7,17].

The significance of selenoproteins for optimal human health is mainly due to their antioxidant activity, protecting against harmful effect of free radicals [21,22]. They also have anti-inflammatory, chemopreventive, and antiviral properties and are related to the improvement of immune responses. The health-related characteristics of selenoproteins

include protection against cancer and cardiovascular dysfunctions, proper thyroid function, and muscle disorders [21]. Twenty-five selenoprotein genes have been identified in sequenced mammalian genomes [21–24]. Selenoprotein family in humans include the following members: glutathione peroxidases (GPX1–GPX4 and GPX6), thioredoxin reductases (TXNRD1-2), thioredoxin-glutathione reductase (TXNRD3), iodothyronine deiodinases (DIO1–3), selenophosphate synthetase (SEPHS2), 15-kDa selenoprotein (SELENOF), selenoprotein H (SELENOH), selenoprotein M (SELENOM), selenoprotein N (SELENON), selenoprotein P (SELENOP), selenoprotein T (SELENOT), selenoprotein V (SELENOV), selenoprotein W (SELENOW), selenoprotein S (SELENOS), methionine sulfoxide reductase B1 (MSRB1), selenoprotein O (SELENOO), selenoprotein I (SELENOI), and selenoprotein K (SELENOK) [21,23].

An appealing candidate for curtailed expression in SAMS is selenoprotein N (SELENON), an endoplasmic reticulum-localized selenoprotein whose loss-of-function may be linked to skeletal muscle pathology [19,25]. Despite the progress regarding the physiological function(s) of SELENON in muscle, the pathogenesis leading to SELENON-related myopathies remains largely unknown [26]. Studies have been suggested that SELENON plays a decisive role in the maintenance of adult muscle progenitors. In mature fibers, SELENON could become essential under stress conditions. SELENON deficiency could also lead to oxidation of various proteins, promoting their aggregation or degradation and corrupting several cellular functions, contributing to the replacement of muscle cells by connective tissue [25–27]. Other candidates are selenoprotein P (SELENOP) and glutathione peroxidase (GPX). SELENOP is a plasmatic selenoprotein that circulates in various forms with different glycosylation patterns. Its production by hepatocytes is crucial to Se homeostasis as it sustains retention of Se in the body and promotes its distribution from the liver to extra-hepatic tissues, especially under Se-deficient conditions [28,29]. GPX was the first identified selenoenzyme and catalyzes the biosynthesis of glutathione, a tripeptide that plays an important role in protecting organisms against oxidative action of hydrogen peroxide (H_2O_2) and organic peroxides which could also present reduced expression. Hence, GPX is involved in hydrogen peroxide and hydroperoxide detoxification, protecting against oxidative injury [28].

Selenoprotein expression is regulated by Se supply. Nevertheless, different selenoproteins respond differently to available Se, resulting in a hierarchy in the response of selenoproteins to Se supply [30,31]. This difference in regulation of selenoprotein

expression may reflect their physiological importance, and during deficiency states, the ones ranked high in the hierarchy have preference for synthesis [30].

Although Se is an essential element naturally occurring in the body, its endogenous level and supply among populations is very diverse, depending on a number of factors. Se levels could affect the normal course of biochemical and physiological processes [31,32]. Therefore, the relationship between Se status and health effects is represented by a U-shaped curve that suggests prevalence of health pathologies associated with Se deficiency as well as its excess [31,33]. Recommended dietary intake (RDI) value of Se for adults in USA and Europe is 55 μg/day [22,31]. According to the World Health Organization (WHO), 19 μg of Se per day is the minimal requirement to prevent the diseases associated with Se deficiency while a daily dose of 400 μg is considered harmless [22,34]. It is fundamental to highlight that the primary source of Se is an appropriately selected and balanced diet that covers the requirement for this element [22].

Due to the widespread deficiency of Se in humans, different dietary Se compounds from animal foods and plants have been suggested, such as selenite, Sec, and selenomethionine (SeMet) [22,33]. One dietary supplement that has become popular among recent studies is the Brazil nut [33,35]. Brazil nut (*Bertholletia excelsa*, H.B.K.) is an Amazonian native species, which is part of the Lecythidaceae family and has been described as one of the most Se-concentrated food sources [36]. Brazil nuts may contain up to 96% of Se as SeMet and its metabolites as selenocystathionine and γ- glutamyl-SeMet [36]. Such characteristics contribute to Brazil nuts being considered an important food source. Besides being found in high concentration, SeMet also has high bioavailability and low toxicity [33,35,36]. Hence, the potential health benefits of supplementation with Brazil nuts includes antioxidant and anti-inflammatory effects, reduction of cardiovascular disease risk factors, chemoprotective effect, reduction of diabetes risk, brain function, among others [30,35–40].

Although the functional significance of blood Se status is positively related with Se intake, additional modifiers may influence status, such as overall health, interindividual variations (age, sex), environmental exposure, medication, other components of diet, physical activity etc [31]. Moreover, the large individual variation in response to Se supplementation potentially points to the presence of selenoprotein gene variants [31].

Genetic polymorphisms have been recognized as an important source of inter-individual variation in response to nutritional supplementation. Several single nucleotide polymorphisms (SNPs) in selenoprotein genes could give rise to interindividual variations in Se metabolism and response to Se supplementation [30,35,41]. In particular, SNPs in *GPX1* (encoding GPX1) and *SELENOP* (encoding SELENOP) have been shown to affect blood selenium or selenoprotein levels in response to supplementation. The *GPX1* rs1050450 polymorphism leads to a proline (Pro) to leucine (Leu) substitution at position 197, which could result in reduced enzyme activity and higher DNA damage levels [42]. This alteration may be responsible for GPx1 conformational changes because Pro is the only amino acid without a free unsubstituted amino group on the α-carbon atom and it is known to promote unique folding in the secondary structure of peptides [43,44].

The results of studies that have investigated the associations between GPX activity and the *GPX1* rs1050450 polymorphism are inconsistent. Erythrocyte GPX activity has been examined in some populations and no significant differences among the genotype profiles could be demonstrated in some studies, whereas others showed this association [43,44]. The impact of variation rs3877899 in codon 234 of *SELENOP*, conferring an alanine (Ala) to threonine (Thr) change, and the rs7579 variation that leads to a guanine (G) to adenine (A) transition within the 3' untranslated region (UTR) of the SELENOP mRNA, may result in changes in Se metabolism [41,45]. Méplan et al. (2007), suggested that SNP rs3877899 affects the stability of SELENOP, possibly through some post-translational modifications, which in turn could affect protein levels [41]. On the other hand, the SNP rs7579 could affect the efficiency of SELENOP synthesis in response to increased intake in Se by inducing a change in the SECIS function [41]. Alterations in SELENOP concentration or function would be expected to change Se supply to different tissues and therefore expression of other selenoproteins. Nevertheless, the involved mechanisms are not fully elucidated [41].

Taken together, the information presented herein indicates that SAMS characterization is complex and involve several factors that could interfere in the mechanism which statins promote adverse muscle symptoms. Alterations in selenoprotein biosynthesis as a consequence of statin use may account for the side effects of statins, an observation confirmed by modifications in the selenoprotein mRNA levels. Although selenoprotein homeostasis are dependent on Se status, the response to Se supplementation vary among individuals, indicating the occurrence of selenoprotein gene variants. Understanding of the interaction between SAMS, selenoproteins and genetic

variations could, therefore, lead to a more individual approach to Se supplementation in clinical practice.

Graphic abstract

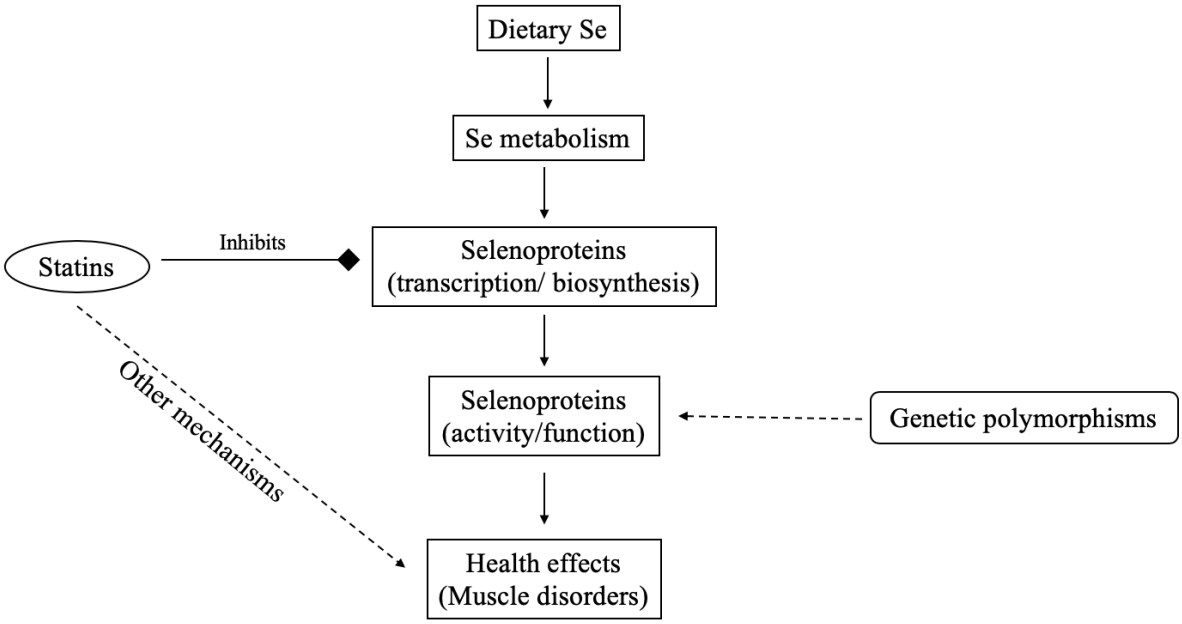


Diagram 1. Schematization of the influence of Se supplementation, statin and genetic polymorphisms on selenoproteins homeostasis with consequences for individual's health conditions, specifically muscle related.

1.2 Selenium: Which form is that?

Selenium (Se), name derived from the Greek word "Selene," was first identified as a by-product of sulfuric acid production by a Swedish chemist Jacob Berzelius in 1817 [22,29,32,34]. Increased interest in the biological role of Se was observed in the 1950s, when it was discovered that this element exerts toxic effects [22]. In 1973, it was discovered that Se is a component required for GPX activity, an enzyme playing a major role in the protection against oxidative injury. Since then, Se has been the subject of numerous scientific investigations, that in general have demonstrated its crucial role in human health and disease [29,34].

Recently, studies have considered different aspects of Se speciation and biological activity, including the pharmacology of synthetic organoselenium compounds, the

antioxidant and pro-oxidant properties of inorganic selenium and oxo-Se compounds, and the speciation of Se in food and supplements and their metabolism in mammals [33].

Most of the Se intake derives from the diet [46]. Food of plant and animal origin are the main sources of this element. Plants are able to take up from the soil both selenate and selenite. Selenate can be easily reduced to selenide. Then selenide can be converted to Sec that can follow two pathways: in the first one, it can enter the Met pathway in order to synthesize SeMet via selenocystathionine and selenohomocysteine. The second possibility consists in the methylation of Cys with the formation of methylselenocysteine [46]. Sec is predominant in products of animal origin [22]. SeMet, Sec, selenite, and selenate constitute the bioavailable Se in our food intake, and are the most intensively studied Se compounds in laboratory and epidemiological studies of disease prevention and treatment [23,33]. The dietary Se compounds differ significantly in their metabolic pathways and in their abilities to produce various Se metabolites [29,33].

The metabolic routes of Se compounds could be divided into two parts: downstream and upstream of the selenide intermediate [33]. In the downstream metabolic pathways, selenoprotein synthesis and excretion pathways are common to all dietary Se compounds [33]. Selenoprotein synthesis is unique due to the insertion of a Sec residue at the UGA codon, which is often read as a stop codon in protein synthesis . Two distinct pathways are available for excretion of Se: the methylation pathway and the selenosugar pathway [28,33]. The biological significance of selenosugars is unknown, but methylation is considered a detoxification pathway due to the low toxicity of the methylated metabolites [28]. The methylation pathway generates dimethyl selenide, which is excreted via the breath, and trimethyl selenonium in the urine [33].

The upstream metabolic pathways of the dietary Se compounds converge on selenide but are very distinct [33]. Selenite and selenate are inorganic Se forms commonly used for Se supplementation and have slightly different metabolic properties [28]. Selenite was the first Se compound to undergo extensive investigation of its metabolism. Reduction of selenite is dependent on the availability of glutathione (GSH) and the presence of GSH-metabolizing enzymes. The reaction of selenite and GSH was shown to produce selenodiglutathione, which could then be reduced by nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione reductase (GR) to glutathione selenenyl sulfide. Finally, selenide is produced in a system containing selenite, GSH, NADPH and GR suggesting that the selenide could act as a precursor for the biosynthesis of other Se compounds [33].

SeMet is the principal chemical form of Se in most human diet [28,33]. Interestingly, Brazil nut (*Bertholletia excelsa*, H.B.K.) has been described as one of the most Se-concentrated food sources and may contain up to 96% of Se as SeMet, and its metabolites as selenocystathionine and γ- glutamyl-SeMet [36]. Once ingested, SeMet is absorbed via intestinal methionine transporters and enters the methionine pool in the body [28]. Any SeMet that is not immediately metabolized is incorporated into organs with high rates of protein synthesis such as the skeletal muscles, erythrocytes, pancreas, liver, kidney, stomach and the gastrointestinal mucosa [47]. As the Se analogue of methionine (Met), SeMet can be non-specifically incorporated into proteins in place of Met. Thus, proteins act as a SeMet storage until degradation. Another major SeMet metabolic pathway is the methionine cycle and transsulfuration pathway. These pathways lead to the production of Sec [33]. Sec ingested with the diet or derived from SeMet cannot be used in its original form to synthesize human selenoproteins in our bodies. Sec has to be first converted into selenide by the action of the enzyme Sec lyase (SCLY) and then phosphorylated to selenophosphate by the enzyme selenophosphate synthetase 2 (SEPHS2) [46].

The essential biological importance of Se is associated with its occurrence in proteins and enzymes, generally with Sec at the active site [22,48]. Multiple, specialized alterations to the translational process are required to maintain the ability to incorporate Sec into proteins [48]. The first challenge to selenoprotein expression is that Sec is encoded by the UGA stop codon and therefore requires a translational recoding event to synthesize the protein. The UGA recoding is strictly dependent on an RNA stem-loop structure, the Sec Insertion Sequence (SECIS), which is found in the 3' untranslated region of eukaryotic selenoprotein messages [22,48]. It also requires a specific elongation factor (EFSec), a Sec-tRNA and SECIS-binding protein 2 (SBP2). Several other proteins involved in the tRNA synthesis, Sec recycling and regulation of recoding efficiency have also been identified [48].

Knowledge of the metabolic products of Se compounds and their biological activities is crucial to understanding Se biology. Research on Se during the last few years has produced a greater evidence demonstrating the important role that Se and its metabolites play in human health and diseases. These findings could be relevant to direct future nutritional interventions aiming towards a more individual and specific approach to Se supplementation.

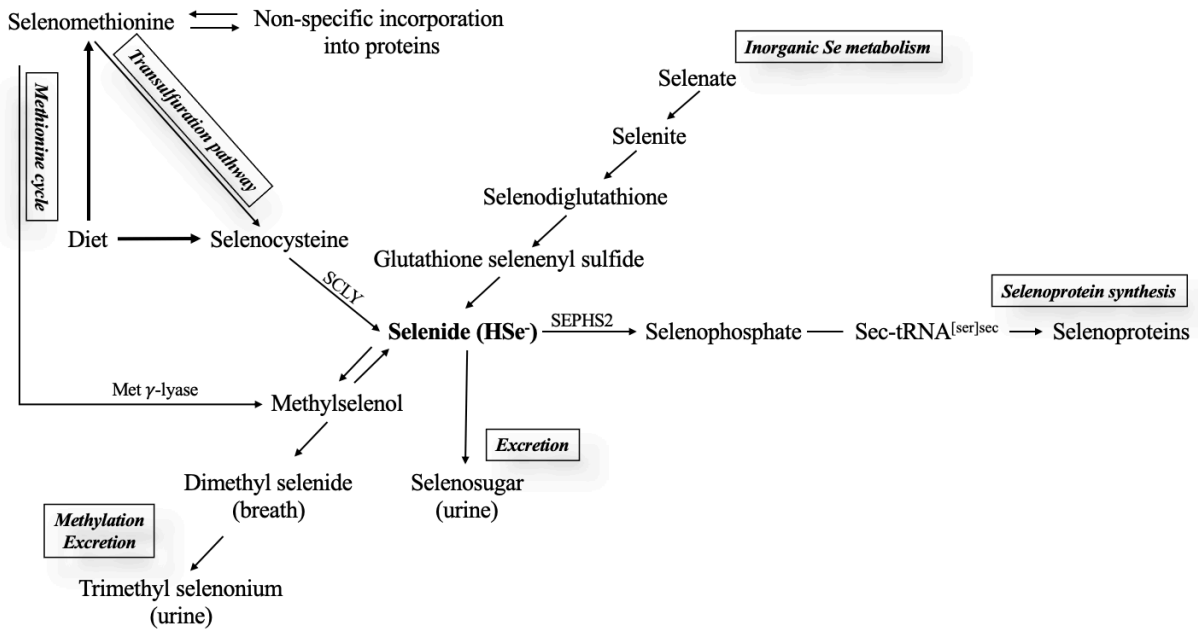


Diagram 2. Metabolic pathway of dietary Se compounds. Adapted from [28,33,49]. Abbreviations: Se, selenium; SCLY, selenocysteine lyase; SEPHS2, selenophosphate synthetase-2; Met γ-lyase: methionine γ-lyase.

2 Aims

2.1 General

This study aimed to investigate if Se supplementation could modulate biomarkers of Se status such as blood Se, GPX activity, and mRNA expression of selenoproteins.

2.2 Specific

- ✓ To investigate if consumption of Brazil nuts modulates serum creatine kinase (CK) activity of patients regularly using statins.

- ✓ To evaluate if nucleotide variations in selenoprotein genes modulate the response to Brazil nuts in patients regularly using statins.

3 Material and Methods

3.1 Ethic Statement

All procedures followed in this study have been performed in accordance with the ethical standards as laid down in the Declaration of Helsink. The study protocol was approved by the Ethics Committee of the Ribeirão Preto Medical School at the University of São Paulo, Brazil (protocol number CAAE: 56221916.5.0000.5440). Informed consent was obtained from all individual participants. The trial was registered at Brazilian Clinical Trial Registry under identification number (RBR-7rwgzt).

3.2. Study population

This was an open, non-randomized, controlled, single-center study conducted at the Ribeirão Preto Medical School University Hospital, University of São Paulo, Brazil, from January 2017 to July 2017. Inclusion criteria were both sexes with age between 18-60 years old and in regular use of statin. Out of the initial 55 eligible participants identified, 7 participants were excluded because they were not using the statin continuously or had the type or dosage of medication changed by the doctor in recent months, and other 4 refused to participate.

This study has performed two main distinct evaluations. In the first one (**Figure 1**), all the 44 participants were allocated according with measurements of CK activity previously obtained from medical records into Group 1 (n= 22), patients with increased CK activity (>189 U/L), or Group 2: (n= 22), patients with normal CK activity (<189 U/L). In the second one, 32 eligible participants were previously genotyped and allocated according to their genotypes (wild type, heterozygous, and variant) for SNPs in GPX1 (rs1050450), SELENOP (rs3877899), and SELENOP (rs7579).

Patients with nuts allergy, taking multivitamins and mineral supplements, in use of antibiotics or other medications that are also metabolized by cytochrome P450 3A4 (CYP3A4), in current tobacco and alcohol consumption, athletes or individuals practicing intense physical activity, with serious cardiac complications, thyroid disorders, liver disease, kidney failure and, neoplasia were not included in the study.

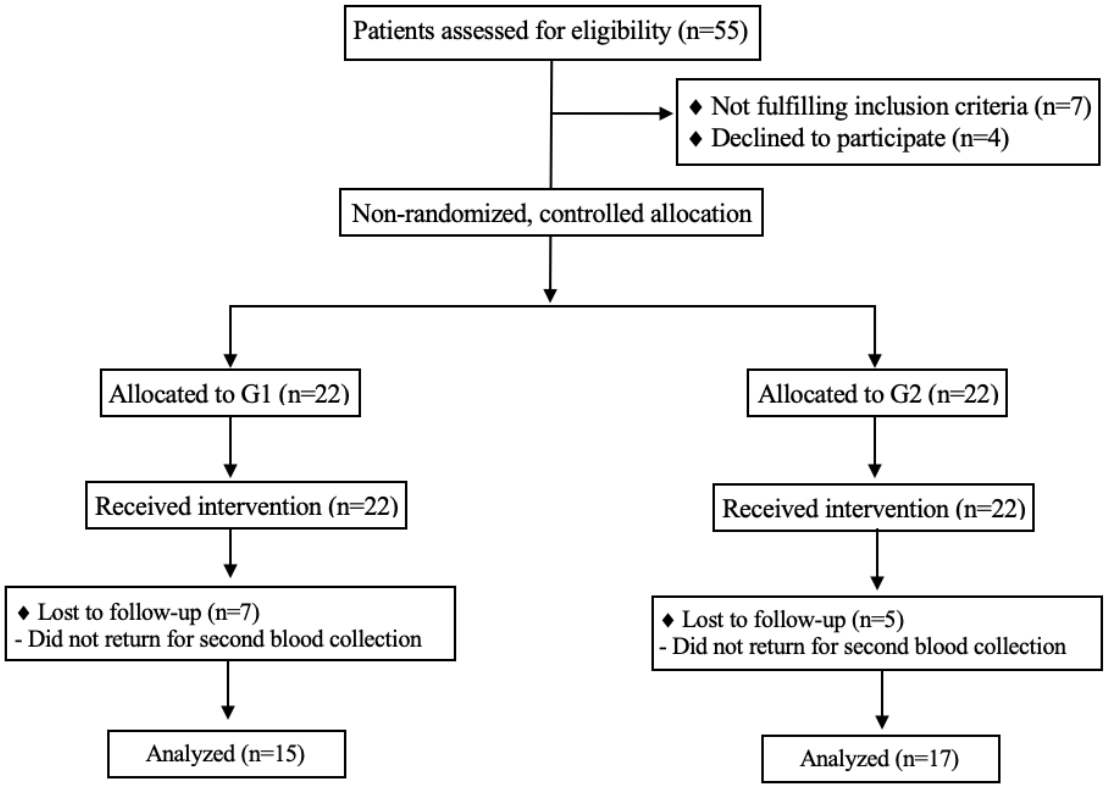


Figure 1. Flowchart of inclusion and follow-up.

3.3. Study Procedures

At the beginning of the study clinical evaluation was performed and an interview was conducted in order to obtain general information for anamnesis. During this baseline visit, height and weight were measured for each participant and these values were used to calculate body mass index (BMI). We also collected venous blood samples to perform biochemical evaluations. Then, all the participants were supplemented with Brazil nuts. The volunteers received, at no cost, three vacuum sealed bags containing Brazil nuts enough for all intervention period. They were oriented to consume one Brazil nut daily for 3 months. Periodically, researchers contacted the participants to monitor the compliance of Brazil nuts consumption. All subjects were instructed to maintain their normal diet and to avoid additional nuts during the study. Once the 3-month study period was completed, we again assessed anthropometric data and collected a second blood sample to perform post supplementation biochemical measurements.

3.4. Centesimal Composition of Brazil nuts

Brazil nuts were originally from the Brazilian state of Amazon and were acquired in partnership with the Excelsa Institute. A random sample of Brazil nuts used in the study was analyzed in triplicate according to AOAC [50] for determining humidity, ash, protein, and lipids. The total carbohydrates were calculated by difference (100—total grams of humidity, protein, lipids, and ash), including fiber fraction.

3.5. Sample collection

The collection of blood samples occurred before and after 3 months supplementation with Brazil nuts and was performed in the University Hospital Clinical Research Unit (UPC) after 8 hours of fasting and the separation of whole blood to obtain plasma and erythrocytes occurred immediately after collection. An aliquot of 500 µL of whole blood was stored into 1.5 mL sterile plastic tubes used for RNA extraction and subsequent gene expression. The samples were stored at -80° until the time of analysis.

3.5.1. Biochemical evaluation

Commercially available kits by Labtest (Minas Gerais, Brazil) were performed following the manufacturer protocol to measure serum CK activity (Cat. No. 117) by UV kinetic increasing reaction according to the International Federation of Clinical Chemistry and Laboratory Medicine and quantitative end point colorimetric assay of total cholesterol in serum (Cat. No. 76).

3.5.2. Selenoprotein gene expression

Total RNA was isolated from whole blood using TRIzol reagent (Invitrogen Life Technologies) and final concentration was measured in a NanoDrop ND 1000 spectrophotometer (NanoDrop ND 1000, Thermo Scientific, Wilmington, DE, USA). RNA integrity was considered acceptable when the absorbance ratios in 260 and 280 nm wavelengths were between 1.8 and 2. cDNA was synthesized by reverse transcription polymerase chain reaction (PCR) using the High Capacity Reverse Transcriptase kit (Applied Biosystems, Thermo Scientific, Foster City, CA, USA). Analysis of gene expression was performed by real-time quantitative PCR using Taqman Gene expression Assays for *GPX1* (Hs00829989_gH), *SELENOP* (Hs01032845_m1) and, *SELENON*

(Hs00898723_ml). *β-acti*n (4352935E) mRNA expression was used as a reference gene. The relative expressions were calculated using the $2^{-\Delta\Delta Ct}$ method.

3.5.3. Genotyping of the rs1050450, rs7579 and rs3877899 SNPs

Isolation of DNA from whole blood was carried out using a PureLink Genomic DNA kit (Invitrogen, Life Technologies Inc., Carlsbad, CA, USA), and the concentration was measured using a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Polymorphisms in GPX1 (rs1050450) and SELENOP (rs7579 and rs3877899) genes were determined by real-time quantitative polymerase chain reaction (qPCR) with TaqMan SNP Genotyping assays (Life Technologies, Foster City, CA, USA). The assays were obtained as pre-designed from Applied Biosystems for rs7579 and rs3877899 (ID Assays C___8806056_10 and C___2841533_10, respectively) and custom-made through Custom TaqMan® Genomic Assays service for rs1050450 (GPX1/rs1050450: primers: F: 5′-TGT GCC CCC TAC GCA GGT ACA-3′, R: 5′-CCC CCG AGA CAG CAG CA-3′, T-allele: 5′-FAM-CTG TCT CAA GGG CTC AGC TGT-MGB-3′, C-allele: 5′-VIC-CTG TCT CAA GGG CCC AGC TGT-MGB- 3′). The qPCR reaction contained 3.125 µL of 1Å~ GoTaq® Master Mix (Promega, Madison, WI, USA), 0.156 µL of the 20Å~ SNP Genotyping Assay (Life Technologies, Foster City, CA, USA) and 20 ng of genomic DNA in a 6.741 µL total reaction volume. Samples were assayed along with no-template and HapMap controls and run on Applied Biosystems 7500 real-time PCR system (Applied Biosciences/Thermo Fisher Scientific, Waltham, MA, USA) under the following conditions: an initial enzyme activation step at 50° for 2 min, 95 °C for 10 min followed by 40 cycles at 92 °C for 15 s and 60 °C for 1 min for annealing and extension. The validity of qPCR results was verified by direct sequencing 5% of the samples using the BigDye FN Sequencing Kit (PE Applied Biosystems) and using the following primers: primer forward 5′-CATCGAAGCCCTGCTGTCT-3′; primer reverse 5′-CACTGCAACTGCCAAGCA-3′. The agreement of the genotypes determined for the blinded quality control samples was 100%

3.5.4. Evaluation of oxidative damage: Malondialdehyde (MDA)

For dosage of malondialdehyde 100µl plasma sample were used. Three hundred microliters of 10mM solution of 1-methyl-phenylindole was added in acetonitrile and

methanol (2:1, v/v) and 75µl of hydrochloric acid (HCl) pure (37%). The tubes were vortexed and incubated in water bath at 45 ° C for 40 minutes. After the bath, samples were cooled in ice and then centrifuged at 4000rpm for 10 minutes. Supernatant was read for absorbance at a wavelength of 586nm. Malondialdehyde concentration was calculated by comparing it to a curve 1,1,3,3 - tetramethoxypropane (TMP) hydrolyzate.

3.5.5. Glutathione Peroxidase activity (GPX)

GPX activity was measured in erythrocytes according to Paglia and Valentine (1976) [51]. The method is based on the reaction in which GPX catalyzes the oxidation of reduced GSH by a hydroperoxide. In the presence of GPX nicotinamide adenine dinucleotide phosphate (NADPH), oxidized GSH is converted to the reduced form with a concomitant oxidation of NADPH NADPH +. The decrease in absorbance at 340 nm was then measured.

3.5.6. Superoxide dismutase (SOD)

Plasma SOD levels was evaluated using a commercially available kit (19160, Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's instruction. The assay utilizes a tetrazolium salt with superoxide anion. The rate of WST-1 reduction is linearly related to the inhibition activity of xanthine oxidase (XO) by SOD. SOD catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen resulting in decrease of WST-1 reduction. This inhibition activity of SOD was measured by colorimetric method at OD 450 nm.

3.5.7. Selenium

The determination of the total concentration of selenium in plasma and erythrocytes was performed by an Inductively Coupled Plasma Mass Spectrometry (ICP-MS), fitted with a dynamic reaction cell (DRC) (Perkin Elmer Sciex Norwalk, CT USA). Samples were diluted in the ratio 1:50 with a solution containing Triton X-100 0.01% (v/v), HNO_3 0.05% (v/v) and 10mg / L-1 rhodium (Rh) as an internal standard. The concentration of the analytical calibration standards ranged from 0 to 50 µg/L [52].

3.6. Sample size calculation

The sample size calculation was based on CK activity of patients using cholesterol-lowering medications. We conducted a pilot study with 104 patients selected at University Hospital and from who we evaluated serum CK activity. Patients were classified according to the assay range (Labtest, Minas Gerais, Brazil) as normal: when CK activity were up to 189 U/L or out of range: when CK activity were greater than 189 U/L. Considering mean CK activity in normal group (66.7U/L) and out of range group (229.3 U/L), standard deviation (SD) 114 U/L, alpha 5%, and 99% power the sample size was calculated as 12 for each group.

3.8. Statistical analysis

In the first segment of the study, continuous variables were tested for normality using the Shapiro-Wilk test and nonparametric tests were used when appropriate. The data were presented as mean ± SD with its respective p-values. Baseline characteristics and baseline measured outcomes were compared with post-treatment using paired t-test or Wilcoxon test. Pearson's correlation coefficient or Spearman's correlation was calculated according to the presence or absence of a normal distribution. To compare averages of gene expression results Wilcoxon test was applied. Values are expressed as median, minimum and maximum, and interquartile range. Data were plotted in Statistical Package for the Social Sciences software version 14.0 (SPSS, Chicago, IL, USA) and GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Differences were considered significant if $p<0.05$.

In the second segment of the study, for the analysis of selenoproteins gene variants, volunteers were selected for gene expression analysis based on their genotype that had been determined previously. For all statistical analysis, individuals who were homozygous (wild type or variant) and heterozygous for the rare alleles were combined together in one category, leaving the homozygous dominant in another category in order to increase the statistical power. The Chi-square test was used to determine whether genotype frequencies followed the Hardy–Weinberg Equilibrium. A repeated-measures analysis of variance (ANOVA) adjusted for multiple comparison with Bonferroni test was used to determine the intragroup-effect between genotype and erythrocyte GPX enzyme activity, erythrocyte or plasma selenium, CK activity and MDA. The post-Se

supplementation data was compared with baseline according genotype and was assessed by the paired Student's t-test or Wilcoxon test. Pearson's test was applied to determine the associations between genotype, GPX enzyme activity, and erythrocyte selenium during the intervention period. Data were plotted in Statistical Package for the Social Sciences software version 14.0 (SPSS, Chicago, IL, USA) and GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Differences were considered significant if $p<0.05$.

4 Results

4.1. Changes in CK activity, oxidative stress, and selenoprotein mRNA expression after Brazil nut consumption

The Se content and centesimal composition of Brazil nuts are shown in **Table 1**.The average weight of Brazil nuts was 5 g, therefore each nut provided approximately 290µg of Se, which is higher than the RDA (Recommended Dietary Allowances) for adults (55µg/d) but less than upper limit (400µg/d) established by the Institute of Medicine (IOM), 2000 [53].

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

Nutrient	**Mean ± SD**
Energy (kcal)	696.0 ± 4.3
Carbohydrates (g)	12.9 ± 0.4
Proteins (g)	10.9 ± 0.51
Lipids (g)	71.6 ± 0.09
Ash (%)	2.91± 0.23
Humidity (%)	1.75 ± 0.33
Selenium (µg/g)	58.1 ± 2.1

Values are mean ± standard deviation (SD). Except for selenium, all nutrients were calculated considering 100g of Brazil nut.

A total of 32 patients completed the entire study protocol. Compliance of Brazil nuts consumption was reported by patients at the end of the study. Moreover, increased Se levels in plasma and erythrocyte post treatment (**Table 2**) confirmed the adherence to the supplementation. According to the analysis of non-consecutive dietary food records, at baseline, the energy intake of participants in G1 (2050 ± 180.2 kcal) and G2 (2130 ± 237.3 kcal) was comparable (p=0.9591) and, all participants maintained their normal diet during the period of supplementation (data not shown). At the beginning of the study, participants were asked about the practice of physical activity. Most of the participants in G1 and G2 reported not practicing regular physical activity and, they did not change this habit during the study. Regarding the type of cholesterol-lowering medication used by study participants, 71.9% was using Simvastatin (40mg) and 28.1% Atorvastatin (40mg).

When stratifying the type of statin according to the study group, in G1, 80% was using Simvastatin and 20% Atorvastatin, and in G2, 64.7% was using Simvastatin and 35.3% Atorvastatin. We did not find differences in the type of statin between groups (p=0.3369). It is relevant to consider that during the 3-month period of supplementation there were no changes in medical prescription of statin dosage or type. Females constituted 40.6% of the group and mean age was 50.1 ± 7.6 years. The study groups were not different in relation to sex (p=0.07) or age (p=0.78).

The body composition, CK activity, total cholesterol, Se status, and oxidative stress parameters of the volunteers pre and post supplementation are summarized in **Table 2**. According to BMI, patients in G1 were classified as with obesity while in G2 patients were classified as overweight. After supplementation with Brazil nuts, individuals in both groups lost a significantly amount of weight with consequent reduction in BMI. In both G1 and G2 groups, the supplementation contributed to decreased levels of CK activity in serum. The levels of total cholesterol were adequate at the baseline and remained unchanged in both groups after supplementation.

Table 2: Body composition, CK, lipid profile, Se status, and oxidative stress parameters according to group after 90 days of Brazil nut supplementation

	Group 1 (n=15)	Group 2 (n=17)

Parameters	*Pre* Mean ± SD	Post Mean ± SD	*P-value*	*Pre* Mean ± SD	Post Mean ± SD	*P-value*
Weight (kg)	95.1 ± 21.6	92.2 ± 20.3	0.04	81.1 ± 20.4	78.1 ± 20.9	0.0001
BMI (kg/m^2)	33.1 ± 5.9	31.9 ± 5.2	0.04	29.4 ± 7.4	28.3 ± 7.4	0.0002
CK (U/L)†	347.9 ± 133.3	228.2± 122.5	0.005	102.9 ± 45.7	78.3 ± 34.2	0.005
Cholesterol (mg/dL)	177.0 ± 39.3	176.5 ± 45.4	0.97	181.2 ± 44.3	152.8 ± 25.3	0.056
Plasma Se (µg/L)	81.5 ± 3.7	258.9 ± 73.8	<0.0001*	88.5 ± 6.9	286.5 ± 146.5	<0.0001*
Erythrocyte Se (µg/L)	82.33 ± 11.5	298.3 ± 84.5	<0.0001	91.3 ± 18.2	388.1 ± 208.4	<0.0001
MDA (nmol/mL)	3.3 ± 1.1	2.1 ± 1.1	0.003	2.8 ± 0.6	1.7 ± 0.9	<0.0001
GPX activity (U P/g)	24.7.5 ± 3.0	29.8 ± 6.0	0.01*	23.5 ± 4.4	35.4 ± 29.5	0.005*
SOD (U/L)	1.4 ± 0.4	0.3 ± 0.1	<0.0001	1.6 ± 0.8	0.4 ± 0.3	0.0001

Note: Paired t-test; Wilcoxon *; t test for independent samples †. Values are expressed as mean ± standard deviation. Pre: pre-treatment; Post: post-treatment; SD: standard deviation. BMI: body mass index; CK: creatine kinase activity; Se: selenium; MDA: malondialdehyde; GPX: glutathione peroxidase; SOD: superoxide dismutase.

Biomarkers of selenium status and oxidative stress

The biomarkers of Se status and oxidative stress measured at baseline and post treatment are shown in **Table 2**. At baseline, the majority of patients showed adequate Se levels in plasma (>80-95 µg/L), only 3 individuals were slightly deficient [54]. Changes in plasma and erythrocyte Se concentrations were significantly positive in both groups after supplementation ($p<0.0001$) and enough to restore selenium deficiency. The levels of GPX activity also increased in both groups post treatment (G1 $p<0.01$, G2 $p<0.005$).

Among the parameters related to oxidative stress, we observed reduced levels of MDA and SOD in both groups after supplementation. Changes in biomarkers of Se status after supplementation were associated with changes in antioxidant parameters in different ways (**Table 3**). While increased levels of erythrocyte selenium were positively associated with erythrocyte GPX activity ($r=0.54$; $p<0.002$) higher levels of plasma selenium were negatively associated with SOD ($r=-44$; $p<0.01$). No correlations were

observed between changes in Se status and changes in CK activity but, interestingly, we observed a moderately negative association between CK activity and GPX activity (r=-41; p<0.02) which in turn, was associated with increased selenium levels (**Table 3**).

Table 3. Correlation analysis between changes in the oxidative stress parameters and changes in Se status

	Correlation (r)					
Parameters	Erythrocyte Se	Serum Se	CK	GPX activity	MDA	SOD
Erythrocyte Se[b]		0.78***	0.05	0.55**	0.15	-0.49**
Serum Se[b]	0.78***		-0.03	0.46*	0.16	-0.44*
CK[b]	0.05	-0.03		-0.41*	0.18	-0.17
GPX[b]	0.55**	0.46*	-0.41*		0.07	-0.40
MDA[a]	0.15	0.16	0.18	0.07		-0.07
SOD[b]	-0.49*	-0.44*	-0.17	-0.40	-0.07	

***p<0.0001; **p<0.005; *p<0.05. [a]Pearson's correlation coefficient. [b]Spearman's correlation. CK: creatine kinase activity; Se: selenium; MDA: malondialdehyde; GPX: glutathione peroxidase; SOD: superoxide dismutase.

Selenoproteins gene expression

Gene expression of three selenoproteins (*GPX1, SELENOP*, and *SELENON*) was analyzed before and after 3 months of Brazil nut supplementation. The results are shown in **Table 4**. Surprisingly, we did not detect changes in the expression of selenoproteins *GPX1, SELENOP*, and *SELENON* after Brazil nut supplementation in both groups.

Table 4: Gene expression of selenoproteins according to study group assessed by quantitative polymerase chain reaction (qPCR) after three months of Brazil nut supplementation

Group 1	
Pre	Post

Gene	Median (min – max)	IQR	Median (min – max)	IQR	p-value
GPX	0.67 (0.30 – 1.3)	0.41	0.61 (0.14 – 2.3)	0.78	0.30
SELENOP	0.05 (0.03 – 0.14)	0.08	0.11 (0.02 – 0.83)	0.36	0.07
SELENON	0.035 (0.001 – 0.08)	0.07	0.03 (0.001 – 0.12)	0.06	0.90
Group 2					
	Pre		Post		
Gene	Median (min – max)	IQR	Median (min – max)	IQR	p-value
GPX	0.23 (0.05 – 0.59)	0.31	0.28 (0.15 – 0.74)	0.21	0.36
SELENOP	0.03 (0.01 – 0.07)	0.04	0.03 (0.01 – 0.31)	0.19	0.44
SELENON	0.03 (0.01 – 0.06)	0.03	0.04 (0.01 – 0.08)	0.03	0.34

Note: Values are expressed as median (minimum – maximum) and interquartile range (IQR) and were normalized to *β-actin* mRNA levels. Wilcoxon test was used to compare averages. Pre: pre-treatment; Post: post-treatment. GPX: glutathione peroxidase; *SELENOP*: selenoprotein P; *SELENON*: selenoprotein N.

4.2. Nucleotide variations in selenoprotein genes (*GPX1* and *SELENOP)*

Thirty-two participants were included in this segment of the study and completed the protocol. The mean age was 50.1 ± 7.6 years, and 59.4% were man. According with BMI, patients were classified as with obesity (31.1 ± 3.8 kg/m^2). According to the analysis of non-consecutive dietary food records, at baseline, the energy intake of participants was 2027.3 ± 302.5 and, all participants maintained their normal diet during the period of supplementation (data not shown). At the beginning of the study, participants were asked about the practice of physical activity. Most of the participants were not practicing regular physical activity (84.4%) and, they did not change this habit during the study. Regarding the type of cholesterol lowering medication used by study participants, 71.9% was using

Simvastatin (40mg) and 28.1% Atorvastatin (40mg). The baseline variables of study population were not different among genotype groups (data not shown).

Genotype distribution and variant allele frequencies for the *GPX1 rs1050450, SELENOP rs7579* and *SELENOP rs3877899* polymorphisms are shown in **Table 5**. The genotype distributions of the studied polymorphisms were in Hardy–Weinberg equilibrium (data not shown).

Table 5. Genotypes and allele frequencies for polymorphisms in *GPX* and *SELENOP* genes of participants (n = 32)

Polymorphism	Wild type	Heterozygous	Variant	Heterozygous + variant	MAF
GPX					
rs1050450	**CC**	**CT**	**TT**	**CT+TT**	**T**
	20 (62.5%)	8 (25%)	4 (12.5%)	12 (37.5%)	0.2500
SELENOP					
rs7579	**GG**	**GA**	**AA**	**GA+AA**	**A**
	17 (54.8%)	12 (38.7%)	2 (6.5%)	14 (45.2%)	0.2580
rs3877899	**CC**	**CT**	**TT**	**CT+TT**	**T**
	7 (21.9%)	23 (71.8%)	2 (6.3%)	25 (78.1%)	0.4218

Note: Categorical variables are presented as n (%). C: cytosine; T: thymine; A: adenine; G: guanine; GPX: glutathione peroxidase; *SELENOP*: selenoprotein P; MAF: minor allele frequency.

The pre and post-Se supplementation and intragroup (between SNP's genotypes) effects in the levels of GPX enzyme activity, plasma Se, whole blood Se and, MDA for each SNP are shown in **Table 6**. We reported previously (Watanabe et al. 2020; accepted article) that the intake of one Brazil nut increased GPX activity and blood Se levels, while influenced the decreased levels of MDA. In the present study, an intragroup comparison demonstrated that the genotype did not influence Se-related variables (blood Se and GPX activity) and MDA levels (p_{Gen}>0.05). Besides that, the highly significant post-supplementation changes for blood Se and MDA levels occurred despite the genotypes. However, in the presence of one or two variant alleles for *SELENOP rs3877899* (CT or TT), and *SELENOP rs7579* (GA or AA) the levels of erythrocyte GPX activity post-supplementation did not increase significantly (p=0.25 and p=0.08 respectively) and the

decreased CK activity levels was not significant (p=0.23 and p=0.13 respectively) when compared to pre-supplementation.

The correlation between GPX activity and erythrocyte Se concentration was influenced by genotype. When we stratified the erythrocyte GPX1 activity by rs1050450 polymorphism, we observed a moderately positive association (r=0.4946; p=0.031) only in CC genotypes.

Table 6. Pre and post-supplementation of GPX activity, plasma Se, erythrocyte Se and, MDA levels according to the studied genotypes

GPX activity (U/g Hb)						
Polymorphism	**Pre**	**Post**	**Change**	**2way ANOVA**		
				P_{int}	P_{Gen}	P_{Sup}
rs1050450						
CC	24.6 ± 4.3	29.1 ± 4.5	4.5 (0.9;8.1)*	0.65	0.34	<0.01
CT+TT	22.8 ± 2.7	28.4 ± 6.8	5.6 (0.9; 10.3)*			
rs3877899						
CC	24.1 ± 3.9	29.2 ± 5.5	5.1 (1.9; 8.2)*	0.78	0.42	<0.01
CT+TT	23.3 ± 3.8	27.5 ± 4.7	4.2 (-2.1; 10.6)			
rs7579						
GG	23.7 ± 4.6	29.9 ± 5.5	6.2 (2.2; 10.2)*	0.29	0.53	<0.01
GA+AA	24.2 ± 2.8	27.8 ± 5.2	3.6 (-0.3; 7.5)			
Plasma selenium (ng/mL)						
Polymorphism	**Pre**	**Post**	**Change**	**2way ANOVA**		
				P_{int}	P_{Gen}	P_{Sup}
rs1050450						
CC	82.9 ± 12.4	294.4 ± 128.6	211.5 (149; 274)*	0.29	0.40	<0.01
CT+TT	88.9 ± 16.0	252.6 ±80.2	163.7 (79; 248)*			

rs3877899						
CC	86.1 ± 14.3	291.1 ± 119.1	205 (147; 263)*	0.36	0.26	<0.01
CT+TT	82.9 ± 12.7	240.0 ± 91.0	157 (50; 264)*			
rs7579						
GG	84.6 ± 12.7	286.3 ± 70.6	201.8 (130; 273)*	0.73	0.75	<0.01
GA+AA	85.5 ± 15.4	272.3 ± 149.8	186.8 (113; 261)*			

Erythrocyte selenium (ng/mL)

Polymorphism	**Pre**	**Post**	**Change**	**2way ANOVA**		
				P_{int}	P_{Gen}	P_{Sup}
rs1050450						
CC	86.4 ± 18.5	399.0 ± 173.0	312.6 (228; 397)*	0.11	0.09	<0.01
CT+TT	85.9 ± 11.1	302.3 ± 103.3	216.5 (105; 328)*			
rs3877899						
CC	87.5 ± 16.7	358.4 ± 177.0	270.9 (190; 322)*	0.46	0.40	<0.01
CT+TT	85.5 ± 13.4	301.7 ± 121.7	216.1 (62; 370)*			
rs7579						
GG	88.1 ± 16.3	367.1 ± 143.5	279 (179; 379)*	0.7	0.61	<0.01
GA+AA	84.9 ± 15.7	340.9 ± 184.1	256 (153; 359)*			

Malondialdehyde (nmol/mL)

Polymorphism	**Pre**	**Post**	**Change**	**2way ANOVA**		
				P_{int}	P_{Gen}	P_{Sup}
rs1050450						
CC	3.0 ± 0.8	2.1 ± 0.9	-0.8 (-1.3; -0.3)*	0.005	0.23	<0.01
CT+TT	3.1 ± 1.0	1.2 ± 0.3	-1.9 (-2.6; -1.1)*			
rs3877899						

CC	3.1 ± 0.9	1.9 ± 0.9	-1.1 (-1.6; -0.6)*	0.36	0.11	<0.01
CT+TT	2.8 ± 0.6	1.2 ± 0.3	-1.6 (-2.6; 0.6)*			
rs7579						
GG	3.0 ± 0.8	1.7 ± 0.8	-1.2 (-1.9; -0.6)*	0.79	0.54	<0.01
GA+AA	3.1 ± 0.9	1.9 ± 0.9	-1.2 (-1.8; -0.5)*			

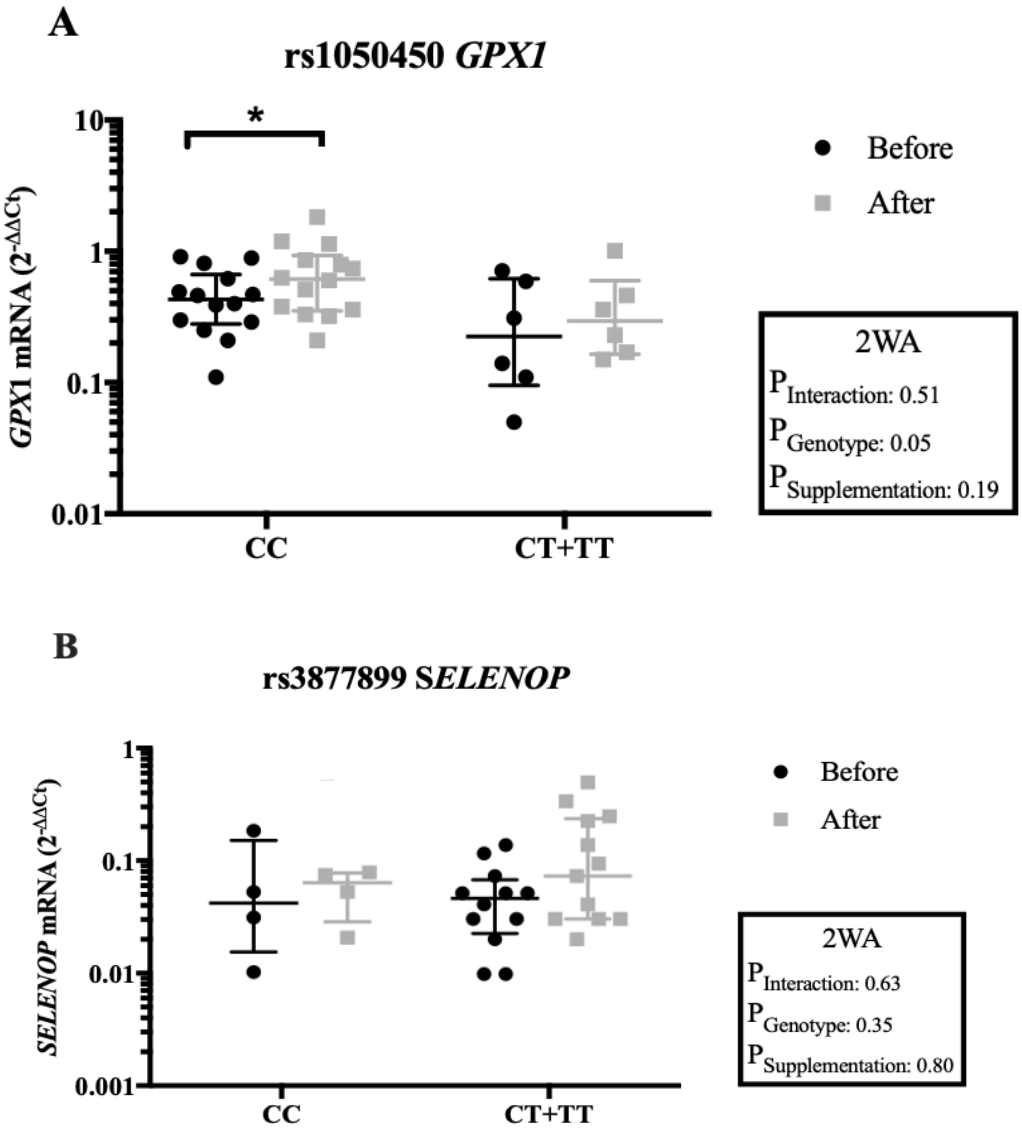
Creatine kinase activity (nmol/mL)

Polymorphism	**Pre**	**Post**	**Change**	**2way ANOVA**		
				P_{int}	P_{Gen}	P_{Sup}
rs1050450						
CC	228.5 ± 35	174.6 ± 30.4	59.7 (0.6; 119.5)*	0.38	0.37	<0.01
CT+TT	206.0 ± 49	109.3 ± 13.47	96.7 (18.2; 175)*			
rs3877899						
CC	220.8 ± 35	152.5 ± 25.3	68.3 (14; 122)*	0.36	0.11	<0.01
CT+TT	218.2 ± 25	163.3 ± 20.2	54.8 (-33; 183)			
rs7579						
GG	192.6 ± 33	116.8 ± 16.5	75.9 (7.8; 143.7)*	0.74	0.75	<0.01
GA+AA	198.3 ± 44	136.7 ± 29.3	61.6 (-13; 137)			

Values are mean ± SEM. Change is represented as n (95% confidence interval of the difference). Repeated measures two-way analysis of variance (ANOVA) adjusted for multiple comparison with Bonferroni test (*significantly different from baseline). $_{Int}$: Interaction; $_{Sup}$: selenium supplementation; $_{Gen}$: Genotype; C: cytosine; T: thymine; A: adenine; G: guanine; GPX: glutathione peroxidase; *SELENOP*: selenoprotein P.

Gene expression of *GPX1* and *SELENOP* is shown in **Figure. 2**. After Brazil nut supplementation, *GPX1* expression was affected by rs1050450 polymorphism with increased expression observed only in CC individuals (p=0.0361) but not in CT+TT individuals (p = 0.4375). In carriers of a variant allele (GA+AA) for rs7579, *SELENOP* mRNA expression was higher when compared with the GG genotype either pre or post-

treatment. SELENOP mRNA expression was not influenced by rs3877899 polymorphism in response to Brazil nut supplementation.



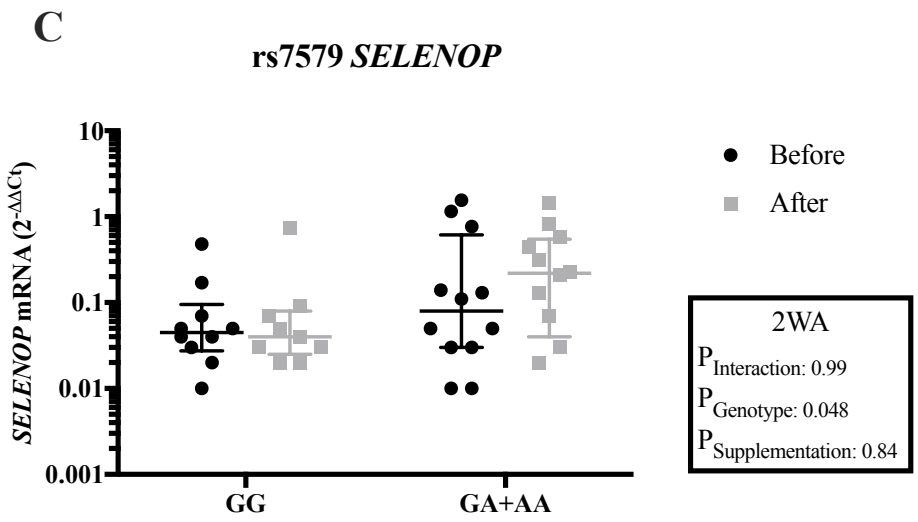


Figure. 2. Effects of dietary consumption of Brazil nuts on selenoprotein expression in previously genotyped volunteers. A) *GPX1* mRNA expression as a function of genotype for rs1050450; B) *SELENOP* mRNA expression as a function of genotype for rs3877899and C) for rs7579. Values are median with interquartile range, plotted as individual values (log10). Two-way ANOVA (2WA) repeated measures adjusted for multiple comparisons with Bonferroni. *p<0.05, Wilcoxon test.

5 Discussion

5.1. Brazil nut consumption modulates the CK activity, oxidative stress and mRNA expression of selenoproteins in patients using statins

Previous studies in other populations have demonstrated that supplementation with one unit of Brazil nut significantly improved Se status [26–28]. Supporting these studies, one Brazil nut daily for 3 months outstandingly enhanced Se intake, did not exceed the tolerable upper intake level (400 μg/day) [54] and was sufficient to increase plasma and erythrocyte Se for all participants.

Our patients were in regular and chronic use of statin, and most of them were using simvastatin. Simvastatin is the most common and cost-effective medication prescribed in the Brazilian Unified Health System (SUS) [55]. However, statin-associated muscle symptoms are more common with simvastatin than other available statins [2]. This side effect occurs due to the high lipophilicity that statins display at greater levels,

with passive diffusion across cellular membranes, increasing the body distribution of the medication [15].

Interestingly, we observed a significant decrease in body weight after Brazil nut consumption. Nevertheless, we did not find an association between Se status or oxidative stress parameters and body mass index (BMI). In the SELGEN study, participants received 100 μg sodium selenite/day for 6 weeks, and they found that the association between Se status and BMI was canceled by Se supplementation, indicating that the effect on BMI only occurs at suboptimal Se intake [41]. Studies investigating the direct effect of nuts on body weight are limited and show varying results [56]. Natoli et al. (2007), proposed mechanisms for the lack of weight gain with increased nut consumption based on clinical trials: 1) Nuts exert a satiating effect therefore reduce the energy intake and 2) The composition of nuts may affect the energy metabolism (monounsaturated fat and antioxidant components) [56].

In our study, after Se supplementation with Brazil nut, the levels of CK activity decreased in both groups indicating that consumption of one Brazil nut could benefit the muscle homeostasis of patients using statins. In contrast with our results, Bosgrud et al. (2012) found a significant elevation of CK levels after supplementation with 200 μg of Se and 400 mg of coenzyme Q10 [57]. Nevertheless, the authors concluded that increases in CK levels probably reflects the effect of multiple comparisons rather than a true effect of supplementation [57]. We did not find an association between higher levels of Se and CK activity, but interestingly, a moderately negative association between CK activity and GPX activity was observed. Thus, attenuation of statin-related muscle damage measured by the decrease of CK activity may be directly associated with improvement in curbing oxidative stress. Indeed, some studies have demonstrated that statin may increase the free radical load in the skeletal muscles causing oxidative damage and myopathy in these tissues [58]. Therefore, administration of statins with dietary antioxidants might be beneficial in reducing the muscular side effects [58].

In the present study, the enhancement of Se status after Brazil nut supplementation reduced oxidative stress. We observed that one Brazil nut supplementation daily was effective in decreasing lipid peroxidation by reducing MDA levels, one of the most commonly and widely used oxidative stress biomarkers [59]. Elevated GPX activity after supplementation was also associated with higher levels of erythrocyte Se. Considering that lipid peroxidation is closely related to oxidative stress [37], these results may indicate a protective effect of Brazil nut consumption connecting the GPX activity and MDA

levels. Thereby, the elevation of GPX activity levels lead to decreases in hydrogen peroxide, lipid and phospholipid hydroperoxides [60], reducing the stimulus to lipid peroxidation and consequently decreasing MDA synthesis [61]. Other studies with different populations, however, have shown controversial results about the effects of Brazil nut supplementation on MDA levels [37–39,62]. In these studies, the general characteristics of patients, such as advanced age and a pre-existing health condition, could have contributed to the distinct oxidative stress response after Se supplementation. Moreover, according to Cardoso et al. (2015), in a context of low levels of free radicals, the consumption of antioxidants may be protective and an additional intake may not be relevant [37].

On the other hand, increased GPX activity after Brazil nut supplementation is observed in most studies, corroborating our findings. Dietary components may interact to reflect different responses to the intervention regarding the modulation of the antioxidant response. In the present study, levels of SOD decreased significantly after supplementation and were negatively associated with higher levels of Se. A possible explanation would be that by increasing Se intake, we also increased metabolites of Se and Se-containing organic molecules, contributing to antioxidant mechanisms decreasing the synthesis of endogenous antioxidants such as SOD [33,63]. Also, Brazil nuts have additional exogenous antioxidant agents such as bioactive compounds (phenolics, flavonoids, tocopherol, and phytosterols) and other minerals (magnesium, copper, and zinc) that could contribute to SOD decreased levels [36].

Studies have suggested that statin-induced myopathy might be associated with the reduction in the expression and activity of selenoproteins [2,12,17,19,64]. This reduction occurs due to the inhibition of the mevalonate pathway, which also inhibits a whole series of endogenous metabolites, among them isopentenyl pyrophosphate. This metabolite is responsible for isopentenylation of adenosine at position 37 whereupon creates isopentenyl adenosine (i6A), required for efficient translation of Sec codon by $tRNA^{[Ser]Sec}$ and synthesis of selenoproteins [7,17–20]. Moosmann and Behl (2004) hypothesized that dietary Se supplementation could decrease the statin-induced side-effects in patients since higher levels of Se could increase the catalytic efficacy and turnover of the remaining, mature Sec-tRNA molecules [19]. Furthermore, Se supplementation, by organic and inorganic Se compounds, has been shown to increase selenoprotein expression and activity [33]. In the present study, after supplementation, we confirmed increases in GPX activity, but we failed to find differences in mRNA

expression of *GPX1, SELENOP*, and *SELENON* post-treatment. A possible explanation could be that a saturation effect of selenoprotein gene expression occurs at optimum Se levels. Any excess supply of Se results in increased metabolism, but marginal or no further increases in selenoprotein biosynthesis [65]. A rreview of Reska et. al. (2012) indicated that mRNA expression of different selenoproteins in leukocytes after Se supplementation in different populations does not result in changes in selenoprotein mRNA levels after Se supplementation, corroborating our findings, and indicating that protein synthesis may already be saturated at sufficient Se concentration [31].

5.2. Selenoprotein gene variations modulate the response of selenium supplementation in patients using statins

Individual variation in response to Se supplementation may indicate the occurrence of selenoprotein gene variants affecting expression [29,31,42]. Recent studies have demonstrated that selenoprotein single nucleotide polymorphisms (SNPs), including *GPX1* rs1050450, and *SELENOP* rs3877899 and rs7579, may interfere with Se utilization and effectiveness [29,31]. In the present study, levels of blood Se were comparable between individuals with variant genotypes. Furthermore, significant changes between pre- and post-supplementation of Se were observed in blood Se and MDA, regardless of the genotype. Corroborating our results, Cardoso et al. (2016) did not find associations between *SELENOP* polymorphisms rs7579 and rs3877899 and Se levels or oxidative stress parameters (MDA and ORAC) in patients with mild cognitive impairment [38].

Nucleotide variation in genes involved in oxidative stress pathways may interact with the redox role of Se. However, evidences on the association of Se and oxidative stress biomarkers and the interacting role of genetic polymorphisms are scarce in population-based studies [66]. When stratifying GPX activity according to genotype, we found that carriers of the variant *SELENOP* rs7579 and rs3877899 alleles did not respond to Se supplementation, indicating that these SNPs modulated the effect of Brazil nut supplementation on antioxidant defense. Studies have demonstrated that the presence of *SELENOP* variant alleles could alter the efficiency of Sec incorporation into SELENOP, the stability of SELENOP, and cellular Se uptake, affecting directly Se metabolism, blood concentrations, and synthesis of other selenoproteins, including antioxidant enzymes [35,38,41,42,67]. Moreover, as seen in the participants of the SELGEN study, the

presence of obesity in patients could interfere in the response of Se supplementation according to *SELENOP* variant alleles [41].

To our knowledge, this is the first study connecting selenoprotein polymorphisms and CK activity. Interestingly, CK activity of patients using statin was less responsive to Se supplementation in the presence of *SELENOP* rs7579 and rs3877899 variant alleles, emphasizing that genetic components may influence interindividual differences in Se metabolism, interfering with treatment outcome.

We observed a positive correlation between erythrocyte GPX activity and erythrocyte Se levels only in the CC genotype for the *GPX1* rs1050450 SNP. This finding agrees with other studies' conclusions in which the presence of variant alleles in selenoproteins might affect the way that the body metabolizes the available Se suggesting a different response of GPX1 activity to Se supplementation [31,35,38,42].

Alterations in selenoprotein activity and concentration during Se depletion and repletion are accompanied by changes in the mRNA level [31]. In the present study, Brazil nut supplementation was effective in increasing GPX1 mRNA expression in circulating blood leukocytes only in individuals with the CC genotype at rs1050450, corroborating with Cardoso et al. (2016), Donadio et al. (2017 and 2019), and Kopp et al. (2018) [30,35,38,42]. However, other studies have not confirmed the hypothesis that Se supplementation affects selenoprotein transcript levels in circulating leukocytes and in whole blood [31,68,69]. A possible explanation for these conflicting findings could be the chemical form of Se supplementation since Se compounds differ significantly in their metabolic pathways and in their abilities to produce various Se metabolites [28,33]. Interestingly, studies with similar conclusions as ours [30,35,38,42] used also organic selenium (Brazil nut and fish/mussels) as we used, which has a higher bioavailability and lower toxicity [33,35] than inorganic forms.

The rs7579 SNP in the *SELENOP* gene also resulted in different changes on mRNA expression. The presence of the less common allele A for rs7579 was associated with an increase in *SELENOP* expression at baseline and after supplementation when compared with the wild genotype. Previous studies have described the impact of SELENOP genetic variations on Se metabolism before and after Se supplementation. One possibility is that SELENOP polymorphisms could influence the ratio of SELENOP isoforms in plasma affecting the SELENOP mRNA expression, specially the 60 kDa isoform, found in higher amounts in the presence of the less common allele A for rs7579 [31,35,38,67]. SELENOP is a glycoprotein with a C-terminal domain composed of nine

Sec residues, and another Sec residue in the N-terminal region in a redox-active domain [45]. In humans, at least two isoforms of approximately 60 kDa and 50 kDa are present in the plasma. The 60 kDa form is generally accepted to be a selenium transporter for tissues of the body while the shorter isoforms are speculated to be involved in redox reactions or signaling [45]. The absence of changes in *SELENO*P mRNA expression between rs3877899 genotypes before and after supplementation is following the following the Supplementation with Brazil nuts study (SU.BRA.NUT) findings, in which a four-month interventional trial with Brazil nuts supplementation of healthy volunteers did not change SELENOP mRNA expression independently of the genotype group [35].

The absence of a control group is one limitation of our study. However, since the main goal of this study was to investigate the effect of the dietary intervention in CK activity, oxidative stress, and gene expression, we decided that each person before treatment would be a better control. The other limitation was the non-performance of biopsy in the skeletal muscle that could enhance our understanding about the specific mechanisms involving Se metabolism and statin side effects on muscle.

6 Conclusion

The present study showed that supplementation with one unit of Brazil nut was sufficient to increase the levels of Se biomarkers and contribute to the improvement of oxidative stress parameters, whereas the latter was directly associated with decreased serum CK activity that could benefit the muscle homeostasis of patients using statins. However, the improvement of Se status post-treatment did not contribute to the enhancement of the expression of selenoprotein genes, indicating a possible saturation effect at optimum Se levels. Our findings indicate that genetically determined variation in selenoproteins, especially SELENOP polymorphisms, modulates the response of GPX activity to Se supplementation in patients using statins. However, levels of blood Se or MDA were not affected. The CK activity response was also dependent on the presence of SELENOP variants after Brazil nut supplementation, emphasizing the importance of inter-individual aspects on treatment outcomes. We also demonstrated the influence of selenoprotein polymorphisms rs1050450 and rs7579 in the mRNA expression of the selenoproteins GPX1 and SELENOP.

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ANNEX I: Association between creatine kinase activity, oxidative stress, and selenoprotein mRNA expression changes after Brazil nut consumption in patients using statins (https://doi.org/10.1016/j.clnu.2020.02.012)

TITLE: Association between creatine kinase activity, oxidative stress, and selenoprotein mRNA expression changes after Brazil nut consumption in patients with obesity using statins

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ABSTRACT

Background and aims: Although the mechanisms by which statins promote muscle disorders remain unclear, supplementation with dietary antioxidants may mitigate statins' side effects. This study aimed to investigate if the consumption of Brazil nuts modulates serum creatine kinase (CK) activity in patients with obesity regularly using statins. **Methods**: The study was performed in the Ribeirão Preto Medical School University Hospital. Thirty-two patients in regular use of statins were divided according to CK activity levels (G1: increased or G2: normal) and received one unit of Brazil nut daily for 3 months. Body composition, blood selenium (Se) concentrations, erythrocyte glutathione peroxidase (GPX) activity, oxidative stress parameters, and CK activity were evaluated before and after supplementation. **Results:** In both groups, supplementation with one Brazil nut daily for 3 months decreased levels of CK activity in the serum, with positive changes in plasma and erythrocyte Se concentrations ($p<0.0001$), and increased levels of GPX activity. Among the parameters related to curbing of oxidative stress, we observed reduced levels of malondialdehyde (MDA) and superoxide dismutase (SOD) in both groups after supplementation. We also found a negative association between CK and GPX activity ($r=-41$; $p<0.02$). Expression of selenoproteins *GPX1*, *SELENOP*, and *SELENON* after Brazil nut supplementation was unchanged. **Conclusion:** Brazil nut consumption enhanced the control of CK activity by improving oxidative stress biomarkers in patients using statins but did not modulate mRNA expression of selenoproteins.

Keywords: Statin, selenium, selenoproteins, creatine kinase activity, oxidative stress

INTRODUCTION

Statins are the most prescribed and effective pharmacological therapy for the treatment of hypercholesterolemia and the prevention of cardiovascular events [1]. They are specific and potent inhibitors of cholesterol biosynthesis in the mevalonate pathway [2–4]. This pathway is also important for the maturation of selenocysteine tRNA (Sec tRNA), which is responsible for the expression of selenoproteins [5,6]. When this pathway is inhibited, diminished availability of selenoproteins can occur [4,7]. Selenoprotein deficits have been associated with side effects of statins, such as myopathies and increased oxidative stress [4,7]. An appealing candidate for curtailed expression in statin-induced myopathy is selenoprotein N (*SELENON)* [8,9]. Other candidates are selenoprotein P (*SELENOP*), important to transport selenium from the liver to target tissues [10], and glutathione peroxidase (*GPX*), which could also present reduced expression, and is involved in hydrogen peroxide and hydroperoxide detoxification, protecting against oxidative injury [10].

Creatine kinase (CK) is an enzyme found in both cytosol and mitochondria of tissues where energy demands are high, most notably skeletal muscle. Elevated serum CK activity can indicate tissue damage and are observed in several pathological conditions, including statin-induced myopathy [3,11,12]. According to Consensus Statements and Clinical Practice Guidelines established by International Health Societies, statin-related myopathy characterization is complex and has been defined as myalgia or muscle weakness associated with serum CK levels ranging between 5 to 10-fold the upper limit of normal [3,13–15]. However, some studies have described the presence of statin-associated muscle disorders in patients even with normal or slightly elevated levels of serum CK [3,14,16]. Symptoms of statin-related myopathy usually occur soon after initiation of statin therapy, but sometimes may appear even after years of treatment [17].

Dietary Selenium (Se) supplementation was shown to mitigate statins' side effects [4,7,18]. However, the mechanisms underlying the beneficial effects have not yet been fully elucidated. One dietary supplement that has become popular among recent studies is Brazil nut (*Bertholletia excelsa*, family Lecythidaceae), especially due to its great amount of Se in the main form of Selenomethionine (SeMet), which has a high bioavailability and low toxicity [19,20]. Supplementation with Brazil nuts has been shown to improve Se status in different conditions and populations [19,21–26].

In the present study, we sought to investigate whether the consumption of Brazil nuts assists in the control of serum CK activity of patients in regular use of statins. We

hypothesized that the daily consumption of Brazil nuts would have benefits on Se status, increase antioxidant enzyme activity, and modulate mRNA expression of selenoproteins involved in the antioxidant process, muscle homeostasis and Se transportation.

METHODS

Ethic Statement

All procedures followed in this study have been performed in accordance with the ethical standards as laid down in the Declaration of Helsink. The study protocol was approved by the Ethics Committee of the Ribeirão Preto Medical School at the University of São Paulo, Brazil (protocol number CAAE: 56221916.5.0000.5440). Informed consent was obtained from all individual participants. The trial was registered at Brazilian Clinical Trial Registry under identification number (RBR-7rwgzt).

Study population

This was an open, non-randomized, controlled, single-center study conducted at the Ribeirão Preto Medical School University Hospital, University of São Paulo, Brazil, from January 2017 to July 2017. Inclusion criteria were being between 18-60 years of age, both sexes, and in regular use of statin. Out of the initial 55 eligible participants identified, seven participants were excluded because they were not using the statin continuously or had the type or dosage of medication changed by the doctor in recent months (n = 7) or refused to participate (n= 4). Thus, according with measurements of CK activity previously obtained from medical records, 44 participants were allocated into Group 1: (n= 22) patients with increased CK activity (>189 U/L) or Group 2: (n= 22) patients with normal CK activity (<189 U/L), either to receive Brazil nut supplementation (**Figure 1**). Patients with nuts allergy, taking multivitamins and mineral supplements, in use of antibiotics or other medications that are also metabolized by cytochrome P450 3A4 (CYP3A4), in current tobacco and alcohol consumption, athletes or individuals practicing intense physical activity, with serious cardiac complications, thyroid disorders, liver disease, kidney failure and, neoplasia were not included in the study.

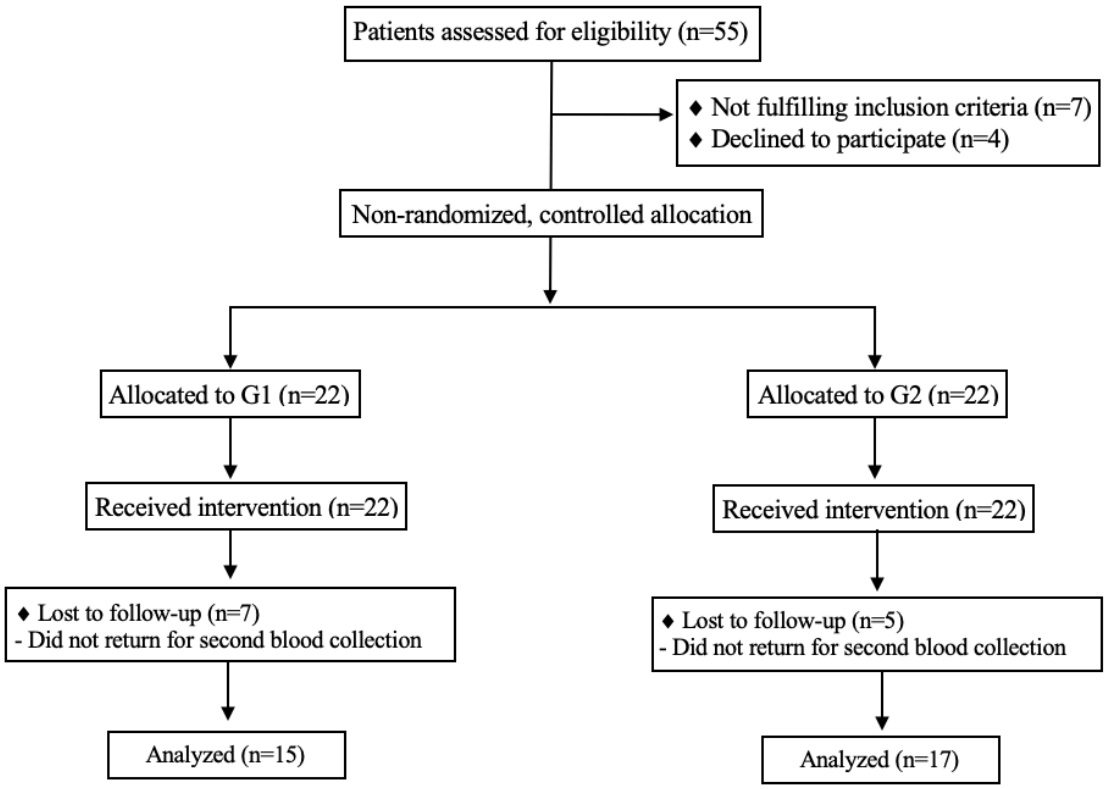

Figure 1. Flowchart of inclusion and follow-up.

Study Procedures

At the beginning of the study clinical evaluation was performed and an interview was conducted in order to obtain general information for anamnesis. During this baseline visit, height and weight were measured for each participant and these values were used to calculate body mass index (BMI). We also collected venous blood samples to perform biochemical evaluations. The volunteers received, at no cost, three vacuum sealed bags containing Brazil nuts enough for all intervention period. They were oriented to consume one Brazil nut daily for 3 months. Periodically, researchers contacted the participants to monitor the compliance of Brazil nuts consumption. All subjects were instructed to maintain their normal diet and to avoid additional nuts during the study. Once the 3-month study period was completed, we again assessed anthropometric data and collected a second blood sample to perform post supplementation biochemical measurements.

Centesimal Composition of Brazil nuts

Brazil nuts were originally from the Brazilian state of Amazon and were acquired in partnership with the Excelsa Institute. A random sample of Brazil nuts used in the study

was analyzed in triplicate according to AOAC [27] for determining humidity, ash, protein, and lipids. The total carbohydrates were calculated by difference (100—total grams of humidity, protein, lipids, and ash), including fiber fraction.

Sample collection

The collection of blood samples occurred before and after 3 months supplementation with Brazil nuts and was performed in the University Hospital Clinical Research Unit (UPC) after 8 hours of fasting and the separation of whole blood to obtain plasma and erythrocytes occurred immediately after collection. An aliquot of 500 µL of whole blood was stored into 1.5 mL sterile plastic tubes used for RNA extraction and subsequent gene expression. The samples were stored at -80° until the time of analysis.

Biochemical evaluation

Commercially available kits by Labtest (Minas Gerais, Brazil) were performed following the manufacturer protocol to measure serum CK activity (Cat. No. 117) by UV kinetic increasing reaction according to the International Federation of Clinical Chemistry and Laboratory Medicine and quantitative end point colorimetric assay of total cholesterol in serum (Cat. No. 76).

Selenoprotein gene expression

Total RNA was isolated from whole blood using TRIzol reagent (Invitrogen Life Technologies) and final concentration was measured in a NanoDrop ND 1000 spectrophotometer (NanoDrop ND 1000, Thermo Scientific, Wilmington, DE, USA). RNA integrity was considered acceptable when the absorbance ratios in 260 and 280 nm wavelengths were between 1.8 and 2. cDNA was synthesized by reverse transcription polymerase chain reaction (PCR) using the High Capacity Reverse Transcriptase kit (Applied Biosystems, Thermo Scientific, Foster City, CA, USA). Analysis of gene expression was performed by real-time quantitative PCR using Taqman Gene expression Assays for *GPX1* (Hs00829989_gH), *SELENOP* (Hs01032845_m1) and, *SELENON* (Hs00898723_ml). *β-acti*n (4352935E) mRNA expression was used as a reference gene. The relative expressions were calculated using the $2^{-\Delta\Delta Ct}$ method.

Evaluation of oxidative damage: Malondialdehyde (MDA)

For dosage of malondialdehyde 100µl plasma sample were used. Three hundred microliters of 10mM solution of 1-methyl-phenylindole was added in acetonitrile and methanol (2:1, v/v) and 75µl of hydrochloric acid (HCl) pure (37%). The tubes were vortexed and incubated in water bath at 45 ° C for 40 minutes. After the bath, samples were cooled in ice and then centrifuged at 4000rpm for 10 minutes. Supernatant was read for absorbance at a wavelength of 586nm. Malondialdehyde concentration was calculated by comparing it to a curve 1,1,3,3 - tetramethoxypropane (TMP) hydrolyzate.

Glutathione Peroxidase activity (GPX)

GPX activity was measured in erythrocytes according to Paglia and Valentine (1976) [28]. The method is based on the reaction in which GPX catalyzes the oxidation of reduced GSH by a hydroperoxide. In the presence of GPX nicotinamide adenine dinucleotide phosphate (NADPH), oxidized GSH is converted to the reduced form with a concomitant oxidation of NADPH NADPH +. The decrease in absorbance at 340 nm was then measured.

Superoxide dismutase (SOD)

SOD levels were determined by the indirect method of NBT (nitroblue tetrazolium) using the SOD® 19160 kit (Sigma-Aldrich Chemie GmbH®). Twenty microliters of the serum were added to white solution 2 plus 20µl of double distilled water in white solutions 1 and 3 were added to 20µl of WST solution provided in the kit in each white solution and mixed. A further 20µl of buffer solution also supplied by the kit in white solutions 2 and 3 were added to 20µl of the enzyme solution (provided by manufacturer) and the white in the sample solution 1 and then mixed continuously. The samples were incubated at 37 ° C for 20 minutes. Finally, absorbance was read at 450nm.

Selenium

The determination of the total concentration of selenium in plasma and erythrocytes was performed by an Inductively Coupled Plasma Mass Spectrometry (ICP-MS), fitted with a dynamic reaction cell (DRC) (Perkin Elmer Sciex Norwalk, CT USA). Samples were diluted in the ratio 1:50 with a solution containing Triton X-100 0.01% (v/v), HNO_3 0.05% (v/v) and 10mg / L-1 rhodium (Rh) as an internal standard. The concentration of the analytical calibration standards ranged from 0 to 50 µg/L [29].

Sample size calculation

The sample size calculation was based on CK activity of patients using cholesterol-lowering medications. We conducted a pilot study with 104 patients selected at University Hospital and from who we evaluated serum CK activity. Patients were classified according to the assay range (Labtest, Minas Gerais, Brazil) as normal: when CK activity were up to 189 U/L or out of range: when CK activity were greater than 189 U/L. Considering mean CK activity in normal group (66.7U/L) and out of range group (229.3 U/L), standard deviation (SD) 114 U/L, alpha 5%, and 99% power the sample size was calculated as 12 for each group.

Statistical analysis

Continuous variables were tested for normality using the Shapiro-Wilk test and nonparametric tests were used when appropriate. The data were presented as mean ± SD with its respective p-values. Baseline characteristics and baseline measured outcomes were compared with post treatment using paired t-test or Wilcoxon test. The Pearson's correlation coefficient or the Spearman's correlation was calculated according to the presence or absence of a normal distribution. To compare averages of gene expression results Wilcoxon test was applied. Values are expressed as median, minimum and maximum, and interquartile range. Data were plotted in Statistical Package for the Social Sciences software version 14.0 (SPSS, Chicago, IL, USA) and GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Differences were considered significant if $p<0.05$.

RESULTS

The Se content and centesimal composition of Brazil nuts are shown in **Table 1**.The average weight of Brazil nuts was 5 g, therefore each nut provided approximately 290μg of Se, which is higher than the RDA (Recommended Dietary Allowances) for adults (55μg/d) but less than upper limit (400μg/d) established by the Institute of Medicine (IOM), 2000 [30].

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

Nutrient	Mean ± SD

Energy (kcal)	696.0 ± 4.3
Carbohydrates (g)	12.9 ± 0.4
Proteins (g)	10.9 ± 0.51
Lipids (g)	71.6 ± 0.09
Ash (%)	2.91± 0.23
Humidity (%)	1.75 ± 0.33
Selenium (μg/g)	58.1 ± 2.1

Values are mean ± standard deviation (SD). Except for selenium, all nutrients were calculated considering 100g of Brazil nut.

A total of 32 patients completed the entire study protocol. Compliance of Brazil nuts consumption was reported by patients at the end of the study. Moreover, increased Se levels in plasma and erythrocyte post treatment (**Table 2**) confirmed the adherence to the supplementation. According to the analysis of non-consecutive dietary food records, at baseline, the energy intake of participants in G1 (2050 ± 180.2 kcal) and G2 (2130 ± 237.3 kcal) was comparable (p=0.9591) and, all participants maintained their normal diet during the period of supplementation (data not shown). At the beginning of the study, participants were asked about the practice of physical activity. Most of the participants in G1 and G2 reported not practicing regular physical activity and, they did not change this habit during the study. Regarding the type of cholesterol-lowering medication used by study participants, 71.9% was using Simvastatin (40mg) and 28.1% Atorvastatin (40mg).

When stratifying the type of statin according to the study group, in G1, 80% was using Simvastatin and 20% Atorvastatin, and in G2, 64.7% was using Simvastatin and 35.3% Atorvastatin. We did not find differences in the type of statin between groups (p=0.3369). It is relevant to consider that during the 3-month period of supplementation there were no changes in medical prescription of statin dosage or type. Females constituted 40.6% of the group and mean age was 50.1 ± 7.6 years. The study groups were not different in relation to sex (p=0.07) or age (p=0.78). The body composition, CK activity, total cholesterol, Se status, and oxidative stress parameters of the volunteers pre and post supplementation are summarized in **Table 2**. According to BMI, patients in G1 were classified as with obesity while in G2 patients were classified as overweight. After

supplementation with Brazil nuts, individuals in both groups lost a significantly amount of weight with consequent reduction in BMI. In both G1 and G2 groups, the supplementation contributed to decreased levels of CK activity in serum. The levels of total cholesterol were adequate at the baseline and remained unchanged in both groups after supplementation.

Table 2: Body composition, CK, lipid profile, Se status, and oxidative stress parameters according to group after 90 days of Brazil nut supplementation

Parameters	Group 1 (n=15)			Group 2 (n=17)		
	Pre Mean ± SD	Post Mean ± SD	*P-value*	*Pre* Mean ± SD	Post Mean ± SD	*P-value*
Weight (kg)	95.1 ± 21.6	92.2 ± 20.3	0.04	81.1 ± 20.4	78.1 ± 20.9	0.0001
BMI (kg/m^2)	33.1 ± 5.9	31.9 ± 5.2	0.04	29.4 ± 7.4	28.3 ± 7.4	0.0002
CK (U/L)†	347.9 ± 133.3	228.2± 122.5	0.005	102.9 ± 45.7	78.3 ± 34.2	0.005
Cholesterol (mg/dL)	177.0 ± 39.3	176.5 ± 45.4	0.97	181.2 ± 44.3	152.8 ± 25.3	0.056
Plasma Se (µg/L)	81.5 ± 3.7	258.9 ± 73.8	<0.0001*	88.5 ± 6.9	286.5 ± 146.5	<0.0001*
Erythrocyte Se (µg/L)	82.33 ± 11.5	298.3 ± 84.5	<0.0001	91.3 ± 18.2	388.1 ± 208.4	<0.0001
MDA (nmol/mL)	3.3 ± 1.1	2.1 ± 1.1	0.003	2.8 ± 0.6	1.7 ± 0.9	<0.0001
GPX activity (U P/g)	24.7.5 ± 3.0	29.8 ± 6.0	0.01*	23.5 ± 4.4	35.4 ± 29.5	0.005*
SOD (U/L)	1.4 ± 0.4	0.3 ± 0.1	<0.0001	1.6 ± 0.8	0.4 ± 0.3	0.0001

Note: Paired t-test; Wilcoxon *; t test for independent samples †. Values are expressed as mean ± standard deviation. Pre: pre-treatment; Post: post-treatment; SD: standard deviation. BMI: body mass index; CK: creatine kinase activity; Se: selenium; MDA: malondialdehyde; GPX: glutathione peroxidase; SOD: superoxide dismutase.

Biomarkers of selenium status and oxidative stress

The biomarkers of Se status and oxidative stress measured at baseline and post treatment are shown in **Table 2**. At baseline, the majority of patients showed adequate Se levels in plasma (>80-95 µg/L), only 3 individuals were slightly deficient [6].

Changes in plasma and erythrocyte Se concentrations were significantly positive in both groups after supplementation ($p<0.0001$) and enough to restore selenium deficiency. The levels of GPX activity also increased in both groups post treatment (G1 $p<0.01$, G2 $p<0.005$). Among the parameters related to oxidative stress, we observed reduced levels of MDA and SOD in both groups after supplementation. Changes in biomarkers of Se status after supplementation were associated with changes in antioxidant parameters in different ways (**Table 3**). While increased levels of erythrocyte selenium were positively associated with erythrocyte GPX activity ($r=0.54$; $p<0.002$) higher levels of plasma selenium were negatively associated with SOD ($r=-44$; $p<0.01$).

No correlations were observed between changes in Se status and changes in CK activity but, interestingly, we observed a moderately negative association between CK activity and GPX activity ($r=-41$; $p<0.02$) which in turn, was associated with increased selenium levels (**Table 3**).

Table 3. Correlation analysis between changes in the oxidative stress parameters and changes in Se status

	Correlation (r)					
Parameters	Erythrocyte Se	Serum Se	CK	GPX activity	MDA	SOD
Erythrocyte Se[b]		0.78***	0.05	0.55**	0.15	-0.49**
Serum Se[b]	0.78***		-0.03	0.46*	0.16	-0.44*
CK[b]	0.05	-0.03		-0.41*	0.18	-0.17
GPX[b]	0.55**	0.46*	-0.41*		0.07	-0.40
MDA[a]	0.15	0.16	0.18	0.07		-0.07
SOD[b]	-0.49*	-0.44*	-0.17	-0.40	-0.07	

***$p<0.0001$; **$p<0.005$; *$p<0.05$. [a]Pearson's correlation coefficient. [b]Spearman's correlation. CK: creatine kinase activity; Se: selenium; MDA: malondialdehyde; GPX: glutathione peroxidase; SOD: superoxide dismutase.

Selenoproteins gene expression

Gene expression of three selenoproteins (*GPX1, SELENOP*, and *SELENON*) was analyzed before and after 3 months of Brazil nut supplementation. The results are shown in **Table 4**. Surprisingly, we did not detect changes in the expression of selenoproteins *GPX1, SELENOP*, and *SELENON* after Brazil nut supplementation in both groups.

Table 4: Gene expression of selenoproteins according to study group assessed by quantitative polymerase chain reaction (qPCR) after three months of Brazil nut supplementation

Group 1					
	Pre		Post		
Gene	Median (min – max)	IQR	Median (min – max)	IQR	p-value
GPX	0.67 (0.30 – 1.3)	0.41	0.61 (0.14 – 2.3)	0.78	0.30
SELENOP	0.05 (0.03 – 0.14)	0.08	0.11 (0.02 – 0.83)	0.36	0.07
SELENON	0.035 (0.001 – 0.08)	0.07	0.03 (0.001 – 0.12)	0.06	0.90
Group 2					
	Pre		Post		
Gene	Median (min – max)	IQR	Median (min – max)	IQR	p-value
GPX	0.23 (0.05 – 0.59)	0.31	0.28 (0.15 – 0.74)	0.21	0.36
SELENOP	0.03 (0.01 – 0.07)	0.04	0.03 (0.01 – 0.31)	0.19	0.44
SELENON	0.03 (0.01 – 0.06)	0.03	0.04 (0.01 – 0.08)	0.03	0.34

Note: Values are expressed as median (minimum – maximum) and interquartile range (IQR) and were normalized to *β-actin* mRNA levels. Wilcoxon test was used to compare averages. Pre: pre-treatment; Post: post-treatment. GPX: glutathione peroxidase; *SELENOP*: selenoprotein P; *SELENON*: selenoprotein N.

DISCUSSION

Previous studies in other populations have demonstrated that the supplementation with one unit of Brazil nuts significantly improved Se status [8–10]. Supporting these studies, one Brazil nut daily for 3 months outstandingly enhanced Se intake, did not exceed the tolerable upper intake level (400 μg/day) [6] and, was sufficient to increase plasma and erythrocyte Se for all participants.

Our patients were in regular and chronic use of statin, and most of them were using simvastatin. Simvastatin is the most common and cost-effective medication prescribed in the Brazilian Unified Health System (SUS) [31]. However, statin-associated muscle symptoms are more common with simvastatin than other available statins [3]. This side effect occurs due to the high lipophilicity that displays at greater levels, with passive diffusion across cellular membranes, increasing the body distribution of the medication [1].

Interestingly, we observed a significant decrease in body weight after Brazil nut consumption. Nevertheless, we did not find an association between Se status or oxidative stress parameters and body mass index (BMI). In the SELGEN study, participants received 100 μg sodium selenite/day for 6 weeks, and they found that the association between Se status and BMI was canceled by Se supplementation, indicating that the effect on BMI only occurs at suboptimal Se intake [32]. Studies investigating the direct effect of nuts on body weight are limited and show varying results [33]. Natoli et al. (2007), proposed mechanisms for the lack of weight gain with increased nut consumption based on clinical trials: 1) Nuts exert a satiating effect therefore reduce the energy intake and 2) The composition of nuts may affect the energy metabolism (monounsaturated fat and antioxidant components) [33].

In our study, after Se supplementation with Brazil nut, the levels of CK activity decreased in both groups indicating that consumption of one Brazil nut could benefit the muscle homeostasis of patients using statins. In contrast with our results, Bosgrud et al. (2012) found a significant elevation of CK levels after supplementation with 200 μg of Se and 400 mg of coenzyme Q10 [34]. Nevertheless, the authors concluded that increases in CK levels probably reflects the effect of multiple comparisons rather than a true effect of supplementation [34]. We did not find an association between higher levels of Se and CK activity, but interestingly, a moderately negative association between CK activity and GPX activity was observed. Thus, attenuation of statin-related muscle damage measured by the decrease of CK activity may be directly associated with the improvement in curbing oxidative stress. Indeed, some studies have demonstrated that

statin could increase the free radical load in the skeletal muscles causing oxidative damage and myopathy in these tissues [35]. Therefore, administration of statins with dietary antioxidants might be beneficial in reducing the muscular side effects [35].

In the present study, the enhancement of Se status after Brazil nut supplementation reduced oxidative stress. We observed that one Brazil nut supplementation daily was effective in decreasing lipid peroxidation by reducing MDA levels, one of the most commonly and widely used biomarkers [36]. Elevated GPX activity after supplementation was also associated with higher levels of erythrocyte Se. Considering that lipid peroxidation is closely related to oxidative stress [21], these results may indicate a protective effect of Brazil nut consumption connecting the GPX activity and MDA levels. Thereby, the elevation of GPX activity levels lead to decreases in hydrogen peroxide, lipid and phospholipid hydroperoxides [37], reducing the stimulus to lipid peroxidation and consequently decreasing MDA synthesis [38]. Other studies with different populations, however, have shown controversial results about the effects of Brazil nut supplementation on MDA levels [21,23,25,39]. In these studies, the general characteristics of patients, such as advanced age and a pre-existing health condition, could have contributed to the distinct oxidative stress response after Se supplementation. Moreover, according to Cardoso et al. (2015), in a context of low levels of free radicals, the consumption of antioxidants may be protective and an additional intake may not be relevant [21]. On the other hand, increased GPX activity after Brazil nut supplementation is observed in most studies , corroborating with our findings. Dietary components may interact to reflect different responses to the intervention regarding the modulation of the antioxidant response. In the present study, the levels of SOD decreased significantly after supplementation and were negatively associated with higher levels of Se. A possible explanation would be that by increasing Se intake, we also increased metabolites of Se and Se-containing organic molecules, contributing to antioxidant mechanisms decreasing the synthesis of endogenous antioxidants such as SOD [20,40]. Also, Brazil nuts have other exogenous antioxidant agents such as bioactive compounds (phenolics, flavonoids, tocopherol, and phytosterols and other minerals (magnesium, copper, and zinc) that could contribute to SOD decreased levels [22].

Studies have suggested that statin-induced myopathy might be associated with the reduction in the expression and activity of selenoproteins [2,3,7,17,18]. This reduction occurs due to the inhibition of the mevalonate pathway, which also inhibits a whole series of endogenous metabolites, among them isopentenyl pyrophosphate. This metabolite is

responsible for isopentenylation of adenosine at position 37 whereupon creates isopentenyl adenosine (i6A), required for efficient translation of Sec codons by tRNA[Ser]Sec and synthesis of selenoproteins [4,7,18,41,42]. Moosmann and Behl (2004) hypothesized that dietary Se supplementation could decrease the statin-induced side-effects in patients since higher levels of Se could increase the catalytic efficacy and turnover of the remaining, mature SectRNA molecules [18]. Furthermore, Se supplementation, by organic and inorganic selenium compounds, has been shown to increase selenoprotein expression and activity [20]. In the present study, after supplementation, we confirmed increases in GPX activity, but we failed to find differences in mRNA expression of *GPX1, SELENOP*, and *SELENON* post-treatment. A possible explanation could be that a saturation effect of selenoproteins gene expression occurs at optimum Se levels. Any excess supply of Se results in increased metabolism, but marginal or no further increases in selenoprotein biosynthesis [43]. A review of Reska et. al. (2012) indicated that mRNA expression of different selenoproteins in leukocytes after Se supplementation in different populations does not result in changes in selenoprotein mRNA levels after Se supplementation, corroborating our findings, and indicating that protein synthesis may already be saturated at sufficient Se concentration [44].

The absence of a control group is one limitation of our study. However, since the main goal of this study was to investigate the effect of the dietary intervention in CK activity, oxidative stress, and gene expression, we decided that each person before treatment would be a better control. The other limitation was the non-performance of biopsy in the skeletal muscle that could enhance our understanding about the specific mechanisms involving Se metabolism and statin side effects on muscle.

In conclusion, the present study showed that the supplementation with one unit of Brazil nut was sufficient to increase the levels of Se biomarkers and contribute to the improvement of oxidative stress parameters, whereas the latter was directly associated with decreased serum CK activity that could benefit the muscle homeostasis of patients using statins. However, the improvement of Se status post-treatment did not contribute to the enhancement of selenoproteins gene expression indicating a possible saturation effect at optimum Se levels. Thus, more studies are required to further address the beneficial effects of Se supplementation to muscle homeostasis of patients using statins, mainly including the molecular and genetic analysis of muscle cells.

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CONFLICT OF INTEREST

The authors declare they have no financial conflict of interest.

STATEMENT OF AUTHORSHIP

LMW, FBJ and AMN made the conception and design of the study. LMW and LFL did the collection and carried out the sample analyses. RFB, DT, ACB, MCFF, and TMBC participated in the sample analyses and revision of the manuscript. LMW did all the data analysis and interpretation with help of ACB. All authors read and approved the final manuscript.

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ANNEX II: Nucleotide variations in selenoprotein genes modulate the response to selenium supplementation in patients using statins

TITLE: Nucleotide variations in selenoprotein genes modulate the response to selenium supplementation in patients using statins

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ABSTRACT

Background and aims: Several single nucleotide polymorphisms (SNPs) have been recognized as important sources of inter-individual variations in selenium (Se) metabolism in response to Se supplementation. This study aimed to investigate whether the selenoproteins gene variations could modulate the response to Brazil nuts of patients in regular use of statins. **Methods**: The study was performed in the Ribeirão Preto Medical School University Hospital. Thirty-two patients in regular use of statins received one unit of Brazil nut daily for 3 months. Body composition, blood Se concentrations, erythrocyte glutathione peroxidase (GPX) activity, oxidative stress parameters, and CK activity were evaluated before and after supplementation. The gene expression evaluation of *GPX1* and *SELENOP* was performed before and after supplementation. The volunteers were previously genotyped for SNPs in *GPX1* (rs1050450) and *SELENOP* (rs3877899 and rs7579). **Results:** The increased concentrations of Se status biomarkers and decreased levels of malondialdehyde after 3 months of supplementation were not influenced by selenoproteins genotype. The presence of variant alleles for *SELENOP rs3877899* and *SELENOP rs7579* modulated the response of Se supplementation for GPX activity and CK levels. Brazil nut supplementation significantly increased *GPX1* mRNA expression only in subjects with rs1050450 CC genotype. *SELENOP* mRNA expression was significantly lower in subjects with rs7579 GG genotype before and after supplementation. **Conclusion:** Selenoproteins gene variations modulate the response of GPX activity and CK levels to Brazil nut supplementation. We also demonstrated that selenoprotein polymorphisms lead to different responses in mRNA expression of selenoproteins following Se supplementation.

Keywords: Selenium, selenoproteins, single nucleotide polymorphisms, oxidative stress

INTRODUCTION

3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors, known as statins, are the most prescribed and effective pharmacological therapy for the treatment of hypercholesterolemia and the prevention of cardiovascular events [1–3]. Despite their favorable overall safety profile, important adverse effects of statin treatment have been reported, especially statin-associated muscle symptoms (SAMS), defined as myalgia or muscle weakness with or without an elevation in serum creatine kinase (CK) levels [1,2,4–7]. It has been suggested that the SAMS are associated with the oxidative stress, in which the imbalance between antioxidants and the over-generation of free radicals, including reactive oxygen species (ROS), could induce apoptosis in the skeletal muscles [4]. The inclusion of foods with antioxidant characteristics may be a worthwhile strategy to improve antioxidant capacity [8] and reduce the risk of SAMS in patients using statins.

Brazil nuts supplementation (*Bertholletia excelsa*, family Lecythidaceae) has become popular among recent studies due to its great amount of selenium (Se) in the main form of Selenomethionine (SeMet), being a viable strategy to increase dietary intake of Se in different conditions and populations [9,10]. In human population, the individual variation in the response to Se supplementation, irrespective of baseline Se status, may indicate the occurrence of gene variants [11]. Genetic polymorphisms have been recognized as an important source of inter-individual variation in response to nutritional supplementation. Several single nucleotide polymorphisms (SNPs) in selenoproteins genes could give rise to interindividual variations in Se metabolism and response to Se supplementation [9,12,13]. In particular, SNPs in *GPX1* (encoding GPX1) and *SELENOP* (encoding SELENOP) have been shown to affect blood Se or selenoprotein levels in response to supplementation. The *GPX1 rs1050450* polymorphism is a Proline (Pro) to Leucine (Leu) substitution at codon 197 which could result in reduced enzyme activity and higher DNA damage levels [14]. The impact of *SELENOP* variation in codon 234, associated with Alanine (Ala) to Threonine (Thr) change (*rs3877899*) and Guanine (G) to Adenine (A) transition within 3' untranslated region (UTR) of *SELENOP* mRNA (*rs7579*), may result in changes in Se metabolism, but the involved mechanisms are not fully elucidated [13]. Moreover, polymorphisms or mutations in selenoproteins' genes and synthesis cofactors may negatively influence the normally protective roles against oxidative stress increasing the risk for many diseases [8,15].

In a previous dietary intervention with patients using statins regularly, we found that the intake of one Brazil nut for 3 months increased GPX activity and blood Se levels,

while influenced the decreased levels of malondialdehyde (MDA) in patients using statins. Thus, in the present study we aimed to investigate whether the selenoproteins gene variations (*GPX1 rs1050450, SELENOP rs3877899* and *rs7579*) modify the effect of increased dietary Se intake on markers of Se status, oxidative stress biomarkers, CK activity, and selenoproteins mRNA expression in the same study population.

MATERIAL AND METHODS

Study design

This was an open, non-randomized, controlled, single-center study performed at the Ribeirão Preto Medical School University Hospital, University of São Paulo, Brazil, during the period of January 2017 to September 2018. Among the patients, 31 fulfilled the inclusion criteria: age between 18-60 years, both sexes, and using statin continuously. Patients with nuts allergy, taking multivitamins and mineral supplements, in use of antibiotics or other medications that are also metabolized by cytochrome P450 3A4 (CYP3A4), in current tobacco and alcohol consumption, engaging in intense physical activity, with serious cardiac complications, thyroid disorders, liver disease, kidney failure and, neoplasia were not included in the study. To investigate the influence of SNPs in the response to daily Brazil nut consumption, all patients received one unit of Brazil nut for 3 months. The study procedures and centesimal composition of Brazil nuts were described previously (Watanabe et al. 2020; accepted article). The study protocol was approved by the Ethics Committee of Ribeirão Preto Medical School at the University of São Paulo, Brazil (protocol number CAAE: 56221916.5.0000.5440). Informed consent was obtained from all individual participants. The trial was registered at Brazilian Clinical Trial Registry under identification number (RBR-7rwgzt).

Biochemical assays

Commercially available kits by Labtest (Minas Gerais, Brazil) were performed following the manufacturer protocol to measure serum CK activity (Cat. No. 117) by UV kinetic increasing reaction according to the International Federation of Clinical Chemistry and Laboratory Medicine. For dosage of MDA 100µl plasma sample were used. 300µl of 10mM solution of 1-methyl-phenylindole was added in acetonitrile and methanol (2:1, v/v) and 75µl of hydrochloric acid (HCl) pure (37%). After tubes were vortexed and incubated in water bath at 45 ° C for 40 minutes. After the bath, samples were cooled in ice and then centrifuged at 4000rpm for 10 minutes. Supernatant was read

for absorbance at a wavelength of 586nm. MDA concentration was calculated by comparing it to a curve 1,1,3,3 - tetramethoxypropane (TMP) hydrolyzate. GPX activity was measured in erythrocytes according to the method described by Paglia and Valentine, 1967 [16]. In the GPX assay protocol, GPX oxidizes GSH to produce GSSG as part of the reaction in which it reduces cumene hydroperoxide. Glutathione reductase (GR) then reduces the GSSG to produce GSH, and in the same reaction consumed nicotinamide adenine dinucleotide phosphate (NADPH). The decrease of NADPH (measured at OD=340 nm) is proportional to GPX activity. The determination of total Se concentration in plasma and erythrocytes was performed according to Batista et al. (2009) by an Inductively Coupled Plasma Mass Spectrometry (ICP-MS), fitted with a dynamic reaction cell (DRC) (Perkin Elmer Sciex Norwalk, CT USA). Samples were diluted in the ratio 1:50 with a solution containing Triton X-100 0.01% (v/v), HNO3 0.05% (v/v) and 10mg / L-1 rhodium (Rh) as an internal standard. The concentration of the analytical calibration standards ranged from 0 to 50 µg/L [17].

Genotyping of the rs1050450, rs7579 and rs3877899 SNPs

Isolation of DNA from whole blood was carried out using a PureLink Genomic DNA kit (Invitrogen, Life Technologies Inc., Carlsbad, CA, USA), and the concentration was measured using a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Polymorphisms in GPX1 (rs1050450) and SELENOP (rs7579 and rs3877899) genes were determined by real-time quantitative polymerase chain reaction (qPCR) with TaqMan SNP Genotyping assays (Life Technologies, Foster City, CA, USA). The assays were obtained as pre-designed from Applied Biosystems for rs7579 and rs3877899 (ID Assays C___8806056_10 and C___2841533_10, respectively) and custom-made through Custom TaqMan® Genomic Assays service for rs1050450 (GPX1/rs1050450: primers: F: 5′-TGT GCC CCC TAC GCA GGT ACA-3′, R: 5′-CCC CCG AGA CAG CAG CA-3′, T-allele: 5′-FAM-CTG TCT CAA GGG CTC AGC TGT-MGB-3′, C-allele: 5′-VIC-CTG TCT CAA GGG CCC AGC TGT-MGB- 3′). The qPCR reaction contained 3.125 µL of 1Å~ GoTaq® Master Mix (Promega, Madison, WI, USA), 0.156 µL of the 20Å~ SNP Genotyping Assay (Life Technologies, Foster City, CA, USA) and 20 ng of genomic DNA in a 6.741 µL total reaction volume. Samples were assayed along with no-template and HapMap controls and run on Applied Biosystems 7500 real-time PCR system (Applied Biosciences/Thermo Fisher Scientific, Waltham, MA, USA) under the following conditions: an initial enzyme activation step at 50° for 2 min, 95 °C

for 10 min followed by 40 cycles at 92 °C for 15 s and 60 °C for 1 min for annealing and extension. The validity of qPCR results was verified by direct sequencing 5% of the samples using the BigDye FN Sequencing Kit (PE Applied Biosystems) and using the following primers: primer forward 5′-CATCGAAGCCCTGCTGTCT-3′; primer reverse 5′-CACTGCAACTGCCAAGCA-3′. The agreement of the genotypes determined for the blinded quality control samples was 100%

GPX1 and _SELENOP_ gene expression

Total RNA was isolated from whole blood using TRIzol reagent (Invitrogen Life Technologies) and final concentration was measured in a NanoDrop ND 1000 spectrophotometer (NanoDrop ND 1000, Thermo Scientific, Wilmington, DE, USA). RNA integrity was considered acceptable when the absorbance ratios in 260 and 280 nm wavelengths were between 1.8 and 2. cDNA was synthesized by reverse transcription polymerase chain reaction (qPCR) using the High Capacity Reverse Transcriptase kit (Applied Biosystems/Thermo Scientific, Foster City, CA, USA). Analysis of gene expression was performed by qPCR using Taqman Gene expression Assays for *GPX1* (Hs00829989_gH) and *SELENOP* (Hs01032845_m1). β-actin (4352935E) mRNA expression was used as a reference gene. The relative expressions were calculated using the $^{2-\Delta\Delta Ct}$ method [18].

Statistical analysis

Volunteers were selected for gene expression analysis based on their genotype that had been determined previously. For all statistical analysis, individuals who were homozygous and heterozygous for the rare alleles were combined together in one category, leaving the homozygous dominant in another category in order to increase the statistical power. The Chi-square test was used to determine whether genotype frequencies followed the Hardy–Weinberg Equilibrium. A repeated-measures analysis of variance (ANOVA) adjusted for multiple comparison with Bonferroni test was used to determine the intragroup-effect between genotype and erythrocyte GPX enzyme activity, erythrocyte or plasma selenium, CK activity and MDA. The post-Se supplementation data was compared with baseline according genotype and was assessed by the paired Student's t-test or Wilcoxon test. Pearson's test was applied to determine the associations between genotype, GPX enzyme activity, and erythrocyte selenium during the intervention period. Data were plotted in Statistical Package for the Social Sciences software version 14.0

(SPSS, Chicago, IL, USA) and GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Differences were considered significant if $p<0.05$.

RESULTS

Thirty-two participants were included in this study and completed the study protocol. The mean age was 50.1 ± 7.6 years, and 59.4% were man. According with BMI, patients were classified as with obesity (31.1 ± 3.8 kg/m^2). According to the analysis of non-consecutive dietary food records, at baseline, the energy intake of participants was 2027.3 ± 302.5 and, all participants maintained their normal diet during the period of supplementation (data not shown). At the beginning of the study, participants were asked about the practice of physical activity. Most of the participants were not practicing regular physical activity (84.4%) and, they did not change this habit during the study. Regarding the type of cholesterol lowering medication used by study participants, 71.9% was using Simvastatin (40mg) and 28.1% Atorvastatin (40mg). The baseline variables of study population were not different among genotype groups (data not shown).

Genotype distribution and variant allele frequencies for the *GPX1 rs1050450, SELENOP rs7579* and *SELENOP rs3877899* polymorphisms are shown in **Table 1**. The genotype distributions of the studied polymorphisms were in Hardy–Weinberg equilibrium (data not shown).

Table 1. Genotypes and allele frequencies for polymorphisms in *GPX* and *SELENOP* genes of participants (n = 32)

Polymorphism	Wild type	Heterozygous	Variant	Heterozygous + variant	MAF
GPX					
rs1050450	**CC**	**CT**	**TT**	**CT+TT**	**T**
	20 (62.5%)	8 (25%)	4 (12.5%)	12 (37.5%)	0.2500
SELENOP					
rs7579	**GG**	**GA**	**AA**	**GA+AA**	**A**
	17 (54.8%)	12 (38.7%)	2 (6.5%)	14 (45.2%)	0.2580
rs3877899	**CC**	**CT**	**TT**	**CT+TT**	**T**
	7 (21.9%)	23 (71.8%)	2 (6.3%)	25 (78.1%)	0.4218

Note: Categorical variables are presented as n (%). C: cytosine; T: thymine; A: adenine; G: guanine; GPX: glutathione peroxidase; *SELENOP*: selenoprotein P; MAF: minor allele frequency.

The pre and post-Se supplementation and intragroup (between SNP's genotypes) effects in the levels of GPX enzyme activity, plasma Se, whole blood Se and, MDA for each SNP are shown in **Table 2**. We reported previously (Watanabe et al. 2020; accepted article) that the intake of one Brazil nut increased GPX activity and blood Se levels, while influenced the decreased levels of MDA. In the present study, an intragroup comparison demonstrated that the genotype did not influence Se-related variables (blood Se and GPX activity) and MDA levels (p_{Gen}>0.05). Besides that, the highly significant post-supplementation changes for blood Se and MDA levels occurred despite the genotypes. However, in the presence of one or two variant alleles for *SELENOP rs3877899* (CT or TT), and *SELENOP rs7579* (GA or AA) the levels of erythrocyte GPX activity post-supplementation did not increase significantly (p=0.25 and p=0.08 respectively) and the decreased CK activity levels was not significant (p=0.23 and p=0.13 respectively) when compared to pre-supplementation.

The correlation between GPX activity and erythrocyte Se concentration was influenced by genotype. When we stratified the erythrocyte GPX1 activity by rs1050450 polymorphism, we observed a moderately positive association (r=0.4946; p=0.031) only in CC genotypes.

Table 2. Pre and post-supplementation of GPX activity, plasma Se, erythrocyte Se and, MDA levels according to the studied genotypes

GPX activity (U/g Hb)						
Polymorphism	**Pre**	**Post**	**Change**	**2way ANOVA**		
				P_{int}	P_{Gen}	P_{Sup}
rs1050450						
CC	24.6 ± 4.3	29.1 ± 4.5	4.5 (0.9;8.1)*	0.65	0.34	<0.01
CT+TT	22.8 ± 2.7	28.4 ± 6.8	5.6 (0.9; 10.3)*			
rs3877899						

CC	24.1 ± 3.9	29.2 ± 5.5	5.1 (1.9; 8.2)*	0.78	0.42	<0.01
CT+TT	23.3 ± 3.8	27.5 ± 4.7	4.2 (-2.1; 10.6)			
rs7579						
GG	23.7 ± 4.6	29.9 ± 5.5	6.2 (2.2; 10.2)*	0.29	0.53	<0.01
GA+AA	24.2 ± 2.8	27.8 ± 5.2	3.6 (-0.3; 7.5)			

Plasma selenium (ng/mL)

Polymorphism	**Pre**	**Post**	**Change**	**2way ANOVA**		
				P_{int}	P_{Gen}	P_{Sup}
rs1050450						
CC	82.9 ± 12.4	294.4 ± 128.6	211.5 (149; 274)*	0.29	0.40	<0.01
CT+TT	88.9 ± 16.0	252.6 ±80.2	163.7 (79; 248)*			
rs3877899						
CC	86.1 ± 14.3	291.1 ± 119.1	205 (147; 263)*	0.36	0.26	<0.01
CT+TT	82.9 ± 12.7	240.0 ± 91.0	157 (50; 264)*			
rs7579						
GG	84.6 ± 12.7	286.3 ± 70.6	201.8 (130; 273)*	0.73	0.75	<0.01
GA+AA	85.5 ± 15.4	272.3 ± 149.8	186.8 (113; 261)*			

Erythrocyte selenium (ng/mL)

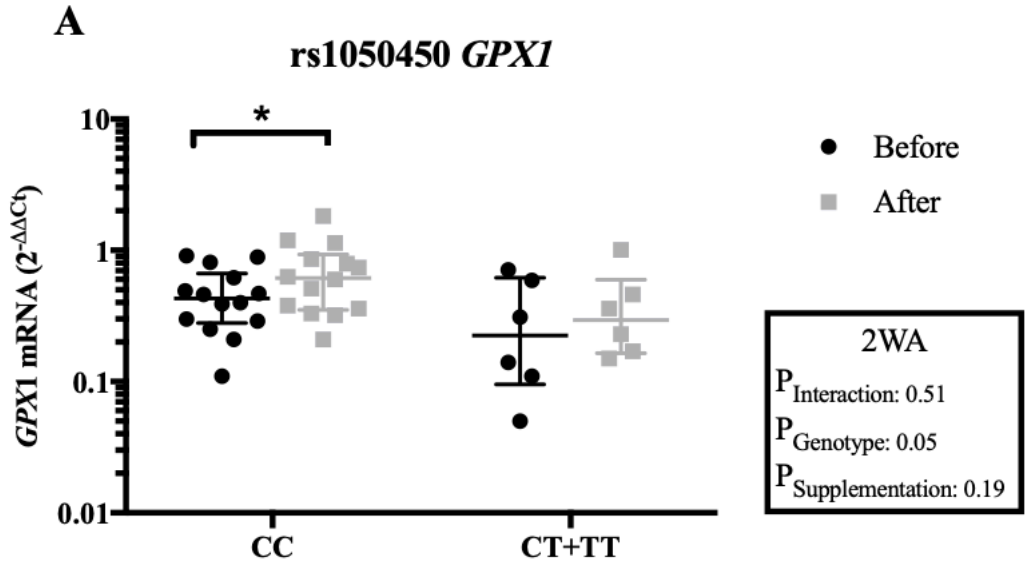
Polymorphism	**Pre**	**Post**	**Change**	**2way ANOVA**		
				P_{int}	P_{Gen}	P_{Sup}
rs1050450						
CC	86.4 ± 18.5	399.0 ± 173.0	312.6 (228; 397)*	0.11	0.09	<0.01
CT+TT	85.9 ± 11.1	302.3 ± 103.3	216.5 (105; 328)*			
rs3877899						
CC	87.5 ± 16.7	358.4 ± 177.0	270.9 (190; 322)*	0.46	0.40	<0.01

CT+TT	85.5 ± 13.4	301.7 ± 121.7	216.1 (62; 370)*			
rs7579						
GG	88.1 ± 16.3	367.1 ± 143.5	279 (179; 379)*	0.7	0.61	<0.01
GA+AA	84.9 ± 15.7	340.9 ± 184.1	256 (153; 359)*			
Malondialdehyde (nmol/mL)						
Polymorphism	**Pre**	**Post**	**Change**	**2way ANOVA**		
				P_{int}	P_{Gen}	P_{Sup}
rs1050450						
CC	3.0 ± 0.8	2.1 ± 0.9	-0.8 (-1.3; -0.3)*	0.005	0.23	<0.01
CT+TT	3.1 ± 1.0	1.2 ± 0.3	-1.9 (-2.6; -1.1)*			
rs3877899						
CC	3.1 ± 0.9	1.9 ± 0.9	-1.1 (-1.6; -0.6)*	0.36	0.11	<0.01
CT+TT	2.8 ± 0.6	1.2 ± 0.3	-1.6 (-2.6; 0.6)*			
rs7579						
GG	3.0 ± 0.8	1.7 ± 0.8	-1.2 (-1.9; -0.6)*	0.79	0.54	<0.01
GA+AA	3.1 ± 0.9	1.9 ± 0.9	-1.2 (-1.8; -0.5)*			
Creatine kinase activity (nmol/mL)						
Polymorphism	**Pre**	**Post**	**Change**	**2way ANOVA**		
				P_{int}	P_{Gen}	P_{Sup}
rs1050450						
CC	228.5 ± 35	174.6 ± 30.4	59.7 (0.6; 119.5)*	0.38	0.37	<0.01
CT+TT	206.0 ± 49	109.3 ± 13.47	96.7 (18.2; 175)*			
rs3877899						
CC	220.8 ± 35	152.5 ± 25.3	68.3 (14; 122)*	0.36	0.11	<0.01
CT+TT	218.2 ± 25	163.3 ± 20.2	54.8 (-33; 183)			

rs7579						
GG	192.6 ± 33	116.8 ± 16.5	75.9 (7.8; 143.7)*	0.74	0.75	<0.01
GA+AA	198.3 ± 44	136.7 ± 29.3	61.6 (-13; 137)			

Values are mean ± SEM. Change is represented as n (95% confidence interval of the difference). Repeated measures two-way analysis of variance (ANOVA) adjusted for multiple comparison with Bonferroni test (*significantly different from baseline). $_{Int}$: Interaction; $_{Sup}$: selenium supplementation; $_{Gen}$: Genotype; C: cytosine; T: thymine; A: adenine; G: guanine; GPX: glutathione peroxidase; *SELENOP*: selenoprotein P.

Gene expression of *GPX1* and *SELENOP* is shown in **Figure. 1**. After Brazil nut supplementation, *GPX1* expression was affected by rs1050450 polymorphism with increased expression observed only in CC individuals (p=0.0361) but not in CT+TT individuals (p = 0.4375). In carriers of a variant allele (GA+AA) for rs7579, *SELENOP* mRNA expression was higher when compared with the GG genotype either pre or post-treatment. SELENOP mRNA expression was not influenced by rs3877899 polymorphism in response to Brazil nut supplementation.



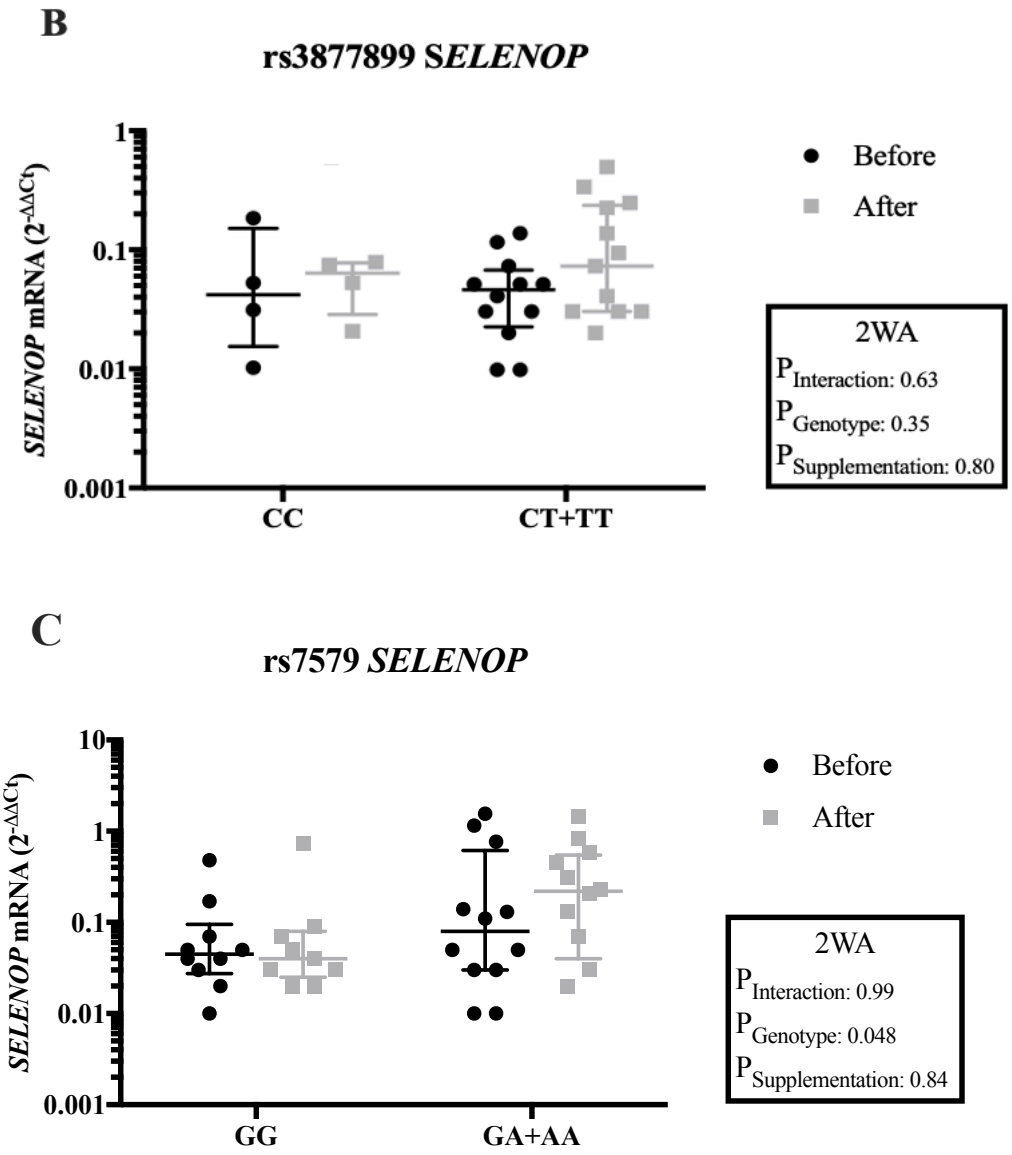


Figure. 1. Effects of dietary consumption of Brazil nuts on selenoprotein expression in previously genotyped volunteers. A) *GPX1* mRNA expression as a function of genotype for rs1050450; B) *SELENOP* mRNA expression as a function of genotype for rs3877899and C) for rs7579. Values are median with interquartile range, plotted as individual values (log10). Two-way ANOVA (2WA) repeated measures adjusted for multiple comparisons with Bonferroni. *p<0.05, Wilcoxon test.

DISCUSSION

The individual variation in response to Se supplementation may indicate the occurrence of selenoprotein gene variant [11,14,19]. Recent studies have been demonstrated that selenoprotein SNPs, including GPX1 rs1050450, SELENOP

rs3877899, and SELENOP rs7579, may interfere with Se utilization and effectiveness [11,19]. In the present study, levels of blood Se were comparable between individuals with wild-type and variant genotypes. Furthermore, significant changes between pre- and post-supplementation of Se were observed in blood Se and MDA, regardless of the genotype. Corroborating our results, Cardoso et al. (2016) did not find associations between *SELENOP* polymorphisms rs7579 and rs3877899 and Se levels or oxidative stress parameters (MDA and ORAC) in patients with mild cognitive impairment [8].

Nucleotide variation in genes involved in oxidative stress pathways may interact with the redox role of selenium. However, evidences on the association of Se and oxidative stress biomarkers and the interacting role of genetic polymorphisms are scarce in population-based studies [20]. When stratifying GPX activity according to genotype, we found that carriers of the variant *SELENOP* rs7579 and rs3877899 alleles did not respond to Se supplementation, indicating that these SNPs did not modulate the effect of Brazil nut supplementation on antioxidant defense. Studies have demonstrated that the presence of *SELENOP* variant alleles could alter the efficiency of Sec incorporation into SELENOP, the stability of SELENOP, and cellular Se uptake, affecting directly Se metabolism, blood concentrations, and synthesis of other selenoproteins, including antioxidant enzymes [8,9,13,14,21]. Moreover, as seen in the participants of the SELGEN study, the presence of obesity in the study patients could interfere in the response of Se supplementation according to *SELENOP* variant alleles [13].

To our knowledge, this is the first study connecting selenoproteins polymorphisms and CK activity. Interestingly, the CK activity of patients using statin was less responsive to Se supplementation in the presence of SELENOP rs7579 and rs3877899 variant alleles, emphasizing that genetic components may influence interindividual differences in Se metabolism and could interfere with treatment outcome.

We observed a positive correlation between erythrocyte GPX activity and erythrocyte Se level only in CC genotype for the *GPX1* rs1050450 SNP. This finding agrees with other studies' conclusions in which the presence of variant alleles in selenoproteins might affect the way that the body metabolizes the available Se suggesting a different response of GPX1 activity to Se supplementation. [8,9,11,14].

Alterations in selenoprotein activity and concentration during Se depletion and repletion are accompanied by changes in the mRNA level [11]. In the present study, Brazil nut supplementation was effective in increasing GPX1 mRNA expression in circulating blood leukocyte only in individuals with the CC genotype at rs1050450,

corroborating with Cardoso et al. (2016), Donadio et al. (2017 and 2019), and Kopp et al. (2018) [8,9,12,14]. However, other studies have not confirmed the hypothesis that Se supplementation affects selenoprotein transcript levels in circulating leukocytes and in whole blood [11,22,23]. A possible explanation for these conflicting findings could be the chemical form of Se supplementation since Se compounds differ significantly in their metabolic pathways and in their abilities to produce various Se metabolites [24,25]. Interestingly, in studies with similar conclusions as ours [8,9,12,14], used also organic selenium (Brazil nut and fish/mussels) as we used, which has a higher bioavailability and lower toxicity [9,24] than inorganic forms.

The rs7579 SNP in *SELENOP* gene also resulted in different changes on mRNA expression. The presence of the less common allele A for rs7579 was associated with an increase in *SELENOP* expression at baseline and after supplementation when compared with wild genotype. Previous studies have described the impact of SELENOP genetic variations on Se metabolism before and after Se supplementation. One possibility is that SELENOP polymorphisms could influence the ratio of SELENOP isoforms in plasma affecting the SELENOP mRNA expression, specially the 60 kDa isoform, found in higher amounts in the presence of the less common allele A for rs7579 [8,9,11,21]. SELENOP is a glycoprotein with a C-terminal domain composed of nine Sec residues, and another Sec residue in the N-terminal region in a redox-active domain [26]. In humans, at least two isoforms of approximately 60 kDa and 50 kDa are present in the plasma. The 60 kDa form is generally accepted to be a selenium transporter for tissues of the body while the shorter isoforms are speculated to be involved in redox reactions or signaling [26]. The absence of changes in *SELENO*P mRNA expression between rs3877899 genotypes before and after supplementation is following the following the Supplementation with Brazil nuts study (SU.BRA.NUT) findings, in which a four-month interventional trial with Brazil nuts supplementation of healthy volunteers did not change SELENOP mRNA expression independently of the genotype group [9].

Due to the small size sample, it was not possible to detect the effect of gender on biochemical parameters. The other limitation was the absence of a control group. However, since the main purpose of this study was to investigate the effect of the genotypes on the response to the dietary intervention, we decided that the same person before treatment would be the best control. Considering the selenoprotein level and activity more useful in determining Se status than Se itself, to not measure the levels of selenoprotein P could be another limiting factor.

In conclusion, our findings indicate that genetically determined variation in selenoproteins, especially SELENOP polymorphisms, modulates the response of GPX activity to Se supplementation in patients using statins, although it did not affect the levels of blood Se or MDA. The response of CK activity was also influenced by the presence of SELENOP variants after supplementation, emphasizing the importance of inter-individual aspects on treatment outcomes. We also demonstrated the influence of selenoprotein polymorphisms rs1050450 and rs7579 in the mRNA expression of the selenoproteins GPX1 and SELENOP. However, more studies are required to understand the specific molecular mechanisms, in which the genetic components interact with other inter-individual variations, affect the response of Se supplementation, and consequently, the Se metabolism. These perceptions could assist in a more individual approach to selenium supplementation in clinical practice.

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CONFLICT OF INTEREST

The authors declare they have no financial conflict of interest.

STATEMENT OF AUTHORSHIP

LMW, FBJ and AMN made the conception and design of the study. LMW and LFL did the collection and carried out the sample analyses. RFB, RD, MPG, ACB, and MCFF participated in the sample analyses and/or revision of the manuscript. LMW did all the data analysis and interpretation with help of ACB. All authors read and approved the final manuscript.

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ANNEX III: Research Internships Abroad (BEPE) - Effects of statin treatment in mice with a disruption of the selenocysteine lyase gene

TITLE: Effects of statin treatment in mice with a disruption of the selenocysteine lyase gene

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Abstract

Introduction: Statins inhibit the first step of cholesterol biosynthesis in the mevalonate pathway. This pathway is also important for the maturation of selenocysteine tRNA (Sec tRNA), responsible for the expression of selenoproteins. When this pathway is inhibited, diminished availability of selenoproteins occurs. Selenoprotein deficits have been associated with side effects of statins, such as myopathies and increased oxidative stress. Selenoprotein synthesis depends on the actions of the enzyme selenocysteine lyase (Scly). **Objective:** Investigate the effects of the use of statins in an animal model lacking the enzyme Scly. **Methodology:** Age-matched homozygous littermates wild-type C57BL/6J (WT) and Scly knockout (Scly KO) mice were used in experiments. Blood samples were collected for analysis of cholesterol levels, oxidative stress and creatine kinase activity. Quantitative real-time polymerase chain reaction (RT-PCR) was performed in livers and soleus muscle to assess for the gene expression of selenoproteins and enzymes involved in creatine metabolism. Using Ampli seq analysis we identified differentially expressed genes and metabolites in the livers of Scly KO mice. **Results:** A high-fat, Se-supplemented diet modulates the obesity in mice treated with statins in different ways, in a sex-dependent manner. Scly KO male mice were more susceptible to side effects from statin use. Scly KO male mice had worsened obesity, and potential muscle damage indicated by higher levels of CK activity in serum and lower levels in soleus, as well as lower Gpx1 expression in the soleus when compared with females. The differentially expression of genes in WT and Scly KO complemented our findings regarding the sexual dimorphism. **Conclusion:** In summary, sexual dimorphism exists upon statin treatment as this aspect may interfere with metabolic outcomes in obese, Se-supplemented mice.

Keywords*:* selenium, selenocysteine lyase, diet-induced obesity

Introduction

HMG-CoA reductase inhibitors (statins) are the most prescribed and effective pharmacological therapy for the treatment of hypercholesterolemia and prevention of cardiovascular events [1,2]. They are specific and potent inhibitors of the rate-limiting step of cholesterol biosynthesis, namely the reduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonic acid by the enzyme HMG-CoA reductase [2,3]. Despite their favorable overall safety profile, statins evoke distinct side effects whose molecular origins have remained unsettled [2]. Muscle disorders are the most commonly reported adverse effects of statins, ranging from muscle weakness, fatigue, pain, to rhabdomyolysis [4]. Creatine kinase (CK) is an enzyme found in both cytosol and mitochondria of tissues where energy demands are high, most notably skeletal muscle. Elevated serum CK activity can indicate tissue damage and are observed in several pathological conditions, including statin-induced myopathy [9–11]. Within skeletal muscle cytosol, the majority of CK activity is attributed to the homodimeric muscle isozyme of CK (CKm) [5].

Recent studies have suggested that the side effects of statins, mainly myopathy, may be related to the reduction in the expression and activity of selenoproteins. This reduction occurs because the inhibition of the mevalonate pathway also decreases the production of downstream cholesterol synthetic pathway products such as the isoprenoids (isopentenyl pyrophosphate, geranyl pyrophosphate, farnesyl pyrophosphate [farnesyl-PP]) [6]. This metabolite is required for the post-transcriptional maturation of the selenocysteine-tRNA (Sec-tRNA), an essential player in selenoprotein synthesis [1,2,4,7]. An appealing candidate for curtailed expression in statin-induced myopathy is selenoprotein N (selenoN) [1]. Other candidates are selenoprotein P (selenoP), important to transport selenium from liver to target tissues [8], glutathione peroxidases (GPX) and thioredoxin reductases (*Txnrds*), which could also have their expression reduced, and are involved in hydrogen peroxide and hydroperoxide detoxification, protecting against oxidative injury [8].

Selenium (Se) is an essential nutritional trace element [9]. It is present in nature as organic and inorganic forms. The most common organic forms are selenomethionine (Semet) and selenocysteine (Sec), and inorganic forms are selenite (SeO3-2) and selenate (SeO4-2) [10]. In our bodies, Se is largely used to produce the amino acid Sec, which is co-translationally incorporated into selenoproteins [11,12]. The Sec sources could be

dietary, by products of Se metabolism, or following selenoprotein degradation [13]. Sec lyase (Scly) is an enzyme that specifically recognizes the Sec residue and breaks it down into L-alanine and selenide [13]. Decomposition of Sec releases selenide which re-enters Sec synthesis by binding to a phosphate to generate selenophosphate, which is utilized to synthesize Sec on tRNA[Ser]Sec and incorporated into selenoproteins [13]. Previous studies have demonstrated that Scly is involved in energy metabolism, however the specific molecular mechanism remains unknown [14]. Mice lacking Scly (Scly KO) develop metabolic syndrome and become obese, with hyperinsulinemia, hyperleptinemia, and hypercholesteremia, aggravated by a selenium-deficient diet [15].

Elucidation of the basic molecular mechanisms leading to side-effects associated with statins could have a considerable effect on clinical practice and research. To investigate the biochemical and functional consequences of statin treatment on selenoprotein expression and biomarkers of creatine metabolism, we treated Scly KO mice with simvastatin and supplemented their diets with a blend of selenite and selenomethionine. The study proposed herein improved our understanding regarding the use of statins in animal model of obesity.

Materials and Methods

Chemicals

All reagents are from Sigma-Aldrich/MilliporeSigma (Burlington, MA, USA) unless otherwise noted.

Animals

Age-matched, littermates homozygous C57BL/6J wild-type (WT; The Jackson Laboratory) and Scly KO mice were born and raised in our vivarium and used in experiments after weaning in accordance with the Institutional Animal Care and Use Committee of the University of Hawaii. Animals were euthanized by CO_2 asphyxiation prior to collection of serum, liver, soleus and gastrocnemius muscle from each experimental mouse.

Diets and drug treatment

Animals were fed for 8 weeks with a customized high-fat diet containing 45 kcal% fat as lard and 35 kcal% carbohydrate as a mixture of sucrose and corn starch (Research

Diets, Inc., New Brunswick, NJ) supplemented with a blend of sodium selenite plus SeMet containing a fixed amount of selenite (0.15 ppm). Mice received simvastatin (Enzo Life Sciences International, Farmingdale, NY) at a dose of 5 mg/kg body weight/day by oral gavage for 21 days. Simvastatin was used for our investigation as it has a well-established link to adverse effects on skeletal muscle and is currently the most prescribed statin drug in the U.S. The simvastatin dose selected was both clinically relevant and physiologically appropriate. Simvastatin is insoluble in water; therefore, it was first dissolved in ethanol. Control mice received an ethanol solution as vehicle.

Serum measurements

Serum cholesterol was assayed by a commercial kit (Cayman Chemical Company, Ann Harbor, MI). Oxidative stress was determined by measuring lipid peroxidation end products with an OxiSelect HNE (4-hydroxynonenal)-His Adduct ELISA kit (Cell Biolabs, Inc., San Diego, CA). Creatine and creatine kinase (CK) activity were determined using a colorimetric assay kit (Sigma-Aldrich/MilliporeSigma, Burlington, MA, USA). For all assays, we followed the manufacturer's protocol.

GPX activity assay

Total serum or liver GPX activity was measured using a colorimetric assay kit (Abcam, Cambridge, UK, catalogue no. Ab102530) following the manufacturer's protocol. In the glutathione peroxidase assay protocol, glutathione peroxidase (GPx) oxidizes GSH to produce GSSG as part of the reaction in which it reduces cumene hydroperoxide. Glutathione reductase (GR) then reduces the GSSG to produce GSH, and in the same reaction consumed NADPH. The decrease of NADPH (measured at OD=340 nm) is proportional to GPx activity.

Quantitative real-time PCR (qPCR)

One microgram of total RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kits (Applied Biosciences/ Thermo Fisher Scientific, Waltham, MA, USA), with 10 ng of resulting cDNA used for qPCR with PerfeCTa SYBR Green FastMix (Quantabio, Beverly, MA, USA) and 45 amplification cycles in a 384-well plate platform of a LightCycler 480 II (Roche, *Basel,* Switzerland). Relative quantification used the Δ^{-CT} method, normalized to hypoxanthine-guanine

phosphoribosyltransferase (*Hprt1*) expression levels. All primers were evaluated for their efficiency prior to use in experiments.

Western blotting

Livers were pulverized and resuspended in CelLytic MT (Sigma-Aldrich/ MilliporeSigma - Burlington, MA, USA), sonicated, and centrifuged for 10 min at 12,000 x *g*, and protein supernatant collected. Samples consisting of 5 to 40 μg of total protein were separated in 4-20% SDS-PAGE (Bio-Rad, Hercules, CA), transferred to Immobilon-FL polyvinylidene difluoride (IPFL) membranes (Sigma-Aldrich/MilliporeSigma - Burlington, MA, USA), and probed for 1.5 h with 1:1,000 dillution of of rabbit polyclonal anti-SQS (Acris Antibodies, San Diego, CA). Detection and analysis of Western blots were performed via an Odyssey Infrared Imager (Li-Cor Biosciences, Lincoln, NE).

Targeted Transcriptome Profiling

We used the Ion AmpliSeq Transcriptome Mouse Gene Expression panel (Life Technologies) for gene expression profiling of liver samples. The panel is designed to target 20,802 RefSeq genes. 100 ng of total RNA was used to construct sequencing libraries according to manufacturer's instructions. Indexed sequencing libraries were constructed using the Mouse Transcriptome AmpliSeq kit and quantified them using the Ion Library Quantification kit on a StepOnePlus Real-Time PCR system. Twenty-four multiplexed samples were templated using the Ion 540 Kit-Chef with a Chef Instrument, enriched, loaded on two Ion 540 semiconductor sequencing chips (12 samples per chip) and analyzed on the Ion GeneStudio S5 System Sequencer using the Ion 540 Chip-Kit. An Ion TorrentSuite was used for signal processing and basecalling and the Ion ampliSeqRNA plugin used to obtain read counts for each targeted gene for each sample. Differential gene expression analysis was performed, and the read counts assigned to each amplicon for all samples using the R statistical programming language package DESeq2, which estimates variance-mean dependence in count data and tests for differential expression using a model based on negative binomial distribution. Significant expression differences were tested between the groups of samples. The 'apeglm' method was employed for log-fold change shrinkage, and the Wald test to detect the differential expression of genes and 0.05 as a significance threshold for the adjusted p-values.

Statistical analysis

Data were plotted in GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Applied statistical tests varied depending on the experiment and differences were considered significant if reaching $p \leq 0.05$.

Results

Statin treatment and diet-induced obesity

In the present study, Scly KO mice fed a high-Se, 45% kcal high-fat diet developed obesity and this phenotype was aggravated by the use of simvastatin in male mice (**Figure 1).** Interestingly, female Scly KO mice treated with statin decreased their body weight. We did not observe differences in body weight for WT mice with or without statin, males or females.

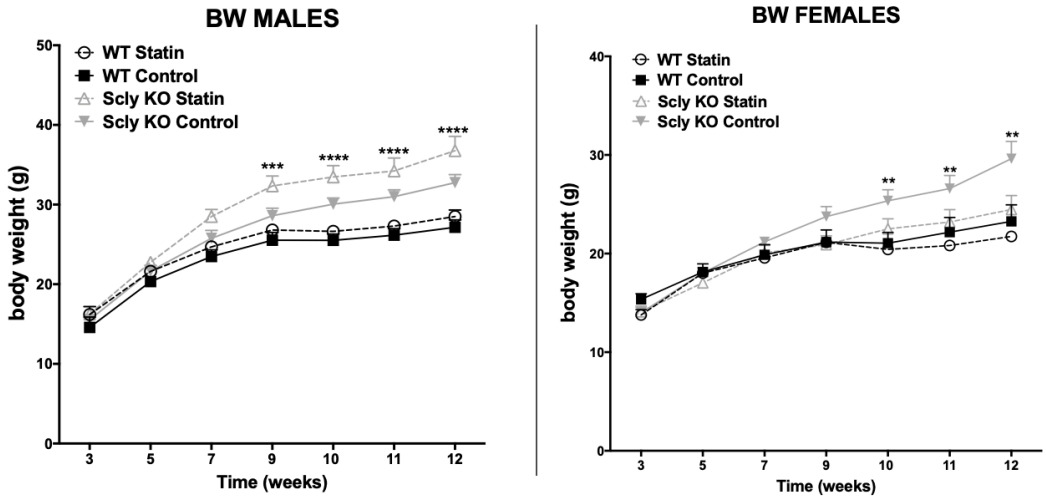

Figure 1: Body weight increases from age 3 to 12 weeks. Two-way analysis of variance (ANOVA) was applied followed by Bonferroni's post-hoc test. **, P <0.01; ***, P <0.001, ****, P<0.0001.

Effectiveness of statin treatment

Serum cholesterol levels at the beginning of the statin treatment and at the time of euthanasia (4 months on diet and 3 weeks on statin) were decreased by 28% (males) and 20% (females) in the Scly KO mice and 21% (males) and 27% (females) in the WT mice. In order to confirm the effectiveness of statin treatment, we measured the squalene

synthase (SQS) in the liver of treated mice, since this enzyme is downstream of the mevalonate synthesis in the cholesterol biosynthesis pathway. Treatment with statin decreased levels of SQS this enzyme in both WT and Scly KO groups, with stronger decreases in female mice (**Figure 2**). Therefore, a genotype effect was not observed for the response to statin.

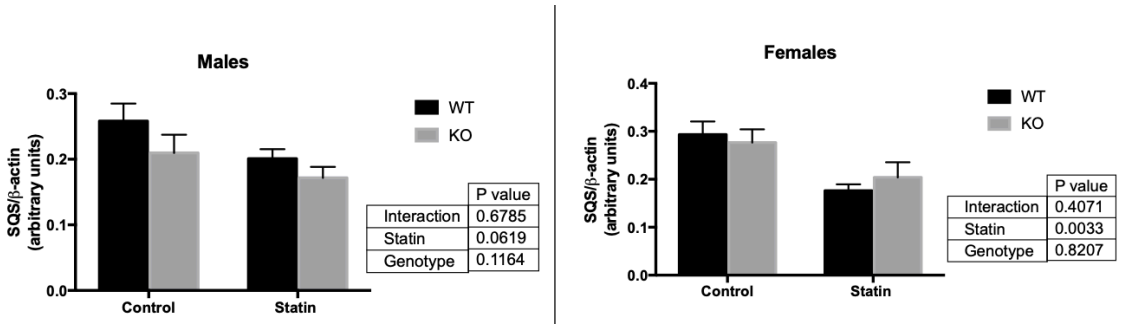

Figure 2: Hepatic squalene synthase (SQS) expression measured by Western blot and normalized by expression levels of b-actin n = 3-4 per group. Two-way analysis of variance (ANOVA) was applied followed by Bonferroni's post-hoc test.

Statin and oxidative stress

Scly KO mice were previously reported to present increased oxidative stress [15]. Statin treatment differentially affected circulating GPX activity between male and female Scly KO mice (**Table** 1). Additionally, we observed an improved hepatic oxidative status in WT and Scly KO mice for both sexes as indicated by serum measurements of hydroxynonenal (HNE)-his adduct after statin treatment.

Table 1. Serum and hepatic parameters of oxidative stress in WT and Scly KO mice fed a high-fat diet and 1.00 ppm of selenomethionine for 3 months.

MALES	**Control**		**Statin**		**2way ANOVA**		
	WT	KO	WT	KO	P_{int}	P_{Statin}	P_{Gen}
GPX act serum (nmol/mL^{-1})	40.7±1.5	43.0±2.4	41.0±1.0	41.9±1.0	0.342	0.6052	0.0399
GPX act liver (nmol/mg^{1})	1.24±0.04	1.20±0.04	1.17±0.03	1.23±0.05	0.0132	0.2036	0.6066
HNE liver (μg/mL)	12.8±5.4	18.9±10.9	6.2±3.5	7.3±4.1	0.3531	0.0023	0.1874
HNE serum (μg/mL)	3.4±1.6	8.5±8.2	2.6±1.6	5.0±3.4	0.4234	0.2151	0.0322

FEMALES	Control		Statin		2way ANOVA		
	WT	KO	WT	KO	P_{int}	P_{Statin}	P_{Gen}
GPX act serum (nmol/mL^{-1})	49.4±1.2	52.0±1.0	46.7±0.9	50.9±1.9	0.1945	0.0053	<0.0001
GPX act liver (nmol/mg^{1})	1.33±0.11	1.25±0.04	1.26±0.07	1.23±0.04	0.3226	0.1726	0.093
HNE liver (μg/mL)	14.5±14.2	31.7±16.7	11.1±3.7	9.4±3.6	0.0504	0.014	0.1148
HNE serum (μg/mL)	3.9±1.4	7.7±4.4	3.4±0.8	5.6±2.2	0.4948	0.2437	0.0157

Values are mean ± SEM. Two-way analysis of variance (ANOVA) was applied followed by Bonferroni post hoc test. p-Values under 0.05 deemed significant; n = 5–8. WT: wild type, Scly KO; selenocysteine lyase knockout, GPX: glutathione peroxidase, HNE: 4-Hydroxy-Trans-2-Nonenal; Act: activity.

Statin treatment and muscle metabolism

Interestingly, statin treatment differentially affected muscle parameters between male and female mice (**Table 2**). After statin treatment, we observed increased CK activity in the serum and decreased in the soleus of male mice, a finding that could indicate development of muscle disorders. In female mice, on the other hand, treatment with statin led to higher levels of CK activity in the serum without affecting the CK activity in the soleus. A genotype effect was observed only for CK activity in the liver of female Scly KO mice.

Table 2. Creatine Kinase (CK) activity in WT and Scly KO mice fed a high-fat diet and selenomethionine for 3 months.

MALES	**Control**		**Statin**		**2way ANOVA**		
	WT	KO	WT	KO	P_{int}	P_{Statin}	P_{Gen}
CK act serum (U/L)	102.3±69.8	118.0±43.7*	101.0±44.8	165.1±31.2*	0.2471	0.2738	0.0637
CK act liver (U/L)	236.3±15.2	225.6±37.1	228.4±36.0	248.5±20.8	0.1878	0.5154	0.6809
CK act soleus (U/L)	224.0±31.1	213.2±34.1*	138.3±62.0	159.6±36.3*	0.3999	0.0015	0.7799

FEMALES	**Control**		**Statin**		**2way ANOVA**		
	WT	KO	WT	KO	P_{int}	P_{Statin}	P_{Gen}
CK act serum (U/L)	92.5±56.2	101.6±66.2*	185.7±60.5	203.2±48.2*	0.8739	0.0016	0.6174
CK act liver (U/L)	154.1±34.4	82.7±57.7	167.2±56.5	80.2±43.0	0.7214	0.808	0.0019
CK act soleus (U/L)	240.9±31.9	192.2±34.8	206.1±44.0	205.5±68.3	0.2395	0.5918	0.2288

Values are mean ± SEM. Two-way analysis of variance (ANOVA) was applied and p-Values under 0.05 were deemed significant. Bonferroni's *post-hoc* test was performed and (*) represented $p < 0.05$; n = 5–8. WT: wild type; Scly KO: selenocysteine lyase knockout; Act: activity.

Statin treatment and hepatic mRNA expression

Surprisingly, the livers of mice did not present changes in mRNA expression of selenoproteins SelenoP, Gpx1, and Txnrd1 (**Table 3**). Guanidinoacetate N-methyltransferase (GAMT), known to be involved in creatine metabolism in the liver, was differentially expressed according to sex. We found decreased expression of *Gamt* in the liver of Scly KO male mice but not in Scly KO female mice. We did not find

differences for GATM or Ck-b after statin supplementation or between genotypes for males and females.

Table 3. Hepatic gene expression of selenoprotein and metabolic enzymes of wild-type and selenocysteine lyase knockout mice after high-fat, high se diet exposure, assessed by quantitative polymerase chain reaction (qPCR).

MALES	**Control**		**Statin**		**2-way ANOVA (p-value)**		
	WT	**KO**	**WT**	**KO**	**Interaction**	**Statin**	**Genotype**
SelenoP	332.4±65.7	438.7±125.8	366.7±74.0	315.0±74.4	0.0321	0.2075	0.4348
Gpx1	40.0±11.1	54.8±7.5	48.5±7.7	44.1±11.4	0.0001	0.062	0.2543
Txnrd1	1.8±0.13	2.2±0.7	2.1±0.4	1.7±0.2	0.0116	0.5111	0.9703
Ck-b	0.10±0.04	0.13±0.03	0.14±0.03	0.07±0.02	0.0015	0.3496	0.1708
Gamt	4.4±1.0	6.2±0.7*	6.0±1.3	4.3±1.2*	0.0019	0.8037	0.9861
Gatm	0.22±0.04	0.25±0.03	0.25±0.02	0.17±0.02	0.0002	0.0611	0.0895
FEMALES	Control		Statin		2-way ANOVA (p-value)		
	WT	**KO**	**WT**	**KO**	**Interaction**	**Statin**	**Genotype**
SelenoP	642.8±68.8	586.1±140.6	510.9±74.8	573.5±134.3	0.2384	0.1572	0.9496
Gpx1	66.8±11.5	63.7±15.2	72.4±20.2	76.9±22.8	0.6367	0.2431	0.9308
Txnrd1	2.2±0.4	2.8±0.8	2.3±0.7	2.1±0.4	0.2105	0.2478	0.4508
Ck-b	0.2±0.03	0.3±0.1	0.2±0.04	0.3±0.1	0.8664	0.7909	0.0783
Gamt	9.7±2.8	7.1±1.7*	6.5±2.1	12.8±1.8*	0.0002	0.2045	0.0646
Gatm	0.5±0.2	0.4±0.2	0.3±0.04	0.4±0.1	0.1441	0.1151	0.8755

Values are mean ± SEM and were normalized to HPRT mRNA levels. Two-way analysis of variance (ANOVA) was applied and p-Values under 0.05 were deemed significant. Bonferroni's *post-hoc* test was performed and (*) represented $p < 0.05$; n = 4–6.

Statin treatment and soleus mRNA expression

Reinforcing the sexual dimorphism**,** mRNA expression of *Gpx1, SelenoN* and *SelenoP* in soleus were different between males and females. Whereas statin treatment led to lower *Gpx1* expression in males, in females we found increased expression of selenoproteins (*SelenoN*, *SelenoP* and *Gpx1*) and *Ckm* genes.

Table 4. Gene expression of selenoprotein and metabolic enzymes in soleus muscle of wild-type (WT) and selenocysteine lyase knockout (Scly KO) mice after high-fat, high se diet exposure, assessed by quantitative polymerase chain reaction (qPCR).

MALES	**Control**		**Statin**		**2-way ANOVA (p-value)**		
	WT	**KO**	**WT**	**KO**	**Interaction**	**Statin**	**Genotype**
SelenoN	0.097±0.03	0.138±0.06	0.162±0.03	0.125±0.04	0.0497	0.1697	0.9106

SelenoP	6.6±1.3	8.2±3.0	8.6±1.7	8.7±02.2	0.4367	0.1910	0.3785
Gpx1	0.55±0.08	0.96±0.1*	0.77±0.1	0.59±0.1*	<0.0001	0.1738	0.0464
Ck-m	142.1±23.4	203.5±42.3	177.5±50.1	180±45.7	0.1351	0.7538	0.1074
FEMALES	**Control**		**Statin**		**2-way ANOVA (p-value)**		
	WT	**KO**	**WT**	**KO**	**Interaction**	**Statin**	**Genotype**
SelenoN	0.12±0.02	0.10±0.02*	0.09±0.01	0.15±0.01*	0.0003	0.2436	0.0115
SelenoP	10.3±2.1	6.2±1.0*	9.0±2.8	10.4±2.0*	0.0112	0.1379	0.1841
Gpx1	0.54±0.14	0.48±0.07	0.74±0.30	0.73±0.24	0.7524	0.0269	0.6926
Ck-m	126.7±15.6	128.5±28.1*	144.9±39.7	187.4±27*	0.1363	0.0087	0.1067

Values are mean ± SEM and were normalized to HPRT mRNA levels. Two-way analysis of variance (ANOVA) was applied p-Values under 0.05 deemed significant. Bonferroni's *post-hoc* test. * ($p < 0.05$) was used; n = 4–6.

Ampli-Seq analysis

Ampli-Seq analysis revealed 18 genes in which the statin effect is differently expressed in the liver of Scly KO and WT mice when compared with control (**Table 5**), wherein 3 genes are expressed only in male mice and 15 genes in female mice. Genes differentially expressed in male mice were cytochrome P450, family 3, subfamily a, polypeptide 11 (*Cyp3a11*), cytochrome P450, family 3, subfamily a, polypeptide 44 (*Cyp3a44*), insulin-like growth factor-binding protein 2 (*Igfbp2*), transcript variant 1. In female mice, genes differentially expressed included Mus musculus AT-rich interactive domain 5B (MRF1-like) (*Arid5b*), transcript variant X3 and lipocalin 2 (*Lcn2*).

Table 5. Differentially expressed genes between control and statin unveiled by Ampli-Seq analysis with livers from Scly KO and WT mice fed a high-fat diet containing 1 ppm of selenite/selenomethionine blend.

Males - WT and KO (Control x Statin)					
Gene ID	**Symbol**	**P-adj**	**P-value**	**lfcSE**	**log2 Fold change**
16008	*Igfbp2*	7.28E-08	0.0E+00	0.1736	-1.1322
13112	*Cyp3a11*	2.15E-02	2.2E-06	0.0014	-0.00001
337924	*Cyp3a44*	4.3E-02	6.7E-06	0.2664	-1.0012
Females - WT and KO (Control x Statin)					
Gene ID	**Symbol**	**P-adj**	**P-value**	**lfcSE**	**log2 Fold change**
12323	*Camk2b*	1.2E-07	0.0E+00	0.3700	-2.3449
71371	*Arid5b*	5.8E-04	1.0E+07	0.2992	1.4011
16819	*Lcn2*	5.8E-04	2.0E-07	0.5383	-2.5148
14219	*Ctgf*	6.1E-03	2.4E-06	0.4614	1.8642
266645	*Acmsd*	1.0E-02	5.0E-06	0.3160	1.2195

18626	*Per1*	1.9E-02	1.3E-05	0.5064	1.8320
229700	*Rbm15*	1.9E-02	1.7E-05	0.3355	1.1832
78889	*Wsb1*	1.9E-02	1.7E-05	0.3814	1.3518
50708	*Hist1h1c*	1.9E-02	1.7E-05	0.3597	-1.2772
100637	*N4bp2l1*	2.0E-02	2.0E-05	0.2578	0.8973
72962	*Tymp*	4.5E-02	4.9E-05	0.3456	-1.1233
331004	*Slc9a9*	4.9E-02	5.9E-05	0.4911	1.5539

Discussion

In the present study, we aimed to better understand the effects of the use of statins in an animal model of obesity. It was previously reported that Scly KO mice are more susceptible to diet-induced obesity than their WT counterparts, despite receiving adequate Se supply [14]. Moreover, male and female Scly KO mice were equally prone to high-fat diet-induced obesity [16]. The results presented herein suggest that the use of statin aggravate weight gain only in male Scly KO mice while female decreased their body weight after statin use. Famer and Crisby (2007), demonstrated that the treatment with rosuvastatin did not reduce the accelerated weight gain in apolipoprotein E (ApoE) knockout mice submitted to the high chloesterol diet. The authors connected the absence of rosuvastatin effect in the bodyweight of mice to the impairment of metabolic pathways caused by ApoE deficiency [17]. It was demonstrated that male mice lacking Scly not only become obese but also develop metabolic syndrome after a high-fat diet [15], indicating a strong influence of the genotype in energy metabolism that could affect the response to the statin treatment. Moreover, our Ampli-Seq analysis revealed a downregulation of *Igfbp2* in male Scly KO mice using statin that could be connect with the worsened obesity state after statin treatment. IGF-binding protein-2 (IGFBP-2) is a 36-kDa protein that binds to the insulin-like growth factors (IGF) with high affinity [18]. IGFBP-2 concentrations correlate inversely with body mass index and lower adiposity. Recently, Haywood et al. (2018) demonstrated that mice overexpressing human *Igfbp-2* had reduced susceptibility to obesity and also improved insulin sensitivity. This was also associated with decreased leptin levels, increased glucose sensitivity and lower blood pressure compared with obese wild type mice [19].

In contrast, the use of statin was connected with positive effects in bodyweight of female mice, especially the Scly KO mice. The *Lcn2* may be linked with this effect since was expressed only in female mice and the expression was proportional to the bodyweight

gain. Interestingly, Scly KO mice treated with a statin had a lower weight gain, similar to WT (control and statin), and a downregulation of *Lcn2* expression. On the other hand, Scly KO controls had increased weight gain and an up-regulation of *Lcn2* expression. LCN2 (*Lcn2* in mice), also known as neutrophil gelatinase-associated lipocalin (NGAL), is a 25 kDa secreted glycoprotein encoded by a gene located at the chromosome locus 9q34 [20]. Among the members of the adipokines superfamily, LCN2 has emerged as a pleiotropic molecule involved in a variety of physiological and pathophysiological processes, such as metabolic homeostasis, apoptosis, infection, immune response, or inflammation [21]. Recently, Ishii et al. (2017), showed that *Lcn2* KO female mice were resistant against diet-induced obesity and circulating LCN2 possesses an endocrine activity to inhibit BAT activity [22].

Our results indicated that the statin treatment influenced the susceptibility of muscle disorders measured by CK activity levels in a sex-dependent fashion. The Ampli-Seq analysis complemented our findings regarding the effects of statin and sexual dimorphism. In the liver of male mice, regardless of the genotype, we observed differentially expression of *Cyp3a11* and *Cyp3a44*. CYPs are a superfamily of phase-I enzymes which play a leading role in drug metabolism and detoxification. Up to 75% of drugs are metabolized by CYP enzymes. Of all CYP enzymes, CYP3A4 (*Cyp3a11* in mice) catalyzes the initial step in the detoxification pathways of many foreign compounds, including prescription drugs. Atorvastatin, lovastatin, and simvastatin are metabolized primarily by CYP3A4 [23–25]. The variability in CYP3A expression creates a potential for harmful drug interactions involving these isozymes [26]. Studies have suggested that gender differences could affect the expression of CYP3A4 [26,27]. Unlike other CYP3As, murine *Cyp3a44* is expressed predominantly in the female liver, with lower levels in male livers [6]. In the present study, apart from gender-biased regulation, the up-regulation of male CYP3A expression appears to be related to the statin side-effects. In accordance with our results, Bhadhprasit et al. (2007) indicated that not only gender, but also the xenobiotic activators, and nuclear receptors act to control *Cyp3a44* gene expression [27].

In the current study, we performed the analysis of SQS to confirm the effectiveness of statin treatment since studies have reported different effects of treatment with statins on plasma and lipoprotein cholesterol levels in mice [28]. SQS measurement has been suggested as a biomarker of statin treatment, since this enzyme is downstream of the mevalonate synthesis in the cholesterol biosynthesis pathway, hence

downregulated by statins [6]. SQS is one of the key enzymes in the synthesis of cholesterol [29]. SQS catalyzes the condensation of two molecules of farnesyl-PP, yielding squalene. Squalene undergoes a two-step cyclization to yield lanosterol. Through a series of 19 additional reactions, lanosterol is finally converted to cholesterol [6].

The effect of statin on oxidative stress still is unclear [30]. While numerous studies have reported pleiotropic antioxidant effects of statins in vitro and in vivo, other studies have implicated the opposite, with some of them even demonstrating massive prooxidant effects in atherosclerotic patients [30]. In the present study, the hepatic oxidative status was improved by statin use regardless of genotype or sex influence. Interestingly, we previously demonstrated that Scly KO mice were further susceptible to oxidative stress when compared with WT [15], and now we demonstrated that the use of statin could mitigate the effect of genotype on oxidative stress. The influence of genotype was determinant for the circulating levels of GPX activity in both sexes, wherein the statin uses only affected the female Scly KO mice.

The absence of results in the hepatic mRNA expression of selenoproteins in mice suggested that the isopentenylation reduction due to the use of statins does not decrease selenoprotein gene expression, contradicting Moosmann and Behl (2004) who hypothesized that the cause of statin-induced myopathy could be found in a depletion of selenoproteins [1]. Considering that our mice were supplemented with a high Se diet, a possible explanation could be that a saturation effect of selenoproteins gene expression at optimum Se levels. Continuing the analyzes of gene expression in the liver, we found differences in the mRNA expression of GAMT in a sex-specific manner, in which Scly KO males mice had a decreased expression when compared with Scly KO females. GAMT, known to be involved in creatine metabolism in the liver, is responsible for the transfer of a methyl group from S-adenosylmethionine (SAM) to guanidinoacetate (GAA) to produce creatine [31]. Decreased expression of GAMT could affect the levels of creatine and creatine fractions in skeletal muscle [31]. Therefore, our results indicated that the use of statin may impair creatine levels in males without affecting the females.

The gene expression in the soleus muscle is also sex-dependent. For male mice, the reduced *Gpx1* expression in soleus could indicate that statins invariably induce a pro-oxidative activity in muscle cells [30]. Excessive reactive molecules and free radicals derived from molecular oxygen and/or nitric oxide unchecked by antioxidant defenses have negative impact on muscle contractile proteins, mitochondrial phospholipids, or DNA, and have been involved primarily or secondarily in the pathophysiology of muscle

aging (sarcopenia), of muscle fatigue and various muscular disorders [32]. For females, the increased expression of selenoproteins (*SelenoN*, *SelenoP* and *Gpx1*) and *Ckm* genes, could indicate a protective factor for muscle homeostasis. Each selenoprotein (*SelenoN*, *SelenoP,* and *Gpx1*) and *Ckm* play a specific role that contributes to proper muscle function. SelenoN is an endoplasmic reticulum-localized selenoprotein whose loss-of-function may be linked to skeletal muscle pathology [1,33]; SelenoP is crucial to Se homeostasis as it sustains retention of Se in the body and promotes its distribution from the liver to extra-hepatic tissues, especially under Se-deficient conditions [8,34]; and GPX is involved in hydrogen peroxide and hydroperoxide detoxification, protecting against oxidative injury. Moreover, CKm, a muscle isoenzyme of CK, has the unique property of binding with the M-line of the sarcomere and maintains energetic homeostasis as variations in energy requirements dictate that ATP be readily available. The reduction of CKm activity may be a major contributor to the gradual loss of muscle function [5].

Taken together, these results indicated that statins could modulate obesity in mice fed a high-fat, Se-supplemented diet and lacking Scly in a sex-dependent manner. Statin treatment worsened obesity in male Scly KO mice while led to weight loss in female mice. Statin treatment was associated with an improvement of hepatic oxidative status in WT and Scly KO mice for both sexes. However, alterations in CK activity levels in serum and muscle may indicate muscle damage in male mice. Statin treatment influenced muscle metabolism also in a sex-dependent way, independent of selenoprotein expression, but influenced by the expression of *Gamt*. Statin treatment improved selenoprotein expression in the soleus, as well as expression of *Ckm*, in Scly KO female mice, while worsening *Gpx1* expression in males. Moreover, the transcriptomics analyses complemented our findings regarding the sexual dimorphism in the current study. In summary, sexual dimorphism should be considered in the prescription of statins as this aspect may interfere with treatment outcomes.

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Author contributions

Conceptualization, LAS and MJB; data collection and analysis, LMW, LAS, ACH and DJT; writing, LMW and LAS; review and editing, all authors; funding acquisition, MJB, LAS, and LMW.

Conflict of Interest

The authors declare no conflicts of interest.

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ANNEX IV: Research Internships Abroad (BEPE) - Effects of selenium supplementation in diet-induced obesity of mice with a disruption of the selenocysteine lyase gene

Ph.D. Internship Project - Research Internships Abroad (BEPE)
Regular PhD FAPESP Fellowship: 2018/09478-4

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TITLE: Effects of Selenium Supplementation in Diet-Induced Obesity of Mice with a Disruption of the Selenocysteine Lyase Gene

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Running head: Selenium supplementation and the Scly KO mouse model

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Abstract

The amino acid selenocysteine (Sec) is an integral part of selenoproteins, a class of proteins mostly involved in strong redox reactions. The enzyme Sec lyase (SCLY) decomposes Sec into selenide to reenter the selenoprotein synthesis machinery, allowing for the recycling of the selenium (Se) atom. We previously demonstrated that disruption of the *Scly* gene (Scly KO) in mice leads to the development of obesity and metabolic syndrome, with effects on glucose homeostasis, worsened by Se deficiency or a high-fat diet, and exacerbated in male mice. Our objective was to determine whether Se supplementation could ameliorate obesity and glucose homeostasis in the Scly KO mice. Age-matched male and female Scly KO mice were fed a diet containing 45% kcal fat and either 0.25 ppm (adequate) or 1 ppm (high) of either sodium selenite or a mixture of sodium selenite and selenomethionine (selenite/SeMet) for 12 weeks. Se supplementation was not able to reverse obesity and elevated epididymal white adipose tissue weights in male Scly KO mice. Strikingly, female Scly KO mice became even more obese when selenite-supplemented, despite unchanged glucose intolerance. Serum glutathione peroxidase activity in Scly KO mice was unchanged regardless of sex or dietary Se intake; however, supplementation with a mixture of selenite/SeMet improved total oxidative stress in the male Scly KO mice. These results suggest that dietary chemical forms of Se are metabolized differently and in a sex-specific manner, with Scly demonstrating to be involved in the control of whole-body energy metabolism regardless of Se levels.

Introduction

Selenium (Se) is a trace element essential to health and acquired from dietary sources such as Brazil nuts, seafood and plants growing in Se-rich soils. In our diets, Se is available as various chemical forms, with the predominance of inorganic selenite or selenate, and organic selenomethionine (SeMet) or selenocysteine (Sec) [1]. Dietary selenocompounds differ significantly in their metabolic pathways and in their abilities to produce various Se metabolites [2,3], and to become bioavailable, selenocompounds are metabolized in cells to selenide. Selenide is utilized to produce the amino acid Sec, present in the primary structure of proteins deemed selenoproteins, and allowing for a strong redox potential. Interestingly, Sec needs to be synthesized from selenide in its $tRNA^{[Ser]Sec}$ in order to be co-translationally incorporated into selenoproteins [4,5]. The mechanism of Sec synthesis and selenoprotein production has been well-characterized by several groups [6–9].

In the liver, inorganic selenite and selenate are reduced via the glutathione/thioredoxin redox cycle. Organic SeMet is metabolized by the same enzymes of the methionine-decomposing transulfuration pathway, such as cystathione beta-synthase (CBS) [10] and cystathione gamma-lyase (CGL, also known as Cth) [11], or can be transaminased by kynurenine aminotransferase III (KYAT3, also known as Ccbl2) into α-ketomethylselenobutyrate [12]. Sec is decomposed into alanine and selenide by the enzyme Sec lyase (SCLY) [13], while other Se-conjugates of Sec can be metabolized by the same KYAT3 into β-methylselenopyruvate [12]. Sec may also be decomposed, in a less efficient manner, by cysteine desulfurases such as NIFS [14].

SCLY utilizes Sec, either from dietary sources, selenoprotein degradation or SeMet metabolism [15]. Sec has not been found freely in cells, suggesting that after selenoproteins exert their biological function and are degraded, the Sec residue should be promptly decomposed to yield selenide. This Se recycling process may be a significant source of Se when this micronutrient is limiting.

We previously uncovered that mice with a disruption in the *Scly* gene (Scly KO) became obese, glucose intolerant and hyperinsulinemic, marked characteristics of metabolic syndrome development, and with reduced hepatic Se levels [16]. Obesity in the Scly KO mice was sexually dimorphic [17], and exacerbated by feeding of a Se-adequate, high-fat diet [18] or by Se deficiency [16,17], conditions in which mild hepatic Se deficiency was observed when amino acid pathways were altered, possibly to cope

with the disruption in Se recycling [19]. Nevertheless, it is unknown whether diet-induced obesity in the Scly KO mice could be amelliorated by dietary Se supplementation.

In this study, we investigated whether Se supplementation can rescue the obese phenotype of the Scly KO mouse model fed a high-fat diet. We also determined that Se supplementation delivers a differential metabolic response depending on the selenocompound present in diets. We found that male Scly KO mice experienced aggravated obesity after Se supplementation, a phenotype worsened in female Scly KO mice. Moreover, expression of selenoproteins glutathione peroxidase 1 (GPX1) and selenoprotein P (SelenoP) in the Scly KO mice was differentially regulated by the dietary Se chemical form ingested.

2. Materials and Methods

Chemicals

All reagents are from Sigma-Aldrich/MilliporeSigma (Burlington, MA, USA) unless otherwise noted.

Animals and diets

Age-matched homozygous Scly KO mice and C57BL/6N wild-type (WT) homozygous animals derived from The Jackson Laboratory (Bar Harbor, ME) were born and raised in our vivarium and used in experiments after weaning in accordance with the Institutional Animal Care and Use Committee of the University of Hawaii (Protocol #17-2616). Animals were euthanized by CO_2 asphyxiation prior to collection of tissues from each experimental mouse. Animals were fed for 8 weeks with a customized high-fat diet containing 45 kcal% fat as lard and 35 kcal% carbohydrate as a mixture of sucrose and corn starch (Research Diets, Inc., New Brunswick, NJ) supplemented with either sodium selenite or a blend of sodium selenite plus SeMet containing a fixed amount of selenite (0.15 ppm).

Glucose tolerance test

For the glucose tolerance test (GTT), mice were fasted for 4 h and injected intraperitoneally with glucose (1 g/kg of body weight). Tail vein blood was collected at 0, 30, 60, and 120 min post-injection and tested for glucose using glucose strips and a

glucometer (LifeScan, Milpitas, CA, USA). The area under the curve (AUC) was calculated for individual mice, averaged for the group and plotted as bar graphs.

Glutathione peroxidase (GPX) activity assay

Total serum or liver GPX activity was measured using a colorimetric assay kit (Abcam, Cambridge, UK, catalog no. Ab102530) following the manufacturer's protocol. In the GPX assay protocol, GPX oxidizes GSH to produce GSSG as part of the reaction in which it reduces cumene hydroperoxide. Glutathione reductase (GR) then reduces the GSSG to produce GSH, and in the same reaction consumed NADPH. The decrease of NADPH (measured at OD=340 nm) is proportional to GPX activity.

Measurement of oxidative stress\

Oxidative stress status was assayed by measuring lipid peroxidation in the serum and liver tissues using the OxiSelect HNE-his Adduct ELISA Kit (Cell Biolabs, Inc., San Diego CA, USA, catalog number: STA – 838-5), according to the manufacturer's protocol.

Quantitative real-time PCR (qPCR)

One microgram of total RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kits (Applied Biosciences/ Thermo Fisher Scientific, Waltham, MA, USA), with 10 ng of resulting cDNA used for qPCR with PerfeCTa SYBR Green FastMix (Quantabio, Beverly, MA, USA) and 45 amplification cycles in a 384-well plate platform of a LightCycler 480 II (Roche, **Basel,** Switzerland). Relative quantification used the Δ^{-CT} method, normalized to hypoxanthine-guanine phosphoribosyltransferase (*Hprt1*) expression levels. All primers were evaluated for their efficiency prior to use in experiments. Primer sequences are provided in **Table 1**.

Statistical analysis

Data were plotted in GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Applied statistical tests varied depending on the experiment and differences were considered significant if reaching p-values under 0.05. Body weight assessment was evaluated after mixed-effects analysis to include the three experimental variables.

Results

We previously reported that Scly KO mice are more susceptible to diet-induced obesity than their WT counterparts, despite receiving adequate Se supply. In the present study, male Scly KO mice fed 45% kcal high-fat diet supplemented with Se developed obesity regardless of dietary Se levels or chemical form (**Figures 1A and 1B**). Nevertheless, female Scly KO mice presented aggravated weight gain when receiving Se supplementation (**Figures 1C and 1D**). We did not observe differences in body weight for WT mice, regardless of sex. The observed increases in body weight in the Scly KO mice were reflected in the weight of the epididymal and gonadal white adipose tissues of mice, which were also significantly higher in comparison to WT mice (**Figure 2).**

After eight-weeks on a Se-supplemented high-fat diet, Scly KO mice of both sexes were more severely glucose intolerant when compared with their WT counterparts (**Figure 3**). Selenite/SeMet blend (**Figure 3C and 3D**), but not selenite (**Figure 3A and 3B**), supplementation improved glucose tolerance in males and females Scly KO mice.

Different forms of Se supplementation influenced the oxidative stress parameters in distinct ways. Selenite supplementation elevated serum GPX activity regardless of sex or dietary chemical form of Se (**Table 2 and 3).** This effect occurred despite unchanged hepatic GPX activity in most groups except female Scly KO mice fed selenite (**Table 2)**. Nevertheless, selenite supplementation did not enhance GPX activity of male Scly KO mice, although they had higher GPX activity when compared with WT (**Table 2**). Selenite supplementation did improve, however, male WT oxidative stress status as assessed by lipid peroxidation (**Table 2**), On the other hand, supplementation with selenite/SeMet blend not only increased circulating GPX activity of Scly KO mice but also improved the hepatic oxidative status of Scly KO mice measured by HNE adduct formation (**Table 3**).

To further investigate the effects of distinct forms of Se supplementation in the Scly KO mice we determined mRNA expression of selenoprotein genes, as well as genes involved in Se and energy metabolism (**Tables 4 and 5**). We did not detect changes in hepatic expression of selenoprotein genes (*Selenop, Gpx1, Txnrd1*) in WT or Scly KO mice, males and females, after feeding a selenite diet (**Table 4**). Nevertheless, when fed a selenite/SeMet blend, we observed downregulation of *Selenop* in males at adequate Se levels, and this effect disappeared after supplementation. In female Scly KO mice fed a selenite/SeMet blend, the opposite occurred, with an upregulation of *Selenop* gene expression at adequate levels, and downregulation after supplementation (**Table 5**). Moreover, Se-sensitive selenoprotein *Gpx1* expression was unaffected by diets containing selenite in male mice, but downregulated in female Scly KO mice, (p value of

0.0582; **Table 4**). Selenite/SeMet dietary intake led to the same gene expression pattern as *SelenoP* in female Scly KO mice (**Table 5**). Combined, these results demonstrate that saturation of selenoprotein gene expression at optimum Se levels depends on the chemical form used in diets.

Particularly, we have noted that expression of the master regulator of lipid metabolism peroxisome proliferator-activated receptor gamma (*Pparg*) and selenium-binding protein 2 (*Selenbp2*) were differentially regulated in the livers of Scly KO mice when compared to their WT countersparts whne dietary Se was limiting [19]. With Se supplementation, we observed that male Scly KO mice decreased expression levels of *Selenbp2*, a result not influenced by the concentration of Se supplementation (**Tables 4 and 5**). *Pparg* expression was upregulated in the liver of Scly KO mice regardless of Se form or sex as well. In terms of Se metabolism, lower levels of *Ccbl2* gene expression occurred only in the selenite group. In female Scly KO mice, *Nifs* expression was higher, and also independent of the Se form fed. Nevertheless, the increased levels of *Selenbp2* and *Cth* for female Scly KO mice were observed only with selenite/SeMet consumption.

Discussion

This is the first study to characterize the effects of Se supplementation in mouse lacking the *Scly* gene and fed a high-fat diet. We previously noted that Scly KO mice had aggravated obesity when fed either a high-fat diet [18] or a Se-deficient diet, with a milder phenotype when dietary Se levels were adequate [16]. Here we attempted to assess whether Se supplementation could rescue, at least to some extent, the metabolic phenotype of the Scly KO mice.

To our surprise, Se supplementation with selenite did not rescue neither the weight gain nor the glucose tolerance of Scly KO mice, even worsening obesity in females. Interestingly, diets with a blend of selenite/SeMet still led to obesity in male Scly KO mice, regardless of Se concentration, despite improving their glucose tolerance. This differential effect of dietary intake of Se according to the chemical form on the development of obesity in the Scly KO mice attests for the different pathways that inorganic and organic Se undergo [20]. It also adds a pivotal role to SCLY in the transselenation pathway that metabolizes SeMet [15,21] at least in males. Notably, only female Scly KO livers presented elevated expression of the gene for transselenation enzyme *Cth*, indicating that not only these levels of dietary Se intake are activating the transselenation pathway in a sex-specific manner, but raising the possibility that male Scly

KO mice are either favoring the metabolism of SeMet through the alternative transamination pathway that produces α-ketomethylselenobutyrate [3,22], via α,γ elimination by Cth [11], or having a higher rate of misincorporation of SeMet into Met residues of proteins as it has been suggested to occur when the SeMet pool is larger [23]. Cth contributes to the elevation of GPX activity in a dose dependent manner following SeMet supplementation [24]. As we did not observe changes in hepatic GPX activity for the male Scly KO mice after supplementation under a high-fat diet, it is possible that the enhanced oxidative stress of the energy overload already induces and maximizes GPX actions, and the Scly requirement for its synthesis is circumvented by the limiting amount of selenite the diet contains. Another possibility for the fate of SeMet is that excess of this selenocompound leads to misincorporation of Sec in place of cysteine via mechanisms independent of oxidative stress levels [25,26], however this possibility has still been determined only in yeast. Regardless of the pathway being favored in mice with disruption of the Scly gene, it is clear that their prevalence varies with sex.

Male WT mice enhance their plasma GPX activity when supplemented with 0.75 ppm of SeMet, but not selenite, and hepatic GPX activity follows this pattern [27]. Our current results add a layer of complexity, as mice were fed a high-fat, Se-supplemented diet. In this paradigm, we uncovered the same patterns for GPX activity in male WT mice. Moreover, we found that serum activity of GPX in female WT mice does not respond to supplementation of both selenite or selenite/SeMet blend, and it remains unchanged in the liver as well. Notably, in the Scly KO mice, neither sex nor supplementation level affects GPX activity in the serum. Most of serum GPX activity comes from GPX3, produced by the kidneys [28]. Hence, it is possible that Scly participation in the synthesis of GPX3 in the kidneys is crucial to gauge and maintain levels of circulating GPX. Interestingly, in the liver, selenite/SeMet blend supplementation was sufficient for maintaining the redox status in female while male Scly KO mice considerably improved their status with supplementation, highlighting that both metabolic pathways of SeMet andmaintenance of redox status require different players according to sex.

SCLY has been postulated to providing selenide to selenoprotein synthesis [29] possibly via delivery to selenophosphate synthetases [20]. Selenoproteins, thus, are considered the main effectors of the biological actions of Se in organisms [8]. Previous studies observed that, for maximized selenoprotein expression, Se concentration requirements were lower than utilized in our study [30,31]. At 1 ppm dietary intake level, Se should not be a limiting factor for appropriate selenoprotein expression. Nevertheless,

when selenite was the dietary source of Se, we observed, for instance, that *Selenop* expression was still diminished in the female Scly KO mouse, but not in the males, as we observed before [16,18]. The expression of *Scly* gene in the male mouse liver has been shown to depend on the presence of *SelenoP* [32]. Differential regulation of *Selenop* expression according to sex has also been determined [33], however it was unclear which Se chemical form was used then. Based on our results, expression of *Selenop* showed the same pattern as previously reported only when feeding selenite, suggesting that varying dietary chemical forms of Se may trigger differential regulatory pathways to coordinate gene expression, and these differences have been investigated in WT mice at the proteome level [27].

Selenop expression was not regulated by sex when a mixture of selenite/SeMet at adequate levels was fed to WT mice. However, a sharp increase in expression occurred at supplemented levels in females but a decrease in males. Interestingly, this pattern was not maintained in the Scly KO mice fed a blend of selenite/SeMet. These results combined suggest that, even though selenide is the common element to provide Se for selenoprotein synthesis, the upstream reactions that form selenide are relevant to the regulation of selenoproteins, possibly even to the hierarchy of expression that this group of proteins present [31,34].

In conclusion, we found that the weight gain and glucose intolerance displayed by the Scly KO mice on a hypercaloric diet are not rescued by Se supplementation. Also, depending on the dietary chemical form of Se, lack of Scly produces a differential metabolic response that is attuned to the role of this enzyme in the transselenation pathway. As an energy-rich, Se-sufficient diet has become common in several countries, including the United States [35-37], our results steer future studies in human populations to also account for the differential, sex-dependent metabolism of Se, particularly when involving Scly, in improving our understanding of obesity development.

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Author contributions

Conceptualization, LAS and MJB; data collection and analysis, LMW, LAS, ACH and DJT; writing, LMW and LAS; review and editing, all authors; funding acquisition, MJB, LAS, and LMW.

Conflict of Interest

The authors declare no conflicts of interest.

Table and Figure legends

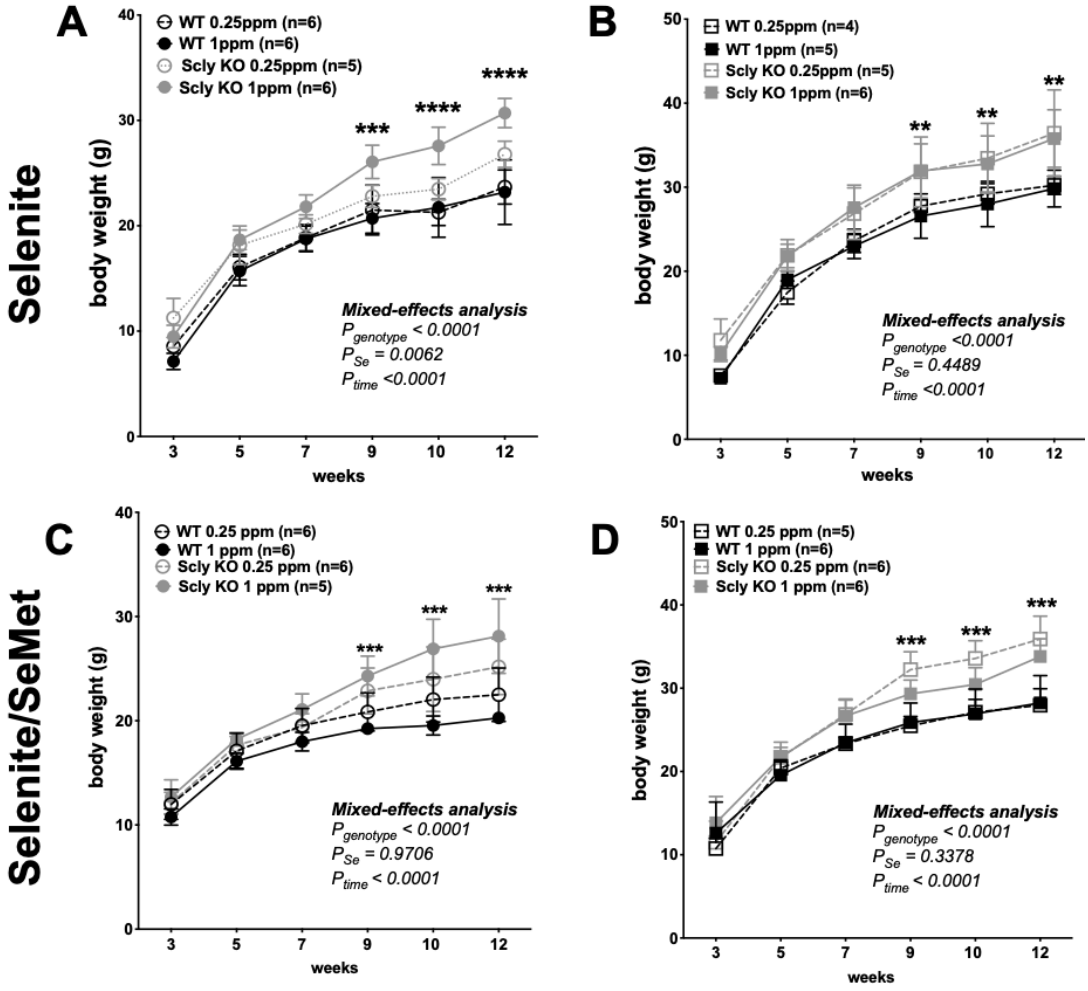

Figure 1. Body weight increases upon feeding a Se-supplemented high-fat in female mice fed with selenite (A) or selenite/SeMet (C), and male mice fed with selenite (B) or with selenite/SeMet (D). Mixed-effects analysis of variance was applied followed by Bonferroni's *post-hoc* test. **, p <0.01; ***, p <0.001, ****, p<0.0001, *n*= 4-6 per group. Circle, female mice; square, male mice; open circle or square, 0.25 ppm of selenocompound; filled circle or square, 1 ppm of selenocompound; black, WT; gray, Scly KO.

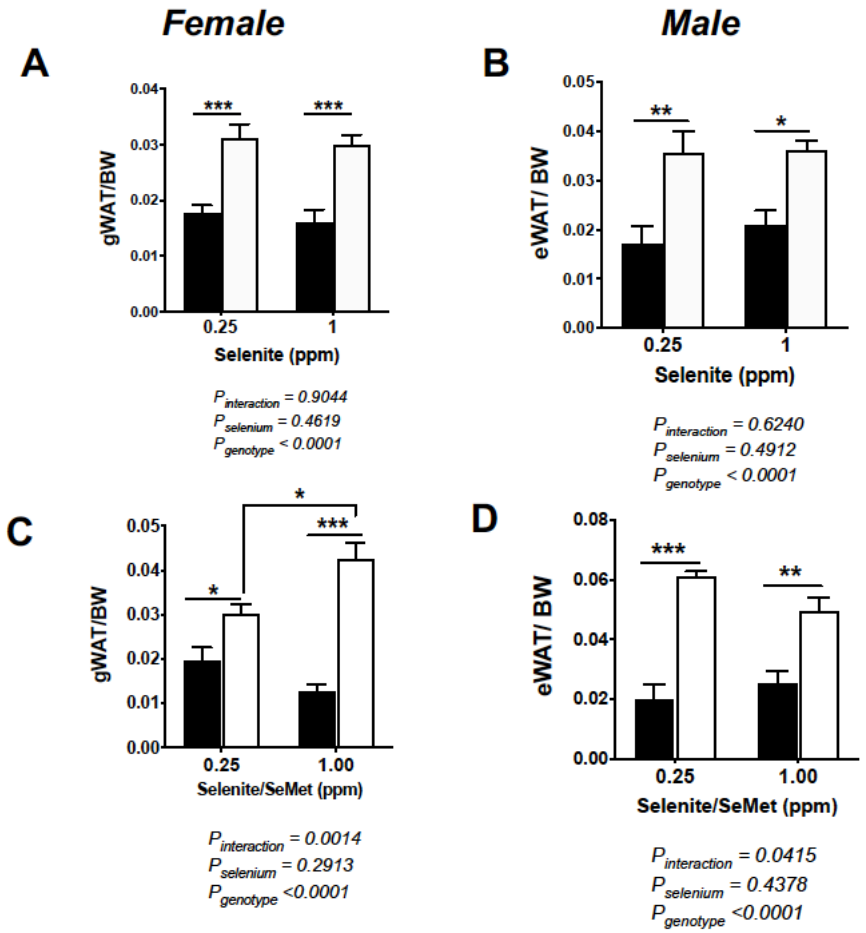

Figure 2. White adipose tissue weights after a high-fat diet in female 277 mice supplemented with selenite (A) or selenite/SeMet (C), and male mice supplemented with selenite (B) or with selenite/SeMet (D). Values are mean + SEM and were normalized by total body weight per mouse. Two-way analysis of variance was applied, followed by Bonferroni's post-hoc test. *,p<0.05; **, p <0.01; ***, p <0.001, n=9-11 per genotype. eWAT, epididymal white adipose tissue; gWAT, gonadal white adipose tissue. Filled bars, WT; open bars, Scly KO.

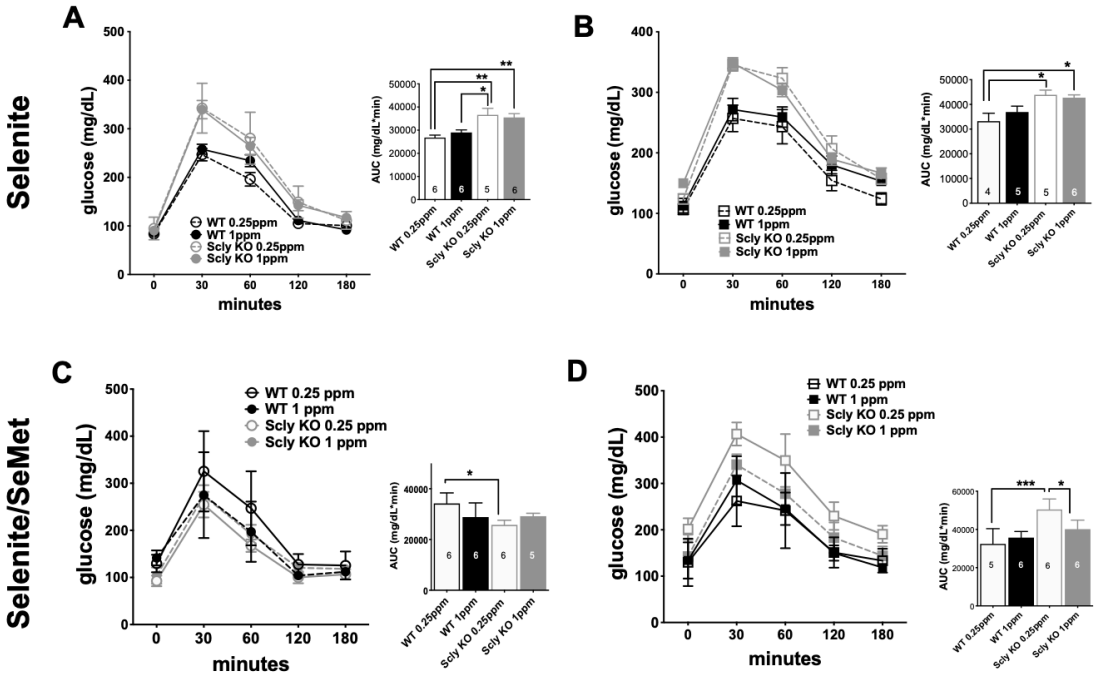

Figure 3. Glucose tolerance test after a glucose overload with area under the curve (AUC) quantification plotted as bar graph. GTT was performed at 10-weeks of age, after mice were fed a high-fat diet for 7 weeks. Female (A,C) and male (B,D) mice were fed with selenite (A, C) or a mixture of selenite/SeMet (B,D). Two-way analysis of variance (ANOVA) was applied followed by Bonferroni's *post-hoc* test. *, $p<0.05$, **, $p<0.01$; ***, $p<0.001$; $n = 4$-6 per group. Circle, females ; square, males; open circle or square, 0.25 ppm of selenocompound; filled circle or squares, 1 ppm of selenocompound; black, WT; gray, Scly KO.

Gene	Forward	Reverse
Ccbl2	5'-CCGTAGAAAATGGCTTTGAAAT	5'-AGTAAATTCAACCCACACATTG
Cth	5'-TGCCACCATTACGATTACCCAT	5'-TTGGTGCCTCCATACACTTCAT
Gpx1	5'-ACAGTCCACCGTGTATGCCTTC	5'-CTCTTCATTCTTGCCATTCTCCTG
Hprt1	5'-TCCTCCTCAGACCGCTTTT	5'-CCTGGTTCATCATCGCTAATC
Nifs	5'-AGTGGAGCTACTGAGTCCAA	5'-CACATTTGTGTTCTGTCTGG
Pparg	5'-TTGATTTCTCCAGCATTTCT	5'-TGTTGTAGAGCTGGGTCTTT

Selenbp2	5'-ATACTGCCTGGTCTCATGTC	5'-AAGGTGTTGACCATGACTTC
Selenop	5'-CCTTGGTTTGCCTTACTCCTTCC	5'-TTTGTTGTGGTGTTTGTGGTGG
Txnrd1	5'-CCTATGTCGCCTTGGAATGTGC	5'-ATGGTCTCCTCGCTGTTTGTGG

Table 1. Primer sequences used for qPCR analysis in this study.

MALES							
	WT		Scly KO		2way ANOVA		
	0.25	1.00	0.25	1.00	P_{int}	P_{Se}	P_{Gen}
GPX activity serum ($nmol/mL^{-1}$)	37.6±0.6	37.0±0.7[a]	38.7±1.2	40.9±1.9[b]	0.0264	0.1613	0.0004
GPX activity liver ($nmol/mg^{-1}$)	13.5±0.09	12.7±1.1	13.3±0.3	13.2±0.2	0.1940	0.1606	0.6859
HNE liver (μg/mL)	16.9±4.9[a]	9.98±3.3[b]	11.5±4.8	13.6±7.5	0.097	0.3514	0.734
FEMALES							
	WT		Scly KO		2way ANOVA		
	0.25	1.00	0.25	1.00	P_{int}	P_{Se}	P_{Gen}
GPX activity serum ($nmol/mL^{-1}$)	39.8±1.6	40.75±0[a]	41.76±1.6	44.29±1.0[b]	0.2466	0.02	0.0008
GPX activity liver ($nmol/mg^{-1}$)	12.8±0.2	12.9±0.3[a]	12.8±0.3	12.4±0.2[b]	0.0425	0.2502	0.1150
HNE liver (μg/mL)	11.7±7.7	6.6±1.5	10.4±1.4	10.73±5.2	0.2367	0.2921	0.5294

Table 2. Serum and hepatic parameters of oxidative stress in WT and Scly KO mice fed a high-fat diet and selenite for 9 weeks. Values are mean ± SEM. Two-way ANOVA was applied followed by Bonferroni *post-hoc* test. P-values under 0.05 were deemed significant; n = 4–6. WT, wild type; KO, knockout; GPX, glutathione peroxidase; HNE, 4-Hydroxy-Trans-2-Nonenal.

MALES							
	WT		Scly KO		2way ANOVA		
	0.25	1.00	0.25	1.00	P_{int}	P_{Se}	P_{Gen}
GPX activity serum ($nmol/mL^{-1}$)	41.2±3.8[a]	43.6±1.8	46.6±1.2[b]	45.8±2.4	0.1666	0.4905	0.0027
GPX activity liver ($nmol/mg^{-1}$)	13.3±0.2	13.3±0.1	13.4±0.08	13.4±0.3	0.9080	0.7292	0.3062
HNE liver (μg/mL)	6.8±1.4[a]	7.6±3.4	12.8±1.8[b]	7.9±2.1[a]	0.008	0.0495	0.0043
FEMALES							

	WT		Scly KO		2way ANOVA		
	0.25	1.00	0.25	1.00	P_{int}	P_{Se}	P_{Gen}
GPX activity serum (nmol/mL^{-1})	40.4±0.8^{a}	40.9±1.3^{a}	42.2±1.1^{b}	45.2±0.8^{c}	0.0155	0.001	<0.001
GPX activity liver (nmol/mg^{-1})	13.3±0.3	13.3±0.2	13.4±0.08	13.5±0.3	0.6771	0.6771	0.4564
HNE liver (µg/mL)	10.4±2.2	9.4±7.3	20.4±4.3	16.2±7.2	0.293	0.524	0.0025

Table 3. Serum and hepatic parameters of oxidative stress in WT and Scly KO mice fed a high-fat diet and selenite + selenomethionine for 9 weeks. Values are mean ± SEM. Two-way ANOVA was applied followed by Bonferroni *post-hoc* test. P-values under 0.05 were deemed significant; n = 4–6 per group. WT, wild type; KO, knockout; GPX, glutathione peroxidase; HNE, 4-Hydroxy-Trans-2-Nonenal.

MALES	**SELENITE**						
	0.25		**1**		**2-way ANOVA (p-value)**		
	WT	**KO**	**WT**	**KO**	**Interaction**	**Se**	**Genotype**
SelenoP	413.8±42.9	382.1±24.6	390.3±33.1	388.8±61.2	0.7291	0.8478	0.7050
Gpx1	80.4±3.7	64.7±6.4	67.7±6.1	65.2±3.8	0.2289	0.2593	0.1024
Trxr1	1.8±0.6	1.6±0.05	2.1±0.7	1.6±0.7	0.5055	0.4433	0.1887
Pparg	0.1±0.02	0.4±0.1	0.2±0.05	0.5±0.1	0.7851	0.7558	0.0063
Selenbp2	3.2±0.8	1.7±0.4	3.9±0.7	1.3±0.3	0.3871	0.8573	0.0023
Nifs	0.3±0.05	0.3±0.04	0.4±0.05	0.4±0.05	0.4112	0.0830	0.9016
Slc6a8	0.05±0.02	0.04±0.004	0.07±0.01	0.06±0.11	0.8394	0.2607	0.3934
Ccbl2	2.5±0.9	1.2±0.3	2.9±0.5	1.5±0.4	0.9311	0.2877	0.0001

FEMALES	**SELENITE**						
	0.25		**1**		**2-way ANOVA (p-value)**		
	WT	**KO**	**WT**	**KO**	**Interaction**	**Se**	**Genotype**
SelenoP	874.0±186.8	517.7±104.4	757.2±355.9	584.2±55.7	0.3369	0.7895	0.0113
Gpx1	66.7±4.8	55.6±13.1	105.0±50.5	67.4±13.0	0.2976	0.0582	0.0653
Trxr1	1.6±0.7	2.1±0.3	2.4±1.6	2.8±1.1	0.9452	0.1451	0.2847
Pparg	0.3±0.1	0.5±0,3	0.3±0.2	0.6±0.2	0.3533	0.4922	0.0083
Selenbp2	2.6±1.4	3.7±1.0	2.5±1.6	3.6±1.2	0.9897	0.8237	0.0851
Nifs	0.3±0.06	0.5±0.05	0.4±0.08	0.5±0.05	0.2237	0.3922	0.0300
Slc6a8	0.04±0.03	0.06±0.02	0.06±0.04	0.04±0.03	0.4253	0.8896	0.9038
Ccbl2	2.2±0.6	4.0±1.1	4.9±2.5	3.6±0.8	0.0444	0.1007	0.7041

Table 4. Hepatic gene expression of selenoprotein and metabolic enzymes of WT and Scly KO mice after feeding on a high-fat diet supplemented with selenite, assessed by qPCR. Values are mean ± SEM and were normalized to *Hprt1* mRNA levels. Two-way

ANOVA was used to compare averages. p-Values under 0.05 were deemed significant; n = 4–6 per group.

MALES	SELENOMETHIONINE						
	0.25		1		2-way ANOVA (p-value)		
	WT	KO	WT	KO	Interaction	Se	Genotype
SelenoP	618.0±47.6	406.6±48.1	425.1±65.7	441.9±56.1	0.0552	0.1744	0.0978
Gpx1	73.1±4.7	48.0±6.3	53.0±8.1	51.6±8.3	0.1066	0.2441	0.0749
Trxr1	4.3±1.4	4.7±1.5	4.5±2.5	5.5±2.6	0.6958	0.5343	0.4542
PPARg	0.2±0.04	0.4±0.05	0.2±0.09	0.4±0.08	0.7356	0.7312	0.0006
Selenbp2	10.9±3.6	3.1±0.4	8.2±1.8	3.2±0.5	0.4553	0.4925	0.0028
Nifs	0.5±0.09	0.4±0.08	0.4±0.03	0.5±0.02	0.6673	0.5710	0.9033
Slc6a8	0.05±0.01	0.04±0.004	0.04±0.009	0.05±0.01	0.3223	0.7695	0.9385
Cth	6.0±0.7	5.7±0.5	5.3±0.3	5.0±0.5	0.9093	0.2389	0.5703
Ccbl2	3.8±1.6	3±0.9	3.3±1.2	3.9±1.3	0.2109	0.7612	0.8552

FEMALES	SELENOMETHIONINE						
	0.25		1		2-way ANOVA (p-value)		
	WT	KO	WT	KO	Interaction	Se	Genotype
SelenoP	658.8±86.1	958.4±145.6	999.5±122.3	713.2±30.4	0.0132	0.6593	0.9508
Gpx1	76.3±36.0	125.7±41.6	109.4±222.2	81.6±25.2	0.0116	0.6916	0.4410
Trxr1	2.9±0.7	4.3±2.2	3.9±0.9	2.6±1	0.0294	0.5586	0.8339
PPARg	0.3±0.1	0.7±0.2	0.3±0.1	0.6±0.2	0.9564	0.5791	0.0021
Selenbp2	2.7±0.8	4.1±0.5	2.8±+0.8	3.6±0.5	0.3160	0.7191	0.0016
Nifs	0.5±0.2	0.8±0.08	0.6±0.1	0.7±0.08	0.0939	0.7087	0.0066
Slc6a8	0.04±0.01	0.04±0.008	0.07±0.02	0.07±0.05	0.9620	0.0328	0.7873
Cth	5.9±1.5	11.1±2.3	6.5±0.8	9.0±2.7	0.1138	0.3548	0.0001
Ccbl2	3.7±0.9	5.5±2.5	5.9±0.9	3.4±1.0	0.0037	0.9297	0.5748

Table 5. Hepatic gene expression of selenoprotein and metabolic enzymes of WT and Scly KO after feeding on a high-fat diet supplemented with a mixture of selenite + SeMet assessed by qPCR. Values are mean ± SEM and were normalized to *Hprt1* mRNA levels. Two-way ANOVA was used to compare averages. p-Values under 0.05 were deemed significant; n = 4–6.

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