SELENIUM, HUMAN HEALTH AND CHRONIC DISEASE

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SELENIUM, HUMAN HEALTH AND CHRONIC DISEASE

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Editorial: Selenium, Human Health and Chronic Disease

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Editorial on the Research Topic

Selenium, Human Health and Chronic Disease

Dietary selenium is a critical factor determining the mineral bioavailability for the synthesis of selenocysteine (Sec)-containing proteins, selenoproteins, which play essential roles in pivotal physiological pathways (1). Selenium deficiency has been implicated in a wide range of chronic diseases, such as cancer, Alzheimer's disease, and thyroid dysfunction (2). Nonetheless, some studies investigate the association between selenium biomarkers and chronic diseases, in an attempt to identify the biological effects of both insufficient and excessive selenium status. Furthermore, with the advance of clinical trials in the field, conflicting information has emerged regarding the benefits of high selenium consumption, and recent research has indicated that supplementation to selenium-replete individuals may be associated with negative health outcomes (3–5).

Considering the aforementioned aspects, this Research Topic aimed to collect scientific articles that bring insights into the fundamental biological role of selenium and shed light on the trade-off between the necessary and harmful levels of selenium intake. In this special e-collection, 12 original and review articles report on selenium metabolism and distribution, suggesting that either deficiency or excess selenium levels may lead to a variety of chronic health issues, including type 2 diabetes *mellitus* (T2DM), non-alcoholic fatty liver disease (NAFLD), and depression.

The review by Ferreira et al. explored the association between gut microbiota and selenium status, focusing on how dietary selenium affects gut microbiome. It provides particular insights into how selenium deficiency may jeopardize the human-microbiota symbiotic relationship, with these symbionts becoming potential competitors and priming the microbiota to be more susceptible to the development of diseases such as cancer, thyroid dysfunctions, and cardiovascular disorders. This comprehensive review also brings novel layers of complexity to selenium metabolism according to the various ingested selenocompounds that can shape future research and the interpretation of selenium biology.

The association between selenium and T2DM was explored in three different studies in this e-collection. The study by Santos et al., conducted with young Brazilian adults with Normal-Weight Obesity (NWO) syndrome, demonstrated that individuals with selenium consumption below the Estimated Average Requirement (EAR; $\leq$ 45 μg/day) had higher concentrations and a higher prevalence of disturbances in glycated hemoglobin (HbA_{1c}) when compared to those with selenium intake above the EAR. In addition, dietary selenium intake was inversely associated with HbA_{1c}

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concentrations. Contrastingly, Dias et al. observed no significant association between selenium intake and the prevalence of T2DM in a sample of highly educated Brazilian adults. Although these two studies were conducted in the same country, a significant difference was noted in the reported selenium intakes: while in the study of Santos et al. the average intake was below 60 μg/day, the participants in the study by Dias et al. presented a median energy-adjusted selenium intake of 143.5 μg/day, which illustrate geographical and possibly social disparities in the dietary selenium intake. The study by Cardoso et al. investigated the association between blood selenium concentration and glucose markers in US selenium-replete adults. As the main findings, the authors reported a positive association between selenium status, insulin, and the Homeostatic Model Assessment for Insulin Resistance. These studies bring insight into the hypothesis of a U-shaped association between selenium status/exposure and T2DM and corroborate the idea of not promoting supplementation strategies amongst selenium-replete populations.

A U-shaped dose-response in selenium metabolic effects was also raised by Zhang et al. when investigating the association between dietary selenium and incident fracture in Chinese adults. In this 20-year longitudinal study, an increased risk of fracture was found at high selenium intakes as well as at intakes below 30 μg/day. The findings from this study contribute to the current evidence demonstrating that adequate selenium levels, rather than high, are more favorable for health.

Day et al. used publicly available human global gene expression datasets to assess gene expression levels of known selenoprotein pathways in individuals with a healthy liver in comparison to those with NAFLD, whose major risk factor is insulin resistance. The bioinformatics analysis indicates that the NAFLD liver may present lower selenium levels, and that a gene expression variation associated with the metabolism of selenoproteins and iron progresses along with the risk of NAFLD. The findings from this study open avenues for future research that aim to explore the role of selenoproteins in NAFLD pathogenesis.

Almeida et al. investigated the association between selenium intake and depression in a cross-sectional analysis of Brazilian farmers, a rural population poorly assessed. Using a food composition software tailored to the typical Brazilian diet, the authors calculated selenium intake and correlated with the occurrence of depressive episodes, concluding that the prevalence of depression was lower among farmers with the highest intake of selenium. The results align with previous studies in different populations that also demonstrate the same inverse association, and may substantiate improved public health policies toward selenium supplementation to an underserved population.

Three studies were dedicated to the investigation of selenoprotein P (SELENOP), the main selenium transporter. Isobe et al. focused on the association between alcohol intake, serum selenium, and SELENOP concentrations in participants from a rural Japanese town, and found a positive relationship that was independent of age, sex, concentrations of liver enzymes or occurrence of fatty liver. Intriguingly, dose-dependence of this positive association was observed in men, but not in women. The authors argue that a seafood rich diet may contribute to the establishment of this relationship, especially in men.

Kiyohara et al. evaluated the yet unreported biological significance of the interaction between SELENOP1 and Zn^{2+}, by examining changes in brain Zn^{2+} in *Sepp1* knockout animals. Changes in the intracellular hippocampal distribution of Zn^{2+} were found in *Sepp1*$^{-/-}$ mice compared to wildtype mice (WT) and this may have been due to a down-regulation of antioxidant selenoproteins. In addition, an increased phosphorylation of tau protein was found in the hippocampus of *Sepp1*$^{-/-}$ mice, possibly resulting from intracellular changes in Zn^{2+}. These observations suggested important roles of SELENOP1 in neuronal function, maintenance of synaptic physiology, and prevention of tau hyperphosphorylation, with possible associations with Alzheimer's disease.

Saito elaborated a comprehensive review focused on the molecular mechanisms and role of SELENOP in selenium metabolism, emphasizing that this selenoprotein is not just a selenium transporter, but has a multifunctional role in the maintenance of cellular selenoproteins and in the regulation of cellular redox homeostasis.

The study by Evenson and Sunde uncovers an intriguing perspective regarding how selenium is metabolized. Using rats fed diets containing 0–5 μg of selenite and pulse-injected with 0.5 μg of radiolabeled selenium as various inorganic and organic selenocompounds, the authors could trace the fate of selenium in the body in a dose- and time-dependent manner. Intriguingly, this study demonstrated that all selenocompounds were metabolized into selenium for selenoprotein incorporation without a particular preference. In addition, a portion of selenium was metabolized into "missing" selenocompounds, possibly selenosugars, particularly at shorter time-points, and the distribution may be linked to selenium toxicity and the development of other disease conditions. Despite the limitation of sample size for each radiolabeled compound, there is a breadth of information that this analysis provides on selenium metabolism for the elaboration of new hypotheses and insights that will strengthen the understanding of how selenium and selenoproteins contribute to diseases and health status.

Sex differences in selenium metabolism were explored in the study by Kremer et al. In previous studies the authors produced knockout mouse strains for both *Scly* and *Selenop* genes, the double knockout (DKO) mice. DKO mice showed significant neurological disorders (6), which although less severe in females than in males, were aggravated by the removal of selenium supplementation during puberty (7). In the current special issue, the authors noted that female DKO mice exhibited a particular metabolic phenotype, with significantly higher total body weight and white adipose tissue deposits when compared to WT mice, which was not observed in male counterparts. As the phenotype was reversed by removing selenium from drinking water shortly before puberty, the authors suggested that restricting access to selenium during this period may prevent excessive body weight gain and gonadal fat deposits. In addition, DKO mice have been proven useful as a model for studying the

underlying mechanisms and relationships between selenium and energy homeostasis.

In summary, the studies in this e-collection bring novel perspectives and intriguing results on selenium intake, metabolism, distribution, and association with several chronic disease states that burden the health system. The range of unveiled findings will possibly contribute to exciting new hypotheses about the contribution of selenium to human health.

AUTHOR CONTRIBUTIONS

All authors contributed to this editorial article and approved the submitted version.

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ORIGINAL RESEARCH
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Alcohol Intake Is Associated With Elevated Serum Levels of Selenium and Selenoprotein P in Humans

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Selenoprotein P is a hepatokine with antioxidative properties that eliminate a physiologic burst of reactive oxygen species required for intracellular signal transduction. Serum levels of selenoprotein P are elevated during aging and in people with type 2 diabetes, non-alcoholic fatty liver disease, and hepatitis C. However, how serum levels of full-length selenoprotein P are regulated largely remains unknown, especially in the general population. To understand the significance of serum selenoprotein P levels in the general population, we evaluated intrinsic and environmental factors associated with serum levels of full-length selenoprotein P in 1,183 subjects participating in the Shika-health checkup cohort. Serum levels of selenium were positively correlated with liver enzymes and alcohol intake and negatively correlated with body mass index. Serum levels of selenoprotein P were positively correlated with age, liver enzymes, and alcohol intake. In multiple regression analyses, alcohol intake was positively correlated with serum levels of both selenium and selenoprotein P independently of age, gender, liver enzymes, and fatty liver on ultrasonography. In conclusion, alcohol intake is associated with elevated serum levels of selenium and selenoprotein P independently of liver enzyme levels and liver fat in the general population. Moderate alcohol intake may exert beneficial or harmful effects on health, at least partly by upregulating selenoprotein P. These findings increase our understanding of alcohol-mediated redox regulation and form the basis for the adoption of appropriate drinking guidelines.

Keywords: alcohol, selenium, selenoprotein P, diabetes, fatty liver, hepatokine

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INTRODUCTION

Selenoprotein P (encoded by the *SELENOP* gene in humans) is a secretory protein that contains multiple selenocysteine residues per polypeptide and functions as a selenium transport protein (1). Selenoprotein P exerts antioxidative properties directly via an N-terminal thioredoxin domain and indirectly by supplying selenium to antioxidative glutathione peroxidases (2). We have re-discovered selenoprotein P as a hepatokine that causes multi-signal resistances leading to type 2 diabetes, such as insulin resistance (3), angiogenesis resistance (4), ischemia-reperfusion injury (5),

insulin secretory failure (6), and exercise resistance (7). Selenoprotein P eliminates a physiologic burst of reactive oxygen species required for intracellular signal transduction, a condition referred to as "reductive stress" (2). Serum levels of selenoprotein P are elevated during aging (8), and in people with type 2 diabetes (3), non-alcoholic fatty liver disease (9), and chronic hepatitis C (10).

The full-length selenoprotein P is digested with plasma kallikrein, which generates N-terminal and C-terminal fragments of selenoprotein P (11). Indeed, the selenium content of selenoprotein P was reported to be 5.4 ± 0.5 (mean ± SD) in humans (12), which seems lower than its theoretical ten selenocysteine residues per polypeptide. Therefore, we previously developed a sol particle homogeneous immunoassay method that selectively measures a full-length form of selenoprotein P in human serum (13, 14). Using this assay system, we reported selenium and full-length selenoprotein P status in a general Japanese population as follows: (1) serum levels of selenium and selenoprotein P are significantly correlated with each other; (2) both serum levels of selenium and selenoprotein P increase with aging; (3) serum levels of selenoprotein P, but not those of selenium, correlated positively with glucose levels and negatively with initial insulin secretion capacity in oral glucose tolerance tests; and (4) elevated serum levels of selenoprotein P, but not those of selenium, predict future onset of hyperglycemia (8). These findings suggest that serum levels of selenoprotein P and selenium, to some extent, serve as surrogate markers for redox and health status in humans.

Selenium supply and its incorporation into selenocysteine upregulate the *SELENOP* gene expression (1). Besides, several transcription factors are involved in *SELENOP* expression. Insulin downregulates *SELENOP* by phosphorylating and inactivating FoxO1 (15), whereas antidiabetic metformin activates AMP-activated protein kinase (AMPK) and phosphorylates and inactivates FoxO3a, subsequently downregulating *SELENOP* in hepatocytes (16). Eicosapentaenoic acid, one of the ω-3 polyunsaturated fatty acids, downregulates *SELENOP* by inhibiting nuclear transport and promoter binding of SREBP-1c (17). Hepatitis C viral infection upregulates *SELENOP* in the liver through C/EBPα (10). However, the regulation of serum levels of full-length selenoprotein P is poorly understood, especially in the general population.

In the present study, we aim to assess the intrinsic and environmental factors associated with serum levels of selenium and full-length selenoprotein P in the general population. We found that moderate alcohol intake is associated with serum levels of selenium and selenoprotein P regardless of liver injury.

MATERIALS AND METHODS

Study Population

We used cross-sectional data from participants in the "Shika study" project of 2013–2017, conducted in the Noto Peninsula, Ishikawa, Japan, since 2011. The Shika study is an ongoing population-based survey that seeks to develop advanced preventive methods for lifestyle-related diseases. It includes interviews, self-administered questionnaires, and comprehensive health examinations. Shika town is located in a rural area of the Ishikawa prefecture, Japan. The town has over 20,000 residents (18). Data were collected from adults above 40 years of age in the model districts. All subjects with no reported gender ($n = 8$) were excluded from the study. All subjects gave their written informed consent for inclusion in the study. The study was conducted following the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Kanazawa University (No.1491).

Measurements

Age, sex, height, weight, waist circumference, and systolic and diastolic blood pressure (SBP and DBP) were measured at health checkups for all participants. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters.

Nutrient Assessment

We assessed alcohol intake from the validated food frequency questionnaire. We used the brief-type self-administered diet history questionnaire (BDHQ) developed in Japan for large-scale nutritional epidemiology studies (19). BDHQ asks for the dietary history for fifty-eight food items taken in the preceding month. These food items are commonly consumed in Japan and are mainly from the food list used in the National Health and Nutrition Survey of Japan. Participants who reported an energy intake of <600 kcal/day (half of the required energy for the lowest physical activity category) or more than 4,000 kcal/day (1.5 times the energy intake required for the highest physical activity category) were excluded from the analyses because they were either extremely low or extremely high energy intakes.

Alcohol Consumption

Alcohol consumption was estimated using the BDHQ (19). The BDHQ asks for the consumption frequency and amount of Japanese sake, beer, wine, whiskey, and brandy, commonly consumed in Japan. Estimated alcohol consumption was calculated using an *ad hoc* computer algorithm, which included weighting factors for BDHQ. Alcohol intake levels were stratified as 0, <30, and ≥30 (g/day) in men and 0, <20, and ≥20 (g/day) in women (**Supplementary Table 1**).

Smoking

Smoking status was stratified as non/past-smokers and current smokers.

Assessment of Fatty Liver

Hepatic steatosis was determined using B-mode ultrasonography performed by experienced hepatologists. The presence of hepatic steatosis was determined by at least one of the following findings: increased hepatorenal contrast, liver brightness, deep attenuation, and vascular blurring. The severity of fatty liver was semi-quantitatively graded as mild (liver brightness or hepatorenal contrast), moderate (mild plus deep attenuation or vascular blurring), and severe (all four findings) (20).

Blood Collection and Assays

Fasting blood samples were collected between 0800 and 1,200 h from the forearm vein of each participant. The serum samples were delivered to Kanazawa University through a commercial laboratory (SRL Kanazawa Laboratory, Kanazawa, Japan). The sera were frozen and stored at −30°C until the assay. Serum concentrations of full-length selenoprotein P were specifically measured by sol particle homogeneous immunoassay using two monoclonal antibodies, as previously established (13, 14). Serum concentrations of selenium were measured by atomic absorption spectrophotometry (8).

Statistical Analyses

Normally distributed data were presented as means ± standard deviations, and the differences between the two groups were analyzed using the Student's *t*-test; the paired *t*-test was used for paired samples. Non-normally distributed data were presented as medians and ranges, and the differences between these groups were assessed using the Mann–Whitney *U*-test. Relationships were determined using regression analyses, and a $P < 0.05$ was considered statistically significant. Multivariate logistic regression analyses (forced entry method) were performed using age, gender, and liver enzymes as explanatory factors and selenoprotein P/selenium as dependent variables. All the

TABLE 1 | Characteristics of the study participants.

	All		Men				Women			
			No drinking		Drinking		No drinking		Drinking	
	N	Mean ± S.D.	*N*	Mean ± S.D.	*N*	Mean ± S.D.	*N*	Mean ± S.D.	*N*	Mean ± S.D.
Age (year)	1,183	62.1 ± 11.2	98	64.3 ± 12.0	349	61.6 ± 10.8	327	65.1 ± 11.2	176	58.1 ± 10.5
BMI (kg/m^2)	1,181	23.3 ± 3.3	98	24.5 ± 3.6	349	23.8 ± 3.0	326	23.0 ± 3.3	175	22.2 ± 3.1
Selenium (μg/L)	752	158.74 ± 27.98	77	154.27 ± 24.73	273	165.48 ± 25.28	251	153.65 ± 32.37	134	155.99 ± 13.38
Selenoprotein P (μg/mL)	948	3.93 ± 0.90	98	3.83 ± 0.62	336	4.16 ± 0.85	318	3.76 ± 1.01	173	3.83 ± 3.1
Aspartate aminotransferase (U/L)	1,181	24.21 ± 10.456	98	24.24 ± 9.62	348	26.93 ± 14.66	326	22.02 ± 6.61	176	22.78 ± 1.84
Alanine aminotransferase (U/L)	1,181	22.29 ± 13.633	98	27.68 ± 21.48	348	25.92 ± 15.68	326	18.35 ± 8.87	176	19.66 ± 1.44
γ-glutamyl transpeptidase (IU/L)	1,181	41.63 ± 53.997	98	36.35 ± 33.95	348	68.33 ± 79.40	326	23.81 ± 22.13	176	30.84 ± 17.95
Energy intake (kcal/day)	950	1864.54 ± 632.58	98	1874.76 ± 587.14	349	2154.92 ± 652.04	327	1658.22 ± 568.01	176	1666.36 ± 3.4
Alcohol intake (g/day)	950	12.96 ± 21.64	98	–	349	30.17 ± 25.89	327	–	176	10.13 ± 3.0
Alcohol intake (% energy)	950	4.50 ± 7.14	98	–	349	10.04 ± 8.12	327	–	176	4.37 ± 3.0
HbA1c (%)	1,182	5.91 ± 0.66	98	6.24 ± 0.91	348	5.88 ± 0.68	327	5.92 ± 0.59	176	5.77 ± 3.5
Fasting plasma glucose (mg/dL)	1,068	96.42 ± 18.229	90	101.92 ± 25.58	314	99.35 ± 19.88	295	93.65 ± 14.66	160	92.24 ± 14.48
Insulin (μU/mL)	1,140	5.73 ± 4.90	92	6.05 ± 5.37	334	5.53 ± 5.77	313	5.77 ± 4.03	169	5.02 ± 3.0

TABLE 2a | Univariate correlation between clinical parameters and selenium in participants consuming and not consuming alcohol.

	Selenium																	
	All						Men						Women					
			No drinking		Drinking				No drinking		Drinking				No drinking		Drinking	
	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*
Age (year)	−0.043	0.244	−0.028	0.609	0.009	0.861	−0.157	0.003	−0.096	0.405	−0.151	0.013	0.050	0.327	−0.011	0.863	0.262	0.002
BMI (kg/m^2)	−0.079	0.031	−0.135	0.014	−0.027	0.583	−0.063	0.230	−0.046	0.691	−0.061	0.318	−0.148	0.003	−0.163	0.010	−0.109	0.208
Smoking	0.126	<0.001	0.061	0.275	0.122	0.014	0.138	0.009	0.174	0.131	0.118	0.052	0.009	0.862	0.015	0.808	−0.023	0.792
Log AST	0.112	0.002	0.073	0.187	0.115	0.020	0.063	0.237	0.033	0.775	0.048	0.426	0.101	0.046	0.086	0.174	0.155	0.073
Log ALT	0.122	<0.001	0.091	0.099	0.108	0.029	0.064	0.223	0.124	0.282	0.042	0.489	0.094	0.063	0.086	0.174	0.119	0.172
log γGTP	0.249	<0.001	0.129	0.019	0.281	<0.001	0.286	<0.001	0.164	0.155	0.269	<0.001	0.121	0.016	0.126	0.046	0.118	0.175
Energy intake (kcal/day)	0.017	0.648	−0.064	0.251	0.027	0.592	−0.039	0.468	−0.273	0.016	−0.022	0.718	−0.034	0.501	−0.014	0.825	−0.095	0.275
Alcohol intake (g/day)	0.228	<0.001	–	–	0.262	<0.001	0.269	<0.001	–	–	0.238	<0.001	0.078	0.126	–	–	0.147	0.091
Alcohol intake (%energy)	0.234	<0.001	–	–	0.271	<0.001	0.283	<0.001	–	–	0.255	<0.001	0.082	0.109	–	–	0.156	0.072

AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGTP, γ-glutamyl transpeptidase.

explanatory variables were tested for collinearity, and only those that were confirmed to have no collinearity using the values of variance inflation factor and tolerance were used as independent explanatory variables in the multiple regression analyses. A two-way ANCOVA was performed to compare mean selenoprotein P/selenium levels and alcohol intake levels among participants with different liver enzyme levels. A simple main effect test was performed for selenoprotein P/selenium levels, in which interactions were assessed. All statistical analyses were conducted using SPSS software, version 16.0 (IBM, Armonk, NY, USA).

RESULTS

Participant Characteristics

A total of 1,183 subjects (551 men) were included in the analyses. Of these, 525 (349 men) had a drinking habit, and the average amount of alcohol consumption was 12.96 g/day (30.17 g/day for men and 10.13 g/day for women) (**Table 1**). There were no significant differences in BMI, selenium, selenoprotein P, HbA1c, and fasting plasma glucose between genders or between drinkers and non-drinkers. In men, drinkers had significantly lower BMI than non-drinkers ($P = 0.019$). Insulin levels were not different between drinkers and non-drinkers and were significantly higher in women than in men ($P < 0.001$, data not shown).

Factors Associated With Serum Selenium Levels

In all participants, selenium levels were correlated positively with AST, ALT, γGTP, and alcohol intake and negatively with BMI (**Table 2a**).

In men, selenium levels were correlated positively with GGT and alcohol intake and negatively with age in the drinkers, whereas they were correlated negatively with energy intake in the non-drinkers. In women, selenium levels were correlated positively with age in the drinkers, whereas they were correlated positively with γGTP and negatively with BMI in the non-drinkers (**Table 2a**).

Serum selenium levels in men were increased by alcohol intake in a dose-dependent manner (**Figure 1-i**) but not in women (**Figure 1-ii**). In multiple regression analyses (**Table 3a**, **Figure 2A**), alcohol intake was positively correlated

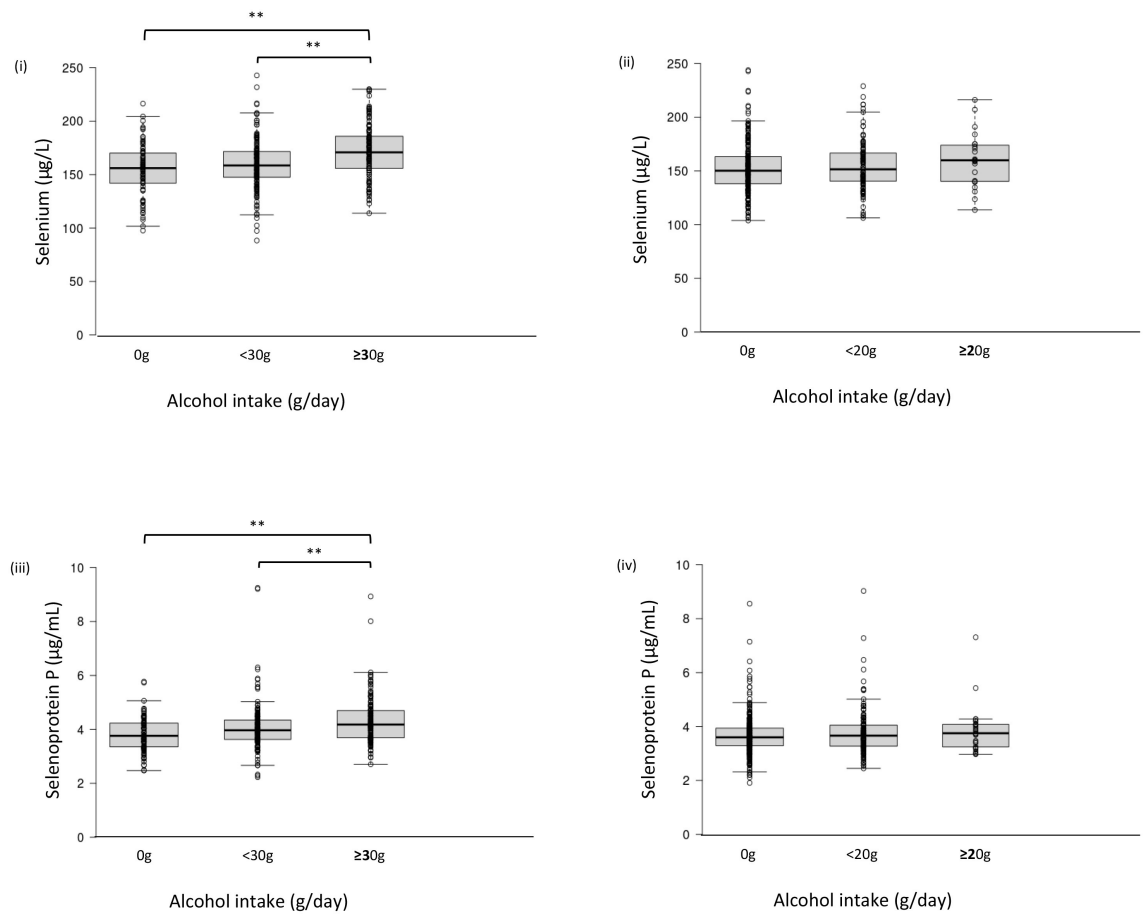

FIGURE 1 | Serum selenium/selenoprotein P levels in each group of alcohol intake. Serum level of selenium (i, ii) and selenoprotein P (iii, iv) in men (i, iii) and women (ii, iv). In box-plots, center lines show the medians, and box limits indicate the 25th and 75th percentiles; whiskers extend 1.5x the interquartile range from the 25th and 75th percentiles; data points are plotted as dots. $^{**}p < 0.01$.

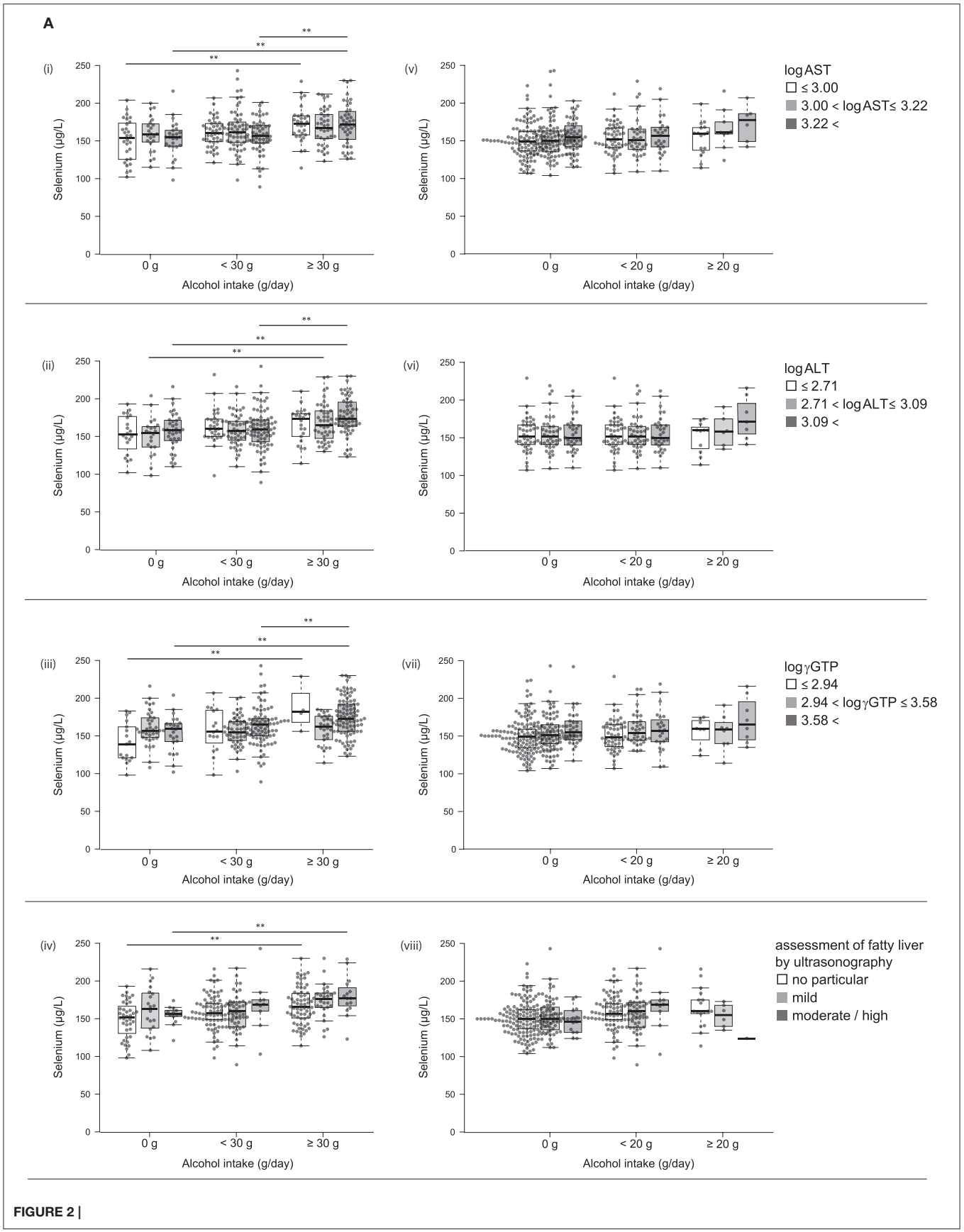
A
(i)
(ii)
(iii)
(iv)
(v)
(vi)
(vii)
(viii)
Selenium (μg/L)
0 g
< 30 g
≥ 30 g
< 20 g
≥ 20 g
Alcohol intake (g/day)
**
logAST
≤ 3.00
3.00 < logAST≤ 3.22
3.22 <
logALT
≤ 2.71
2.71 < logALT≤ 3.09
3.09 <
logγGTP
≤ 2.94
2.94 < logγGTP ≤ 3.58
3.58 <
assessment of fatty liver by ultrasonography
no particular
mild
moderate / high

FIGURE 2 |

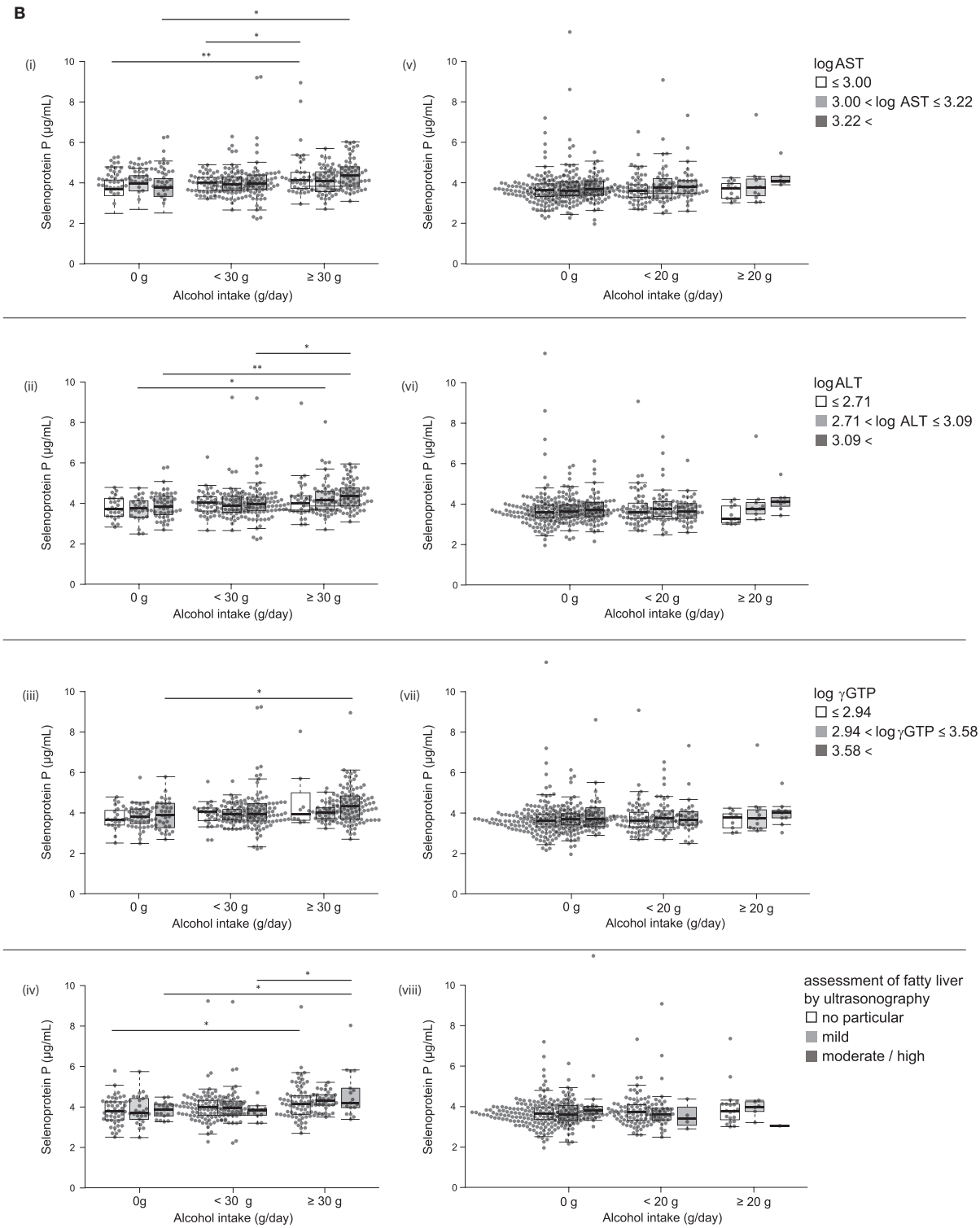

FIGURE 2 | (A) Serum levels of selenium and selenoprotein P in participants with different alcohol intake levels and different liver enzyme levels. Serum levels of selenium in participants according to various levels of log AST (i,v), log ALT (ii,vi), log γGTP (iii,vii), and fatty liver by ultrasonography (iv,viii) in men (i∼iv) and women (v∼viii). **(B)** Serum levels of selenoprotein P in participants with different alcohol intake levels and different liver enzyme levels. Serum levels of selenoprotein P in participants according to various levels of log AST (i,v), log ALT (ii,vi), log γGTP (iii,vii), and fatty liver by ultrasonography (iv,viii) in men (i∼iv) and women (v∼viii). In box-plots, center lines show the medians, and box limits indicate the 25th and 75th percentiles; whiskers extend 1.5x the interquartile range from the 25th and 75th percentiles; data points are plotted as dots. $*p < 0.05$; $**p < 0.01$.

TABLE 2b | Univariate correlation between clinical parameters and selenoprotein P in participants consuming and not consuming alcohol.

	Selenoprotein P																	
	All						Men						Women					
			No drinking		Drinking				No drinking		Drinking				No drinking		Drinking	
	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*
Age (year)	0.099	0.002	0.095	0.054	0.167	<0.001	0.030	0.528	−0.016	0.874	0.064	0.243	0.160	<0.001	0.121	0.031	0.300	<0.001
BMI (kg/m^2)	−0.032	0.333	−0.084	0.086	0.022	0.618	−0.010	0.839	0.037	0.715	0.002	0.970	−0.106	0.018	−0.121	0.031	−0.070	0.360
Smoking	0.158	<0.001	0.142	0.004	0.124	0.005	0.154	0.001	0.24	0.017	0.118	0.031	0.067	0.137	0.133	0.017	−0.018	0.819
Log AST	0.136	<0.001	0.059	0.232	0.167	<0.001	0.128	0.007	0.075	0.464	0.126	0.021	0.085	0.057	0.054	0.337	0.157	0.040
Log ALT	0.095	0.003	0.040	0.419	0.104	0.019	0.073	0.123	0.141	0.167	0.069	0.207	0.024	0.596	0.005	0.923	0.051	0.504
Log γGTP	0.197	<0.001	0.103	0.035	0.188	<0.001	0.196	<0.001	0.174	0.087	0.154	0.005	0.079	0.076	0.085	0.128	0.053	0.491
Energy intake (kcal/day)	0.057	0.085	−0.019	0.702	0.060	0.178	0.008	0.869	−0.190	0.062	0.008	0.889	−0.006	0.894	0.007	0.898	−0.041	0.593
Alcohol intake (g/day)	0.212	<0.001	–	–	0.221	<0.001	0.237	<0.001	–	–	0.193	<0.001	0.058	0.200	–	–	0.091	0.232
Alcohol intake (%energy)	0.212	<0.001	–	–	0.219	<0.001	0.244	<0.001	–	–	0.201	<0.001	0.058	0.200	–	–	0.091	0.232

AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGTP, γ-glutamyl transpeptidase.

TABLE 3a | Relationship between alcohol intake and selenium in participants taking alcohol.

		Selenium								
		All			Men			Women		
		β	t	*p*	β	t	*p*	β	t	*p*
Alcohol intake (g/day)	Adjusted for age, gender, and log AST	0.220	4.262	<0.001	0.235	3.987	<0.001	0.200	2.370	0.019
	Adjusted for age, gender, and log ALT	0.228	4.431	<0.001	0.239	4.094	<0.001	0.201	2.389	0.018
	Adjusted for age, gender, and log γGTP	0.172	3.256	0.001	0.176	2.941	0.004	0.184	2.113	0.037
	Adjusted for age, gender, and assessment of fatty liver by ultrasonography	0.239	4.461	<0.001	0.248	4.087	<0.001	0.212	2.436	0.016
Alcohol intake (g/day)	Adjusted for age, gender, smoking, and log AST	0.179	4.351	<0.001	0.246	4.686	<0.001	0.094	1.713	0.088
	Adjusted for age, gender, smoking, and log ALT	0.189	4.626	<0.001	0.252	4.871	<0.001	0.100	1.825	0.069
	Adjusted for age, gender, smoking, and log γGTP	0.136	3.189	0.001	0.178	3.255	0.001	0.080	1.453	0.147
	Adjusted for age, gender, smoking, and assessment of fatty liver by ultrasonography	0.196	4.617	<0.001	0.262	4.899	<0.001	0.106	1.836	0.067

AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGTP, γ-glutamyl transpeptidase.

with serum levels of selenium independently of age, gender, liver enzymes, and fatty liver on ultrasonography in both men and women. Furthermore, in men, similar results were obtained with an adjustment for smoking (**Table 3a**). In women, however, the correlation between alcohol intake and selenium disappeared with a smoking adjustment (**Table 3a**).

We compared selenium levels among participants' alcohol intake levels and different liver enzyme levels using a two-way ANCOVA (**Table 4a**). The results showed a significant main effect of alcohol consumption on selenium levels only in men. A significant interaction was observed between alcohol intake levels and log γGTP levels for selenium.

Factors Associated With Serum Selenoprotein P Levels

In all participants, serum levels of selenoprotein P were positively correlated with age, AST, ALT, γGTP, and alcohol intake (**Table 2b**). In men, selenoprotein P levels were correlated positively with AST, γGTP, and alcohol intake in the drinkers. In women, selenoprotein P levels were correlated positively with age and AST in the drinkers, whereas they were correlated positively with age and negatively with BMI in the non-drinkers (**Table 2b**).

Among drinkers, alcohol intake higher than 30 g/day increased serum levels of selenoprotein P in men (**Figure 1-iii**) but not in women (**Figure 1-iv**). In multiple regression analyses (**Table 3b**, **Figure 2B**), alcohol intake was positively correlated

TABLE 3b | Relationship between alcohol intake and selenoprotein P inparticipants taking alcohol.

		Selenoprotein P								
		All			Men			Women		
		β	t	*p*	β	t	*p*	β	t	*p*
Alcohol intake (g/day)	Adjusted for age, gender, and log AST	0.174	3.742	<0.001	0.181	3.333	<0.001	0.147	2.001	0.047
	Adjusted for age, gender, and log ALT	0.184	3.965	<0.001	0.191	3.560	<0.001	0.148	2.014	0.046
	Adjusted for age, gender, and log γGTP	0.162	3.361	<0.001	0.163	2.898	0.004	0.146	1.944	0.054
	Adjusted for age, gender, and assessment of fatty liver by ultrasonography	0.196	3.943	<0.001	0.205	3.509	<0.001	0.162	2.077	0.040
Alcohol intake (g/day)	Adjusted for age, gender, smoking, and log AST	0.155	4.260	<0.001	0.204	4.295	<0.001	0.079	1.666	0.096
	Adjusted for age, gender, smoking, and log ALT	0.166	4.604	<0.001	0.220	4.702	<0.001	0.081	1.729	0.084
	Adjusted for age, gender, smoking, and log γGTP	0.138	3.664	<0.001	0.179	3.594	<0.001	0.072	1.524	0.128
	Adjusted for age, gender, smoking, and assessment of fatty liver by ultrasonography	0.170	4.405	<0.001	0.228	4.551	<0.001	0.085	1.675	0.095

AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGTP, γ-glutamyl transpeptidase.

with serum levels of selenoprotein P independently of age, gender, liver enzymes, and fatty liver on ultrasonography in both men and women. Furthermore, in men, similar results were obtained after adjusting for smoking. In women, however, the correlation between alcohol intake and selenoprotein P disappeared with a smoking adjustment (**Table 3b**).

We compared selenoprotein P among participants' alcohol intake levels at different liver enzyme levels using a two-way ANCOVA (**Table 4b**). The results showed a significant main effect of alcohol consumption on the selenoprotein P levels only in men. No significant interaction was observed between alcohol intake levels and liver enzyme levels for selenoprotein P.

Intake of Macro/Micronutrients and Foods Associated With Serum Levels of Selenium or Selenoprotein P

Alcohol intake was associated with an estimated intake of total energy, protein, carbohydrate, protein ratio to total energy, zinc, copper, and manganese (**Supplementary Table 2**). However, selenium levels were not associated with these nutrients, and selenoprotein P levels were associated only with protein ratio to the total energy.

Alcohol intake was associated with relatively selenium-rich foods, such as seafood, especially in men (**Supplementary Table 3**).

DISCUSSION

In the present study, we found that moderate alcohol intake was associated with elevated serum levels of selenium and selenoprotein P independently of liver enzyme levels and liver fat in the general population. This finding provides the first evidence of a positive correlation between alcohol intake and serum levels of selenoprotein P in the general population.

Too much alcohol consumption should be avoided due to its health hazard aspects (21). Since any alcohol use is associated with short-term and long-term health risks, it seems difficult to define universally applicable population-based thresholds for low-risk drinking (22). Therefore, the WHO aims to prevent and reduce the harmful use of alcohol as a public health priority (21). On the other hand, alcohol intake has both positive and negative effects on health. It remains unresolved whether alcohol intake elevates or reduces oxidative stress (23, 24). Accumulating evidence suggests that alcohol consumption can elevate or reduce cardiovascular risk, depending on the dosage (25–27). Selenium and selenoprotein P also exert beneficial or adverse health effects on the development of diabetes (28) and cardiovascular diseases (29) depending on their concentrations and circumstances. Selenoprotein P is a crucial redox protein in the body, but in excess, it induces reductive stress leading to various forms of intracellular signal resistance, such as resistance to insulin, VEGF, and exercise (2). Therefore, selenoprotein P may interfere with alcohol-induced alterations in oxidative stress and mediate the known alcohol-mediated deleterious effects on health by impairing vasculogenesis (25, 26) and exercise performance (30).

To date, the reported effects of alcohol intake on selenium and selenoprotein P appear to be inconsistent. Contrary to the present findings, mounting evidence suggests that binge alcohol consumption or alcohol abuse is associated with lower serum selenium levels and downregulated selenoproteins in humans (31–34) and animals (35, 36). Lower selenium levels may cause the reduced selenoprotein P levels observed in cases of alcoholism. At least in rats, binge alcohol consumption reduces selenium absorption and downregulates hepatic expression of selenoproteins, such as *Gpx1* and *Gpx4* (encoding glutathione peroxidases one and four), but not *SELENOP* (36).

Reduced hepatic reserve caused by alcoholic hepatitis/cirrhosis may cause low serum levels of selenium (34). Among the selenoprotein family members, selenoprotein P is a primary source of selenium in the plasma as systemic removal of selenoprotein P reduces plasma levels of selenium to <10% (37). Although most of the organs produce selenoprotein P ubiquitously, the liver produces most of the selenoprotein P

TABLE 4a | Comparison of serum selenium levels (μg/L) among participants with different alcohol intake levels and different liver enzyme/fatty liver levels.

		Men							Women						
		Alcohol intake			*p*-value				Alcohol intake			*p*-value			
		0 g	<30 g	≥30 g	Between alcohol intake levels	Between liver enzyme levels	Interaction between alcohol intake levels and liver enzyme levels	Between alcohol intake levels in each liver enzyme levels	0 g	<20 g	≥20 g	Between alcohol intake levels	Between lilver enzyme levels	Interaction between alcohol intake levels and liver enzyme levels	Between alcohol intake levels in each liver enzyme levels
Log AST	≤3.00	151.214	160.690	172.533	<0.001	0.710	0.330	–	149.769	153.226	151.000	0.360	0.064	0.691	–
	3.00–3.22	159.174	163.789	168.282				–	153.250	156.281	165.875				–
	3.22<	153.231	156.192	174.132				–	161.909	157.240	177.750				–
Log ALT	≤2.71	152.600	163.310	165.864	<0.001	0.241	0.452	–	151.227	153.951	151.273	0.540	0.083	0.561	–
	2.71–3.09	151.000	159.348	168.386				–	151.425	155.711	159.500				–
	3.09<	157.111	159.750	176.964				–	162.167	155.613	175.571				–
LogγGTP	≤2.94	141.235	157.563	187.250	0.001	0.232	0.006	0.002	149.734	150.981	156.125	0.781	0.210	0.873	–
	2.94–3.58	160.703	156.080	158.885				0.670	158.418	159.514	154.750				–
	3.58<	153.565	163.318	174.870				<0.001	159.966	157.348	170.375				–
Fatty liver	no particular	148.925	158.714	167.426	<0.001	0.039	0.466	–	152.488	154.871	164.706	0.895	0.324	0.786	–
	mild	161.250	158.467	173.846				–	152.827	152.786	154.333				–
	mederate / severe	154.538	168.600	179.667				–	147.769	151.500	124.000				–

TABLE 4b | Comparison of serum selenoprotein P levels (μg/mL) among participants with different alcohol intake leves and different liver enzyme/fatty liver levels.

		Men							Women						
		Alcohol intake			*p*-value				Alcohol intake			*p*-value			
		0 g	<30 g	≥30 g	Between alcohol intake levels	Between liver enzyme levels	Interaction between alcohol intake levels and liver enzyme levels	Between alcohol intake levels in each liver enzyme levels	0 g	<20 g	≥20 g	Between alcohol intake levels	Between lilver enzyme levels	Interaction between alcohol intake levels and liver enzyme levels	Between alcohol intake levels in each liver enzyme levels
Log AST	≤3.00	3.753	3.978	4.417	<0.001	0.477	0.317	–	3.670	3.689	3.620	0.529	0.072	0.851	–
	3.00–3.22	3.920	4.022	4.090				–	3.809	3.943	3.991				–
	3.22<	3.847	4.100	4.431				–	3.872	3.907	4.362				–
Log ALT	≤2.71	3.784	4.027	4.184	<0.001	0.350	0.863	–	3.795	3.822	3.485	0.660	0.376	0.367	–
	2.71–3.09	3.709	4.037	4.291				–	3.720	3.891	4.088				–
	3.09<	3.920	4.045	4.401				–	3.768	3.709	4.194				–
LogγGTP	≤2.94	3.740	3.949	4.586	0.001	0.087	0.465	–	3.706	3.763	3.646	0.949	0.377	0.899	–
	2.94–3.58	3.827	3.953	4.025				–	3.797	3.895	3.994				–
	3.58<	3.901	4.115	4.390				–	3.955	3.807	4.003				–
Fatty liver	No particular	3.823	4.006	4.230	<0.001	0.819	0.412	–	3.766	3.771	3.952	0.903	0.661	0.754	–
	Mild	3.842	4.074	4.295				–	3.705	3.888	3.917				–
	Mederate/Severe	3.844	3.781	4.624				–	3.882	3.525	3.050				–

found in plasma (38). Therefore, the liver is most responsible for circulating selenium levels. We observed that hepatic expression of *SELENOP* significantly declines in the pre-cirrhotic/cirrhotic (F3/4) liver than in the F1/2 liver in patients with chronic hepatitis C (10). We speculate that impaired production of selenoprotein P in the cirrhotic liver reduces circulating levels of selenoprotein P.

Healthy cohort studies evaluating selenium and selenoprotein P are limited. Rasmussen et al. reported that fish intake correlated weakly with serum levels of selenium but not with those of selenoprotein P in 817 randomly selected subjects from two cities in Denmark (39). Smoking status, alcohol intake, exercise habits, BMI, and medicine use did not influence selenium status in their cohort. In a study of selenium status in 391 healthy residents in the south of England (40), daily drinkers showed lower plasma selenium concentrations than non-drinkers and weekend drinkers in men but not in women. In the 966 subjects with colorectal cancer and 966 matched control subjects participating in the European prospective investigation of cancer and nutrition cohort, alcohol intake is not associated with serum levels of selenium and selenoprotein P (41). These studies investigate European people whose blood levels of selenium and selenoprotein P are relatively low compared with the present study. Also, our study evaluated the full-length selenoprotein P levels selectively. These may affect the inconsistent findings between the present study and the previous European studies. On the other hand, our findings are consistent with the study investigated in 124 male and female subjects living in the States, half of whom consumed alcoholic beverages lightly or moderately (42). In that study, alcohol consumption is positively correlated with selenium level and GPx activity in the plasma and whole blood cells. In support of these findings, experimental data in rats has shown that alcohol consumption raises selenium levels in the liver and whole blood without influencing selenium absorption or retention (43). Put together, we can conclude that moderate alcohol intake in healthy individuals raises serum selenium and selenoprotein P levels. In contrast, binge alcohol consumption and alcoholic liver cirrhosis cause reduced serum selenium and selenoprotein P.

Selenium sources are derived from the diet. Also, *SELENOP* gene expression is regulated with macronutrients and hormones, positively with glucose and saturated fatty acids, and negatively with insulin (3). Thus, we investigated the association between intake of alcohol, macro/macronutrients, and foods and serum levels of selenium or selenoprotein P (**Supplementary Tables 2, 3**). Alcohol intake was associated with an estimated intake of total energy, protein, and carbohydrate (**Supplementary Table 2**). However, selenium/selenoprotein P levels were not closely associated with these nutrients, suggesting that nutritional alterations associated with alcohol intake seem unlikely to be involved in the elevated levels of selenium and selenoprotein P.

Selenium is abundant in seafood, animal organs, and eggs, followed by cereals (depending on soil selenium content), meat, and dairy products (Standard tables of food composition in Japan 2015–seventh revised version). In the present study, alcohol intake was positively associated with an intake of fish, squid, octopus, shrimp, shellfish, meat, egg, whole milk, and wheat (noodles, bread), which are abundant in selenium (**Supplementary Table 3**). These findings suggest that moderate alcohol consumption elevates serum levels of selenium and selenoprotein P via preferential intake of selenium-rich foods such as seafood.

In this study, alcohol intake is associated with elevated serum levels of selenium and selenoprotein P more strongly in men than women. One of the causes for such sexual dimorphic findings may be less alcohol intake in women than men (**Supplementary Table 1**, **Figures 1**, **2A,B**). There were only 29 women with alcohol intake $\geq$20 g/day, whereas 155 men with alcohol intake $\geq$30 g/day (**Supplementary Table 1**). Even in men, alcohol intake over 30 g/day exerted apparent effects on selenium and selenoprotein P (**Figures 1**, **2A,B**). Besides, alcohol intake was more frequently associated with an intake of selenium-rich food, such as seafood, in men than women (**Supplementary Table 3**), which may also be one of the causes for the sexual dimorphic findings.

It may be necessary to investigate the molecular mechanisms underlying alcohol intake-induced elevation of serum selenoprotein P levels. Besides selenium status, other environmental factors that affect transcription factor networks determine selenoprotein P levels (2). To date, various ethanol-responsive genes or altered genes in alcoholic hepatitis, including transcription factors, have been identified (44) and may be responsible for alcohol-induced *SELENOP* expression in the liver. Of note, ethanol treatment enhances the nuclear translocation of FoxO3a and upregulates the expression of FoxO3a-target genes in primary hepatocytes and mouse liver (45). Another study showed that alcohol administration downregulates FoxO1 but slightly upregulates FoxO3a in the liver of mice (46). We previously found that metformin activates AMPK and inhibits FoxO3a activity, thereby downregulating *SELENOP* in hepatocytes (16). Therefore, it may be possible that alcohol intake upregulates *SELENOP* expression by activating FoxO3a in the liver, which should be confirmed experimentally in the future.

This study has a limitation that alcohol intake was assessed by self-report, which may not be purely true and accurate.

In conclusion, alcohol intake is associated with elevated serum levels of selenium and selenoprotein P independently of liver enzyme level and liver fat in the general population, especially in men. Therefore, moderate alcohol intake may exert beneficial or adverse effects on health, at least partly by upregulating selenium and selenoprotein P. These findings increase our understanding of alcohol-mediated redox regulation, which leads to increased antioxidative capacity and increased risk for diabetes via reductive stress. The findings should also form the basis for creating appropriate drinking guidelines.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because several studies are ongoing using the datasets in the

present study. Requests to access the datasets should be directed to ttakamura@med.kanazawa-u.ac.jp.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of Kanazawa University (No.1491). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TT designed the study, interpreted the data, and wrote the manuscript with input from all authors. YI analyzed the data with supports by HA, HTs, AH, and HN. TKann and AT built the database for the cohort. HTa, YT, K-aI, TKana, and TT collected the data. TY and SK evaluated the hepatic steatosis. TT is the guarantor of this work and takes responsibility for the integrity of the data and accuracy of the data analysis. YI analyzed the data with supports by HA, HTs, AH, SK, and HN. All authors have read the manuscript and took part in the discussion.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.633703/full#supplementary-material

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BRIEF RESEARCH REPORT
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Sex-Specific Metabolic Impairments in a Mouse Model of Disrupted Selenium Utilization

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The essential micronutrient selenium (Se) provides antioxidant defense and supports numerous biological functions. Obtained through dietary intake, Se is incorporated into selenoproteins *via* the amino acid, selenocysteine (Sec). Mice with genetic deletion of the Se carrier, selenoprotein P (SELENOP), and the Se recycling enzyme selenocysteine lyase (SCLY), suffer from sexually dimorphic neurological deficits and require Se supplementation for viability. These impairments are more pronounced in males and are exacerbated by dietary Se restriction. We report here that, by 10 weeks of age, female *Selenop*/*Scly* double knockout (DKO) mice supplemented with 1 mg/ml sodium selenite in drinking water develop signs of hyper-adiposity not seen in male DKO mice. Unexpectedly, this metabolic phenotype can be reversed by removing Se from the drinking water at post-natal day 22, just prior to puberty. Restricting access to Se at this age prevents excess body weight gain and restriction from either post-natal day 22 or 37 reduces gonadal fat deposits. These results provide new insight into the sex-dependent relationship between Se and metabolic homeostasis.

Keywords: selenium, selenoproteins, sex differences, selenocysteine lyase, metabolic syndrome

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INTRODUCTION

Selenium (Se) has been implicated in a wide range of biological functions that are critical for human health (1). This essential trace element is translationally incorporated into selenoproteins, as the amino acid, selenocysteine (Sec). Selenoproteins, in turn, comprise a major component of the antioxidant defense system of many different tissues. Se is acquired *via* dietary intake and utilized in particularly high levels by the liver, kidneys, brain, testes, and skeletal muscle (2). Distribution of Se throughout the body requires the combined actions of the Se carrier, Selenoprotein P (SELENOP) and the Se recycling enzyme selenocysteine lyase (SCLY). Following absorption by the gut and transport to the liver *via* the portal vein, Se is used to synthesize SELENOP, which contains multiple Sec residues. After being secreted into the bloodstream, SELENOP is taken up by target tissues to be catabolized intracellularly. Proper utilization of the delivered Sec residues is dependent on SCLY, which catalyzes the breakdown of Sec into selenide, to be used for *de novo* selenoprotein biosynthesis (3).

The role of Se in energy homeostasis is complicated, as clinical studies have correlated both Se deficiency and high Se intake with metabolic disease in humans (4). Hepatic SELENOP has been implicated in the development of hyperglycemia (5) and insulin resistance (6) in humans and mice, respectively. Mice with genetic knockout (KO) of *Scly* have increased susceptibility to metabolic

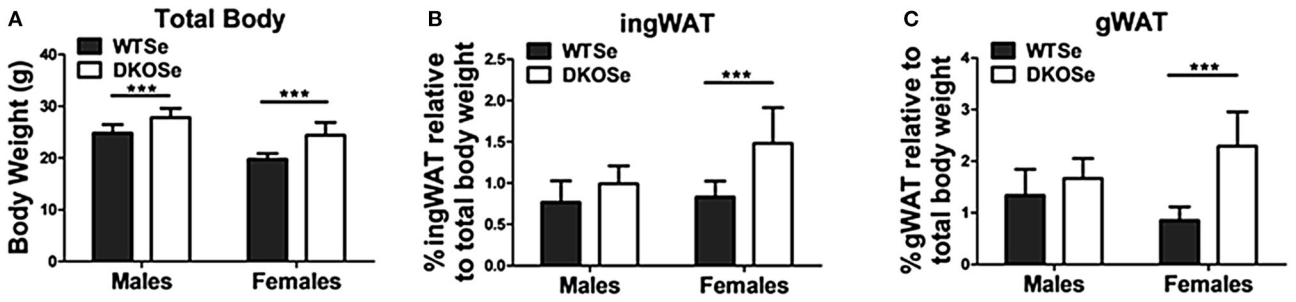

FIGURE 1 | Body composition of wild-type (WT) and double knockout (DKO) mice of both sexes. **(A)** Total body weight, **(B)** percentage of inguinal white adipose tissue (ingWAT), and **(C)** percentage of gonadal white adipose tissue (gWAT) weight of male and female WT and DKO mice at 10 weeks of age. Two-way ANOVA: Total body weight Interaction NS, Genotype $F_{(1,52)} = 60.68$, $p = <0.0001$, Sex $F_{(1,52)} = 75.15$, $p < 0.0001$; ingWAT Interaction $F_{(1,52)} = 7.54$, $p = 0.0083$, Genotype $F_{(1,\ 52)} = 31.51$, $p < 0.0001$, Sex $F_{(1,52)} = 12.64$ $p = 0.0008$; and gWAT Interaction $F_{(1,52)} = 18.64$, $p < 0.0001$, Genotype $F_{(1,52)} = 47.51$, $p < 0.0001$, Sex NS; $n =$ 14 all groups. Bonferroni's multiple comparisons test: $^{***}p < 0.001$. All values are reported as mean ± SEM.

syndrome (7) and diet-induced obesity (8), with more dramatic effects observed in male mice. Additionally, targeted deletion of specific selenoproteins causes differential metabolic disturbances in animal models (9–11), demonstrating the impact of not only dietary Se intake, but also proper Se utilization.

We previously bred *Scly* KO and *Selenop* KO mouse strains to produce double knockout (DKO) mice. DKO mice were found to suffer from severe neurological dysfunction (12), which was subsequently found to be attenuated by prepubescent castration (13). We recently reported that although female DKO mice display less severe neurological deficits than their male counterparts, the phenotype is worsened by the removal of Se supplementation during puberty (14). Here we provide preliminary data showing that female DKO mice exhibit a metabolic phenotype not seen in male DKO mice.

MATERIALS AND METHODS

The data in this report were generated from mice used in our previous publication addressing the sex-specific neurological phenotype of DKO mice (14). Male and female C57/BL6N wild-type (WT) and DKO mice were generated as previously described [9]. Since supplementation with Se is critical for DKO mouse survival, all subjects in this study were maintained on standard mouse chow containing ∼0.25 ppm Se and drinking water containing 1 mg/ml sodium selenite, Na_2SeO_3. Mice were given *ad libitum* access to food and water from weaning (∼18 days of age) until the age of 10 weeks, at which point they were weighed, sacrificed *via* CO_2 asphyxiation and tissues were harvested. Inguinal and gonadal white adipose tissue (WAT) deposits were removed and weighed on a benchtop analytical balance. For some groups, Se-supplemented drinking water was replaced with non-supplemented drinking water at either 22 days or 37 days post-natal (denoted as -NoSeP22 and -NoSeP37, respectively). All procedures and experimental protocols involving animals were approved by the University of Hawaii's Institutional Animal Care and Use Committee. Animal Care and Use Committee (IACUC) Protocol: "Mechanism of Selenoprotein Synthesis and Studies of Selenoprotein Functions:" APN 09-871-9, approved: 18 July 2019. Institutional Biosafety Committee (IBC) Protocol: "Mechanism of Selenoprotein Synthesis and Studies of Selenoprotein Functions:" IBC #18-10-544-02-4A-1R, approved: 23 October 2018.

RESULTS

Female DKO mice had significantly higher total body weights compared to their WT counterparts at 10 weeks of age (**Figure 1A**). WAT deposits were also significantly heavier in female DKO mice compared to WT controls (**Figures 1B,C**), an effect that was not observed in male DKOSe mice. These results indicate a metabolic effect specific to female DKO mice.

Previously, we demonstrated that removal of Se supplementation from female DKO mice prior to puberty at post-natal day 22 (P22) exacerbates the neurological phenotype to a similar level as male DKO mice without Se removal (14). We report here that, surprisingly, total body weight was significantly lower in female DKO when Se water was removed at P22 compared to female DKO mice with constant Se supplementation (**Figure 2A**). Regarding fat deposits, inguinal WAT weights trended lower in female DKO mice when Se was removed at P22 (**Figure 2B**). Removal of Se supplementation at either P22 or P37 significantly reduced gonadal WAT deposits in female DKO mice (**Figure 2C**). These data demonstrate that while female DKO mice develop a metabolic phenotype not seen in male DKO mice, this phenotype is partially attenuated by the removal of Se-supplemented drinking water.

DISCUSSION

Although concurrent KO of *Selenop* and *Scly* in female mice causes a milder neurological phenotype compared to male DKO mice, we report here that female DKO exhibit an obesogenic

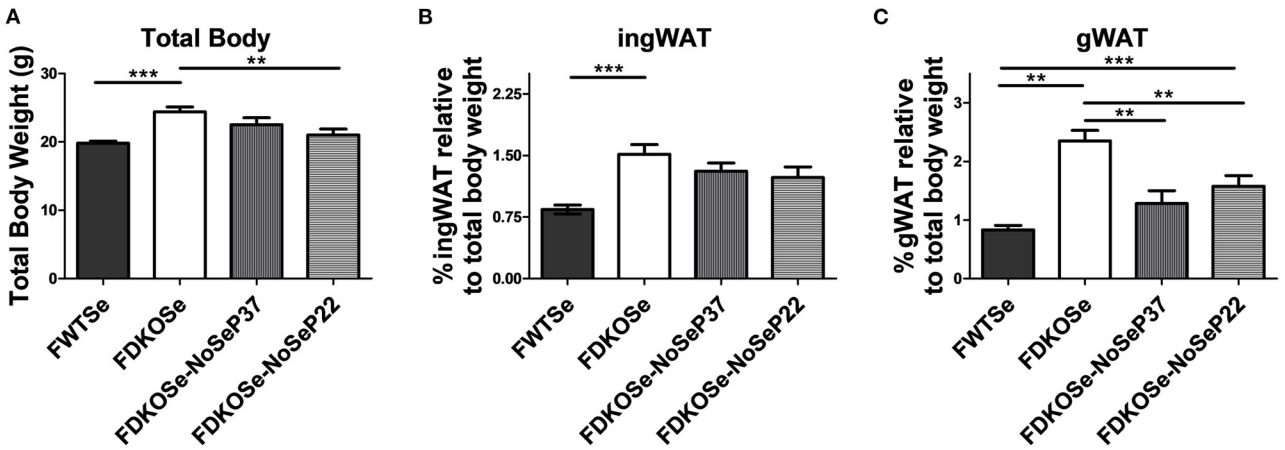

FIGURE 2 | Effect of dietary Se removal on the body composition of female double knockout (DKO) mice. **(A)** Total body weight, **(B)** percentage of ingWAT, and **(C)** percentage of gWAT weight at 10 weeks of age of female wild-type (FWT), female double-knockout (FDKO), and female double knockout with Se supplementation removed at post-natal day 37 (FDKOSe-NoSeP37) or post-natal day 22 (FDKOSe-NoSeP22). One-way ANOVA: Total body weight $F_{(3,39)} = 8.46$, $p = 0.0002$; %ingWAT $F_{(3,39)} = 7.29$, $p = 0.0005$; %gWAT $F_{(3,39)} = 17.08$, $p < 0.0001$; $n = 13$ all groups except P37 $n = 4$; Bonferroni's multiple comparisons test: $^{**}p < 0.01$, $^{***}p < 0.001$. All values are reported as mean ± SEM.

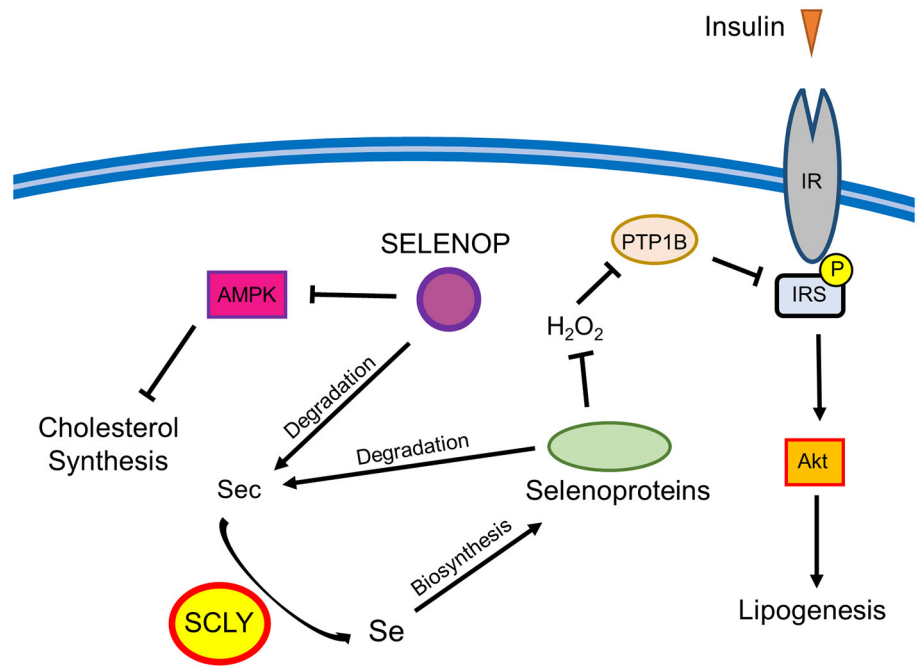

FIGURE 3 | Schematic representation of the possible interactions between Se metabolism and lipid metabolism. Selenoprotein P (SELENOP) has shown an ability to de-activate AMP-activated protein kinase (AMPK) and inhibit insulin signaling in liver and skeletal muscle (6). AMPK limits cholesterol synthesis in the liver, thus representing a potential node through which SELENOP may regulate cholesterol. SELENOP and other selenoproteins can be degraded to produce selenocysteine (Sec) residues that are further metabolized through a process involving selenocysteine lyase (SCLY) to produce Se. This Se can then be used for *de-novo* synthesis of selenoproteins. The antioxidant activity of selenoproteins may promote the negative regulation of insulin signaling by preventing the oxidative inactivation of protein tyrosine phosphatase 1B (PTP1B) by hydrogen peroxide (H_2O_2). PTP1B de-phosphorylates the IR and insulin receptor substrate (IRS), which, in turn, reduces protein kinase B (AKT)-mediated lipogenesis. Through affecting this pathway, SCLY could work to limit lipogenesis (7).

phenotype marked by elevated WAT deposit weights. These results are consistent with past studies showing that *Scly* KO mice are predisposed to similar metabolic deficits (7, 8). This predisposition is more pronounced in male *Scly* KO mice, which contrasts with our current findings that female DKO mice exhibit hyper-adiposity while males do not. Considering

that male DKO mice suffer from severe motor deficits and seizures, even while supplemented with Se, however, it is possible that these symptoms could mask a metabolic phenotype by affecting their ability to ambulate and eat. Thus, further characterization of DKO mice should involve analysis of physical activity and feeding behavior. Future studies should also evaluate core temperature, respiratory metabolism, and a more comprehensive analysis of adiposity, as Se has been shown to regulate adipose tissue thermogenesis (15) and lipid metabolism (16). Finally, a broad assessment of circulating hormones, such as plasma insulin, leptin, and thyroid hormones, as well as nutrients, such as glucose, triglycerides, and free fatty acids, would help detect changes in the endocrine regulation of body fat stores.

Surprisingly, challenging female DKO mice with the removal of Se supplementation in drinking water partially prevented the development of excess weight gain and hyper-adiposity. These results are in contrast with our previous findings that *Scly* KO mice develop signs of metabolic syndrome, including obesity, when raised on a Se-deficient diet (7). This implies that the added effect of *Selenop* deletion, which limits the ability of the body to distribute Se, alters the metabolic response to dietary Se restriction in mice lacking *Scly*. It is possible that the baseline redox environment in DKO mice is dramatically different from *Scly* KO mice, thus altering the compensatory mechanisms implemented in response to changes in antioxidant availability. These changes are likely complex as Se and selenoproteins have shown a capacity to differentially regulate energy metabolism through a variety of physiological processes, such as thyroid hormone metabolism (17) and insulin activity (18), as well as tissues that closely regulate energy metabolism including liver (19), pancreas (20), and the hypothalamus (21). This phenomenon is somewhat reminiscent of the observation that there appears to be a relatively narrow range of Se intake that is beneficial in humans as both low and high levels of Se status have been connected to an increased risk for type 2 diabetes (4). Thus, it is possible that the beneficial window is somehow lowered in female mice under the conditions of S*elenop*/*Scly* double KO, and that the combination of Se intake from both food and water surpasses the upper limit of that window. This could possibly explain why, in regard to metabolic phenotype, removal of Se water, while maintaining some Se intake *via* food consumption, appears to have a beneficial effect on female DKO mice. There are multiple pathways through which Se may affect adiposity by regulating lipid metabolism, including cholesterol synthesis and insulin signaling (7). For example, SCLY-mediated selenoprotein activity may negatively regulate the ability of insulin to induce lipogenesis in the liver by promoting protein tyrosine phosphatase 1B (PTP1B) antagonism of insulin signaling. On the other hand, SELENOP may regulate glucose metabolism in both the liver and skeletal muscle by actin on AMP-activated protein kinase (AMPK) (6). Thus, the deletion of both the *Selenop* and *Scly* genes may disrupt multiple pathways affecting adiposity. The possible intersection between Se metabolism and lipid metabolism is described in **Figure 3**.

On a final note, it is possible that the reduction of the female DKO mouse hyper-adiposity phenotype brought on by Se water removal may be affected by worsening neurological symptoms (14). It is important to note, however, that while gonadal WAT deposits were reduced in female DKO mice by Se removal at either P22 (just prior to puberty) or P37 (latter stages of puberty), the neurological phenotype of female DKO mice was shown to be aggravated only by Se removal at P22, not at P37 (14). Since female DKO mice with Se removed at P37 show no changes in neuromotor function as a result, it is, thus, likely that the reduced gonadal WAT deposits result from a distinct mechanism central to energy homeostasis. Taken together, these data implicate S*elenop*/*Scly* DKO mice as a useful model for investigation of these relationships and warrant comprehensive metabolic characterization of these mice and interrogation of underlying mechanisms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Hawaii's Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

PK, AH, and MB were involved in conceptualization, experimental design, obtaining resources, and interpretation of data. PK and AH were involved in data acquisition and analysis. PK was involved in writing the initial drafts. DT was involved in data validation, interpretation of data, writing the initial drafts, and manuscript revisions. MB was involved in manuscript revisions and providing funding. All authors read and approved of the final manuscript.

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Association of Selenium Intake and Development of Depression in Brazilian Farmers

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Introduction: Depression and deficiency in the consumption of micronutrients are a public health problem, especially in the rural population. The deficiency in selenium consumption affects mental health, contributing to the development of major depressive disorders. Thus, this study aimed to evaluate selenium intake and its association with depressive symptoms in farmers in southeastern Brazil.

Material and Methods: Epidemiological, cross-sectional, and analytical study with 736 farmers aged between 18 and 59. A semistructured questionnaire was used to collect sociodemographic, lifestyle and clinical condition data. For evaluation of food intake, three 24-h recalls were applied, and for identification of depressive episodes, the Mini-International Neuropsychiatric Interview was conducted.

Results: A total of 16.1% ($n = 119$) of the farmers presented symptoms of major depressive episodes, 5.8% ($n = 43$) presented symptoms of current depressive episodes, and 10.3% ($n = 76$) presented symptoms of recurrent major depressive episodes. Sociodemographic factors associated with depression were gender ($p < 0.001$), marital status ($p = 0.004$), and socioeconomic class ($p = 0.015$). The consumption of high doses of selenium was associated with a reduction of ∼54% in the chances of occurrence of depression (OR = 0.461; 95% CI = 0.236–0.901).

Conclusion: High selenium intake is associated with a lower prevalence of depression even after adjusting for sociodemographic variables, lifestyle, and pesticide intoxication. The findings of this study contributed to highlighting the high prevalence of depression in rural areas and its relationship with selenium intake.

Keywords: rural worker, rural population, food consumption, micronutrient, selenium, depression, public health

INTRODUCTION

Depression is a common mental disorder all over the world and the leading cause of inability (1)[1]. It contributes considerably to the global burden of disease and leads to loss of productivity and increased time away from work (2). It is estimated that 322 million people worldwide have this disease. Globally, depressive disorder is ranked as the largest contributor to disability-adjusted life-years (DALYs) with 7.5% of years lived with disability (YLD) (3)[2]. The Global Burden of Disease Study, a systematic analysis of the global burden of disease, examined 369 diseases and injuries in 204 countries and territories between 1990 and 2019, identifying the 10 most important factors that increase the burden of disease—among them, depressive disorders that are common from adolescence to old age (4)[3].

One of the major contributing factors to the progressive increase of chronic non-communicable diseases such as depression is an unhealthy lifestyle, including an inadequate diet (5). There is evidence of the protective effect of certain dietary patterns and nutrients on depression occurrence. Among the mechanisms involved in this relationship are a decrease in oxidative stress, reduction in inflammatory markers, improvement in endothelial function, and alteration in the synthesis and function of serotonin (6). Additionally, it is known that long-term exposure to low levels of some nutrients, like selenium, may affect brain function, such as cognitive function and mood (7).

Selenium is an essential nutrient necessary for the optimal functioning of several selenoproteins. The role of selenium in the development of depression seems to be related to its ability to reduce oxidative stress and inflammatory markers, improving endothelial function and altering the synthesis and functioning of serotonin (6, 8, 9). The modulatory effects of selenium on thyroid metabolism also seem to influence an individual's susceptibility to developing depression, as well as the action of selenoproteins in the dopaminergic, serotonergic and noradrenergic systems (10). A study involving more than 14,000 individuals showed that participants who met the RDA (Recommended Dietary Allowance) for selenium had significantly lower chances of depression (OR: 0.52; 95% CI: 0.39, 0.71) (11). Selenium concentrations were also significantly lower in patients with major depressive disorder compared to controls in Bangladesh ($p < 0.05$) (12). In rats, higher levels of selenium have been shown to be protective against the development of depressive symptoms in response to stress (13). Despite this, the evidence is still contradictory (9–11, 14, 15).

In Brazil, few studies evaluate the consumption of micronutrients in the population (7). Thus, the aim of this study was to evaluate selenium intake and its association with depressive symptoms in Brazilian farmers and to analyze whether farmers with highest quartile selenium intake are less likely to develop depressive symptoms when compared to farmers with the lower quartile of selenium intake.

MATERIALS AND METHODS

Study Design

This was a quantitative analytical epidemiological study derived from a larger population-based project entitled "Health status and associated factors: A study in farmers in Espírito Santo—AgroSaúdES." The target population of this research was farmers of both sexes, aged between 18 and 59 years, working in the municipality of Santa Maria de Jetibá - ES, Brazil. The inclusion criteria were that farmers had to be between 18 and 59 years old, had to not be pregnant, had to have agriculture as the main source of income, and had to be in full employment for at least 6 months. Farmers who did not meet the inclusion criteria, did not participate in one of the stages of data collection, or did not sign the informed consent form (ICF) did not participate in the research.

The sample size was calculated by considering an estimated prevalence of depression in rural populations of 5.6% (16), an error rate of 2%, and a confidence interval of 95%, with a minimum required sample size of 468 individuals. However, to improve the representativeness of the sample and the statistical relevance, data from all farmers who participated in the original project and who had data of interest available were used. The sample size was calculated using the EPIDAT program version 3.1. Farmers who met the inclusion criteria were identified based on the data available in the registers of individuals and families filled out by Family Health Strategy teams responsible for covering 100% of the 11 health regions that made up the municipality.

Through these records, we identified 7,287 farmers belonging to a total of 4,018 families who met the inclusion criteria. Participants were selected through stratified draw after considering the number of families per health region and per community health agent (CHA), and the proportionality among the 11 regions and the 80 CHAs. Only one randomly selected individual per family was admitted, thus avoiding the interdependence of information. In case of refusal or non-attendance, a new participant was called from a reserve list of the draw, based on the sex and health unit of origin of the person who dropped out. A total of 806 farmers were invited to compensate for possible losses. Of these, 790 agreed to participate in the research and signed the ICF. Subjects who did not respond to the three 24-h recordings ($n = 50$) and who had attempted suicide by ingesting pesticides ($n = 4$) were excluded from the study, leaving a total of 736 farmers.

Study Environment

The study was conducted among farmers in the municipality of Santa Maria de Jetibá, a mountainous region of the state of Espírito Santo, Brazil. The study was conducted here because it is the largest producer of horticultural products in Espírito Santo (17).

[1]https://www.paho.org/bra/index.php?option=com_content&view=article&id=5635:folha-informativa-depressao&Itemid=1095

[2]https://apps.who.int/iris/bitstream/handle/10665/254610/WHO-MSD-MER-2017.2-eng.pdf;jsessionid=129DFEF24B5BE7B74114594D24DCA6D4?sequence=1

[3]https://www.thelancet.com/journals/lancet/article/PIIS0140-6736(20)30925-9/fulltext

The population is predominantly rural and carries out family farming as its main economic activity. Its agricultural practices are characterized by the predominance of polyculture and a low degree of mechanization. The population follows a contemporary food pattern characterized by a traditional Brazilian pattern and a local yet industrialized pattern, indicating that the farmers follow a diet with ultra-processed products and low fruit consumption, and that they have habits characteristic of more urbanized rural regions (18). Ultraprocessed foods are characterized by high energy, low fiber, and microscopic minerals and high added or free sugars, sodium, saturated fats, and chemical food additives (19).

Ethics

The Research Ethics Committee of the Federal University of Espírito Santo—Opinion No. 2091172 (CAAE 52839116.3.0000.5060) approved the project and complied with the ethical rules governing research involving human beings. All participants who agreed to participate in the research signed the Free and Informed Consent Form. The research was conducted in partnership with the Municipal Health Secretariat and the Rural Workers Union of the municipality of Santa Maria de Jetibá.

Data Collection

Data were collected from December 2016 to April 2017. It should be noted that, given the predominance of polyculture and the production of short-cycle foods such as vegetables (20), no major differences were identified between the months selected for data collection and the other months of the year with respect to agricultural practices, including those related to pesticide use. The selected farmers were grouped by region of residence and invited by the CHAs to come to the basic health unit on a predefined day and time for data collection. Data collection was structured as follows: after signing the ICF, the farmer answered the semi-structured questionnaire to provide socioeconomic and occupational, lifestyle habit, risk perception, behavior adopted during pesticide handling, and self-reported disease and symptom data. The data collection team was composed of five trained permanent members: one PhD student, two master's students, one graduate student, and one undergraduate student. To minimize inter-observer variability, the researchers remained in the same positions from the beginning to the end of the data collection process, and four interviewers administered the questionnaires.

Selenium Intake Analysis

Selenium intake was assessed using three 24-h recordings (24 h). Given the high variability in nutrient intake on different days, two 24 h recall schedules were applied to 2 days of the week and one 24 h recall schedule on the weekend was added to be more representative of the usual intake since there are significant variations between these days. From the results of the three recall schedules, an average was made that depicts the usual intake of the study population. This analysis methodology is in accordance with the protocol widely used in the literature of de area in several studies that evaluate the food consumption of populations (21, 22). The first recall was applied during the interview, in which individuals reported all the food and beverages consumed, including the respective amounts and portion sizes consumed in the last 24 h. The second recall was performed within 7 days after the first 24 h data collection, and the third recall was performed during the return visit, which took place 8–15 days after the first contact with the interviewee. To ensure greater accuracy of the portions eaten, photo albums were used to facilitate the identification and quantification of the consumed items. In the case of processed products, the brands, type, and quantity of the product were recorded. The nutritional composition analysis of the 24 h was conducted later by means of the AvaNutri 4.1 program and the Brazilian Table of Food Composition (TACO) (23). The typical foods of the region were registered in the software according to manufacturer information or standardized recipes.

After the food and nutrient intake was registered, it was observed that none of the participants had energy consumption equal to or <500 kcal and more than 6,000 kcal (22). Thus, they presented values compatible with the usual food consumption patterns, and it was not necessary to exclude any individual from the analyses. After obtaining the values of each 24 h, deattenuation analysis was conducted using PC-SIDE software (Department of Statistics, Iowa State University, Iowa, United States). Energy adjustment was also performed using the residual method, which, according to Willett, corrects nutrient estimates by total energy intake, thus providing the energy-adjusted selenium value (22). Variation of selenium intake was expressed in micrograms/day (μg/day) and in quartiles, allowing risk trend analysis according to the degree of exposure and the differences between extreme intake concentrations which allows comparison of risk between lower and higher quartiles of food intake (24). Selenium intake in the evaluated sample ranged between 28.15 and 146.83 μg/day. No farmer in the study had a consumption higher than the UL established for this micronutrient. Thus, the first quartile contained individuals with a daily selenium intake of 66.66 μg, the second quartile contained those who had a daily selenium intake of 66.67–80.37 μg, the third quartile contained those who had a daily selenium intake of 80.38–95.25 μg, and the fourth quartile contained those who had a daily selenium intake of above 95.26 μg.

Analysis of Depressive Symptoms

To evaluate the symptoms of depression, we used the Major Depressive Episodic Module of the Brazilian version of the Mini-International Neuropsychiatric Interview (MINI), which is organized into independent diagnostic modules. It is a brief standardized diagnostic interview with good sensitivity and specificity for use in clinical practice and research (25), which aims to diagnose interviewees in a way that is compatible with the criteria of the Diagnostic and Statistical Manual of Mental Disorders (26) and the International Classification of Diseases (27). The version used in the present study corresponded to MINI 5.0 in Portuguese (25). The individuals were classified into two categories: "without depression episode" or "with depression episode." The latter category contained two subcategories, "current depressive episode" or "recurrent depressive episode,"

based on the version MINI 5.5 mentioned above. According to literature data, the results concerning the reliability and validity of this instrument were globally satisfactory (25).

Independent Variables

The independent variables were subdivided into sociodemographic variables, lifestyles, clinical conditions, and selenium consumption. Sociodemographic variables included gender, age group ("up to 29 years," "30–39 years," "40 years or older"), race/color ("white" and "non-White"), education ("<4 years," "4–8 years," "more than 8 years"), marital status ("single," "married/living with a partner," and "separated/divorced/widowed"), and socioeconomic class ("class A or B," "class C," and "class D or E"). National studies use this classification. Socioeconomic classes are estimated according to the purchasing power of individuals and families, allowing the estimation of the average monthly gross family income (A: ∼R$ 11,037.00; B: ∼R$ 6006.00; C: ∼R$ 1,865.00; D/E: ∼R$ 895.00) (28)[4]. For the lifestyle variable, the consumption of alcohol ("consumes" and "does not consume") and tobacco ("smoker or former smoker" and "non-smoker") was analyzed. The clinical condition variable analyzed the diagnosis of pesticide intoxication made by a health professional and involved the question, "Has a doctor or other health professional ever diagnosed you with pesticide intoxication?" The responses were categorized as "yes" and "no."

Statistical Analysis

Statistical analyses and the interpretation of the results followed the consistency of the theoretical model used to investigate the relationship between selenium consumption and depression. The absolute and relative frequencies of the independent variables were calculated according to the presence or absence of depression. To evaluate the qualitative variables and their associations, the Chi-squared test of association was used. Variables with p-value < 5 (5% significance level) in this test were included in the binary logistic regression analysis as the following adjustment factors: sociodemographic variables (gender, marital status, socioeconomic class); lifestyle (alcohol consumption); clinical condition (pesticide intoxication), and selenium consumption. Pesticide poisoning was included as an adjustment variable as it has often been associated with the occurrence of depression (29–31).

The results were expressed as odds ratios (OR) along with the respective confidence intervals. The quality of the model was assessed using the Hosmer-Lemeshow test. All data were organized and analyzed in IBM SPSS® version 22.0 software.

RESULTS

In this study, 378 (51.33%) farmers were male, and 358 (48.64%) were female. The majority (535, 73.1%) were above 30 years old. A total of 497 (67.53%) of them had an education level below 4 years of study, 666 (90.49%) were of White race/color, and 680 (92.39%) belonged to classes C, D, and E.

[4]http://www.abep.org/criterio-brasil

Of the 736 farmers who were assessed, 617 (83.83%) were classified as without depressive episodes. The prevalence of major depressive episode was 16.17% ($n = 119$). Subdivided by recurrence, 43 (5.84%) participants had current major depressive episode, and 76 (10.33%) had "recurrent major depressive episode" (**Figure 1**).

The variables associated with depression that were entered into the logistic regression model as adjustment variables were as follows: sex ($p < 0.01$), marital status ($p = 0.004$), socioeconomic class ($p = 0.015$), alcohol consumption ($p = 0.002$), and pesticide intoxication ($p = 0.001$). Most subjects did not consume alcohol (56.66, $n = 417$), were not smokers (85.33 %, $n = 628$), and had not been diagnosed with pesticide intoxication (93.2%, $n = 686$) (**Table 1**).

When the association of selenium consumption with depression was analyzed, 33 individuals (17.8%) in the lowest quartile of consumption showed depression, whereas 17 individuals (9.2%) in the highest quartile of consumption showed this morbidity (**Table 2**).

Below are the binary logistic regression data between depression and selenium consumption adjusted for sociodemographic variables (gender, marital status, economic class), lifestyle (alcohol consumption), and pesticide intoxication (**Table 3**).

The comparison of the first quartile (lowest consumption) with the fourth quartile (highest consumption) showed that the prevalence of depression was significantly lower in farmers with higher consumption of selenium (Q4), even after adjusting for sociodemographic variables, lifestyle and pesticide poisoning (OR = 0.461; 95% CI = 0.236–0.901) (**Table 3**).

DISCUSSION

This is the first Brazilian study to evaluate the relationship between selenium intake and depression in farmers and to contribute to filling this gap in the literature, especially in rural populations. It has been argued that studies on the role of nutrition in psychiatric diseases are essential to expand the role of health professionals with individuals with this morbidity (32). This study is justified by its relevance to public health, especially given the high incidence and prevalence of depression in recent years in the general population, and the scarcity of information regarding mental health in farmers (30, 33)—especially depression (34, 35)—as well as of nutritional and anthropometric assessments of this population (18, 36, 37). The representative and randomly selected sample of this study allowed us to extrapolate the results to the target rural population.

Importantly, the proportion of the global population with depression in 2015 was estimated to be 4.4%, and the estimated total number of people in the world living with depression increased by 18.4% between 2005 and 2015, totaling 322 million people with depression worldwide. The Americas were ranked fourth in the global population with the highest prevalence of depression (15%), followed by the Southwest Asian (27%), Western Pacific (21%), Mediterranean (16%), European (12%), and African (9%) regions (3). In the population studied, there

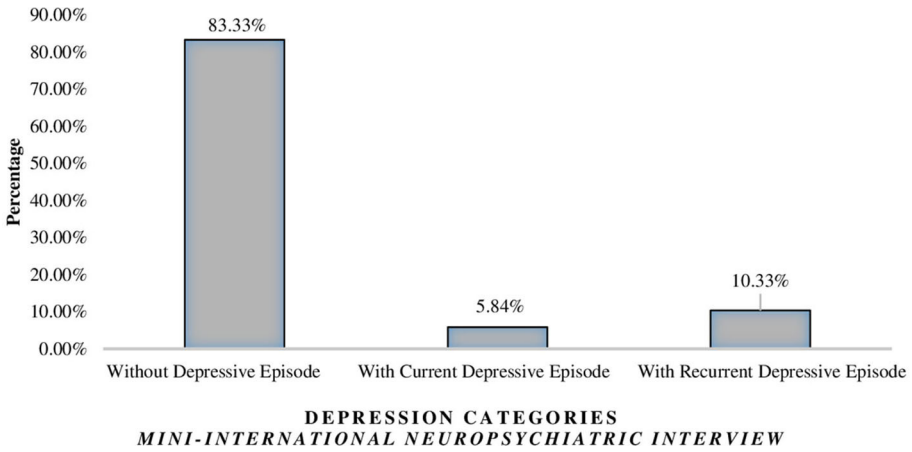

FIGURE 1 | Prevalence of major depressive episodes in farmers according to the MINI scale version 5.0.

was a high prevalence of depression (16.17%) (35). This data is alarming since the prevalence of depression found in this study is higher when compared to some Brazilian studies. This can be verified in the National Health Survey (30), which indicated the prevalence of depression in Brazilian adults (7.6%), namely, the rural population (5.6%) and Espírito Santo (5.5%). Another study conducted in a rural population in southern Brazil found the prevalence of depression to be 8.1% (38). These data were corroborated by international studies carried out among the rural populations of Lithuania, China, and Canada (7, 33, 39).

Further, the sample studied presented a contemporary food pattern characterized by a "traditional Brazilian pattern," "local traditional" and "industrialized," that opts for a diet with ultra-processed products and low fruit consumption—habits characteristic of more urbanized rural regions (18).

This eating behavior results in inadequate micronutrient intake (40), worse health outcomes, and overall poor diet quality (41), and may be directly associated with depressive feelings (29) and poor mental health. These data confirm the trend indicated by the literature of increasing adherence of residents of rural areas to the dietary patterns of urban areas (42). The process of urbanization and globalization have led to the mitigation of regional differences and to the increase of integration, exchange of information, and access to variable and healthy foods (43)[5].

The sociodemographic, lifestyle, and work variables associated with depression were used in this study as adjustment variables to corroborate the data in the literature.

The results demonstrated the association between selenium consumption and the prevalence of depression in rural areas. Farmers in the highest quartile of selenium consumption were 54% less likely to experience depression compared to the lower quartile of consumption. Corroborating these findings, a cross-sectional study with 14,834 adults from the National Health and Nutrition Examination Survey (NHANES) 2009–2014 identified Odds ratios of 0.46 (0.32–0.67) for the highest quartile vs. the lowest quartile of selenium intake (10), values very close to those found in the present investigation. Randomized clinical trials found improvement in mood (44, 45) and improvement in post-partum depression (46) with selenium supplementation in adult populations. In the United States, a rural health study supported the link between exposure to selenium in groundwater and decreased symptoms of depression (47). In the MASHAD study, selenium intake was negatively associated with the relative risk of a high depression score using the Beck scale (48). In contrast, the results of two cross-sectional studies conducted among a geriatric population in rural China (7), as well as a population of patients on hemodialysis (49), found no significant association between selenium levels and depression scores after controlling for chronic kidney disease and cognitive function. Research with the American adult population also found no association between these variables (15).

It should be noted that excessive selenium consumption also seems to be related to a higher risk of developing depression, corroborating the hypothesis of a U-association between these two variables (15). Considering that, among the evaluated farmers, no individual exceeded the intake above the UL established for selenium (400 μg/day), the evaluation of the effects of excessive consumption of this nutrient cannot be performed in this study. The average selenium intake among the evaluated farmers was 81.27 μg/day, higher than that found in other countries such as New Zealand (50) and Poland (51). Only 8.8% ($n = 65$) of farmers had lower consumption than the Recommended Dietary Allowances (RDAs) of 55 μg/day established for this nutrient. These results are possibly due to the high consumption of meat, eggs and minimally processed food sources identified in this population (18, 52). The high intake of this micronutrient in the Brazilian population has also been reported by other studies (53).

[5]https://biblioteca.ibge.gov.br/index.php/biblioteca-catalogo?view=detalhes&id=291110

TABLE 1 | Prevalence of depression according to sociodemographic characteristics, lifestyles, and pesticide intoxication among Brazilian farmers.

Variables	Depression						*P*-value
	Total		No		Yes		
	n	%	*n*	%	*n*	%	
Sex							
Male	378	51.36	336	88.90	42	11.10	**<0.001**
Female	358	48.64	281	78.50	77	21.50	
Age group							
Up to 29 years	201	27.31	171	85.10	30	14.90	0.753
30–39 years	217	29.48	183	84.30	34	15.70	
40 or more	318	43.21	263	82.70	55	17.30	
Marital status							
Single	56	7.61	52	92.90	4	7.10	**0.004**
Married/living with partner	635	86.28	534	84.10	101	15.90	
Separated/divorced/widowed	45	6.11	31	68.90	14	31.10	
Education							
<4 years	497	67.53	418	84.10	79	15.90	0.713
4–8 years	161	21.88	132	82.00	29	18.00	
More than 8 years	78	10.59	67	85.90	11	14.10	
Race/color							
White	666	90.49	561	84.20	105	15.80	0.36
Non-white	70	9.51	56	80.00	14	20.00	
Socioeconomic class							
Class A or B	56	7.61	52	92.90	4	7.10	**0.015**
Class C	375	50.95	322	85.90	53	14.10	
Class D or E	305	41.44	243	79.70	62	20.30	
Alcohol consumption							
Does not consume	417	56.66	334	80.10	83	19.90	**0.002**
Consumes	319	43.34	283	88.70	36	11.30	
Smoking							
Non-smoker	628	85.33	529	84.20	99	15.80	0.473
Current and past smoker	108	14.67	88	81.50	20	18.50	
Intoxication by agrochemicals							
Yes	49	6.65	33	67.30	16	32.70	**0.001**
No	686	93.2	583	85	103	15.00	

Chi-squared test. In bold: Statistically significant values ($p < 0.05$).

TABLE 2 | Prevalence of depression according to quartile of selenium consumption of Brazilian farmers.

Variable	Depression						*P*-value
	Total		No		Yes		
	n	%	*n*	%	*n*	%	
Selenium consumption							
Quartile 1 (≤66.66 μg)	185	25.13	152	82.20	33	17.80	**0.032**
Quartile 2 (66.67–80.37 μg)	184	25	150	81.50	34	18.50	
Quartile 3 (80.38–95.25 μg)	183	24.87	148	80.90	35	19.10	
Quartile 4 (>95.26 μg)	184	25	167	90.80	17	9.20	

Chi-squared test. In bold: Statistically significant values ($p < 0.05$).

TABLE 3 | Binary logistic regression [OR (95% CI)] between depression and highest quartile of selenium consumption, gender, marital status, socioeconomic class, alcohol consumption, and pesticide poisoning in Brazilian farmers.

	OR crude		OR adjusted*	
	OR	**CI 95%**	**OR**	**CI 95%**
Depression				
No	Reference**		Reference**	
Yes	0.469	(0.251–0.876)	0.461	(0.236–0.901)

**Adjusted for gender, marital status, socioeconomic class, alcohol consumption, and pesticide poisoning.*
***Statistically significant values ($p < 0.05$).*
Hosmer-Lemeshow = 0.795.

A case-control study with 1,494 women aged 20–89 years reported that dietary intake of lower selenium (<8.9 μg/day) was associated with an increased risk of developing major depressive disorder (14). In another US study of 30 men on a low or high selenium diet (32.6 μg vs. 226.5 μg/day), mood deteriorated with the poor diet and improved with the rich diet (54). A study in post-partum mothers found that depressive symptoms were lower around serum selenium concentrations of 82–85 mg/L. Below 82 mg/L, depressive symptoms began to increase, culminating in the highest depressive symptoms for participants in the lowest decile of serum selenium (approximate serum selenium concentration of 62 mg/L). Concentrations >110 mg/L have also been associated with an increase in depressive symptoms (50).

It is important to highlight that the understanding of the association of selenium consumption and depression found is due to the essential role of selenium in health, mood, and the physiology of depression (6, 10, 55).

According to WHO (56)[6]. mental disorders result from many factors and have their physical basis in the brain. This is an organ predisposed to oxidative-nitrosative stress. If its antioxidant defenses do not react adequately to radical damage, the neurons may undergo microalteration, microdysfunction, and degeneration, contributing to the pathogenesis of depressive disorders (57). Additionally, depression has been recognized as an inflammatory disorder accompanied by an accumulation of reactive oxygen species that overwhelm the physiological processes of the individual. This suggests that depression is a disease belonging to the (neuro)degenerative disorder spectrum (58). There are several possible physiological hypotheses about the effects of selenium on mood enhancement, including its role in maintaining metabolic, oxidative, and central nervous system function, as well as the potential underlying mechanisms between low serum selenium levels and the development of depression, such as dysregulation of thyroid function and oxidative and inflammatory pathways (10). However, studies examining the relationship between depression and selenium are largely inconclusive (10), and further research is needed to clarify selenium's actions on the other physiological mechanisms of depression.

The implications of this work are that, despite working in rural areas, the food intake of the farmer population is inadequate and needs to be better observed. Broader analyses of food intake are needed to verify the inadequacy of other micronutrients. Dietary adequacy is necessary because depression may be related to inadequate intake of other micronutrients (10, 59, 60) and the lack of important nutrients in a diet creates risk for many non-transmissible chronic diseases (49)[7]. Considering all the economic, social, and health implications resulting from the inadequacy of food intake and depression, such as a loss of productivity, an increase of sick leaves (2), and the disease burden itself (1), it is necessary to reflect on strategies of confrontation and prevention of this problem to improve workers' health.

The implementation of food education programs is essential to strengthen the health surveillance system and promote an adequate and healthy diet for farmers. Furthermore, it is essential to ensure access to mental health care services in primary care to support the prevention and treatment of depression in rural areas from the comprehensive viewpoint of the farmer, because few effective interventions exist that can reduce the vulnerability of rural workers (55). The main finding of the study was the height selenium intake was associated with reduction in the prevalence of depression in Brazilian farmers. The result contribute to the limited literature on mental health and selenium consumption in rural workers by showing evidence of an association between high selenium intake and reduced rates depression.

Final Consideration

Higher selenium intake was associated with a lower prevalence of depression in the rural Brazilian population. Actions to promote adequate nutrition are important to reduce the vulnerability of rural workers to depressive disorders.

To better understand the influence of selenium consumption on the development of depression, studies with a longitudinal design and the inclusion of biochemical measures for selenium measurement are necessary.

Strengths and Limitations

It is important to mention the limitations of this study. Its cross-sectional nature created limitations inherent to this type of epidemiological study. It made reverse causality possible, given that the association between the variables was synchronous. Therefore, it is not possible to infer causality between the variables evaluated. However, the diagnostic scale adopted investigated the symptomatology of depressive disorders in the last 2 weeks, which favored temporal analysis of the observations. Another possible limitation to consider is the information retrieval bias related to recent memory and the inaccurate estimation of ingested portion sizes, amounts, and frequencies in assessing the nutritional status of the participants (61). To minimize the effect of these biases and to ensure greater accuracy of the ingested portions, photographic albums were used to facilitate the identification and quantification of the consumed

[6]http://www.who.int/whr/2001/en/whr01_ch1_po.pdf

[7]https://biblioteca.ibge.gov.br/visualizacao/livros/liv50063.pdf

items, as well as—in the case of industrialized products—to register the brands, types, and quantities of the products. There was also the possibility of respondent bias, in which the individual tends to overestimate the consumption of healthy foods and underestimate that of unhealthy foods because of the stereotype of a healthy lifestyle based on agriculture and health diet based on natural foods (62). However, the occurrence of this bias was unlikely because the study population followed a contemporary dietary pattern, as already described.

Biochemical measurements of selenium were not used. The use of dietary data alone may limit the assessment of selenium intake. However, in an attempt to minimize this weakness, several precautions were adopted, including methodological rigor in obtaining information regarding farmers' food intake, to ensure that, in fact, it could reflect the actual consumption of the assessed population. The calculation of the average consumption was performed based on the information obtained after the application of three 24-h reminders (including weekdays and weekends), expanding the capacity of this instrument to detect variations in consumption between the days of the week. In addition, the average selenium intake value was obtained by calculating the attenuated and energy-adjusted average, as a way of reflecting the usual consumption of the evaluated sample. Photo albums were also used to facilitate the identification and quantification of the items consumed. In addition, data collection was performed by a fixed team of trained researchers in order to reduce the variability between observers. It is also noteworthy that the assessment of the nutritional composition of the foods identified in the 24-h recalls was carried out using a Brazilian food composition table (TACO) (23) as a way to more accurately reflect the content of the nutrient in the food. The selection of a large and representative sample, at random, also attributed greater robustness to the data obtained.

Despite these limitations, we highlight the unprecedented character of the study in the Brazilian literature in relation to the target population involved, the rigorous methodology of recruiting participants and assessing food consumption, the large sample size, the adoption of a validated diagnostic scale to obtain the results of depression and the inclusion, in statistical analysis, of other variables with potential influence on the development of depression.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee on research with human beings - CEP/UFES: n° 2091172 (CAAE - Certificate of Presentation of Ethical Appreciation - 52839116.3.0000.5060). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GP, LS, TF, and MC: conception/design of the work. EZ, GP, LS, MC, and OB: data acquisition. GP, KT, LS, MC, and TF: data analysis/interpretation. TF: drafting of the manuscript. EZ, GP, KT, MC, LS, TF, and OB: substantial revision of the manuscript. All authors: approval of the submitted version and taking of personal responsibility for any part of the work.

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MINI REVIEW
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Selenium Transport Mechanism via Selenoprotein P—Its Physiological Role and Related Diseases

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Selenoprotein P (SELENOP) is selenium (Se)-containing protein in plasma, which is primarily produced in the liver. The "P" in SELENOP originated from the presence in plasma. SELENOP contains selenocysteine, a cysteine analog containing Se instead of sulfur. SELENOP is a multi-functional protein to reduce phospholipid hydroperoxides and to deliver Se from the liver to other tissues, such as those of the brain and testis, playing a pivotal role in Se metabolism and antioxidative defense. Decrease in SELENOP causes various dysfunctions related to Se deficiency and oxidative stress, while excessive SELENOP causes insulin resistance. This review focuses on the Se transport system of SELENOP, particularly its molecular mechanism and physiological role in Se metabolism. Furthermore, the chemical form of Se and its biological meaning is discussed.

Keywords: low-density lipoprotein receptor-related protein, lysozome, selenoprotein synthesis, ApoER2, LRP1, megalin, selenium transport system

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INTRODUCTION

Selenoprotein P (encoded as SELENOP) was first described in 1973, and its character was reported as the major selenium (Se)-containing protein in plasma (1, 2). SELENOP is mainly synthesized in the liver and secreted to the plasma after cleavage of the signal peptide. The "P" in SELENOP denotes its presence in plasma. SELENOP contains the essential trace element Se in the form of selenocysteine (Sec), which is an analog of cysteine that contains Se instead of sulfur (2–4). Twenty-five genes encoding human Sec-containing proteins, i.e., selenoproteins, have been discovered, which play significant roles in several physiological processes such as antioxidant defense and metabolism; five types of glutathione (GSH) peroxidases (GPXs) play significant roles in the removal of several hydroperoxides, three types of thioredoxin reductases (TRXRs) in redox regulation, three types of iodothyronine deiodinases in the regulation of thyroid hormones, and selenophosphate synthetase 2 (SEPHS2) in Sec synthesis (4, 5). Most selenoproteins have a Sec residue, while SELENOP has 10, making it multifunctional (6, 7). SELENOP possesses two different functions: Se transport activity to supply Se to cells and GPX-like activity to reduce phospholipid hydroperoxide (8, 9). SELENOP maintains selenoenzymes in several tissues and plays a crucial role in antioxidative defense and Se metabolism (10, 11). A decrease in SELENOP causes deficiency in selenoproteins and various dysfunctions with oxidative stress, while excess SELENOP induces insulin resistance, which can lead to type 2 diabetes (12, 13). This review focuses on the Se transport system via SELENOP, particularly its molecular mechanism and physiological role in Se metabolism. Furthermore, the chemical form of Se and its biological meaning are discussed.

SELENOPROTEINS IN HUMAN PLASMA

In human plasma, there are two kinds of selenoproteins: SELENOP and extracellular GPX (GPX3), possessing Se as Sec residue (14). SELENOP is primarily secreted from the liver, while GPX3 is synthesized in the kidney. To estimate the Se content derived from each selenoprotein, SELENOP- and GPX3-deficient human plasma were prepared using immobilized specific antibody (9). The absorption of SELENOP resulted in the decrease in Se content to 47% of the total, while the removal of GPX3 decreased it to 81%, indicating that 53 and 19% of plasma Se is derived from SELENOP and GPX3, respectively (**Figure 1**). Similar results on the contributions of SELENOP and GPX3 to plasma Se have been reported by the laboratories of Burk and Schomburg (10), Olson et al. (15), and Brodin et al. (16). The residual 28% of Se might be derived from selenomethionine (SeMet) in albumin, and/or low molecular Se compounds, which have in part been identified as selenosugars (17).

Sec is encoded by the UGA codon, known as a stop (opal) codon, and is called the 21st amino acid in the genetic code (18, 19). In eukaryotes, the Sec insertion sequence (SECIS), which is a specific hairpin structure located in the 3′ untranslated region (3′UTR) of selenoprotein mRNA, is essential for the incorporation of Sec during the biosynthesis of selenoproteins. SECIS binds SECIS-binding protein 2 (SBP2) and forms a complex for Sec incorporation via the recruitment of the Sec-specific eukaryotic elongation factor (eEFsec) and Sec-tRNA^{Sec} (an anticodon complementary to the UGA codon) (20).

The mRNA of SELENOP has a unique property of containing 10 UGA codons in the open reading frame (ORF) and two SECIS in the 3′UTR, while other selenoprotein mRNAs have only one SECIS element (21) (**Figure 1A**). Multiple Sec residues in SELENOP are important for its function; one N-terminal Sec residue forms an active site of enzyme activity to reduce phospholipid hydroperoxide, while the nine C-terminal Sec residues function as Se transporter (22) (**Figure 1A**). The first SECIS element, which is located on the 5′ side near the stop codon, mainly facilitates the processive Sec incorporation, while the second SECIS functions slow decoding at the first UGA codon (23). Plasma kallikrein cleaves SELENOP by limited proteolysis with Arg-235–Gln-236 and Arg-242–Asp-243, generating N-terminal fragments (residues 1–235) with enzyme activity and C-terminal fragment (residues 243–361) exhibiting Se-supply activity (22). N-terminal, a possible catalytic center of SELENOP, has U(Sec)XXC motif, similar to the active-site of thioredoxin (CXXC motif), which suggests the reactivity of SELENOP against protein thiols. Actually, SELENOP has broad thiol specificity (24), and it uses not only GSH but also other thiols, such as TRX, dithiothreitol, and mercaptoethanol, as reducing agents, while the thiol specificity of cellular GPX (GPX1) is narrow, using only GSH as reductant. In human plasma, the concentration of GSH and TRX is ∼5 μM and 2 nM, respectively (25, 26). Based on kinetic analysis, SELENOP uses TRX 500-fold more effectively than GSH (24), but it is still uncertain whether both thiols could contribute to the reduction of SELENOP in plasma.

The mRNA of GPX3 has a UGA codon in the ORF and a SECIS in the 3′UTR, which is a representative feature of selenoproteins, such as the GPX family. GPX3 has catalytic triad composed of Sec, Gln, and Trp, in which the Se of a Sec residue is activated by hydrogen bonding to Trp and Gln residue (27). GPX3, like GPX1, is a homotetramer, while GPX4 (PHGPX) and SELENOP are monomers. GPX3 reduces diverse hydroperoxides, including hydrogen peroxide, tert-butyl hydroperoxide, and phospholipid hydroperoxide (24). GPX3 also possesses broad thiol specificity and uses TRX, dithiothreitol, and mercaptoethanol as reducing agents.

Se incorporated in Sec is specifically regulated via the synthesis of selenoproteins (28, 29). Sec is synthesized on tRNA using inorganic Se (**Figure 1B**). Seryl-tRNA synthetase (SerRS) binds Ser to tRNA^{Sec}, which has an anticodon of UGA, and the hydroxyl residue of Ser undergoes phosphorylation. SEPHS2 produces selenophosphate (H_2SePO_3) from inorganic Se and ATP. SEPHS2 is a selenoprotein, suggesting the self-regulation of the Sec synthesis system. The Sec synthase catalyzes the formation of a selenol residue on tRNA using H_2SePO_3 and phosphorylated Ser-tRNA^{Sec} (**Figure 1B**). Inorganic Se, such as sodium selenite, is recognized as "Se" in mammals and is reduced, phosphorylated, and incorporated into the synthesis pathway of Sec. Sec derived from the diet is also recognized as "Se" and is converted to inorganic Se by Sec lyase (30) (**Figure 1B**). In contrast, SeMet, a methionine analog that contains Se instead of sulfur, is recognized as "Met" in mammals and is incorporated into proteins in the same manner as Met (31). SeMet is also metabolized by Met-metabolizing enzymes. Se in SeMet is recognized as "Se" when it is converted to Sec via metabolism by cystathionine β-synthase and cystathionine γ-lyase, which are also known as Cys-persulfide-producing enzymes (**Figure 1B**). SeMet is considered "masked Se," and the contents of SeMet in the diet influence the concentration of Se in blood. As described above, ∼70% of "Se" is derived from Sec of SELENOP and GPX3, and the other 28% might be from SeMet and low molecular weight Se. Sec lyase is specific for Sec and provides "Se" to SEPHS2. Only through this pathway, Se is used for the synthesis of Sec on the tRNA^{Sec}.

SE TRANSPORT SYSTEM VIA SELENOPROTEIN P AND OTHERS

Serum free-culture of neurons and several cells requires Se addition, because SELENOP functions as a Se carrier in serum-containing culture (32, 33). SELENOP was identified as a survival-promoting factor for cultured neurons in serum-free medium in 1998, suggesting the Se transport system via SELENOP (32). The Se transport system of SELENOP has been described in *in vitro* experiments using human T lymphoma

Abbreviations: ApoER2, apolipoprotein E receptor 2; GPX, glutathione peroxidase; GSH, glutathione; LRP1, low-density lipoprotein receptor-related protein 1; PAH, pulmonary arterial hypertension; PASMC, pulmonary artery smooth muscle cell; PHGPX, phospholipid hydroperoxide GPX; SBP2, SECIS-binding protein 2; Se, selenium; Sec, selenocysteine; SECIS, Sec insertion sequence; SeMet, selenomethionine; SELENOP, selenoprotein P; SEPHS2, selenophosphate synthetase 2; TRX, thioredoxin; TRXR, thioredoxin reductase; 3′UTR, 3′ untranslated region.

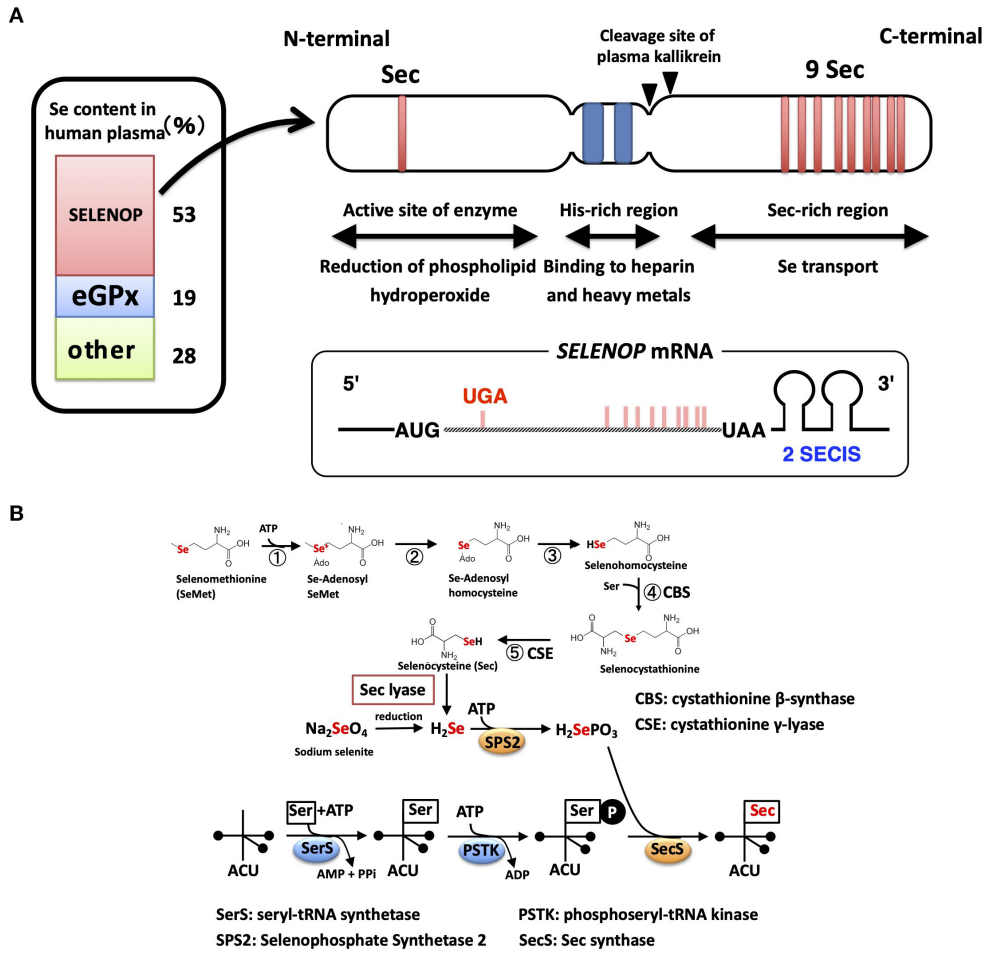

FIGURE 1 | Structure of the SELENOP and selenocysteine synthesis pathway. **(A)** Domain structure of human SELENOP. The Se content estimated from SELENOP- and eGPX-deficient human plasma is shown in the left panel. **(B)** Selenocysteine synthesis pathway. SeMet is converted to Sec with Met-metabolizing enzymes without discrimination with Met. Inorganic Se is produced with Sec lyase and used for the synthesis of Sec.

Jurkat cells and selenoprotein-deficient human serum prepared by the immobilized antibody for SELENOP and GPX3 (9). When cultured with SELENOP-depleted serum, and not GPX3-depleted or the control serum, the activity of GPX1 decreased to 17% compared with that of the control (**Figure 2A**). The activity of two other selenoproteins, GPX4 and TRXR, also decreased to 16 and 38%, respectively (9). When cultured with SELENOP-depleted serum, the whole-cell Se content also decreased to 19% compared with that of control cells. Time-dependent analysis revealed that the GPX1 activity of cells cultured with SELENOP-depleted serum was almost undetectable after 4 days (**Figure 2B**). The addition of 270 ng/ml purified SELENOP (SELENOP concentration of 5% human serum) resulted in the complete recovery of GPX1 activity. Thus, SELENOP functions as a major Se transporter in this culture system.

The comparison of SELENOP with other Se-containing materials as a Se supplier demonstrated that SELENOP was the most effective with a 50% effective dose (ED_{50}) of 5 nM (Se equivalent), followed by GPX3, sodium selenite, selenocystine, SeMet, and albumin (9) (**Figure 2C**). The ED_{50} of the former three reagents was 25 nM, and that of the latter two was 300 and 500 nM, respectively. Ebselen had no effect up to 500 nM. These results suggest the effective Se transport system via SELENOP (9). Next to SELENOP, the inorganic Se and Sec group, which is recognized as "Se," is also a good source of Se. Third group SeMet and albumin, which is classified as "masked Se," is not effective, but it could function as a Se source in high concentration. The Se concentrations of human plasma used in the previous study was 1.5 μM, and the estimated Se concentration of SELENOP, GPX3, and the others were 750, 300, and 450 nM, respectively. Thus, it is considered that the effective Se transport system of SELENOP might be less functional in cells that have direct contact with plasma, and that it will be effective in the interstitial fluid where the plasma is diluted. It needs further characterization in the SELENOP concentration of the interstitial fluid. The biological significance of the effective Se transport system via SELENOP has been demonstrated by SELENOP KO mice (35, 36). The decrease in tissue Se in SELENOP KO mice has been reported in

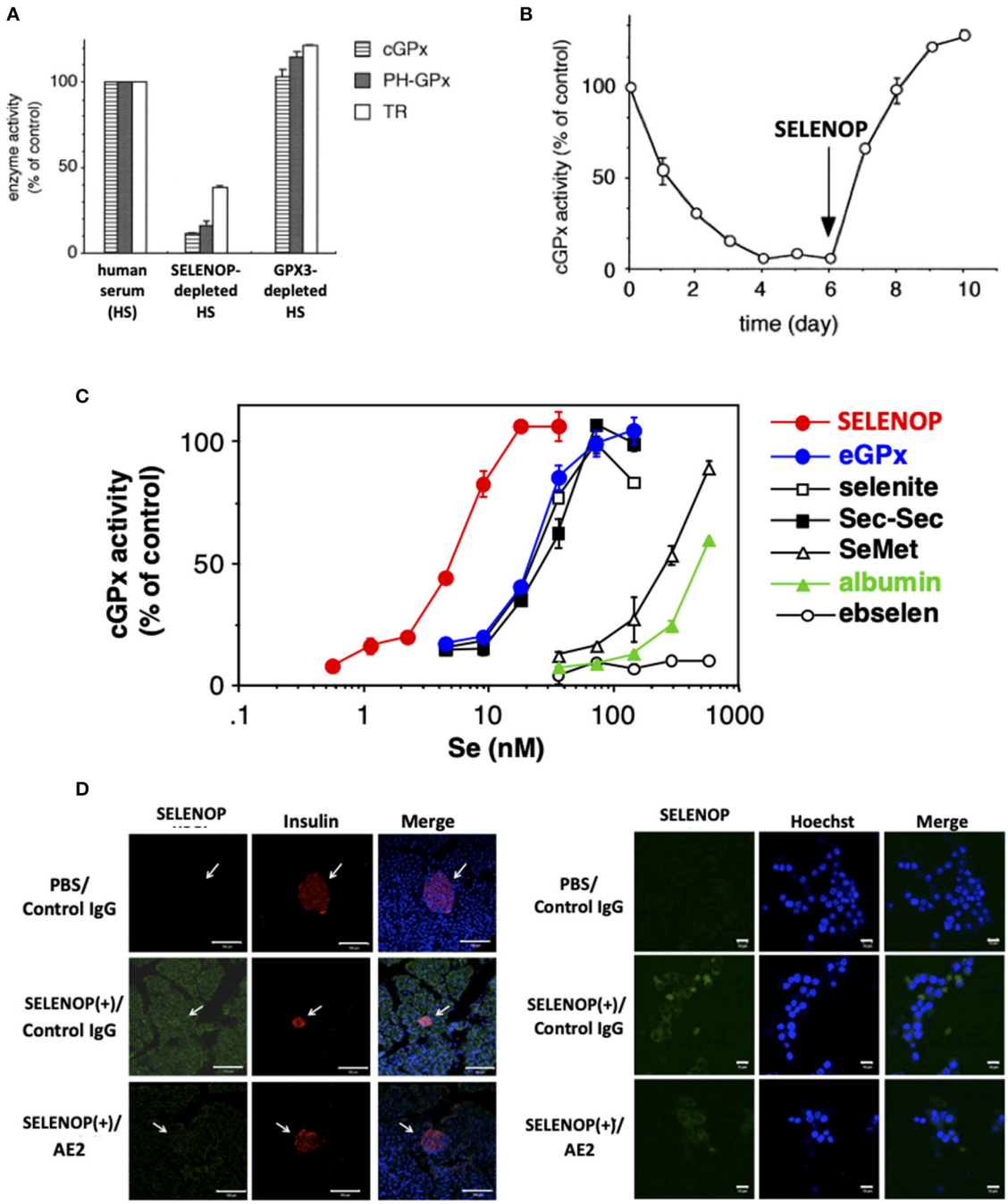

FIGURE 2 | Se transport activity of SELENOP. **(A)** Effect of serum selenoprotein depletion on cellular selenoenzyme activities. The Jurkat cells were cultured for 3 days in a medium containing each human serum. **(B)** Effect of SELENOP on cGPX activity. The Jurkat cells were cultured in a medium containing SELENOP-depleted human serum, and cGPX activity was measured. After 6 days, purified SELENOP was added. **(C)** Effect of the addition of Se-containing materials on cGPX activity. In the presence of variable amounts of Se-containing materials, the Jurkat cells were cultured with SELENOP-depleted serum. **(D)** Immunohistochemical analysis of incorporated human SELENOP. Left panel. SELENOP-neutralizing monoclonal antibody (mAb) AE2 improved pancreatic β-cell area. Pancreas tissues from neutralizing mAbAE2- and human SELENOP-treated mice were examined immunohistochemically using anti-insulin Ab (indicative of β-cells) and anti-human SELENOP Ab. Cell nuclei were stained with DAPI (blue). The distribution of human SELENOP in pancreatic β-cells was decreased in mice administered with AE2. Right panel. MIN6 cells were incubated with 10 μg/ml human SELENOP in the presence of AE2 and control IgG (500 μg/ml) for 48 h. Treated MIN6 cells were examined immunohistochemically using anti-human SELENOP Ab. [**A–C**, (9) with permission and modifications: 5051220115395, D, reference (34) with permission:CC-BY].

the brain, kidney, testis, and bone (35–38). The severe phenotype of SELENOP is spermatogenesis disorder, which could not be recovered by Se supplementation, while the other disorders might be partly recovered by Se supplementation, suggesting the compensatory role of the Se transport system other than SELENOP. It is also notable that SELENOP is contained in mothers' milk and has the function to supply Se to offspring (37). The effective Se transport system of SELENOP is mediated by its receptors, such as apolipoprotein E receptor 2 (ApoER2), megalin, and low-density lipoprotein receptor-related protein 1 (LRP1), which belong to lipoprotein receptors. In addition, Se transport of SELENOP has been observed in a receptor-independent manner (pinocytosis) (39).

THE RECEPTORS FOR SELENOPROTEIN P

Three kinds of SELENOP receptors have been identified, namely, ApoER2 (LRP8), megalin (LRP2), and LRP1. ApoER2 and megalin were first discovered by immobilized SELENOP affinity column chromatography (40, 41), while LRP1 was identified by the siRNA experiment on C2C12 myocytes based on the expression of LRPs (42). SELENOP transports Se to several tissues via these receptors. Based on the phenotype of each receptor KO mice, ApoER2 is associated with SELENOP uptake in the brain, testis, and bone; megalin with the kidney and brain; and LRP1 with the skeletal muscle. It has been reported that megalin mediates brain Se uptake and that Apoer2 mediates neuronal SELENOP uptake (43).

Similar phenotypes of SELENOP KO mice have been reported in the brain and testis of ApoER2 KO mice, indicating the biological significance of receptor-mediated uptake of Se in these tissues (35–37, 44). Similar phenotypes in the brain and testis have been reported in SELENOP$^{\Delta 240-361}$ mice, in which the Sec-rich C-terminal domain of SELENOP had been deleted (34). The interaction between the C-terminal domain of SELENOP and the YWTD β-propeller domain of ApoER2 has been reported, and the importance of this interaction, particularly in maintaining Se levels in the brain and testis, has been manifested in the phenotypes of these KO mice (45). In the normal diet that contains enough Se (0.4 mg Se/kg diet), Se levels in other tissues such as the intestine and lung of SELENOP KO mice, except for the brain, testis, and bone, do not differ greatly from those in WT mice. Selenite in mouse chow is not a normal constituent of diet, but can be imported directly or after metabolism into SELENOP- and ApoER2-deficient cells. In the case of the brain of SELENOP KO mice, Se content was greatly affected by diet; 0.1 mg Se/kg diet induced a significant decrease in brain Se (∼50% of WT) and caused severe motor dysfunction, which needed humane endpoint (44). These observations suggest the role of the Se transport system via not only SELENOP but also SELENOP-independent systems *in vivo*. These observations indicate the role of SELENOP in the Se transport system, which is particularly effective to maintain homeostasis under the Se-deficient condition. This system might be significant to survive in the evolutionary process.

Megalin plays a crucial role in the reabsorption of SELENOP in the kidney, and increase in urine SELENOP has been shown in megalin KO mice (40, 43). Megalin is a large glycoprotein (∼600 kDa), which possesses four large clusters of ligand-binding repeats stabilized with a disulfide bond. In the kidney, proximal tubule epithelial cells highly express megalin, indicating the physiological role of megalin in the reabsorption of SELENOP. LRP1 is identified as SELENOP receptor in the skeletal muscle (42). LRP1 is a super-macromolecule with a size of about 600 kDa and has a diverse set of ligands, such as amyloid β. Skeletal muscle has relatively low affinity for SELENOP, and the Se content does not change in SELENOP KO mice. However, the increase in SELENOP in the case of type 2 diabetes enhances the uptake of SELENOP via LRP1, which is related to the increase in insulin and exercise resistance (42). Thus, the SELENOP–LRP1 axis is an important therapeutic target for the cure of type 2 diabetes. Human embryo rhabdomyosarcoma RD cells express both ApoER2 and LRP1, which show low affinity for SELENOP uptake, and high amount of SELENOP is necessary for the uptake and use of Se in SELENOP (46). Treatment of RD cells with siRNA for either ApoER2 or LRP1 resulted in significant decrease in SELENOP uptake and increase in GPX1, suggesting both receptors coordinately work for SELENOP uptake. Diverse variants of ApoER2 have been known, while the relationship between ApoER2 variants and SELENOP uptake is unknown.

SE TRANSPORT SYSTEM VIA SELENOPROTEIN P

SELENOP possesses Se as Sec, which is covalently bonded to the polypeptide chain. To use the Se in SELENOP for selenoprotein synthesis, several biochemical steps are necessary. Based on previous reports, it has considered that SELENOP is incorporated into the cell, and then is degraded to amino acids in the lysosome (45). It is notable that the life span of incorporated SELENOP is long, which makes it possible to observe incorporated cellular SELENOP by *in vivo* and *in vitro* immunostaining (46) (**Figure 2D**). SELENOP is a glycoprotein, and the carbohydrate chains in SELENOP might function to prevent proteolysis in lysosomes. The generated Sec from SELENOP is cleaved with Sec lyase, converting to hydrogen selenide H_2Se, which is further phosphorylated to H_2SePO_3 with SEPHS2, as described above. The toxicity level of H_2Se is high, and the phosphorylation of H_2Se is important to prevent its toxicity. Notably, the necessity for Sec lyase in Se transport of SELENOP is not clear, because mice lacking Sec lyase do not have selenium deficient phenotypes, such as male sterility (47).

SELENOP is synthesized in peripheral tissues and SELENOP-expressing cells are present in several tissues (48). Based on serum SELENOP concentrations of liver-specific SELENOP KO mice, 60% of SELENOP is estimated to be derived from the liver and the remaining 40% from other tissues (49). SELENOP expression in the brain is significant for maintaining Se and selenoprotein levels, and Se concentrations in the brain are preserved in liver-specific SELENOP KO mice. SELENOP expression has been reported in several cell types of the brain, such as neurons

and ependymal cells, that are responsible for cerebrospinal fluid production (50). SELENOP synthesized in the brain is incorporated into other brain cells and used to synthesize selenoproteins, which help maintain Se concentrations in the brain. This system is called the SELENOP cycle, and it retains selenoproteins in several tissues and cells (51).

ApoER2 is a significant mediator of the SELENOP cycle. The similar phenotype between SELENOP and ApoER2 KO mice implies the role of ApoER2 in this cycle. Details are not fully elucidated, but the role of ApoER2 as a mediator of signal transduction has been known, which is realized by pulmonary arterial hypertension (PAH) where the increased expression of SELENOP in pulmonary artery smooth muscle cells (PASMCs) forms lesions (52). PAH-PASMCs are proliferative, and the pulmonary artery is constricted/occluded by the abnormal proliferation of PAH-PASMCs. The decrease in SELENOP expression by SELENOP-siRNA treatment inhibits the proliferation of PAH-PASMCs, and these effects are mediated by ApoER2 (52). Interestingly, the proliferative effects of SELENOP were not explained by Se transport activity; namely, the addition of selenocystine did not reproduce this effect of SELENOP, and proliferation-promoting effects were observed by the overexpression of the mutant in which all Secs were substituted with Cys. The proliferative effects of increased SELENOP on PAH-PASMCs are considered to be mediated by the cell signal from ApoER2 and HIF-1. Se-independent biological effects of SELENOP-ApoER2 axis have been described in a study on PAH, and it is interesting to speculate about the possibilities to relate to other physiological and/or pathological conditions.

CONCLUSION

This review focuses on the Se transport system via SELENOP, particularly its molecular mechanism and role in Se metabolism. SELENOP is not a mere Se transporter. It plays the role of multifunctional protein to maintain cellular selenoproteins and regulate cellular redox homeostasis. Furthermore, the Se-independent role of SELENOP suggests the diverse biological and pathophysiological significance of this protein. Further research is necessary to understand the various roles of SELENOP.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Dietary Selenium Intake and Type-2 Diabetes: A Cross-Sectional Population-Based Study on CUME Project

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Background and Aim: Previous studies have suggested that the specific association between selenium (Se) and diabetes remains unclear. This study aimed to investigate the association between dietary Se and type-2 diabetes (T2D) in the Brazilian cohort [Cohort of Universities of Minas Gerais (CUME)].

Methods and Results: This cross-sectional study was conducted with a large sample comprising 4,106 participants of the CUME project, a concurrent open cohort restricted to a highly educated population group, composed of graduates of federal institutions of higher education located in the State of Minas Gerais, Brazil. Data on socioeconomic and dietary characteristics, as well as anthropometric measures, were collected from each subject for analysis. The sample was classified into energy-adjusted tertiles of dietary Se intake (μg/day). Differences in the continuous data were evaluated by the Kruskal–Wallis *H*-test (abnormal data), and the χ^2-test assessed differences in qualitative data. As there was no relationship between T2D and Se intake in the bivariate analysis, multivariate analysis was not performed. The prevalence of T2D in the studied population was 2.8%. The mean age was 36 years. Regarding gender, 1,209 are males and 2,807 are females. Among females, the mean Se intake was 165.12 μg/day and the mean intake was 157.4 μg/day. Among males, it was 168.4 μg/day. Significant differences were observed across all Se intake tertiles in terms of age, gender, activity level, alcohol intake, energy intake, sugar, carbohydrates, lipids, fiber, and energy-adjusted meat intake. However, no significant differences were observed across all Se intake tertiles in terms of BMI, smoking status, and T2D. The results indicated that there was no significant association between dietary Se intake and the prevalence of T2D.

Conclusion: Dietary Se intake was not associated with the prevalence of T2D, despite the high intake of this micronutrient in the sample. These results contradict studies that identified the association between Se intake and T2D, with values of Se intake much lower than those observed in this study. Thus, this relationship seems to remain controversial.

Keywords: type 2 diabetes, selenium, food intake, cross-sectional study, adults

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INTRODUCTION

Diabetes is a metabolic disorder that results from an insulin-production deficiency or its action, having hyperglycemia as one of its main signs (1). According to the International Diabetes Federation (IDF), in 2019, about 463 million adults aged between 20 and 79 years were diagnosed with this disease in the world, of which 15.5 million cases occurred in Brazil, which is the 5th country in the world regarding the number of diabetes cases in this age group (2).

Of the diabetes cases, about 90% corresponds to type-2 diabetes (T2D) mellitus (2), which is a multicausal disease mainly related to genetic factors and lifestyle, such as being overweight or obese; sedentary lifestyle; diets low in whole grains, fruits, nuts, and seeds; and diets rich in red meat, sugary drinks, and processed meat (3). Hyperglycemia, a characteristic of diabetes, is associated with acute and chronic complications of this disease, negatively affects the quality of life, and has a higher mortality rate in people with diabetes (1). Furthermore, hyperglycemia has been reported to cause an increase in reactive oxygen species production, causing oxidative stress and impacting the progression of T2D (4).

Antioxidant nutrients play an important role in the defense of our body by reducing the oxidative stress and preventing the emergence of chronic diseases, mainly by neutralizing free radicals and their metabolic effects. Vitamins A, C, and E and minerals, such as zinc, selenium (Se), copper, and manganese, which are present in our diet, are among the nutrients related to antioxidant status (5).

Selenium, one of the essential nutrients for humans and animals with the greatest antioxidant potential, is both organic and inorganic in nature (6). Its inorganic form, selenite salts and selenate, mainly accumulates in plants *via* the sulfur assimilation pathway. Plants absorb these salts from soil and convert them into the organic form, selenomethionine and selenocysteine, which may be incorporated into proteins, originating from selenoproteins (6). Animals and humans cannot synthesize these components and must ingest them as part of their diet (7).

The concentration of Se in plants is directly related to plant species, concentration in soil, type of soil, accumulation capacity, pH, salinity, organic matter, and redox reactions (7). Thus, Se dietary intake varies among countries and regions, and the type of food consumed. In plant tissues, its concentration depends on the geographic area and its level and availability in the soil (7). In animal tissues, it depends on the amount ingested (6).

Foods rich in selenium are meat, cereals, grains, and dairy products. Meat is the main source consumed, since skeletal muscle is the major site of selenium storage, accounting for ∼28–46% of the total selenium pool (8).

Although animal-based foods are the best source of selenium, the Brazil nut (*Berthlletia excelsa*), a plant-based food, stands out as an exception, as it is the richest known source of selenium. The Brazil nut, which is part of the oilseed group, together with walnuts, peanuts, and cashew nuts, has a higher concentration of selenium. However, the concentration of selenium in Brazil nut varies considerably, depending on the geographic region of cultivation and the ability of the plant to absorb the mineral. Cardoso et al. (9) cite concentrations of selenium in Brazil nuts grown in São Paulo, Maranhão, Pará, and Amazonas ranging from 5 to 71.5 μg/g, while Silva Junior et al. (10) identified values ranging from 0.5 to 98 μg/g in Brazil nuts from Acre, Amapá, Amazonas, and Mato Grosso, which are states located in the north, southeast, and midwest regions of Brazil. This wide range among the levels of Se in Brazil nuts is explained by the geographic region where they were grown, and the capacity of each plant to absorb the mineral.

Silva Junior et al. (10) identified that the consumption of Brazil nuts grown in some Brazilian states (Amazonas and Amapá) exceeds the recommended intake value. In contrast, in other states (Acre and Mato Grosso), it does not even offer 10% of the selenium value recommended for consumption. These results demonstrate that it is difficult to define a single number of Brazil nuts to be consumed daily to achieve daily selenium consumption recommendations.

The selenoprotein family consists of 25 eukaryotic genes, with 25 human genes. All of these proteins have selenocysteine residue in their primary predefined structure (11). These selenoproteins are responsible for the function and regulation of thyroid hormones (4, 12), glucose metabolism (12), male fertility improvement (6, 12), and anti-inflammatory actions (6). They also indirectly participate in the mechanism of wound healing as oxidative stress reducers through glutathione peroxidase (GPX). GPX is the major selenoprotein present in the human body, and it assists in the control of excessive production of free radicals at the site of inflammation (6).

Recent research has focused on the relationship between selenium (Se) levels and glucose metabolism. Some observational studies have identified an association between high Se intake and a higher risk for T2D (4, 13, 14). Two meta-analyses (15, 16) have also reported this positive relationship. According to Ogawa-Wong et al. (17), the relationship between Se and T2D is a U-shaped curve, i.e., the onset of T2D occurs with insufficient or very high levels of Se. This finding shows the need to control Se intake so that it is neither scarce nor excessive.

Despite these findings, in the meta-analysis by Kohler et al. (16), the authors have reported that this relationship was found in observational studies and not in clinical trials. Thus, it is still not clear whether these differences are the result of uncontrolled misperception in observational studies or if there is a modest effect of Se and risk of T2D that may vary according to the length of exposure.

However, Vincet et al. (15) have reported that in non-experimental studies, a direct relationship between exposure to Se and risk of diabetes was identified, with a clear and almost linear trend in individuals with plasma or serum Se levels of above 140 μg/L when compared with the reference category of exposure to Se, which is 45 μg/L. A dose–response meta-analysis that focused on studies with direct assessment of dietary Se intake showed a similar trend. In experimental studies, it was shown that Se supplementation increased the risk of diabetes by 11% compared with participants allocated with placebo, regardless of gender.

In this sense, epidemiological studies on Se intake by the Brazilian population have identified that the Se daily intake has been above the recommended daily intake (RDI). The Se RDI for

adults is 55 μg/day with a tolerable upper intake level (UL) of 400 μg/day (18). The Se intake values in a cohort of Brazilian adults, the ELSA study, identified a mean intake of 222 μg/day, being higher among women (19). The National Food Survey of the Family Budget Survey (FBS) (2008–2009) identified a mean intake value of 107.61 μg/day in the population, being higher among male adults (20).

However, research with smaller samples and residents of a single municipality, such as São Paulo, Teresina, and Manaus (21–23), has had different results. In these studies, among other parameters, the mean value of selenium intake and its biochemical parameters were evaluated, and the mean intakes were 41, 61, and 72 μg/day and erythrocyte values were 56.7 and 211 μg/L. Intake values were closer to those of the recommended. Regarding erythrocytes, in the first two studies, they were below the reference values (90–190 μg/L) (24), therefore showing that not all of the selenium ingested is absorbed. According to Soares (23), the mean selenium intake in the Brazilian population is very diverse (30 to 200 μg/day). It depends on the amount of Se in the soil, with foods from the north and northeast regions being richer in Se when compared to other Brazilian regions. However, we have not seen studies that evaluated the association of Se with diabetes in Brazilian populations.

Thus, this study aimed to investigate the association between dietary Se and T2D in a Brazilian cohort (CUME).

SUBJECTS AND METHODS

Design and Study Population

This is a cross-sectional study with participants from the CUME project of two collection waves (March and August 2016, and between March and July 2018) in a virtual environment. In these two collection periods, 4,987 graduates answered the online survey questionnaire.

The CUME project is an open cohort with graduates from federal institutions of higher education in Minas Gerais, Brazil. The objective of the cohort is to assess the impact of specific food groups, nutrients, and dietary factors, and the nutritional transition on non-communicable diseases (NCD). The design, dissemination strategies employed, and project baseline profile have already been detailed in a previous publication (25). The CUME project was approved by the Human Research Ethics Committees of *Universidade Federal de Viçosa* (UFV) and *Universidade Federal de Minas Gerais* (UFMG) (Protocol No. 596,741-0/2013).

In this study, we did not include participants with incomplete questionnaires regarding demographic data ($n = 531$), from other nationalities ($n = 22$), Brazilians living abroad ($n = 173$), and likely type 1 diabetes: she/he was not diagnosed with diabetes in adulthood and uses insulin ($n = 13$). Participants with energy consumption below 500 kcal ($n = 2$) and above 6,000 kcal ($n = 128$) (26), meat intake >600 g/day ($n = 184$), nuts intake >100 g/day ($n = 91$) were also excluded from the study. Thus, the study sample consisted of 4,016 adults who graduated from the referred institutions and answered the 2016 and 2018 baseline CUME questionnaires.

The Study Protocol and Data Collection

Invitations to participate in the research were sent by email to all graduates (graduates and postgraduates) from UFMG and UFV trained in the periods mentioned previously. The email addresses used were those in the Alumni Associations (UFV) databases and the Universities Technology and Information Directorates (UFMG).

For data collection, we used the self-administered baseline online questionnaire (Q_0), which was divided into two parts (accessed at http://www.projetocume.com/questionario). The first part consisted of questions related to sociodemographic and economic characteristics, lifestyle, individual and family referred morbidity, medication use, personal history of clinical and biochemical exams from the last 2 years, and anthropometric data. The second part of the questionnaire was sent a week after completion of the first part and had a quantitative Food Frequency Questionnaire (FFQ) composed of 144 food items, based on an original version previously validated in Brazil (27). As we did some modifications in the original FFQ, a validation study was also developed with a subsample of 146 CUME participants. The results showed a moderate agreement between the self-reported data and those directly measured from 24-h food recalls by telephone (overall intraclass correlation coefficient = 0.44, unpublished results).

Evaluation of Variates

The outcome variable used for the analyses in this article was T2D, based on self-reported data from having a confirmed T2D diagnosis in adulthood and/or having blood glucose above 126 mg/dl in the previous year (1) and/or using oral antidiabetic and/or using insulin.

The exposure variable "selenium (Se) intake" was based on self-reported data on food intake; and subsequently, Se intake (μg/day) was calculated. Daily Se intake was adjusted by caloric intake using the residual method (28) and analyzed according to intake tertile.

Sociodemographic, lifestyle, and food intake were used as adjustment variables. Sociodemographic variables were gender (male, female) and age (years, continuous). Regarding lifestyle variables, smoking status (never smoked, former smoker, and current smoker), alcoholic beverage intake (never or does not consume, consumes), and physical activity (performs or does not perform scheduled physical activities) were assessed.

Food intake data were obtained from a quantitative FFQ. To minimize errors in the data collection process, images of food items and utensils were made available to facilitate the estimation of portion size and filling in of the report, and to obtain a more reliable response regarding the intake of the participants (29). There was a list of items that constituted the food group at the beginning of each page of the questionnaire. The participants were instructed to select the foods consumed in the previous year. For each food chosen, the participants indicated the portion size expressed in homemade measures commonly used in Brazil (teaspoon, tablespoon, ladle, knife tip, tongs, saucer, cup, and glass) or in traditional portions (unit, slices, and pieces) and the usual frequency of intake (day/week/month/year).

Consumption frequency of each food was transformed into daily consumption. Subsequently, daily consumption (grams or milliliters) was calculated by multiplying the portion size by the frequency of consumption. For the calculation of caloric intake (kcal) and nutrients, Brazilian tables of the nutritional composition of foods were used (30) and, if necessary, the table of the United States Department of Agriculture was also used (31).

For food consumption, the following were evaluated: energy intake, protein, meat, total lipids, animal fats, the relationship between saturated and polyunsaturated fatty acids, carbohydrates, sugars, fibers, and alcoholic drinks. For sugars, intake was quantified in grams of table sugar, brown sugar, honey, sweet treats, and soft drinks. Dietary variables were studied as a continuous variable and adjusted for energy using the residual method (28).

Analysis of Results and Statistics

Sample characteristics are expressed as absolute and relative frequencies, or median and interquartile interval (percentile 25 to percentile 75), according to the medical diagnosis of T2D and sociodemographic, anthropometric variables, lifestyle, and food intake. Differences between continuous variables and categorized according to the presence or absence of T2D were assessed by the Mann–Whitney (abnormal data) test, and the χ^2-test was performed to evaluate differences in qualitative data. Analysis of the prevalence of T2D according to the tertile of Se intake adjusted for energy and adjustment variables was carried out using the Kruskal–Wallis H-test (non-parametric data) χ^2-test for categorized data. Hierarchical cluster analysis was carried out to group the Se intake using the centroid method to calculate Euclidean distances. All analyses were conducted using the SPSS statistical software, version 18.0, considering a significance level of 5%.

RESULTS

Of the total participants, 2.8% ($n = 112$) had T2D at the cohort baseline. The median energy-adjusted Se intake was 143.5 μg/day, and it did not differ with the intake of those with or without diabetes. The median, not energy-adjusted Se intake was 137 μg/day, and the average consumption was higher among men (148.9 μg/day) than among women (131.4 μg/day).

The food groups consumed by the participants that most contributed to selenium intake were meat and meat products (58.2%), starch (26.2%), dairy (8.2%), and oilseed, presented in the FFQ as Brazil nuts, walnuts, peanuts, and cashew nuts (7.5%). Age, gender, BMI, smoking cigarettes, and sugar intake differed statistically ($p < 0.05$) between the participants with and without T2D (**Table 1**).

The characteristics of the participants according to daily energy-adjusted Se intake, as well as sociodemographic, lifestyle, and dietary characteristics are shown in **Table 2**. Age, gender, physical activity, alcoholic beverage intake, and all the dietary variables studied differed according to tertiles of Se intake ($p < 0.05$). On the other hand, selenium intake was not associated with the presence of diabetes.

The distribution of the participants according to energy-adjusted Se intake and T2D prevalence is shown in **Figure 1**. Cluster analysis identified two distinct profiles of Se intake among the studied graduates, one with median intake of 139.8 μg/day and another with median intake of 341.3 μg/day ($p < 0.05$), but no significant association with T2D prevalence ($p < 0.05$) was seen.

Figure 2 shows that both people without and with T2D have an average energy-adjusted Se intake at three very similar levels, which makes it very difficult to identify the relationship between Se intake and T2D among the participants in this study.

DISCUSSION

We conducted a cross-sectional study on a sample of 4,016 young adults from a cohort of public University graduates in Brazil, with the primary objective of investigating the association between dietary Se and T2D. The prevalence of T2D in the studied population was 2.8%, which is close to that estimated in a study with a representative sample of Brazilian adults in 2019 for people with over 12 years of schooling (3.5%) (32).

Although the study participants were still young, T2D was seen to be more prevalent among the elderly, confirming that the prevalence of T2D increases with advancing age. However, it is noteworthy that the effect of nutrients on health outcomes may require a long exposure time. Cohort studies are recommended for this evaluation. Thus, although the CUME project is a cohort study, this is a cross-sectional study. In the future, we will verify that a long time of exposure to high consumption of selenium may favor T2D. Furthermore, this study did not evaluate blood selenium biomarkers (such as plasma selenium and erythrocytes), which provide more reliable information on selenium intake and the body reserve of this mineral (33).

It is also noteworthy that not all selenium ingested is absorbed. The Se absorption mechanism differs between consumed sources, and this difference determines its absorption rate. Most selenite is absorbed in the duodenum by passive diffusion, while selenate is actively absorbed in the ileum by cotransport with sodium ions. Selenium from selenomethionine is also absorbed in the small intestine, with the highest absorption rate in the duodenum. Its absorption occurs through the sodium-dependent system and has the same mechanism as the methionine, as well as selenocysteine, which competes with cysteine, lysine, and arginine (34). Furthermore, the organism tries to maintain its homeostasis and generally has higher absorption when its reserves are reduced, and less absorption when inadequate or excessive (35).

The profile of the participants in this study was previously described by Gomes-Domingos et al. (25). It is a group made up predominantly of graduates from undergraduate and graduate courses at public universities in the state of Minas Gerais and residents of all Brazilian states and the federal district. Of the total participants, 72.9% has a postgraduate degree, are young

TABLE 1 | Characteristics of the study population according to type-2 diabetes status ($n = 4,016$).

Characteristics	With T2D	Without T2D	*p*-values
N	112	3,904	
Age (years)*	42 (34; 53)[£]	34 (29; 40)	**<0.001**
Females (%)	64 (57.1)	2,743 (70.3)	**0.003**
Males (%)	48 (42.9)	1,161 (29.7)	
BMI (Kg/m^2)	29 (26–34)[≠]	24 (21–27)	**<0.001**
Performs physical activity regularly (%)			
No	36 (32.1)	965 (24.7)	0.073
Yes	76 (67.9)	2,939 (75.3)	
Smokes cigarettes (%)			
Never smoked	80 (71.4)	3,128 (80.1)	**0.024**
Smoker or former smoker	32 (28.6)	776 (19.9)	
Drinks alcoholic beverages (%)			
No	32 (28.6)	1,072 (27.4)	0.795
Yes	80 (71.4)	2,832 (72.6)	
Energy intake (Kcal/day)	2,342 (1,690; 3,206) [£]	2,168 (1,691; 2,706)	0.052
Energy-adjusted proteins (g/day)	101 (89; 117)[£]	98 (86; 113)	0.097
Energy-adjusted meat (g/day)	207 (156; 287)[£]	194 (144; 258)	0.076
Total energy-adjusted lipids (g/day)	89 (75; 98)[£]	85 (75; 95)	0.176
Animal fat adjusted for energy (g/day)	32 (35; 37)[£]	30 (25; 35)	0.139
SFA/PUFA** adjusted by energy	0.6 (0.5; 0.8)[£]	0.6 (0.5; 0.7)	0.337
Energy-adjusted carbohydrates (g/day)	260 (233; 299)[£]	273 (243; 303)	0.052
Energy-adjusted sugars (g/day)	6 (−2.2; 12.2)[£]	11 (5.5; 19.7)	**<0.001**
Energy-adjusted fibers (g/day)	29 (24; 35)[£]	29 (24; 34)	0.512
Energy-adjusted alcoholic beverage (g/day)	2.8 (0.8; 8.9)[£]	3.1 (1.0; 7.8)	0.959
Energy-adjusted selenium (μg/day)*	142 (121; 184)[£]	145 (121; 183)	0.661

**Median. **SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; [£]25 and 75 percentile. [≠]Minimum and maximum. The difference between continuous variables was assessed using the Mann–Whitney test; the χ^2-test evaluated differences in the categorized data. Bold values mean they are statistically significant.*

adults with a mean age between 30 and 39 years (46.4%), and live on an individual income of up to five minimum wages (49.5%).

The characteristics of the participants according to T2D status are similar to the studies conducted by Wei et al. (14) and Stranger et al. (13) regarding difference in age and BMI of the participants, according to T2D status ($p < 0.05$). However, these studies (13, 14) identified differences in selenium and alcohol intake, consumption of energy, fibers, and animal protein (14) according to T2D status, a fact that was not observed in this study. Nevertheless, it is noteworthy that different from this research, the nutrient intake in these studies (13, 14) was not adjusted for energy consumption.

The results of this research did not identify a positive and significant association between energy-adjusted Se intake and the prevalence of T2D. Thus, our findings corroborate other studies that did not identify an association between dietary Se and T2D (36). Thompson et al. (36) found no significant difference between the appearance of T2D between the control group and the placebo from Se supplementation of 200 μg/day. As mentioned earlier, according to Ogawa-Wong et al. (17), the relationship between Se and diabetes is a U-shaped curve. That is, the onset of the disease occurs in situations of very low or very high levels of Se. In this study, the Se intake of the participants was within the acceptable range. It was above the RDI but below the tolerance limits for this mineral.

The relatively young age of the studied participants and the method used to assess selenium intake could justify the absence of an association between selenium intake and T2D in this research. However, some studies (11, 12, 35, 36) that identified a positive association between selenium intake and T2D were also carried out with young participants (30 to 52 years old), that used a frequency of consumption questionnaire to estimate intake selenium. And unlike the present study, which presented a consumption of selenium adjusted for energy well above the recommendation value (143.5 μg/day), selenium intake in these other studies was much closer to the recommendation value (11, 12, 35), or above (36), but much lower than that observed in our study. Therefore, indicates the need for further research to confirm the association between the consumption of selenium and type 2 diabetes.

According to Parekh et al. (37), an intake of Se between 50 and 400 μg/day is considered a safe range for adults, while 850–900 μg/day may reach a toxicity level. We carried out this analysis to verify whether the graduates who consumed below or above the values considered safe showed higher prevalence of T2D, and no significant association was observed. Among those with T2D,

TABLE 2 | Characteristics of the study population according to dietary selenium intake ($n = 4,016$).

Characteristics	Selenium intake tertile			
	1st tertile (≤ 129.2 μg/day)	**2nd tertile** (> 129.2 <164.2 μg/day)	**3rd tertile** (≥ 164.2 μg/day)	***p*-value**
Energy-adjusted Se intake*	109.6 (92.2; 120.8)	144.5 (136.9; 153.2)	220.2 (183.0; 320.2)	**<0.001**
Age (years)*	34 (29; 41)	33 (29; 39)	34 (30; 42)	**<0.001**
BMI (Kg/m^2)	24.0 (21.5–27.3)£	23.7 (21.4–26.7)	24.1 (21.6–26.8)	0.154
Females (%)	875 (65.4)	959 (71.6)	973 (72.7)	**<0.001**
Males (%)	463 (34.6)	380 (28.4)	366 (27.3)	
Performs physical activity regularly (%)				
Yes	932 (69.7)	998 (74.5)	1,085 (81.0)	**<0.001**
No	406 (30.3)	341 (25.5)	254 (19.0)	
Smokes cigarettes (%)				
Never smoked	1,044 (78.0)	1,095 (81.8)	1,069 (79.8)	0.053
Smoker or former smoker	294 (22.0)	244 (18.2)	270 (20.2)	
Uses alcoholic beverages (%)				
No	413 (39.9)	355 (26.5)	336 (25.1)	**0.002**
Yes	925 (69.1)	984 (73.5)	1,003 (74.9)	
Energy intake (Kcal/day)	2,384 (1,876; 2,984) £	1,861 (1,475; 2,352)	2,241 (1,817; 2,824)	**<0.001**
Energy-adjusted proteins (g/day)	89 (78; 100) £	102 (93–114)	106 (90; 124)	**<0.001**
Energy-adjusted meat (g/day)	155 (110; 205) £	209 (167; 262)	226 (159; 311)	**<0.001**
Total energy-adjusted lipids (g/day)	81 (68–93) £	84 (76–93)	90 (80–101)	**<0.001**
Energy-adjusted animal fat (g/day)	29 (24; 35) £	30 (27; 35)	30 (26; 35)	**<0.001**
SFA/PUFA** ratio adjusted by energy	0.7 (0.5;0.8) £	0.6 (0.6; 0.7)	0.5 (0.5; 0.6)	**<0.001**
Energy-adjusted carbohydrates (g/day)	295 (266–327) £	272 (249–297)	256 (224; 282)	**<0.001**
Energy-adjusted sugars (g/day)	14 (6; 25) £	12 (7; 19)	8 (4; 15)	**<0.001**
Energy-adjusted fibers (g/day)	31 (24; 38) £	28 (23; 32)	29 (24; 34)	**<0.001**
Energy-adjusted alcoholic beverage (g/day)	2.5 (0.3; 7.3) £	3.4 (1.7; 7.8)	3.4 (0.9; 8.2)	**<0.001**
Type-2 diabetes status				
Yes (%)	39 (2.9)	36 (2.7)	37 (2.8)	0.936
No (%)	1,299 (97.1)	1,303 (97.3)	1,302 (97.2)	

**Median. **SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid. £25 and 75 percentiles. The difference between continuous variables was assessed using the Kruskal–Wallis H-test; the χ^2-test evaluated differences between categorized data. Bold values mean they are statistically significant.*

four participants consumed Se above 400 μg/day and one below 50 μg/day.

Although some epidemiological studies have explored the association between Se and T2D, the results are still inconclusive. Some researchers have cited that a high level of Se may reduce the prevalence of diabetes (38, 39). In contrast, others have mentioned that a high serum Se level may be related to the increased prevalence of diabetes (40, 41), or that there is no significant relationship between Se and risk of T2D (42).

In a randomized trial of Se supplementation, Stranges et al. (43) identified a significantly increased risk of T2D in participants who used supplemental Se. These findings are corroborated by a placebo-controlled trial conducted by Faghihi et al. (44), which identified that Se supplementation might be associated with adverse effects on blood glucose homeostasis in patients with T2D.

On the other hand, observational studies on dietary Se intake and diabetes have identified an association between high Se intake and higher risk for T2D. Stranges et al. (13), after monitoring 7,812 women in a 16-year cohort study in northern Italy, found an odds ratio of 1.29 (95% CI: 1.1, 1.52) for an increase of 10 μg/day in Se intake, associating with an increased risk for T2D. The association was also found in a study that assessed 19,931 North American individuals. The same increase in daily intake found in Italy led to a rise in the prevalence of T2D by 12% (OR: 1.12; CI: 95 %: 1.06–1.18) (4). Siddiqi et al. (45) also found a positive linear association in a population of 8,824 adults in Heilongjiang province in northern China. The dietary intake of Se was associated with an elevated risk of T2D in both genders. Wei et al. (14), who carried out a cross-sectional study with 5,423 middle-aged and elderly adults in Hunan province of China, also identified a positive and significant association between Se dietary intake and the prevalence of diabetes.

Meta-analyses published by Vincet et al. (15) and Kohler et al. (16) have reported that this relationship exists. According to Vincet et al. (15), in general, results of experimental and non-experimental studies indicate that Se may increase the risk of T2D in a wide range of exposure levels. The relative increase in risk is slight but of possible importance for public health because of the high incidence of diabetes and exposure to Se. However,

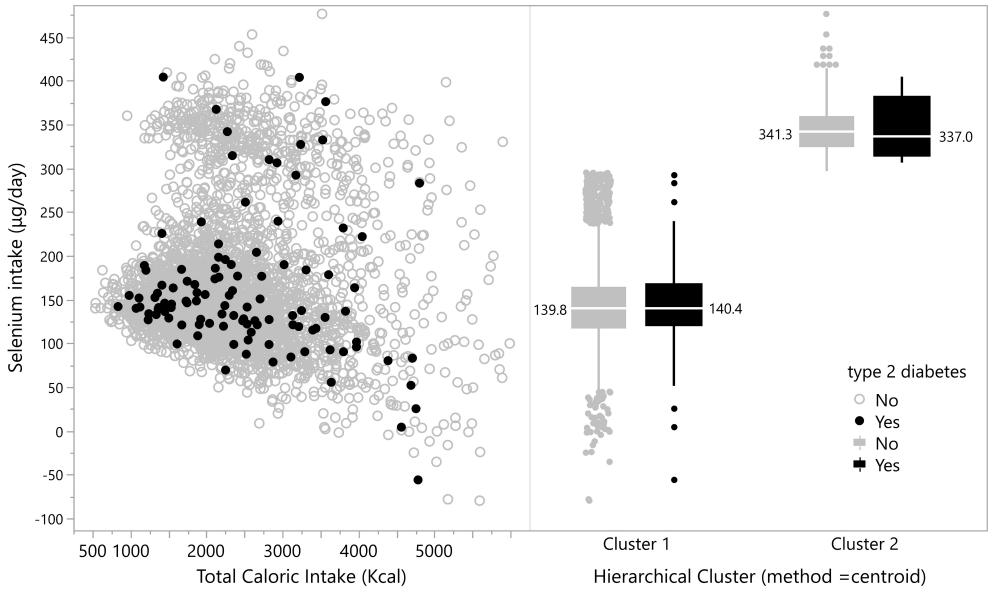

FIGURE 1 | Distribution of participants according to energy-adjusted Se intake and T2D prevalence.

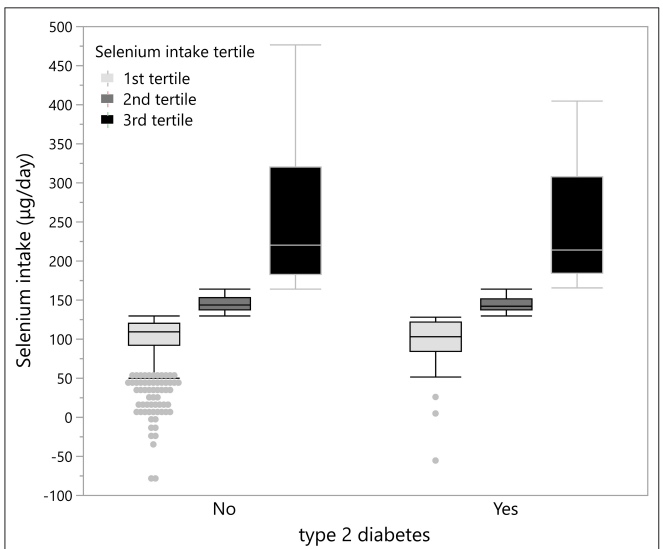

FIGURE 2 | Distribution of participants according to tertile of energy-adjusted Se intake.

according to Kohler (16), in the set of studies evaluated, the relationship between Se and T2D differs between observational studies and randomized clinical trials. This relationship is found in observational studies and not in clinical trials. In randomized clinical trials, a higher risk of T2D was not observed in those who received Se compared to placebo. Thus, it is still not clear whether these differences are the result of an uncontrolled misperception in observational studies or if there is a modest Se effect and T2D risk that may vary according to the length of exposure (16).

Studies on the relationship between Se and T2D have considered different sources: serum Se, nails, urine, diet, plasma, and erythrocyte. Other types of studies, such as cross-sectional, case control, and cohort, have divergent results. Cross-sectional studies with the American population (40, 41, 45) identified that high serum Se levels were positively associated with the prevalence of diabetes. A cohort study found that plasma Se was marginally significantly related to the occurrence of blood glucose changes in males but not in females (46). In contrast, other studies (38, 39) have identified that serum Se values were significantly lower in patients with T2D, or that Se in the diet was not associated with the development of disorders in glucose metabolism or diabetes (47). They have shown that Se supplementation was not associated with elevated plasma glucose levels (48).

It is important to highlight that in this research, selenium intake was estimated, and this may not reflect the status of body selenium. In addition, since the participants are young, perhaps a longer exposure time to high selenium values will be necessary for this outcome (T2D) to be evident. Nevertheless, some epidemiological research on the association between selenium and fT2D mentioned in this article also used an estimate of the selenium dietary intake assessed by means of a food record (13, 14, 45, 49).

Since Se has multiple effects on the human body, it may be both protective and a risk for T2D. According to Mueller et al. (50) and Steinbrenner et al. (51), its antidiabetic effect is due to its antioxidant capacity. However, its therapeutic range is relatively narrow. Some Se compounds may generate reactive oxygen species with toxic effects (39), and overaccumulation of these reactive species may increase insulin resistance and impair the function of pancreatic β cells

(52). Furthermore, higher dietary Se intake may increase the release of glucagon and consequently increase hyperglycemia (53), and increase the expression of glutathione peroxidase 1. The high activity of this enzyme may interfere with insulin signaling, favoring resistance to the action of insulin, and hyperinsulinemia (54).

The main strength of this article lies in the fact that it is the first to assess the association between dietary Se intake and T2D in the Brazilian population. The studies mentioned here have been conducted in the United States, Europe, and China. It is important to assess this association, particularly for the Brazilian population, because of differences in ethnicity, geography, and eating habits, which may affect the results. It is important to know that Se is a very common nutrient in food, especially in Brazil nuts, known in the world as the food with the highest concentration of this nutrient (10). However, in this study, oilseeds were the food that least contributed to the Se ingested by the participants, and meat was the one that stood out as source of selenium. Although the frequency of consumption questionnaire is the most suitable for food consumption studies in population research, it may overestimate consumption, which is not recommended when the objective is to assess nutrient intake quantitatively (55).

Nevertheless, there are some limitations to this research. First, it is a cross-sectional study, which is not able to explain the causal relationship of an outcome; therefore, further prospective studies are needed to confirm the findings. Second, the information is self-reported in an online questionnaire, and the serum Se level has not been measured. Studying the relationship between dietary Se, serum Se, and diabetes may provide a more comprehensive understanding of this topic. On the other hand, the sample studied is extensive, including people from all Brazilian states, which allowed us to examine the association between dietary Se intake and diabetes, thus suggesting a reasonable representation of the Brazilian population in the study.

This study did not identify an association between Se intake and T2D from the studied sample. Thus, this relationship is seen to remain controversial, and further research is required, especially of the cohort type. In addition to selenium intake, blood biomarkers should also be evaluated.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Universidade Federal de Minas Gerais (UFMG) (Protocol No. 596,741-0/2013. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LN and JD designed the study, interpreted the data, and wrote the manuscript. PC analyzed the data with supports by LN and AP. JB, HH, and AP built the database for the cohort. LN takes responsibility for the integrity of the data and accuracy of the data analysis. All authors have read the manuscript and took part in the discussion.

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REVIEW
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Selenium in Human Health and Gut Microflora: Bioavailability of Selenocompounds and Relationship With Diseases

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This review covers current knowledge of selenium in the dietary intake, its bioavailability, metabolism, functions, biomarkers, supplementation and toxicity, as well as its relationship with diseases and gut microbiota specifically on the symbiotic relationship between gut microflora and selenium status. Selenium is essential for the maintenance of the immune system, conversion of thyroid hormones, protection against the harmful action of heavy metals and xenobiotics as well as for the reduction of the risk of chronic diseases. Selenium is able to balance the microbial flora avoiding health damage associated with dysbiosis. Experimental studies have shown that inorganic and organic selenocompounds are metabolized to selenomethionine and incorporated by bacteria from the gut microflora, therefore highlighting their role in improving the bioavailability of selenocompounds. Dietary selenium can affect the gut microbial colonization, which in turn influences the host's selenium status and expression of selenoproteoma. Selenium deficiency may result in a phenotype of gut microbiota that is more susceptible to cancer, thyroid dysfunctions, inflammatory bowel disease, and cardiovascular disorders. Although the host and gut microbiota benefit each other from their symbiotic relationship, they may become competitors if the supply of micronutrients is limited. Intestinal bacteria can remove selenium from the host resulting in two to three times lower levels of host's selenoproteins under selenium-limiting conditions. There are still gaps in whether these consequences are unfavorable to humans and animals or whether the daily intake of selenium is also adapted to meet the needs of the bacteria.

Keywords: selenium, gut microbiota, selenocompounds, selenoproteins, selenium metabolism

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SELENIUM FORMS, FOOD SOURCES, AND BIOAVAILABILITY

The organic forms of Se are found as a sulfur amino acid analog, selenomethionine (SeMet), selenocysteine (SeCys), and as methylated derivatives. The inorganic forms correspond to Se salts such as selenate (SeO_4^{-2}) and selenite (SeO_3^{-2}) (1). SeMet is found in plant- and animal-origin products as well as in some food supplements (2). On the other hand, SeCys is

found primarily in animal-derived food (3), whereas selenium-methylselenocysteine (SeMeCys) is a natural monomethylated organic Se found in some vegetables such as garlic, onion, broccoli, and leeks (2–4). Among the inorganic forms, selenite is present mainly in food supplements, while selenate is found in plant and fish sources (3). These forms of Se have been used to biofortify some vegetables (5, 6).

Brazil nuts, cereals, meat, fish, seafood, milk, and nuts are the best sources of Se (7) (**Figure 1**). The interaction of fish and seafood with mercury results in insoluble Se derivatives that can reduce its bioavailability (8). In fact, the bioavailability of Se depends primarily on its chemical form. In general, the organic forms are more quickly absorbed and are usually used for the biosynthesis of selenoproteins (9). In addition, the amount of protein, fat, and heavy metal in the diet influence the bioavailability of Se (7–10). High levels of Se are present in some herbal plants such as *Astragalus bisulcatus* and *Brassicaceae* (broccoli) (11). *Bertholletia excelsa,* known as Brazil nut, is one of the highest sources of Se with concentrations that range from 1.80 to 320.80 μg Se/g (12). In addition, the content of Se in the soil has a major influence on the amount of this metal in food, being related to its deficiency and toxicity in some regions. The Se content in the soil usually ranges from 1 to 1.5 μg Se/g, reaching 5.0 μg Se/g in seleniferous soils (13) (**Figure 2**).

SELENIUM ABSORPTION, METABOLISM, EXCRETION, AND BIOMEDICAL APPLICATIONS

Dietary Se intake from either organic or inorganic origin is absorbed in the gastrointestinal tract and subsequently transported to the liver, where it is metabolized and used for producing selenoproteins, followed by its distribution to other tissues of the body. Selenoamino acids are actively transported in the duodenum, cecum, and colon through various membrane transport mechanisms, whereas selenate is transported by anion exchangers from the family of the SLC26 gene. On the other hand, there is insufficient evidence on the transport of the other forms of Se (14). Se absorption, metabolism and body distribution are represented in **Figure 3**.

Although the metabolic route differs depending of the Se source, all the absorbed Se is converted to hydrogen selenide (H_2Se) in the enterocytes before the specific incorporation of selenocysteine takes place in the active site of the selenoproteins (15). SeMet undergoes transulfurization reactions, in which cystathionine beta-synthetase catalyzes the formation of selenocystathionin, being further converted to SeCys by cystathionine gamma-lyase followed by conversion to H_2Se by selenocysteine lyase. SeCys, from both food and the SeMet pathway, will also be reduced to H_2Se (16, 17). Alternatively, SeMet can also be incorporated non-specifically into proteins,

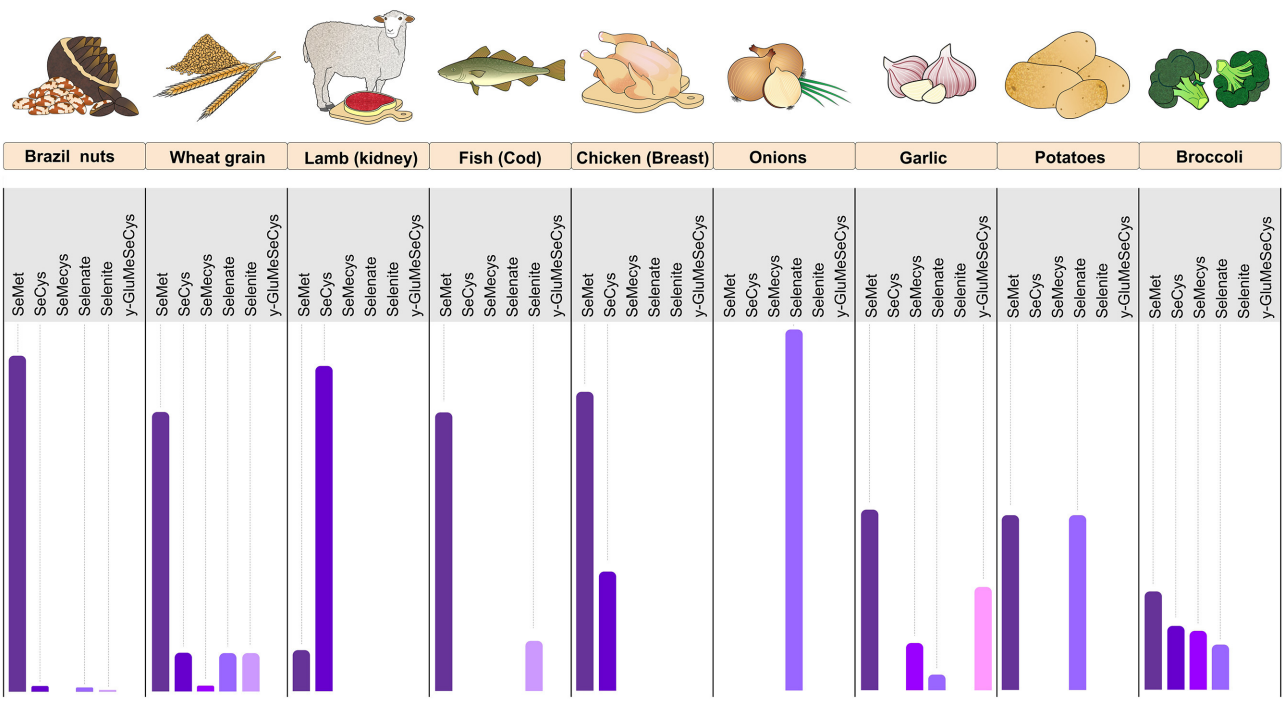

FIGURE 1 | Foods rich in Se with their relative proportions of SeMet, SeCys, SeMeCys, selenate, selenite, and γ-GluMeSeCys. The figure shows the predominance of SeMet in Brazil nuts, wheat grain, fish cod, and chicken (breast). Lamb meat (kidney) is rich in Se mainly in the SeCys form, whereas onion has Se almost exclusively in the form of selenate. Garlic, potato, and broccoli have a balanced proportion of the various forms of Se. Se, selenium; SeMet, selenomethionine; SeCys, selenocysteine; SeMeCys, selenium-methylselenocysteine; γ-GluMeSeCys, γ-glutamyl-Se-methyl-selenocysteine (Figure illustration by Francisco Irochima Pinheiro).

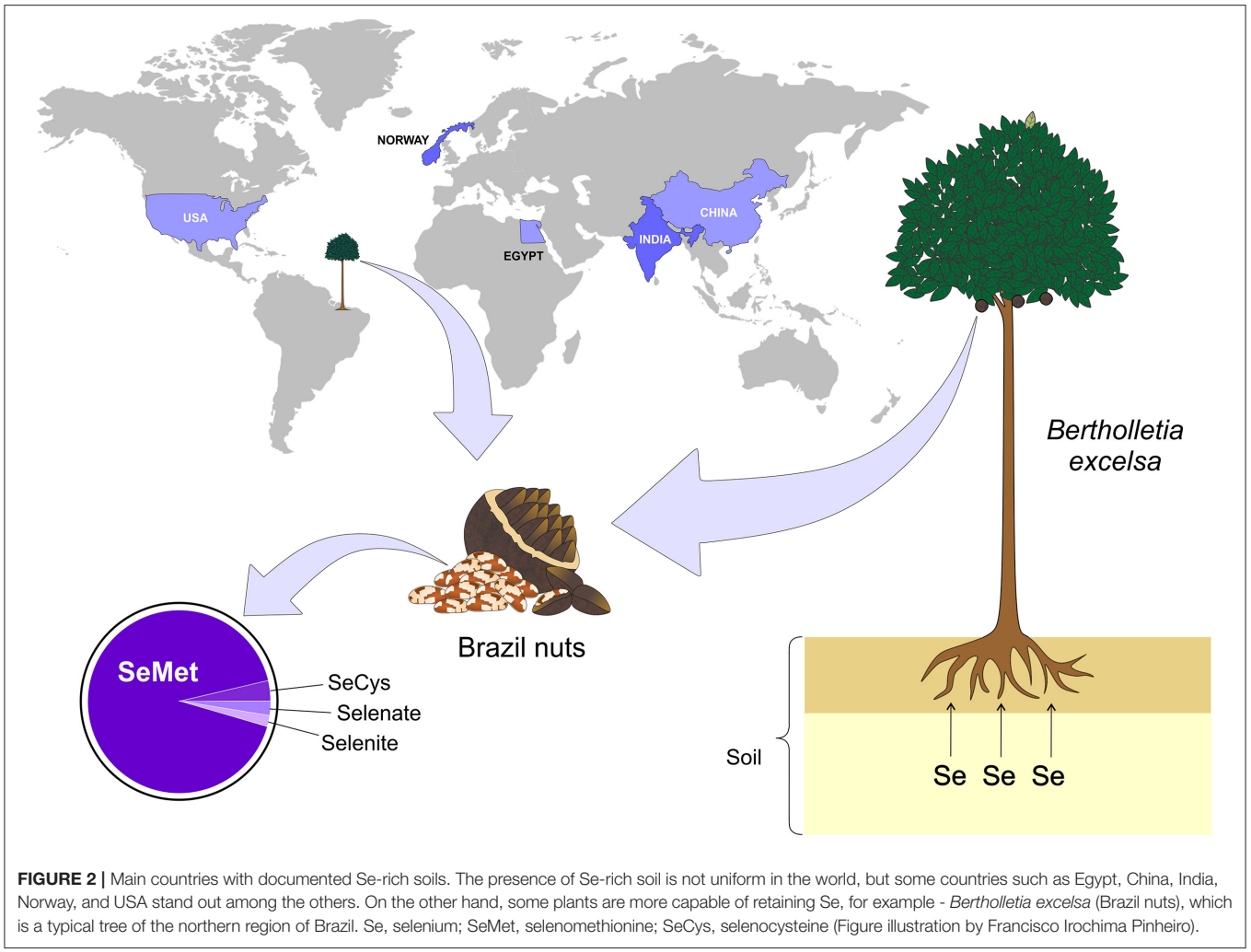

FIGURE 2 | Main countries with documented Se-rich soils. The presence of Se-rich soil is not uniform in the world, but some countries such as Egypt, China, India, Norway, and USA stand out among the others. On the other hand, some plants are more capable of retaining Se, for example - *Bertholletia excelsa* (Brazil nuts), which is a typical tree of the northern region of Brazil. Se, selenium; SeMet, selenomethionine; SeCys, selenocysteine (Figure illustration by Francisco Irochima Pinheiro).

such as albumin and hemoglobin, replacing methionine (3). As for the inorganic forms, selenate is converted to selenite followed by reduction to H_2Se by thioredoxin reductase (TXNRD) and thioredoxin, as well as by glutathione to form selenodiglutathione (GS-Se-SG). Glutathione reductase converts the latter to glutathioselenol (GS-SeH) which reacts with glutathione to form H_2Se (3, 18–20). On the other hand, SeMeCys and the synthetic Se derivatives selenobetaine, methylseleninic acid, and methylselenocyanate are converted into methylselenol $(CH_3)SeH$ through the enzyme cystathione gamma-lyase, followed by demethylation to become H_2Se (21, 22).

In view of this cascade of reactions, all H_2Se regardless of its origin will be transported in the blood linked to VLDL and LDL fractions as well as to other proteins (albumin and alpha-globulin). In the liver, H_2Se is converted to selenophosphate (SePhp) via selenophosphate synthetase (SEPHS), which will be incorporated into selenoproteins in the form of SeCys. For selenocysteine synthesis, the UGA codon (TGA) is used as an initiation codon, requiring a specialized tRNA (ribonucleic acid carrier), which, after several reactions from the seryl-tRNA, provides information in a targeted manner to the ribosomes that translate mRNAs (messenger ribonucleic acid) to selenoproteins (3, 18–20). Se is transported to tissues such as brain, kidneys, and testicles, mainly in the form of selenoprotein P (SELENOP) through endocytosis mediated by apolipoprotein E receptor 2 (apoE2) and megaline (14).

H_2Se can also be methylated by thiol-S-methyltransferase before being excreted. The main form of Se excretion is through urine, however, in cases of excessive consumption, respiratory excretion might occur. Excretion by the lungs occurs when the elimination of Se in the form of trimethyl selenonium $(CH_3)_3Se$ in the urine becomes saturated, whose elimination occurs mainly in the form of volatile dimethyl selenide $(CH_3)_2Se$ (23). In situations of moderate consumption of Se, the main monomethylated compound eliminated through kidneys is a seleno sugar namely 1β-methylseleneN-acetyl-D-galactosamine. The non-absorbed Se from food is incorporated into the bile, pancreatic, and intestinal secretions, being eliminated in the feces (23).

Se functionality occurs in the form of selenoproteins that are encoded by the insertion of SeCys by the UGA codon in mRNA

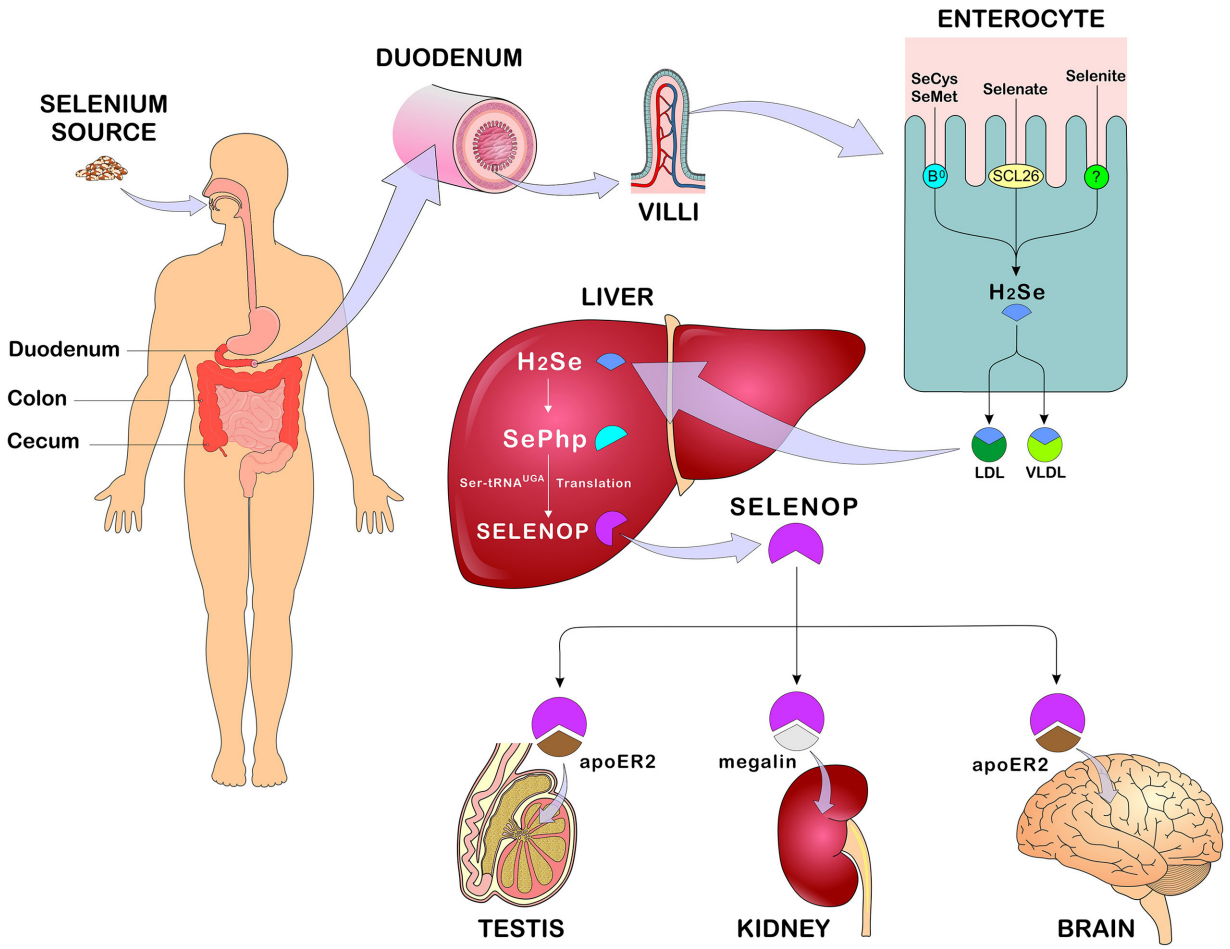

FIGURE 3 | Se absorption, metabolism, and distribution. After eating Se-rich foods in its organic and/or inorganic form, Se absorption occurs in the duodenum, cecum, and colon. In enterocytes, SeMet and SeCys are absorbed by active transport (systems B0 and b0 + rBAT) while selenate is absorbed by passive transport (anion changers of the SLC26 gene family). After absorption, all forms of Se are converted to H_2Se through reactions that occur in the enterocyte and transported in the blood bound LDL, VLDL (mainly). In the liver, H_2Se is converted to SePhp and incorporated into selenoproteins in the form of SeCys. Transport to other tissues such as testis, kidneys, and brain occurs mainly in the form of SELENOP through receptor-mediated endocytosis - apoE2 and megaline. Se, selenium; SeMet, selenomethionine; SeCys, selenocysteine; H_2Se, hydrogen selenide; LDL, low-density lipoprotein; VLDL, Very low-density lipoprotein; SePhp, selenophosphate; SELENOP, selenoprotein P; apoE2, apolipoprotein E receptor 2 (Figure illustration by Francisco Irochima Pinheiro).

under specific conditions. Most of these selenoproteins are involved in the regulation of redox signaling and are grouped into families such as glutathione peroxidases (GPXs), iodothyronine deiodinases (DIOs), TXNRDs, and SELENOP. Thus, the main biomedical applications attributed to Se are related to its antioxidant activity, regulation of thyroid hormone metabolism, anticarcinogenic property, and prevention of cardiovascular diseases. SELENOP acts as the main Se transporter for peripheral tissues in addition to performing extracellular antioxidant function (14, 22).

SELENIUM BIOMARKERS

The evaluation of Se status determines the amount of this biologically active nutrient as a function of intake, retention, and metabolism. Thus, the Se status can be assessed at three levels using biomarkers of intake, retention/excretion, and concentration on tissues as well as biomarkers of functionality (22) (**Figure 4**).

Biomarkers of Intake

The assessment of Se intake can be performed using methods of assessing food consumption, such as the food frequency questionnaire. The Se content on foods is estimated using food composition tables (22). Algeria 's population consumes a wide variety of Se-rich foods, such as seafood, meat, eggs, milk, and legumes, however, no significant associations were found between dietary patterns and Se biomarkers, such as Se in plasma, SELENOP, and GPX. Predicting the Se status from food consumption remains a challenge due to the lack of precision of nutrient content in food composition tables, considering the variation in the Se concentration of foods as a function of Se

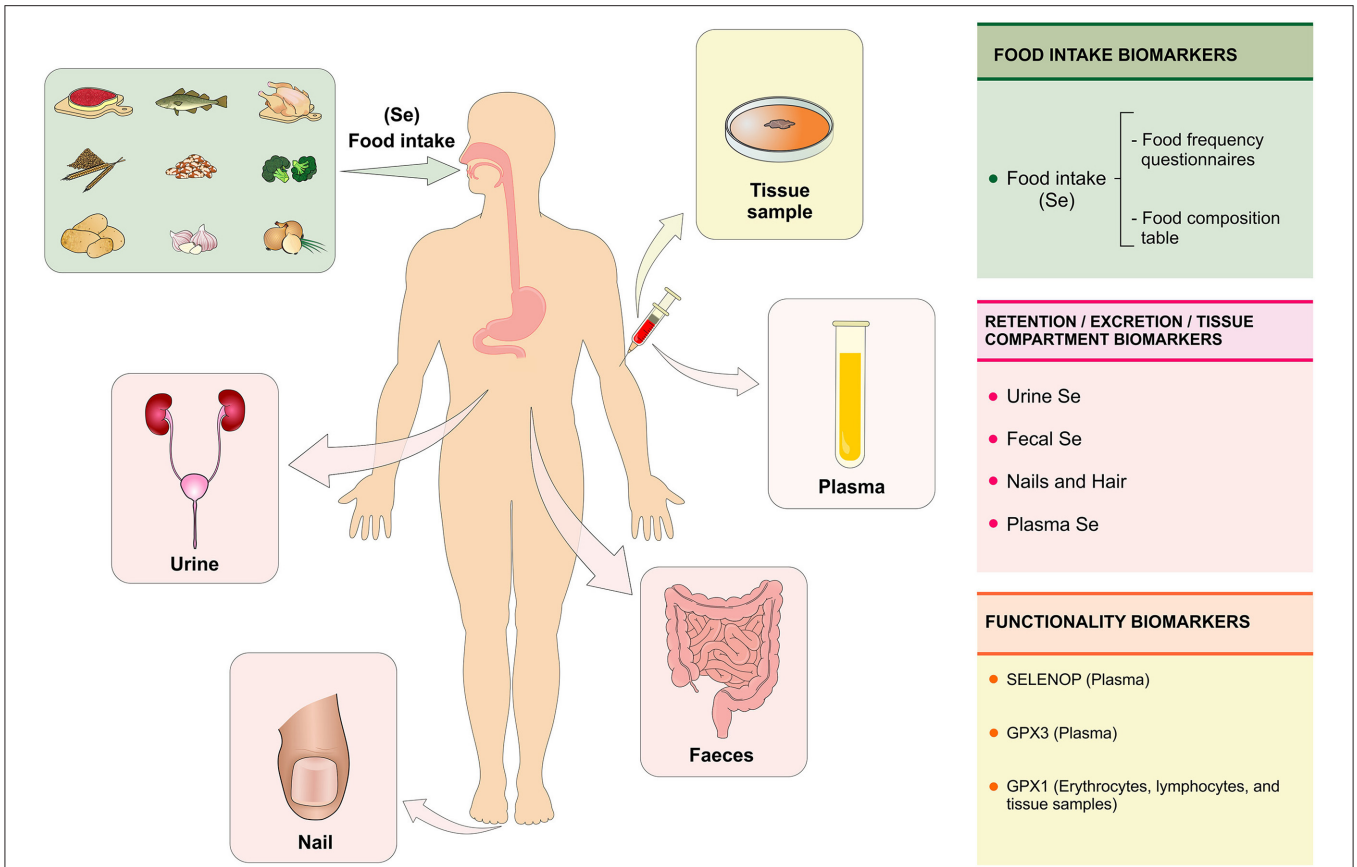

FIGURE 4 | Se biomarkers. The Se status is evaluated with the purpose of quantifying the biologically active or potentially active nutrient as a function of Se intake, retention, and metabolism in the body. The Se status can be assessed at three levels using specific biomarkers: (1) biomarkers of intake (assessment of food consumption through the food frequency questionnaire); (2) biomarkers of retention/excretion and concentration in tissues (urine, feces, nails, hair, and plasma) and (3) biomarkers of selenium functionality (SELENOP and GPX3 in plasma and GPX1 in erythrocytes, lymphocytes, and tissue samples). Se, selenium; SELENOP, selenoprotein P; GPX1, glutationa peroxidase; GPX3, glutationa peroxidase 3 (Figure illustration by Francisco Irochima Pinheiro).

content in the soil (22, 24). In addition, Se from the diet affects colonization of the microbial intestine, which in turn influences the host's Se status and selenoproteoma expression (25).

Biomarkers of Retention/Excretion and Se Concentrations in Tissues

The retention of Se in the body can be assessed by the difference between the amount of Se ingested and the sum of Se in the urine and feces, which requires the collection of total urine and excreted feces for a few days. Alternatively, it is recommended to evaluate the concentration of creatinine in the urine to reduce the error associated with the variation in urinary excretion. Renal excretion is the main route of elimination of absorbed Se (22). Genetic and environmental factors, as well as body size, age, and sex can influence the retention and excretion of Se in the urine (26). However, studies on Se in urine for biological monitoring are scarce, especially with regard to occupational exposure, in which inhalation is the main route of exposure. After inhalation of high concentrations of Se by workers, inflammatory effects were observed in the respiratory tract (27). An increased intake of Se is reflected rapidly in the increased excretion of Se in the urine (28). The evaluation of Se in urine can be a sensitive parameter for occupational exposures of Se in the short term, but the knowledge about specificity and kinetics of this elimination pathway is still little explored (27).

The measurement of the Se concentration in urine is considered as a potentially viable biomarker of Se status in population studies. Additionally, the concentration of Se in the urine can be used to identify regional variations in the status of Se and might reflect differences in the amount of Se in food according to the type of soil. This evidence supports the need for reviewing the policies of national systems for monitoring micronutrient deficiencies including Se (26).

The concentration of Se in the nail is considered a superior biomarker of Se status, as it provides an integrated measurement of long term exposure (up to 1 year), while blood biomarkers indicate a short term exposure (29). Toenails are considered non-invasive matrices and are used in large epidemiological studies because they present slow growth, easy collection and have less influence from external contamination. The standardization of sample collection, quality control, and analytical techniques are important to consolidate the usefulness of this matrix in

epidemiological studies (30). The Se content in nails has a direct relationship with SELENOP and most organic forms of Se, especially SeCys, whereas it has an inverse relationship with the amount of the inorganic forms, such as selenite and selenate. This opposite behavior may be related to the composition of human nails, which are mainly made of proteins rich in cysteine, the latter being able to form complex with Se (31).

There are controversies about the use of Se content in nails and hair as a way to assess the effectiveness of Se supplementation. A systematic review performed with 18 Se supplementation studies found no evidence to support the use of the Se content in the nail and hair as a reliable measurement of effectiveness of Se supplementation (32). Se content in hair has been used to assess long-term Se status in epidemiological studies, offering the advantage of being a low-cost method and easy to store the samples. The Se concentration in hair and nails are excretory forms of Se. Therefore, both reflect the previous status, being more useful as biomarkers in studies of populations with stable dietary patterns (22).

Plasma Se concentration is a more useful biomarker to assess Se status in humans, considering the stability of Se in this compartment (22). A systematic review has recommended the use of plasma Se concentration as a reliable biomarker in supplementation studies with adults of both sexes. The measurement of Se in plasma has shown to be effective in reflecting changes in the amount intake (supplementation) in individuals with intermediate or high Se concentrations at baseline. In addition, this review highlights the usefulness of Se in erythrocytes and whole blood as markers of Se status, both of which are reported as markers of long-term status (32).

Biomarkers of Selenium Functionality

Biomarkers of Se functionality include SELENOP, which comprises 20–70% of Se in plasma; GPX3, which comprises 10 to 25% of Se in plasma and GPX1, which can be tested on erythrocytes, lymphocytes, oral cells, and tissue biopsy specimens (22). Plasma SELENOP has been considered a useful biomarker of Se status in populations with relatively low Se intake, but not in populations with high intake that already had high levels of Se before supplementation began (32). SELENOP has shown to be a reliable and sensitive Se status biomarker, providing dose response that can be used to estimate the Se intake required to reach its plateau in the plasma (33). It seems that SELENOP reaches a plateau after supplementation with selenite at doses around 400 μg/day (34).

GPX is one of the main selenoproteins that belongs to the cellular antioxidant defense system. The recommended Se intake was calculated based on optimal plasma GPX3 activity due to the hierarchy of selenoproteins. It also considers the necessary amounts of Se for normal concentrations of other biologically Se compounds (35). A cohort study conducted with 51 participants with adequate Se intake investigated the association between plasma Se, GPX activity, and SELENOP. The results were discrepant between plasma Se concentrations and GPX activity, suggesting other factors may impact the activity of this enzyme such as genetic polymorphisms (36, 37).

SELENIUM AND DISEASES

Se plays a crucial role in normal physiology and contributes to the pathophysiology of various diseases. Due to its antioxidant and anti-inflammatory properties, several studies have evaluated the impact of Se status in conditions characterized by inflammation and oxidative stress, which includes diabetes, metabolic syndrome, cancer, cardiovascular, and neurodegenerative diseases (38).

Inadequate serum Se levels may increase the risk for the development of several diseases, especially cardiovascular disorders, but it also may lead to cancer, liver diseases, and arthropathies. On the other hand, excessive consumption of Se can cause selenosis, which leads to symptoms such as fatigue, tachycardia, nausea, and diarrhea. Chronic selenosis can cause liver and kidney necrosis, neurological disorders and might compromise the reproductive and immune systems (39).

In three large cohorts, the high serum Se concentration was associated with reduced mortality (40). Another larger study, in which more than 13,000 adults were followed for 12 years, revealed that serum Se greater than or equal to 135 μg/L were associated with reduced cancer mortality (41, 42). Meta-analysis involving 16 prospective studies demonstrated an inverse relationship between Se status and cardiovascular risk (43). Likewise, a systematic review with meta-analysis involving 13 studies revealed that high physiological levels of Se are associated with lower incidence and lower mortality from cardiovascular disease (CVD) (44). In another meta-analysis in which more than 40 thousand participants in randomized clinical trials were included, the authors found that Se supplementation decreases the serum levels of C-reactive protein and increases the levels of GPX, suggesting a positive effect on reduction of inflammation and oxidative stress in cardiovascular diseases (45).

Selenium-binding protein 1 (SELENBP1), an intracellular protein involved in Se metabolism and redox control, has been identified as a circulating biomarker for cardiac events in patients with suspected acute coronary syndrome. At the molecular level, it seems that hypoxia acts as a modulator of SELENBP1, therefore reducing the oxidative stress and controlling the lower oxygen supply (46).

Previous studies have shown that circulating Se plays an important role in the pathogenesis of abnormal glucose metabolism, especially at high concentrations (47, 48). High exposure to Se can affect the expression of the main regulators of glycolysis and gluconeogenesis, through actions mediated by the GPX1 (49), as shown in studies that evidenced that the overexpression of this selenoprotein causes insulin resistance (50).

A review study has elucidated the relationship between Se status and cerebral Se homeostasis via SELENOP. In fact, SELENOP may be involved in some brain disorders, in particular in Alzheimer's disease, providing Se for brain tissue to produce selenoproteins. In addition, it competes with amyloid-β for metal ions and redox-active metals, such as copper and iron. This study points out the involvement of SELENOP in signaling pathways in neuronal and glial tissues, including neuronal calcium homeostasis and excitotoxicity (51).

SELENIUM SUPPLEMENTATION

Brazil nuts (*Bertholletia excelsa, family Lecythidaceae*) are known to be the richest source of Se with high SeMet content and therefore, it has been widely used in studies of Se supplementation. Regular consumption of Brazil nuts results in optimum plasma Se and erythrocytes concentrations, as well as in better activity of selenoenzymes (52–55) antioxidant state (56), muscular retention (57), and inflammation status (54, 58). It is important to consider genetic variants in selenoprotein genes (55) and pre-stratification of the population prior to starting the trials as a way to avoid possible differentiated responses depending on the Se status in each individual (59). Studies on the effects of supplementation with Brazil-nuts on selenium biomarkers are shown in **Table 1**.

The effect of Brazil nuts on the human intestinal microbiota is still unknown. It is well-known, however, that Brazil nuts contain fiber, unsaturated fatty acids, and polyphenols that may impact the composition of the gut microbiota and overall gut health. A systematic review with meta-analysis including randomized controlled trials on nut consumption investigated the intake of almonds ($n = 5$ studies), walnuts ($n = 3$ studies), and pistachios ($n = 1$ study) and demonstrated a significant increase in the gut content of genera *Clostridium, Dialister, Lachnospira,* and *Roseburia*, as well as a significant decrease in *Parabacteroides*. The nuts did not show any significant influence on bacterial phyla, bacterial diversity or stool output (60). Other studies have only reported an increase in the abundance of butyrate-producing bacteria after nuts (61, 62) and pistachios (63) intake, without demonstrating any effect on the overall composition of the microbiome.

The Nutritional Prevention of Cancer (NPC) trial showed the effectiveness of supplementing 200 μg/day of Se, as selenized yeast, in reducing the risk of prostate, lung, and colorectal cancers (64). Se supplementation was also reported to decrease CVD and related mortalities (65, 66). In a group of healthy New Zealand men, Se supplementation as selenized yeast (Selplex, 200 μg/day) in the form of SeMet, significantly increased Se levels, improved the TXNDR activity and enhanced DNA stability (59). Due to the positive results from the NPC trial, the Selenium and Vitamin E Cancer Prevention Trial (SELECT) was undertaken in 35,000 healthy US men randomly assigned to 4 groups (selenium-−200 mcg/d from L-selenomethionine, vitamin E-−400 IU/d of all rac-alpha-tocopheryl acetate, selenium + vitamin E, and placebo). Neither selenium nor vitamin E, alone or in combination, was able to prevent prostate cancer in this population (67).

Observational studies and randomized clinical trials conducted with high-dose Se supplementation have shown controversy over the effects on diabetes mellitus, indicating that both excess and deficiency of Se may be associated with higher risks of diabetes mellitus type 2 (DM2) (68–71). In a NPC trial, participants were randomly assigned to receive 200 μg/day of Se (as high-selenium yeast). This group were more likely to develop DM2 than those assigned to placebo. In a randomized controlled study involving patients with DM2 and cardiovascular disease, supplementation with 200 μg Se/day resulted in a significant

TABLE 1 | Studies on the effects of supplementation with Brazil-nuts on selenium biomarkers.

Studies	Country population	Supplemented dose (μg)	Ingestion period (w)	Se in plasma (μg/L)		SELENOP (ng/mL)		GPX (Ug/Hb) erythrocyte	
				Before	After	Before	After	Before	After
Thomson et al. (52)	New Zealand 59 adult men	100	12	90.8 ± 12.63[a]	150.81 ± 17.4[a]			20.6 ± 4.4[a,b]	22.8 ± 5.0[a]
Cominetti et al. (53)	Brazil 37 obese women	290	8	55.7 ± 13.3[a]	132.5 ± 34.9[a]			36.6 ± 17.0[a]	53.6 ± 20.4[a]
Duarte et al. (54)	Brazil 55 obese women	1261.4	8	87.1 (82–97.7)[c]	244 (226–278)[c]	37.7 (16.1–51.9)[c]	55.5(37.1–150.6)[c]	48.7 (37.5–57.6)[c]	57.2 (45.8–67.5)[c]
Donadio et al. (55)	Brazil 130 healthy adults	300	8	90.7 (86.4–95.2)[d]	267.0 (252.8–282.0)[d]	3.4 (3.2–3.5)[d]mg/L	3.9 (3.7–4.1)[d]	61.8 (58.8–65.1)[d]	61.3 (57.7–65.1)[d]

[a]*Mean ± SD.*
[b]*Whole blood GPx was assayed as a measure of erythrocyte GPx activity.*
[c]*Median (interquartile interval).*
[d]*Geometric means (CI 95%).*

decrease in insulin, HOMA-IR, C-reactive protein and an increase in total antioxidant capacity (70).

SELENIUM TOXICITY

Se toxicity can affect individuals as a result of occasional overdose that usually occurs with intake of incorrectly formulated supplements (72) or due to the excess of Se intake in randomized clinical trials, in which doses of 200 μg/day or more are administered for a substantial period of time (73). Acute toxicity from excessive Se exposure causes stomach pain, headache, respiratory symptoms, changes in blood pressure, vomiting, and nausea. Chronic oral intake of high amounts of Se results in selenosis, a condition characterized by hair loss, deformation and loss of nails, tooth discoloration, garlic breath, gastrointestinal disturbances, skin rash, numbness, paralysis, and occasional hemiplegia (74). Other outcomes have been reported such as dermatitis, increased mortality (73), DM2 (68) and increased incidence of prostate cancer (67), which are also observed in Se deficiency.

Increased mortality has been reported at the highest dose of Se in the Danish PRECISE, a randomized, double-blinded, placebo-controlled, clinical trial performed with four groups treated with 100, 200, or 300 μg Se/day as Se-enriched yeast or placebo yeast. The results of this study warn that a 300-μg/day dose of Se (as Se yeast) taken for 5 years in a country with moderately low Se status can increase all-cause mortality by 10 years later (73).

The levels of dietary exposure that is able to induce selenosis and Se toxicity is difficult to establish due to the fact that toxicity is affected by the chemical form of Se and its bioavailability. Furthermore, interactions of Se with other dietary components, the individual's genotype and intestinal microbiota are also factors that influence the Se toxicity. Even in the face of Danish PRECISE, some populations exposed to excess Se did not develop adverse effects. Such conditions suggest that there are mechanisms for genetic adaptation that might be involved in oscillations in the Se intake, which are mediated by polymorphisms, complexation of SELENOP with toxic elements such as cadmium, arsenic, and mercury forming products of Se excretion (75, 76). The metabolism of Se by intestinal bacteria also favors the excretion of excess of Se (41, 77).

SELENIUM AND GUT MICROBIOTA

The human digestive tract is inhabited by several microorganisms (bacteria, viruses, fungi, and protozoa) named microbiota, that includes ∼100 trillion microorganisms (78). Bacterial cells are distributed unevenly along the gastrointestinal tract with more than 50 types of bacterial phylum. Only *Bacteroidetes* and *Firmicutes* are preserved in practically all individuals (79).

Human microbial colonization begins at birth and it is similar to the maternal vaginal microbiota. It is believed that intestinal colonization during birth and breastfeeding is essential to define the composition of the intestinal microbiota later in adulthood, although the determination of the composition of the microbiota is also influenced by several external and internal factors related to the host (80).

The microbiome is capable of encoding more than three million genes. It carries out a variety of metabolic functions not attainable by the human host, which includes the production of some types of vitamins and bioactive compounds, the synthesis of essential and non-essential amino acids, the metabolism of non-digestible carbohydrates and the activity in neural, hormonal, and immunological signaling through the gut-brain axis. Furthermore, it acts on the absorption of nutrients and as an epithelial barrier for pathogens (78). In this sense, imbalances in the intestinal ecosystem or in two-way communication with the brain are associated with gastrointestinal disorders, metabolic diseases, and neurobehavioral disorders. Therefore, strategies have been developed to manipulate the microbiome, with the aim of preventing and/or reversing conditions that are harmful to health (81).

Comparative genomics provides a powerful tool for investigating genes, pathways and evolutionary changes across multiple lineages (82). In the past decade, studies have been conducted evaluating the use of Sec Trait in ∼600 bacterial and archaeal genomes, in which the organisms rich in selenoproteins were the anaerobic *Deltaproteobacteria* and *Clostridia* classes, especially *Syntrophobacter fumarroxidans*, with the highest prokaryotic selenoproteoma reported (83).

Traces of Se and related key genes have been evaluated in over 2,300 bacterial and archaea genomes, identifying a phylogenetic and genomic mosaic pattern among organisms using Se in different forms. This profile suggests new genes whose encoded proteins participate in Se metabolism and homeostasis in prokaryotes, such as YedE involved in Se transport, YedF which transcribes redox protein and LysR Se known as specific transcriptional Se-regulator (84).

Evolutionary trends in the use of Se and selenoproteins indicate more than 5,200 bacterial genomes, with the majority being related to the host, resulting in the largest Se utilization map in this realm. However, of this total, 2/3 of the bacteria do not use Se, suggesting that this ability has been lost over time. Environmental factors and use of Se were also investigated, revealing that Se-cofactor trait (68%) and Sec Trait (37%) appear to favor the conditions of host-associated bacteria, while SeU trait prefers aquatic species that have been isolated mainly from the sea or freshwater (85). These macro-evolutionary trends extend to cell respiration and temperature characteristics, in which anaerobic conditions can significantly promote the use of the Se-cofactor trait and lead to the evolution of new selenoprotein genes. Temperature seems to affect the use of Se, in which thermophilic (<40°C), mesophilic (20–40°C), and psychrophilic (<20°C) conditions favor the use of Sec Trait, Se-cofactor trait (mostly, but non-significant values) and SeU trait, respectively (85, 86). Thus, the human intestine can be a favorable ecosystem for the use of selenium by prokaryotes as the oxygen level in the colon, which is the site with the highest degree of Se absorption, is low and the optimum temperature varies between 25°C and 30°C (87).

Dietary Factors and Intestinal Modulation

Genetic sequencing data carried out with 1,135 Dutch people detected 126 different environmental factors associated with microbiota, including diet, physical activity, diseases, and use

of medicines (88). Specific foods and dietary patterns can influence the abundance of different types of bacteria in the intestine. For instance, the low intake of FODMAPs (Fermentable Oligosaccharides, Disaccharides, Monosaccharides, and Polyols) has been identified as a nutritional therapy indicated for the relief of gastrointestinal symptoms reported by patients with irritable bowel syndrome (IBS) and non-celiac sensitivity to gluten (89). Foods rich in fructans (wheat, rye, garlic, and onion) lactose (milk and dairy products), fructose (fruits and processed foods containing syrups), sorbitol, xylitol red fruits, and mushrooms are fermented by intestinal bacteria (*Actinobacteria*) and yeasts producing hydrogen and methane gases, resulting in bloating symptoms, abdominal pain, and diarrhea (90). In a meta-analysis study with randomized clinical trials, the low FODMAP diet was beneficial for remission of gastrointestinal symptoms in patients with IBS (91). However, the restriction of several foods may lead to a potential inadequacy of micronutrients in patients who follow this dietary recommendation, resulting in significant changes in the microbiota and metabolome, whose duration and clinical relevance are still unknown (92, 93).

Selenium as a Modulating Agent of Intestinal Flora

Dietary Se influences both the host's selenium status and selenoproteoma expression. The intestinal microbiota can use the ingested Se for the expression of its own selenoproteins. Se affects the composition and colonization of the gut microbiota, which may interfere with the diversity of the microbiota and cause unique effects on microbial composition. About 1/4 of all bacteria have genes that encode selenoproteins. Some of them, such as *Escherichia coli, Clostridia,* and *Enterobacteria* classes, are able to colonize the gastrointestinal tract of humans and animals (94). Selenocysteine synthase (SelA) is a pyridoxal phosphate-dependent enzyme (PLP) (95) which catalyzes the formation of selenocysteinyl-tRNA in bacteria from a UGA decoding tRNASec (SelC) loaded with serine and selenophosphate, the product of the enzyme selenophosphate synthetase (SelD). Along with SelB, a specific translation factor of selenocysteinyl-tRNA, SelA, SelC, and SelD are components of bacterial Sec decoding, allowing the incorporation of Sec into specific UGA codons followed by a sequence of insertion of Sec elements (SECIS) (96).

The composition of the microbiota can also be modulated by metals that participate in microbial growth through respiratory mechanisms, as a source of energy for autotrophic growth, as well as to transfer and storage of electrons between cells (86). Manganese, zinc, selenium, and iron act as critical cofactors for bacterial enzymes responsible for DNA replication and transcription, antioxidant action, and cellular respiration (97). Iron and zinc are the metals used by almost all living organisms in metabolic and oxidation-reduction processes (98). Some species require Se for normal metabolic functions, for instance, *Escherichia coli* has three selenoproteins in its structure (99).

Selenocompounds are found in animal and plant sources with distinct bioavailability. In experimental models using rats, no differences in nutritional availability were observed between selenite, selenate, selenocyanate (SeCN), SeMeCys, SeMet, selenohomolanthionine (SeHLan), selenocenoine (SeCys2), 1β-methyl-acetyl-D-galactosamine (SeSug1), except for trimethylselenonium ion (TMSe) administered orally. The authors discussed these findings based on mechanisms related to gastrointestinal enzymes that can degrade bioselenocompounds into selenocompounds in the intestine (100).

Germ-free mice that were fed with diets with adequate and high Se levels modified their selenoproteoma expression in a similar way to that of the control group but showed higher levels and activity of GPX1 and methionine-R-sulfoxide reductase 1 (MSRB1) in the liver, suggesting partial sequestration of Se by intestinal microorganisms, therefore resulting in limited availability to the host. In these experiments, the genus *Parabacteroides* of the phylum *Bacteriodetes*, showed an opposite correlation with Se dietary supplementation. The study concluded that dietary Se affects both the composition of the gut microflora and the colonization of the gastrointestinal tract (99).

Zhai et al. (101) compared the effects of different levels of Se dietary supplements (deficient, adequate, and supranutritional) on the intestinal microbiota of mice. The animals' fecal microbiota transplantation was performed in one of the experiments. Supplementation conducted with different amounts of Se did not significantly alter the mice's intestinal microbiota. It rather induced significant changes in the composition of the gut microbiota. In comparison to the Se-deficient diet, supranutritional Se supplementation significantly decreased the abundance of *Dorea* sp. and increased the levels of microbes with potential protective effects against colitis and intestinal barrier dysfunction, such as *Turicibacter* and *Akkermansi*. *Dorea* sp. is one of the most common species of the intestinal microbiota that supplies hydrogen and carbon dioxide in the intestine. The authors concluded that Se supplementation can optimize the intestinal flora to protect against intestinal dysfunction.

Microbiota as an Environment That Affects Selenium Status

Although the host and the intestinal microbiota mutually benefit from a symbiotic relationship, these environments can become competitors when the supply of micronutrients becomes limited. On the other hand, the intestinal microbiota favors the biotransformation of Se compounds, characterizing a dubious situation (**Figure 5**). The Se uptake by intestinal bacteria can negatively influence the expression of selenoproteins in the host, which results in a two to three times lower levels of selenoproteins under Se limiting conditions. The unfavorable consequences of this effect for humans and animals have not yet been evidenced. In view of the high propagated intake of probiotics, the metabolism of Se in these organisms should be investigated in order to assess whether a higher Se intake is recommended (94).

A study conducted with animal models indicated that the gut microbiota may affect the status of Se and the expression of selenoproteins. The colonization of germ-free (GF) mice has shown to induce the expression of the gastrointestinal form of several selenoproteins, even under conditions of Se-deficient diet. GF mice showed higher GPX and TXNRD1 activities in the

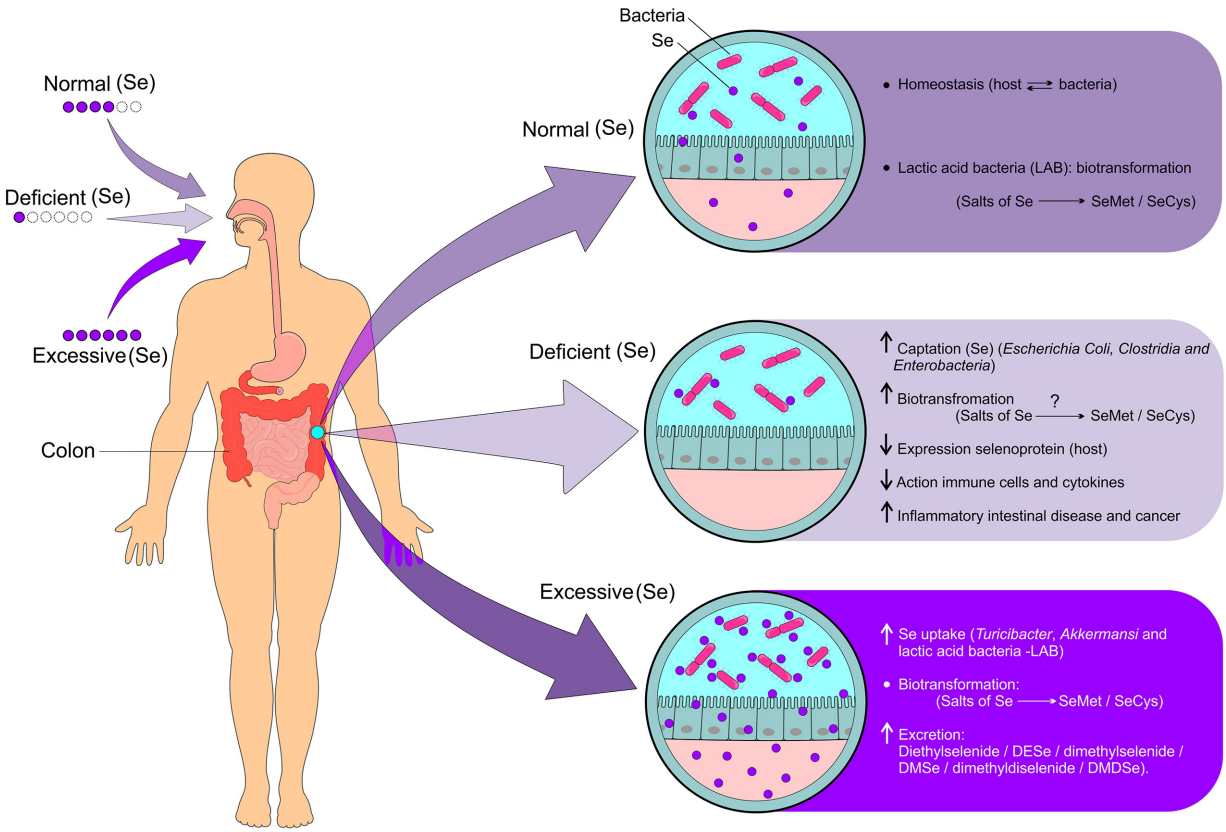

FIGURE 5 | Modulation of the gut microbiota dependent on Se status and biotransformation of Se derivatives. Given the adequate intake of Se, homeostasis occurs due to the beneficial relationship between intestinal and host bacteria resulting in the biotransformation of Se compounds (Se salts metabolized into SeMet and SeCys). Se deficiency results in increased Se uptake by bacteria (*Escherichia coli, Clostridia,* and *Enterobacteria*), biotransformation of Se compounds (Se salts metabolized into SeMet and SeCys), decreased expression of selenoproteins by the host, decreased activation of Se immune cells, increased pro-inflammatory cytokines, and increased risk for IBD and cancer. On the other hand, excessive intake of Se causes increased uptake by bacteria such as *Turicibacter, Akkermansi,* and Lactic acid bacteria (LAB), biotransformation of Se compounds such as selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) which are metabolized into SeMet and SeCys, and increased excretion of volatile compounds from Se. Se, selenium; SeMet, selenomethionine; SeCys, selenocysteine; IBD, inflammatory bowel diseases (Figure illustration by Francisco Irochima Pinheiro).

intestine and liver, greater expression of GPX1 in the liver and GPX2 in the proximal and distal jejunum and colon, as well as greater activity of GPX1 and GPX2 in the colon. The study indicated that GF animals have less need for Se for selenoprotein biosynthesis than conventionally colonized animals. In addition, it has been observed that colonized animals have a higher risk for developing selenoprotein deficiency when the supply of Se becomes limited (94).

Another study has demonstrated that several inorganic and organic selenocompounds were metabolized to SeMet by the gut microflora of rats and that SeMet was incorporated into bacterial proteins. Proteins containing SeMet, available as a Se pool for the host animal, were accumulated in the gut microflora. The main urinary selenometabolite, SeSug1, was transformed into a nutritionally available selenocompound by the intestinal microflora. Finally, positive effects on the bioavailability of some bioselenocompounds, such as SeCN, MeSeCys, and SeSug1, were observed in the gut microflora (102).

Some bacterial species are able to benefit from Se by triggering some effects on bacterial pathogenesis. Faced with an infection by this type of bacteria, a complex interaction takes place between the host's immune response, the microbial pathogen, the microbiota, and the host's Se status. Bacteria that have Se-dependent enzymes can survive under anaerobic conditions in the mammalian gut. As a result, these bacteria benefit from the host by using Se to increase its virulence and pathogenicity (103).

Se deficiency can leave the individual immunocompromised, allowing the survival of bacteria that do not need Se to establish an infection and cause disease. The host's microbiota may also differ in the presence of Se, which can prevent infection by Se-dependent bacteria, either by competition for Se or by the production of toxic metabolites that can be harmful to pathogenic bacteria (103).

Selenium, Microbiota, and Toxicity

The role of the intestinal microbiota in the excretion of SeMet and selenite has been investigated in rats. It has been reported

that the excretion of excess of SeMet and selenite occurs through the production of methylated derivatives of Se and elemental Se from the biotransformation of L-selenomethionine and selenite (104). Another study corroborates this hypothesis by showing that the gut microflora of rats can metabolize L-SeMet to some metabolites (77).

Bacterial count and protein analysis have shown that the number of cells and protein concentrations in the cecum and colon suspensions of rats are similar, but the cecum microbiota of these animals may contain more metabolically active microorganisms for SeMet and selenite compared to those in the colon microbiota. Given the much larger relative size of the colon in humans, the metabolism of Se compounds in the human intestine is likely to occur mainly in the colon. The formation of these volatile compounds of methylated and elemental Se in the intestinal tract points to the role of the microbiota in protecting the host from toxicity due to high doses of Se supplements (104).

Significant increase in the absorption and distribution of cadmium and lead in the blood, gastrointestinal tract, kidneys, liver, and spleen were seen in germ-free mice exposed to cadmium or lead (5, 20, and 100 ppm) for 6 weeks in comparison to non-exposed animals. Thus, it seems that the microbiota act as a protective factor against heavy metals (105).

The role of Se has also been investigated against methylmercury (MeHg) poisoning though the modulation of gut flora and decomposition of this compound. Treatment with selenite for 90 days of rats poisoned with MeHg showed a modulation of flora abundance, specially *Bacteroidetes* and *Firmicutes* phyla. An increase in total mercury (THg) was found in fecal samples after treatment with Se on the 30th day. The percentage of MeHg in the poisoned group was between 81 and 105%, while 65–84% was found in the Se treated group, suggesting an increase in MeHg decomposition after treatment with Se (106).

Selenium, Microbiota, and Diseases

Se and selenoproteins may play an important role in signaling pathways that are involved in the pathogenesis of some diseases, especially IBD (107), cancer (108), thyroid dysfunction (109), and neurogenerative disorders (110). The Se status may impact the expression of nuclear factor-κB (NF-κB) transcription factors and peroxisome proliferator activated receptor (PPAR) γ, which are involved in immune cell activation that ultimately results in various stages of inflammation (107). Thus, Se deficiency and inadequate selenoprotein expression impair innate and adaptive immune responses, especially at the colonic level where an increase in inflammatory cytokines is observed (25). In addition, low intake of Se might result in a phenotype of the gut microbiota that is more susceptible to colitis and infection by *Salmonella typhimurium*. On the other hand, a diet with sufficient or high levels of Se can optimize the gut microflora for protection against intestinal dysfunctions and chronic diseases (101).

Selenium, Microbiota, and Inflammatory Bowel Diseases

Crohn's disease and ulcerative colitis are IBD characterized by microbial dysbiosis that result in changes in intestinal motility and secretion, visceral hypersensitivity (hyperalgesia), and failure in the intestinal-brain communication (111). Se deficiency is common among patients with IBD, reaching 30.9% of cases (112). The importance of Se in improving IBD is attributed to the ability of the selenoproteins in reducing the inflammatory response (113, 114).

The nuclear factor erythroid factor 2-related factor 2 (Nrf2) also appears to contribute to redox homeostasis in epithelial cells (115). In a study conducted using an animal model of IBD, the lack of Nrf2 led to increased expression of inflammatory cytokines, such as TNFα and IL6 and increased expression of COX2 (116). Nrf2 can also stimulate the expression of TXNRD and GPX under adequate concentrations of Se (117). This relationship was explored in another study that found a positive association between plasma concentration of Se and the expression of Nrf2-related genes (118). In addition, a study showed that the lack of Nrf2 increases NF-?B activity, further intensifying oxidative stress (119).

Zhu et al. (120) investigated the protective effect of Se nanoparticles with *Ulva lactuca* polysaccharide (ULP-SeNPs) on DSS-induced acute colitis in mice. The main benefits were the reduction of CD68 in the colon, modulation of IL-6 and TNF-α, inactivation of macrophages and suppression of nuclear translocation of NF-κB.

Bacteria with pro-inflammatory activity, such as *Escherichia* and *Fusobacterium*, are increased in patients with IBD, whereas anti-inflammatory species such as *Faecalibacterium, Roseburia Clostridium coccoides, Clostridium leptum, prausnitzii,* and *Bifidobacterium* are reduced in this disease (121, 122). Other phyla of bacteria have been associated with the ingestion of Se in individuals with IBD. Dietary Se was positively correlated with the presence of *Firmicutes* and negatively correlated with *Verrucomicrobia* in patients with Crohn's disease and ulcerative colitis, respectively (123).

Animals treated with SeCys and selenocystine showed a reduction in the concentration of ROS and malondialdehyde (MDA), as well as an increase in intestinal activity of SOD and GPX, which seems to indicate a protective effect against damage to the gut mucosa. In addition, the levels of IL-1, MCP, IL-6, and TNF-α were significantly reduced in the group treated with SeCys (124).

Xu et al. (125) reported that the administration of Se nanoparticles with *Lactobacillus casei* ATCC 393 (*L. casei* 393-SeNPs) protected mice from intestinal barrier dysfunction and oxidative stress associated with enterotoxigenic *Escherichia coli* infection K88 (ETEC K88), when compared to the animals supplemented with *L. casei* alone. These findings suggest the ability of *L. casei* 393-SeNPs in maintaining intestinal epithelial integrity.

Selenium, Microbiota, and Cancer

The specific link between gut microbiota, selenium status, and cancer is difficult to establish, and multiple mechanisms may be involved in the complex interplay between microbiome, diet, and human host. It has been demonstrated that dietary Se affects both composition of the intestinal microbiota and colonization of the gastrointestinal tract, which, in turn, influence the host

Se status and selenoproteome expression (99). The effect of the gut microbiota on selenoproteins and other molecules linked to redox homeostasis and those linked to the WNT/β-catenin signaling pathway may have an impact on the regulation of oxidative stress, apoptosis, inflammation, and immune response, suggesting a direct influence on increased risk of cancer (108).

Considering that Se uptake by intestinal microbiota occurs in conditions of imbalance, it might negatively impact the supply of Se to the host, therefore predisposing to cancer and gut dysfunctions. Deficiency of selenoproteins and molecules linked to redox homeostasis can lead to a gut microbiota phenotype that is more vulnerable to colitis, pathogen infections, and cancer (101). Lower expression of different selenoproteins have been described in colorectal adenomas and cancer tissues, while higher SELENOP concentrations were inversely associated with colorectal cancer risk (126).

Bacteria of the *Dorea* sp. genus, one of the most common species of the gut microbiota, are increased in conditions of deficiency of Se (101) and are associated with IBS, cancer, multiple sclerosis, and non-alcoholic liver disease (101, 127–130). Se deficiency and inadequate selenoprotein expression impairs innate and adaptive immune responses with higher levels of inflammatory cytokines, especially at colonic level. The effect of the gut microbiota on selenoproteins and other molecules linked to the redox homeostasis may have an impact in the regulation of oxidative stress, apoptosis, inflammation, and immune response, which appears to have a direct influence on cancer risk and development (108, 131).

On the other hand, the administration of probiotics enriched with organic Se seems to by a promising alternative for elimination of pathogenic bacteria in the case of IBD and colon cancer (132). Likewise, Porto et al. (133) showed that oral administration of *Saccharomyces cerevisiae* enriched with Se reduced eosinophil peroxidase activity, histopathological tissue damage and oxidative stress (lipid peroxidation and nitrite production) in the small intestine of mice. Therefore, clinical studies involving the biological function and bioaccessibility/bioavailability/bioactivity of selenoproteins and selenometabolites in different functional foods enriched with Se and nutraceuticals are highly recommended in order to confirm the findings of preclinical studies.

Selenium, Microbiota, and Thyroid Dysfunctions

The thyroid gland contains the highest amount of Se per mg of tissue in the body. Several proteins involved in thyroid metabolism contain Se, namely GPX (type I and II), DIOs, and TXNRD. Resident microbes of the colon metabolize Se, which is not absorbed by the host in the upper gastrointestinal tract. Microbes influence thyroid levels by regulating iodine uptake, degradation, and enterohepatic cycling. In addition, some minerals play an important role on interactions between host and microbiota, particularly selenium, iron, and zinc (134).

Besides having beneficial effects on the activity of the immune system, a healthy gut microbiota positively influences the thyroid function. Although dysbiosis has been found in autoimmune thyroid diseases (AITDs), it has also been reported in patients with thyroid carcinoma, in which an increased number of carcinogenic and inflammatory bacterial strains were observed. In addition, the composition of the gut microbiota has a major influence on the availability of essential micronutrients for the thyroid gland such as Se and zinc, which are co-factors for deiodination reactions that convert thyroxine (T4) into triiodothyronine (T3). Deficiency of these minerals might result from restrictive or unbalanced diets at any stage of life, which leads to a decreased production of thyroid hormones (135, 136).

The microbiota influences the uptake of Se and may alter the availability of L-thyroxine and toxicity of propylthiouracil (PTU) (134). In case of normal levels of Se, the thyoredoxin reductase system and SH-Px protect the thyrocytes from the activity of peroxides, whereas the apoptotic response to H_2O_2 is increased with Se deficiency (136). For instance, the decrease in the levels of *Lactobacillus* can interfere with the formation of iodothyronine deiodinases (DIOs) and, consequently, might result in thyroid dysfunctions (109, 137).

Several species of *Lactobacillus* are able to keep sodium selenite intracellularly as SeCys and SeMet, thus providing a more bioavailable form of Se, whose absorption by human cells is usually poor in its inorganic form (138). Therefore, the decrease in the amount of *Lactobacillus* in patients with thyroid disease might impart the bioavailability of Se and its role in the transformation of activated thyroid hormone. In addition, Se protects against oxidative damage during the synthesis of other hormones (109).

In a cohort study, the relationship between the gut microbiome, thyroid cancer, and thyroid nodules was confirmed. Among the findings, a relative abundance of *Butyricimonas* ($p < 0.001$) and a significant lower amount of *Lactobacillus* ($p < 0.001$) was observed in the group with thyroid cancer and in the group of thyroid nodules, respectively (86). The authors point out to the fact that *Lactobacillus* is an important genus in the human intestine that is able to improve the concentration of various metals in human cells including Se.

In human and rats, it has been proven that large amounts of conjugated iodothyronines can be hydrolyzed in fecal suspension. The diversity and structure of gut microbiota may play several roles in regulating the drug-controlled thyroidal metabolism (139). Some studies have corroborated that thyroid disorders are the causal factor in the relationship with gut microbes. Other studies have demonstrated that bacteria might act as the motivating factor, as thyroid function may be impaired in patients with small intestinal bacterial overgrowth (140, 141). However, the causative role of Se deficiency, thyroid and gut microbiota has not been thoroughly ascertained yet and further clinical studies are highly recommended.

Selenium, Microbiota, and Cardiovascular Diseases

The metabolic potential of gut microbiota has been identified as a contributing factor for the development of CVD (142). The intestinal microbiota produces signaling molecules such as lipopolysaccharide (LPS) and peptidoglycans that interact with host mucosal surface cells, often through the pattern recognition receptors (PRR) (143). In addition, the gut microbiota interacts with the host through the trimethylamine (TMA)/trimethylamine-N-oxide (TMAO) and short-chain fatty

acids routes as well as through other routes related to biliary acids. Some of these molecules have shown to functionally interact with ghrelin, leptin, glucagon-like peptide 1 (GLP-1), and peptide YY (PYY), and to stimulate the parasympathetic nervous system. Such activities impact the metabolic processes related to the development of risk factors for CVD (142).

TMAO has gained considerable attention as a potential promoter of atherosclerosis, cardiometabolic diseases, arterial hypertension, ischemic stroke, atrial fibrillation, heart failure, and acute myocardial infarction (142, 144). Mice supplemented with choline or TMAO showed increased risk of thrombosis, in contrast to germ-free mice under the same diet, suggesting that gut microbiota and specific dietary nutrients that enhance TMAO generation seems to modulate platelet function and thrombosis potential *in vivo* (145).

Phosphatidylcholine and L-carnitine are metabolized by the intestinal microbiota producing trimethylamine gas (TMA), being further metabolized to TMAO by the liver enzymes of the host (144). A variety of enzymes are involved in the production of TMA from dietary components (146). Glycine betaine reductase (GrdH) is an enzyme that requires Se and is responsible for the production of TMA from glycine betaine (147). However, the role of Se in the TMA-generating pathways remains to be elucidated.

The role of gut microbiota in the oxidative stress process occurs through the uric acid metabolism. Higher levels of *Escherichia coli* result in greater uric acid decomposition, whereas elevated serum uric acid levels in patients with coronary heart disease are related to gut microbiota dysfunction. High levels of serum uric acid increase the production of oxygen free radical and induce endothelial dysfunction (148). Circulating Se is inversely associated with acid uric levels, suggesting the role of selenium in regulating the intracellular redox status (149).

Patients with type 2 diabetes and coronary heart disease have shown reduced hs-CRP, fasting blood glucose, insulin levels, HOMA-IR and increased nitric oxide (NO), total antioxidant capacity (TAC), and glutation (GSH) after use of 200 μg/day of Se and 8 x 10^9 CFU/day of *Lactobacillus acidophilus, Lactobacillus reuteri, Lactobacillus fermentum,* and *Bifidobacterium bifidum* (2 x 10^9 CFU/g each) for 12 days (150).

The bioavailability of Se on *Enterococcus faecium* CCDM 922A (EF) and *Streptococcus thermophilus* CCDM 144 (ST) and their respective forms enriched with Se, SeEF, and SeST, improved their antioxidant status in animal models (151). Selenium works by blocking the activation of nuclear factor-kB through modulation of expression of selenoprotein genes and by inhibiting the production of reactive oxygen species (ROS) (152). Moreover, probiotic may reduce inflammatory factors and oxidative damage by producing short chain fatty acids in the gut and by decreasing the production of free radicals (153). Probiotic and Se co-supplementation in diabetic patients with coronary heart disease showed beneficial effects on indicators of metabolic profiles related to cardiovascular disease.

Despite multiple human clinical studies revealing associations between gut microbiota composition with the development of cardiovascular diseases, few studies have provided mechanistic or causal evidence of a direct role of Se in gut microbiota in this context.

Selenium, Microbiota, and Glycemic Disorders

A study has shown that mice fed with a high-fat diet presented high plasma concentrations of LPS, which is a gram-negative bacterial translocation marker that is strongly related to insulin resistance, obesity, and diabetes. However, such LPS-induced metabolic responses were not observed in CD14 mutant mice, suggesting that the LPS/CD14 system may define the intensity of insulin sensitivity and related diseases (154). In this context, the presence of *Bifidobacterium* was associated with lower concentrations of LPS in the intestine, which resulted in a lower incidence of metabolic diseases (155). In addition to reducing the systemic inflammatory response, *Bifidobacterium* reduces intestinal permeability in patients with DM2 (156).

The antidiabetic effects of *Bifidobacterium* were more responsive when administered together with Se. *Bifidobacterium* enriched with sodium selenite (*B. longum* DD98, Se-B) mitigated oral glucose tolerance in diabetic mice, suggesting increased insulin sensitivity and protection of pancreatic β cells. These effects were dose dependent indicating the importance of administering adequate doses for better effectiveness of *B. longum* DD98, Se-B (157). Wei et al. (69) also evaluated the combined supplementation of Se with microorganisms in diabetic C57BL/6 mice, reporting that treatment with aqueous extracts of selenium-enriched *Auricularia auricular* (AESA) relieved liver damage triggered by oxidative stress in mice with DM.

Other mechanism involved in the prevention and treatment of insulin resistance relates to the production of short-chain fatty acids (SCFAs), especially butyrate (158). Increased concentration of butyrate in DM2 mice supplemented with live multi-strain probiotics was able to reduce HbA1C levels, improving glucose tolerance and insulin resistance (159). In addition, the administration of Se nanoparticles (0.9 mg/kg) demonstrated an increase in butyrate and in the amounts of beneficial bacteria such as *Lactobacillus* and *Faecalibacterium* (160). High concentrations of butyric acid, acetic acid, and isobutyric acid were identified in the feces of mice after oral administration of *B. longum* DD98, Se-B (157).

The positive effects of butyrate on insulin seems to be associated with an increase in the levels of GLP-1, which in turn lowers blood glucose in patients with DM2 (161) demonstrated that the administration of probiotic, VSL # 3, prevented and treated obesity and diabetes in mice. The mechanism discussed involves the probiotic-gut flora-butyrate-GLP-1 axis which is capable of promoting enhanced metabolic efficiency. Considering that supplementation with *B. longum* DD98, Se-B also resulted in increased secretion of GLP-1 and protected β cells, it has been speculated whether Se acts on this axis as a modulator of the deleterious effects caused by DM (157).

Selenium, Microbiota, and Neurological Diseases

With the discovery that some bacteria species produce chemicals similar to hormones and monoaminal neurotransmitters in the intestine, the microbiota-intestine-brain axis became evident. This bidirectional interaction allows the brain to influence the gastrointestinal functions as well as the immune functions (162). Oral administration of heat-killed *Candida kefyr* decreased the

severity of experimental autoimmune encephalomyelitis (EAE), significantly reduced Th17 cells and increased regulatory T dendritic cells (DC) and CD103+. In addition to changes in intestinal immunity, changes in the microbiome have been observed such as increase in *Lactobacillales* and decrease in *Bacteroides* contents (163). In another study, the administration of Lactobacillus reuteri DSM 17938 modulated the immune response in EAE, decreasing T_H1/T_H17 cells and cytokines IFN-γ/IL-17 (164).

Neurodegenerative disorders are characterized by an increase in the production of reactive oxygen species (ROS) and a decline in the blood-brain barrier function. Due to the antioxidant property of Se, some selenoproteins play a neuroprotective role (110). TXNRD, for instance, maintains the redox balance and protects the dopaminergic cells, which are prone to oxidative stress in the pathophysiology of Parkinson's disease (165).

Fraga-Silva et al. (166) showed that the administration of Saccharomyces cerevisiae enriched with Se (Selemax) reduced the prevalence of EAE, increased the number of CD103 + dendritic cells and reduced the intestinal inflammatory process compared to the administration of *Saccharomyces cerevisiae* alone. In addition, Selemax supplementation demonstrated a neuroprotective effect by increasing the expression of protein tau in the CNS. Tau is the main protein associated with the stability of neuronal microtubules along with the MAPs (MAP1 and MAP2) (166). Long-term dietary supplementation (3 months) with Se-enriched yeast (Se-yeast) in triple transgenic mouse model of Alzheimer disease (AD), significantly improved spatial learning, retention of neuronal memory and activity (167).

In a study using humans, probiotic containing *Lactobacillus* acidophilus, *Bifidobacterium bifidum* and *Bifidobacterium longum* (2×10^{9} UFC/day each) and selenium co-supplementation (200 μg/day, sodium selenite) administered in patients with AD resulted in improved cognitive function and enhanced metabolic profile (168).

DISCUSSION

Recently, the role of Se in gut health has attracted the interest of the scientific community. This review points out that Brazil nuts, cereals, meat, fish, seafood, milk, and nuts are the best sources of Se and that both deficiency and excess of this metal are related to the occurrence of some diseases. Studies using human and rodent have shown that different doses and sources of Se supplementation can modulate gut microbiota with a positive or negative impact on the host's health (99, 100, 169). The microbial absorption of Se in the large intestine of rats was estimated to be 40–46% of the whole oral dose of Se. In addition, dietetic SeMet increased both fermentation and SCFA production in rats (170). The role of Se in the gut microbiota needs to be better investigated in humans as most studies have been conducted in animal models.

Both the structure and composition of the gut microbiome are significantly affected by genetic and external factors. Among the external factors, dietary pattern is the one that most rapidly alters the gut microbiome in real time, having important role in human health and in the development of chronic diseases (171). Moreover, growth and aging results in physiological changes that modify the gut microbiota (172). The composition of the gut microbiota can also be modulated by metals, therefore requiring a variety of cellular processes such as the system of capture of metal ions by bacteria or high affinity transporters (86). Until now, no specific Se carrier has been identified (84).

A systematic review with meta-analysis including randomized controlled trials on nuts consumption demonstrated a significant increase in the gut content of *Clostridium, Dialister, Lachnospira,* and *Roseburia*, as well as a significant decrease in *Parabacteroides* (60). This finding suggests that high consumption of nuts (a rich source of Se) regulates gut microbiota and promotes the expression of selenoproteins. An *in vivo* study using mice as the experimental model reported that Se supplementation can optimize the gut microbiota for protection against intestinal dysfunction (101). However, randomized clinical trials are necessary in order to investigate the real impact of Se supplementation on the microbiota and selenoprotein synthesis due to the lack of high-level evidence in the scientific literature.

It has been reported that Se plays a key role in cellular and paracellular permeability, as well as in cellular redox balance and inflammatory cell infiltration (173). Consistent with this finding, Se-deficiency adversely affected the gut barrier function and induced disturbances in the intestinal and immune responses in mice (169). Such events has recently been implicated in several chronic diseases ranging from IBD, DM2, and CVD to cancer and thyroid dysfunctions (135, 136, 169, 174, 175).

The metabolic pathways of selenium biotransformation in gut microbiota remain unclear, even though some bioproducts from Se metabolizing organisms enriched with sodium selenite have been manufactured (137). The rationale behind the use of selenium and probiotic co-supplementation is based on the antioxidant and anti-inflammatory effects of this treatment as observed in the metabolic responses of animal models. For instance, it has been reported that a 4-week probiotic and selenium co-administration to mice under a high-fat diet led to a significant decrease in MDA levels (176). Se-enriched probiotics present themselves as a less toxic alternative to supplementation and have demonstrated a protective effect against liver damage in rats (177) and possible antioxidant, anti-inflammatory, and anti-apoptosis properties (178).

This current review has shown that the composition of the microbiota can be modulated by the dietary Se, in which it can influence both the Se status of the host and the expression of the selenoproteoma. In return, the organism provides the nutrients used by bacteria for energy production and maintenance of their metabolic pathways, therefore characterizing a symbiotic relationship. The gut microbiota can interact with Se for the expression of its own selenoproteins. In addition, some species of intestinal microorganisms can improve the bioavailability of Se and protect against its toxicity. One question that remains unanswered is what constitutes an optimal health-promoting microbiome.

Ultimately, determining the full landscape of host-microbiota interactions and Se status will enable advances in the development of bioproducts involving selenium metabolizing

microorganisms, which seems to be a safe alternative for studies about Se supplementation.

AUTHOR CONTRIBUTIONS

LP coordinated the elaboration of the manuscript. RF, KS-E, RC, and LP developed the layout of the manuscript, collected literature, and wrote the manuscript. FP collaborated with the layout of the manuscript and drew all figures. EA translated the entire text to English. LP and EA edited the final version of the manuscript. RF assisted in the reference management. All authors participated in the analysis and interpretation of data as well as in writing the manuscript. All authors approved the submitted version.

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Selenium Status Is Associated With Insulin Resistance Markers in Adults: Findings From the 2013 to 2018 National Health and Nutrition Examination Survey (NHANES)

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Although literature has been consistently showing an increased risk of type 2 diabetes (T2DM) in populations with high exposure to selenium, there is a lack of information quantifying the association between diabetes-related markers and the nutritional status of selenium. Therefore, we aimed to investigate the association between blood selenium concentration and glucose markers in a representative sample of the US population, which is known to have moderate to high exposure to selenium. This cross-sectional analysis included 4,339 participants ≥18 years from the 2013 to 2018 National Health and Nutrition Examination Survey (NHANES). All participants were assessed for whole blood selenium concentration, fasting plasma insulin and glucose, HbA1c, and HOMA-IR (Homeostatic Model Assessment for Insulin Resistance). In this cohort, all participants presented with adequate selenium status [196.2 (SD: 0.9) μg/L] and 867 (15%) had diabetes mellitus. Selenium was positively associated with insulin, glucose and HOMA-IR in models adjusted for age and sex. When the models were further adjusted for smoking status, physical activity, metabolic syndrome and BMI, the associations with insulin and HOMA-IR remained but the association with glucose was no longer significant. A 10 μg/L increase in selenium was associated with 1.5% (95% CI: 0.4–2.6%) increase in insulin and 1.7% (95% CI: 0.5–2.9%) increase in HOMA-IR in fully adjusted models. There was no evidence of an association between selenium and diabetes prevalence. Our findings corroborate the notion that selenium supplementation should not be encouraged in populations with high dietary intake of selenium.

Keywords: selenium, diabetes, glucose, insulin, insulin resistance

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INTRODUCTION

Selenium is an essential micronutrient to human life, as it is required for the synthesis of the 21st amino acid selenocysteine, the defining feature of 25 selenoproteins identified in humans. The selenoproteome is small and functionally diverse. Selenoproteins are recognized as crucial for antioxidant response, as well as for

immune system and thyroid hormone regulation, and heavy metal and xenobiotic detoxification [reviewed by Roman et al. (1)], but around half the identified human selenoproteins remain largely uncharacterized (2).

Diet is the principal source of selenium, and selenium intake is reflected by corresponding concentrations in the soil where crops are grown (3). Inadequate selenium intake, which affects one in seven people in the world (3), has been associated with increased risk of cancer (4), neurodegenerative diseases (5–7) and thyroid dysfunction (8, 9). This has led to empirical health advice recommending increasing selenium intake through diet or supplementation. As a result, selenium supplements have been widely consumed under the understanding that "the more the better." Nonetheless, recent studies have indicated that high selenium consumption is associated with an increased risk of chronic diseases such as diabetes (10) and non-alcoholic fatty liver disease (11), and all-cause mortality (12). These studies corroborate the hypothesis that the metabolic outcomes of selenium in the human body follow a U-shaped curve (13, 14), meaning that selenium intake within the correct range is critical for human well-being, with either too high or too low being prejudicial.

First evidence linking high selenium consumption and type 2 diabetes (T2DM) was derived from a secondary analysis of a trial where selenium (200 μg/day) was provided to over 1,200 non-melanoma skin cancer patients aiming to evaluate the efficacy of selenium supplementation for prevention of cancer. After 7.7 years of intervention, no cancer protective effects were observed and, as an unexpected secondary outcome of the trial, the researchers reported an increased risk for T2DM in the selenium group compared to placebo (hazard ratio: 1.55; 95% CI: 1.03, 2.33) (15). More recently, meta-analyses of human studies revealed that plasma selenium concentrations of 140 μg/L were associated with a 3.6-fold increased risk of T2DM when compared with the reference category (45 μg/L) (10), although data from randomized clinical trials (RCTs) reported by a systematic review did not confirm an eventual negative effect of selenium on the incidence of the disease (Odds Ratio: 1.18; 95% CI: 0.95, 1.47) (16). Despite limited information on selenium supplementation to individuals with T2DM, a systematic review looking into the effectiveness of selenium supplementation in adults with T2DM reported that selenium treatment had no effect on HbA1c, and fasting blood glucose and insulin in four RCTs. However, one study reported an increase in fasting plasma glucose, and two studies reported a decrease in insulin resistance, assessed as fasting plasma insulin, HOMA-IR (Homeostatic Model Assessment for Insulin Resistance) and HOMA-B (Homeostasis Model Assessment of β-cell dysfunction) (17).

Although literature has been consistently showing an increased risk of T2DM in populations with high exposure to selenium, as recently demonstrated in Americans assessed in the National Health and Nutrition Examination Survey (NHANES) (18), there is a lack of information identifying diabetes-related markers associated with nutritional status of selenium. Therefore, we aimed to investigate the association between selenium status, measured as blood selenium, and glucose markers in a representative sample of the US population, which is known to have moderate to high exposure to selenium (19, 20).

METHODS

Study Population

This cross-sectional analysis included participants ≥18 years of age in three National Health and Nutrition Examination Survey (NHANES) cycles: 2013–14, 2015–16, and 2017–18. NHANES was conducted by both the Center for Disease Control and the National Center for Health Statistics, and utilizes a complex, multistage, probability-sampling procedure to provide nationally representative estimates on the health and nutritional status of non-institutionalized US residents (21). The NHANES protocol was approved by the National Center for Health Statistics (NCHS) Research Ethics Review Board. Informed consent was obtained from all participants included in the study (22).

In the NHANES cycles included in this study, a total of 17,961 participants ≥ 18 years old were interviewed. According to the complex NHANES survey design, only one-half sample from participants in the NHANES aged 12 years and older was randomly eligible for blood selenium assessment; those who were examined in the morning session were eligible for fasting glucose and insulin assessment. Individuals were excluded from our analysis if they had missing data for: (i) selenium; (ii) glucose or insulin markers; (iii) any variable necessary for the definition of metabolic syndrome (blood pressure, triglycerides, HDL, waist circumference, blood glucose, with parameters as defined under the Covariates heading, below); (iv) covariates included in the models (physical activity, BMI). After exclusions, the total number of participants for our analysis was 4,339 (**Figure 1**).

Glucose and Insulin Measures

Glycohemoglobin (Hb1Ac) was measured using a Tosoh Automated Glycohemoglobin Analyser HLC-723G8. Fasting glucose was measured in the serum enzymatically by a hexokinase-mediated reaction using a chemistry analyser (Roche/Hitachi Cocas C Chemistry Analyser). Serum insulin was measured by the Elecsys 2010 Insulin chemiluminescent sandwich immunoassay. HOMA-IR was calculated according to the formula: fasting insulin (μU/L) × fasting glucose (mmol/L)/22.5. Individuals were considered as having diabetes if they reported current use of prescribed medication (including insulin) to control glucose levels, or presented with HbA1c ≥ 6.5% or fasting glucose ≥ 126 mg/dL (23).

Selenium

Selenium was measured in whole blood by inductively coupled plasma mass spectrometry (ICP-MS), that monitored the ion intensity at m/z 80 (^{80}Se). Polyatomic interferences in the analysis were reduced by using methane as a reaction gas. The lower limit of detection was 24.5 μg/L; no values below this were reported in the dataset (24).

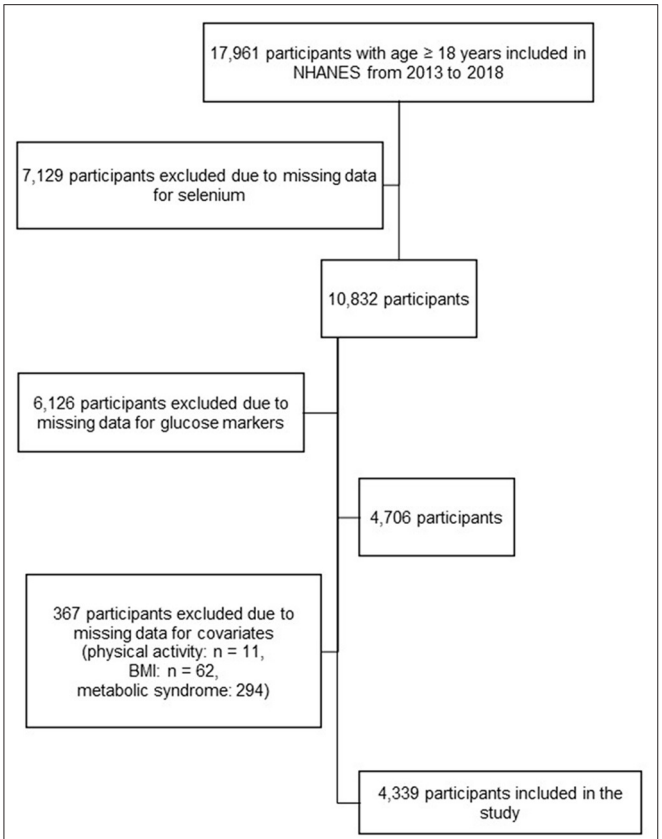

FIGURE 1 | Selection of the study population, from the National Health and Nutrition Examination Survey (NHANES) from 2013 to 2018.

Covariates

Participants completed a self-reported demographic questionnaire that included questions about age, sex, race, and smoking status. Racial groups were categorized as Mexican American, other Hispanic, Non-Hispanic White, Non-Hispanic Black, Non-Hispanic Asian, Other non-Hispanic. Current smokers were identified as those who responded "every day" or "some days" to the question "Do you now smoke cigarettes?." Anthropometric measurements were performed by trained health technicians while the participants wore a standard examination gown. Waist circumference was measured at the superior lateral border of participants' iliac crest and recorded as cm. A digital scale was used to measure body weight, and height was measured using a stadiometer. BMI was calculated as weight (kg) divided by squared height (m^2), and then rounded to one decimal place. Physical activity was assessed using the Global Physical Activity Questionnaire. Sum of daily moderate and vigorous physical activity was calculated by multiplying the frequency per week by the duration (minutes) of the physical activity divided by seven. Z-scores for moderate/vigorous activity were generated using the mean and standard deviation of the NHANES ≥ 18 years population. Metabolic syndrome was defined according to the harmonized definition that considers the presence of at least three of the following conditions: (i) elevated waist circumference (≥88 cm for women or ≥102 cm for men; (ii) elevated triglyceride levels (≥150 mg/dL); (iii) low HDL levels, characterized as ≤50 mg/dL for women, ≤40 mg/dL for men, or current use of prescribed medication to treat high cholesterol; (iv) elevated blood pressure, identified as systolic blood pressure ≥130 mmHg, diastolic blood pressure ≥85 mmHg, or current use of prescribed medication to treat high blood pressure; (v) elevated fasting blood glucose levels, characterized by ≥100 mg/dL or current use of prescribed medication or insulin to treat hyperglycaemia (25).

Statistical Analysis

Data analyses included complete data of survey participants and used sample weights provided by NHANES for blood selenium in order to account for the complex NHANES survey design including nonresponse and oversampling. Demographic and clinical characteristics were presented as mean with standard deviation (SD) for continuous variables, or number (% weighted) for categorical variables. The glucose markers were log (base e) transformed based on inspection of the quantile-quantile plots. The association between selenium concentration and the outcomes (log HbA1c, log glucose, log insulin, and log HOMA-IR) was examined using two multivariable linear regression models. Model 1 was adjusted for age and sex; Model 2 was further adjusted for smoking status (smokers/non-smokers), physical activity (*Z*-score), BMI (kg/m^2), and metabolic syndrome (yes/no). The linearity of the associations was explored visually by fitting a restricted cubic spline with four knots (at 5th, 35th, 65th, and 95th percentiles of selenium concentration). The association between the prevalence of diabetes and selenium concentration was investigated using Poisson regression using selenium as a continuous variable. Selenium concentration was compared between individuals with and without diabetes using linear regression. Two models with the same covariates as mentioned above for models 1 and 2 were tested.

Effect modification analyses were performed in order to explore whether the relationship between selenium and the outcomes was modified according to age, sex, presence of metabolic syndrome or diabetes, smoking status, BMI, or physical activity by including the interaction term between selenium and one of the covariates in model 2.

Statistical analysis was performed with STATA/SE 16.0 for Windows (StataCorp LLC). Confidence intervals and *P*-values were reported two-sided without adjustment for multiple testing.

RESULTS

Table 1 displays the characteristics of NHANES participants included in this analysis. The average age of the participants was 47.3 years. Females accounted for 51% of the study sample, and the majority self-reported as non-Hispanic white (64%). The study population presented with similar demographic characteristics when compared with the excluded individuals (**Supplementary Table 1**). Overall, BMI was in the overweight range, with 1,698 (39%) individuals presenting with obesity (BMI ≥ 30). HDL-cholesterol was below reference cut-offs

TABLE 1 | Demographic and clinical characteristics of US adults in the NHANES 2013–2018.

	Study sample (*n* = 4,339)
Age, years	47.3 (0.4)
Females, *n* (%)	2,242 (50.9%)
Racial group, *n* (%)	
Mexican American	662 (9.4%)
Other Hispanic	474 (6.5%)
Non-Hispanic White	1,581 (64.4%)
Non-Hispanic Black	891 (10.4 %)
Non-Hispanic Asian	549 (5.3%)
Other non-Hispanic	182 (4.0%)
Smoking status, *n* (%)	
Current smoker	780 (16.8%)
Non-smoker	3,559 (83.2%)
Physical activity, *z*-score	−0.03 (0.02)
BMI, kg/m^2	29.3 (0.2)
Waist circumference, cm	100.1 (0.5)
HbA1c, %	5.64 (0.02)
Fasting insulin, U/mL	12.5 (0.3)
Fasting glucose, mg/dL	6.00 (0.03)
HOMA-IR	3.67 (0.10)
HDL-cholesterol, mg/dL	55.0 (0.5)
Blood pressure, mm/Hg[a]	
Systolic	122.4 (0.4)
Diastolic	70.0 (0.4)
Triglycerides, mg/dL	126.0 (1.9)
Metabolic syndrome, *n* (%)	2,012 (42.9%)
Diabetes mellitus, *n* (%)	867 (15.0%)
Selenium, μg/L	196.2 (0.9)

All values are mean with standard deviation (SD) or number (proportions, %). [a]n = 4,287; Metabolic syndrome was defined according to Alberti et al. (25); Diabetes mellitus was defined based on participants' self-reported current use of prescribed medication (including insulin) to control glucose levels, or HbA1c ≥ 6.5% or fasting glucose ≥ 126 mg/dL (23). BMI, Body Mass Index.

(≥60 mg/dL), while blood pressure was considered within normal ranges overall. Prevalence of metabolic syndrome in this population was 43% and 15% of the participants met the criteria for diabetes mellitus.

All the participants were considered selenium-replete given that selenium in blood was above the cut-off established reflecting the minimum concentration to maximize activity of the selenoprotein glutathione peroxidase (GPx; 84–100 μg/L) (26) (**Table 1**).

In the model adjusted for sex and age, selenium concentration was positively and linearly associated with insulin, fasting glucose and HOMA-IR. After further adjustment for smoking status, physical activity and presence of metabolic syndrome, a 10 μg/L increase in selenium was associated with 1.5% (95% CI: 0.4–2.6%) increase in insulin and 1.7% (95% CI: 0.5–2.9%) increase in HOMA-IR (**Table 2**; **Figure 2**). Selenium concentration was not significantly associated with the prevalence of diabetes mellitus in this population in either of the two models (Model 1: Estimate: 1.00, 95% CI: 1.00, 1.01; Model 2: Estimate: 1.00, 95% CI: 1.00, 1.00). No significant difference was observed in selenium concentration between individuals with and without diabetes (mean difference: −3.9 μg/L, 95% CI: −8.8 to 0.85, $P = 0.104$).

Seven models for sensitive analysis were performed containing interaction terms between selenium and one of the covariates included in the fully adjusted model, but no interaction was identified (all $P \geq 0.2$) (**Supplementary Table 2**).

DISCUSSION

Despite current evidence showing an increased risk of diabetes amongst individuals with higher selenium intake and increased concentration in different blood markers, the literature lacks information on the association between selenium status and diabetes-related markers. By investigating diabetes-related markers, our study is designed to shed light on potential mechanisms disturbed by high levels of circulating selenium. In this cross-sectional analysis of a selenium-replete population of US adults, blood selenium concentration was positively associated with insulin concentration and HOMA-IR, markers of insulin resistance. Selenium status was also associated with fasting glucose; however this association was no longer significant when the model was further adjusted for smoking status, physical activity, metabolic syndrome and BMI. Finally, there was no evidence of an association between selenium and diabetes mellitus.

To our knowledge, our study is the first to investigate the association between selenium status and diabetes-related markers. In our analysis, only markers of insulin resistance (insulin concentration and HOMA-IR), but not glucose, were associated with selenium in the fully adjusted model. Our findings corroborate the hypothesis that high selenium status disturbs insulin metabolism leading to hyperinsulinemia (27, 28), but the effect on glucose is not as strong, as it can be mitigated when controlling strong risk factors for T2DM such as body weight and metabolic syndrome. In our study, no association between selenium status and the prevalence of diabetes was observed, which counteracts the findings reported in two other studies conducted in the NHANES population (18, 29). Nonetheless, it is important to mention some key differences between our analysis and those studies. Bleys et al. (29) reported that the highest quintile of selenium in plasma (≥137.66 μg/L) was associated with OR = 1.57 (95% CI: 1.16, 2.13) when compared with the lowest quintile (<111.62 μg/L) in US adults, although no trend was observed for quintiles 2–4. In that study, selenium was assessed in plasma, which represents a short-term marker and therefore is more sensitive to acute dietary changes. Furthermore, the definition of diabetes used in that study was a self-report of a physician diagnosis of diabetes, which may be biased and result in prevalence of diabetes being over-estimated. Our study used more reliable criteria to define diabetes, reducing the risk of bias. More recently, Moon et al. (18) revealed that the increase of 10 μg/L in blood selenium increased the prevalence of diabetes by 12% (OR: 1.12; 95% CI: 1.06–1.18) in adults from the

TABLE 2 | Associations between whole blood selenium concentration and glucose and insulin markers in US adults from NHANES 2013 to 2018.

	Model 1[a,b]		Model 2[a,c]	
	Estimate (95% CI)	***P*-value**	**Estimate (95% CI)**	***P*-value**
HbA1c, %	1.0002 (−1.002, 1.0023)	0.825	−1.0004 (−1.003, 1.002)	0.726
Insulin, U/mL	1.020 (1.007, 1.030)	0.003*	1.015 (1.004, 1.026)	0.007*
Glucose, mg/dL	1.003 (1.00, 1.007)	0.037*	1.002 (−1.001, 1.005)	0.239
HOMA-IR	1.024 (1.009, 1.039)	0.002*	1.017 (1.005, 1.029)	0.007*

*[a] The models were fitted using log-transformed outcomes and are presented on the original scale for 10 μg/L increase in selenium; [b] Model 1 adjusted for age and sex; [c] Model 2 adjusted for age, sex, smoking status (smoker/non-smoker), physical activity, metabolic syndrome, and BMI. * $P < 0.05$.*

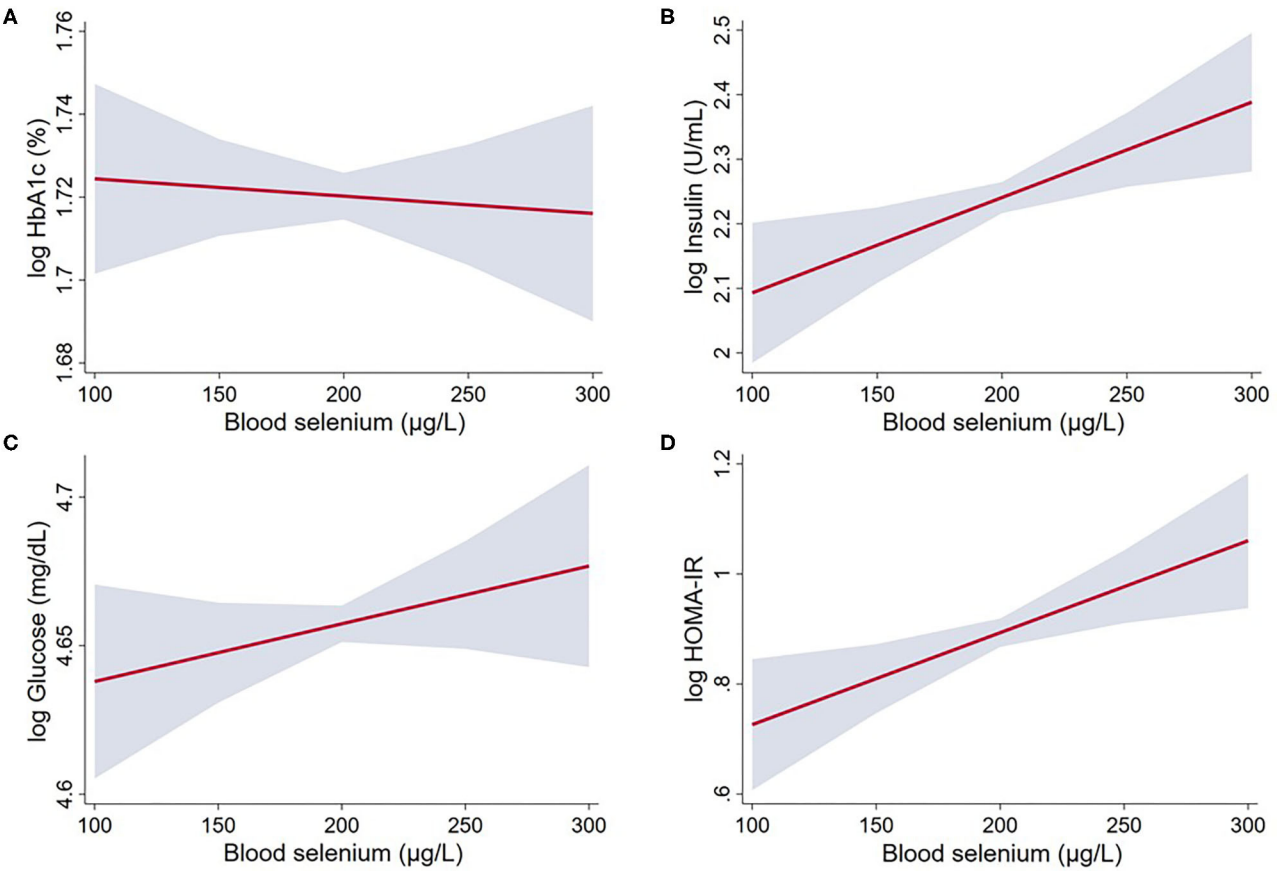

FIGURE 2 | Linear regression between whole blood selenium concentration and glucose and insulin markers in US adults from NHANES 2013 to 2018: **(A)** log HbA1c; **(B)** log Insulin; **(C)** log Glucose; and **(D)** log HOMA-IR. The associations were adjusted for age, sex, smoking status (smoker/non-smoker), physical activity, metabolic syndrome, BMI (Model 2). Gray area represents the 95% confidence intervals. Statistical parameters for these variables are given in **Table 2**.

2011 to 2014 NHANES. Nonetheless, unlike our study, Moon et al. did not consider Hb1Ac in the diagnosis of diabetes; furthermore, they did not consider smoking status or physical activity as covariates, although these are two important risk factors for diabetes. Additionally, while we adjusted our model for metabolic syndrome, they adjusted their models for only some metabolic syndrome-related factors such as hypertension and dyslipidaemia.

Animal experiments have been conducted to elucidate the mechanisms involved in the association between selenium nutritional status and the risk of T2DM. These studies have associated the upregulation of the selenoproteins glutathione peroxidase 1 (GPX1), methionine sulfoxide reductase B1 (MSRB1), selenoprotein S (SELENOS) (27) and selenoprotein P (SELENOP) (30) induced by high selenium intake with hyperglycaemia, decreased insulin sensitivity and liver triglyceride concentrations. It was hypothesized that increased synthesis of these antioxidant selenoproteins diminishes intracellular reactive oxygen species derived from glucose metabolism and disturbs key regulators of pancreatic beta-cells,

leading to chronic hyperinsulinaemia (27, 28). An experimental animal model of diabetes revealed that neutralization of SELENOP improved glucose tolerance and insulin secretion (31), suggesting that this selenoprotein may play a particularly important role in the pathogenesis of T2DM.

Taking a different perspective, Schomburg (32) hypothesized that rather than high selenium being a cause for insulin resistance, diabetes potentially causes increased synthesis of SELENOP by the liver, which leads to increased circulating selenium. Under this hypothesis, the primary cause of the association between selenium and diabetes is the presence of insulin resistance that raises the synthesis of SELENOP, the main selenium transporter. When developing this hypothesis, Schomburg (32) raised concerns around "young autoimmune-disease prone women who wished to supplement and correct their selenium deficiency" but are precluded from doing so because they are afraid of developing diabetes due to the supplementation. Findings from a systematic review that included RCTs on selenium supplementation to individuals with T2DM revealed inconsistent effects of selenium on the main glucose and insulin markers (17). While no effect of selenium was seen in two out of the four included studies, insulin resistance markers (plasma insulin, HOMA-IR and HOMA-B) were decreased after the intervention in comparison to the placebo group in two studies. Nonetheless, no selenium status was assessed in these studies, and therefore it is not possible to establish if the positive effects were due to a recovery from selenium deficient nutritional status.

High selenium intake has been associated with increased risk of diabetes in observational and controlled trial studies. A meta-analysis conducted with four studies which assessed the association between dietary selenium and risk of diabetes revealed that, in comparison with a selenium intake of 23 μg/day, an intake of 50 μg/day was associated with a relative risk (RR) of 1.5 (95% CI: 1.1, 1.9), and an intake of 75 μg/day was associated with an even higher RR (RR: 1.9; 95% CI: 1.4, 2.7) (10). We here emphasize the U-shaped metabolic effects of selenium, where both too little or too much selenium is detrimental to human health (4, 10, 14). We found that the population assessed in this analysis is selenium-replete, in accordance with other studies within the NHANES that demonstrated a high consumption via diet and supplementation (19, 20). Thus, a linear relationship, representing the right-hand arm of a U-shaped association, was observed. Although we cannot extrapolate our findings to selenium-deficient populations, through the lens of a U-shaped dose-response, it is possible that a selenium-deficient population would in fact benefit from supplementation. Further analysis to determine potential benefits of selenium supplementation in selenium-deficient individuals is required.

Our analysis encompassed the examination of sex differences in the association between selenium status and glucose markers, but no significant sex interaction was observed. This analysis is critical due to previously reported sex differences in selenium biology (33, 34), and should be included in every study.

A key strength of this study relies on the survey and analytical methods, as well as the controlled protocols used by the NHANES. Furthermore, we used blood selenium as a marker, which is the preferred option to assess selenium status in populations with high exposure to selenium as it does not plateau like plasma and is very responsive to selenium intake (35). A limitation of this study is associated with the cross-sectional nature of the study design, which precludes inference of causation.

CONCLUSIONS

In this analysis that included a representative sample of US adults with selenium-replete nutritional status, selenium was positively associated with markers of insulin resistance, such as plasma insulin concentration and HOMA-IR, independently of other risk factors for T2DM such as smoking status, physical activity, metabolic syndrome and BMI. Our findings corroborate the notion that selenium supplementation should not be encouraged in populations with high dietary intake of selenium.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://wwwn.cdc.gov/nchs/nhanes/.

AUTHOR CONTRIBUTIONS

BRC: conceptualization, data curation, data analysis, and writing (original and final draft). SB: data analysis and writing (review and editing). RMG: writing (review and editing). All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.696024/full#supplementary-material

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ORIGINAL RESEARCH
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Selenoprotein P Regulates Synaptic Zinc and Reduces Tau Phosphorylation

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Selenoprotein P (SELENOP1) is a selenium-rich antioxidant protein involved in extracellular transport of selenium (Se). SELENOP1 also has metal binding properties. The trace element Zinc (Zn^{2+}) is a neuromodulator that can be released from synaptic terminals in the brain, primarily from a subset of glutamatergic terminals. Both Zn^{2+} and Se are necessary for normal brain function. Although these ions can bind together with high affinity, the biological significance of an interaction of SELENOP1 with Zn^{2+} has not been investigated. We examined changes in brain Zn^{2+} in SELENOP1 knockout (KO) animals. Timm-Danscher and N-(6-methoxy-8-quinolyl)-*p*-toluenesulphonamide (TSQ) staining revealed increased levels of intracellular Zn^{2+} in the SELENOP1$^{-/-}$ hippocampus compared to wildtype (WT) mice. Mass spectrometry analysis of frozen whole brain samples demonstrated that total Zn^{2+} was not increased in the SELENOP1$^{-/-}$ mice, suggesting only local changes in Zn^{2+} distribution. Unexpectedly, live Zn^{2+} imaging of hippocampal slices with a selective extracellular fluorescent Zn^{2+} indicator (FluoZin-3) showed that SELENOP1$^{-/-}$ mice have impaired Zn^{2+} release in response to KCl-induced neuron depolarization. The zinc/metal storage protein metallothionein 3 (MT-3) was increased in SELENOP1$^{-/-}$ hippocampus relative to wildtype, possibly in response to an elevated Zn^{2+} content. We found that depriving cultured cells of selenium resulted in increased intracellular Zn^{2+}, as did inhibition of selenoprotein GPX4 but not GPX1, suggesting the increased Zn^{2+} in SELENOP1$^{-/-}$ mice is due to a downregulation of antioxidant selenoproteins and subsequent release of Zn^{2+} from intracellular stores. Surprisingly, we found increased tau phosphorylation in the hippocampus of SELENOP1$^{-/-}$ mice, possibly resulting from intracellular zinc changes. Our findings reveal important roles for SELENOP1 in the maintenance of synaptic Zn^{2+} physiology and preventing tau hyperphosphorylation.

Keywords: Selenoprotein P, zinc, Alzheimer's disease, tau, selenium

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INTRODUCTION

Within the body, selenium (Se) functions primarily in the form of selenocysteine (Sec), the 21st amino acid, which is incorporated into members of the selenoprotein family (1–3). Selenoprotein P (SELENOP1) is a selenium-rich protein with 10 Sec residues that transports Se in serum from liver to the brain and other organs (4). SELENOP1 is present in the cerebral spinal fluid (CSF) and in the choroid plexus, which releases CSF (5, 6), and in glial cells (7). SELENOP1 has also been described in brain neurons (8, 9), which may be the targets of Se transport. SELENOP1 KO mice have reduced brain selenium and reduced levels of antioxidant selenoproteins such as glutathione peroxidases 1 and 4 (GPX1 and GPX4) (10). Mice with the SELENOP1 gene deletion have deficient hippocampal synaptic function and deficits in spatial learning and long-term potentiation (LTP), a model for learning and memory (11). SELENOP1 is increased in the brain and CSF in Alzheimer's disease (5, 8, 12) and associated with both Alzheimer's and Parkinson's pathology (8, 13).

Se and Zn^{2+} are both essential trace elements required for proper brain function. Selenium deficiency correlates with impaired cognitive and motor function (14, 15), while Zn^{2+} deficiency correlates with decreased nerve conduction and impaired cognitive performance (16). Alzheimer's disease is associated with increased brain Zn^{2+} levels (17). Zn^{2+} can increase tau phosphorylation (18, 19), which contributes to the formation of neurofibrillary tangles, a hallmark of Alzheimer's disease (20). However, studies have yet to address the biological relevance of the interaction between these elements despite their high affinity for each other and their importance in brain function.

Figure 1A shows SELENOP1's two functional, glycosylated domains: (1) a Se-rich C-terminal domain with 9 S residues, (2) an N-terminal domain with 1 S (U) in U-x-x-C redox motif, 2 histidine-rich metal binding sites (located at residue 204–217 and residue 244–250) and a heparin binding site (4, 21). SELENOP1 also has an N-terminal signal peptide for extracellular secretion, which is cleaved in the Golgi (22).

We hypothesized that SELENOP1, as a metal-binding protein, could have a role in brain Zn^{2+} homeostasis. In this study, we investigated whether SELENOP1 influences brain Zn^{2+} by evaluating changes in hippocampal Zn^{2+} in SELENOP1 KO animals. Here we report that deletion of SELENOP1 alters levels of chelatable Zn^{2+} and prevents the release of synaptic Zn^{2+} in mouse hippocampus. These findings indicate an important additional role for SELENOP1 in the regulation of zinc in the brain. They could also have important implications for the treatment of disorders where Zn^{2+} physiology is impaired.

MATERIALS AND METHODS

Animals

Mice were group housed on a 12-h light cycle and provided food and water *ad libitum*. All animals in this study were maintained on diets containing adequate selenium (∼0.25 ppm) and zinc (∼80 ppm). All mice used were 3–6 months of age and included both male and female mice as indicated. All animal procedures were approved by the University of Hawaii Institutional Animal Care and Use Committee.

$SELENOP1^{-/-}$ mice were obtained from the laboratory of Dr. Raymond Burk at Vanderbilt University. The mutant mice were backcrossed to C57BL/6J for at least ten generations with C57BL/6J mice from Jackson Laboratories to ensure congenic strains. Breeding of $SELENOP1^{+/-}$ mice generated littermates of $SELENOP1^{+/+}$ and $SELENOP1^{-/-}$ pups, which were used in this study in addition to $SELENOP1^{+/-}$ mice. Genomic DNA extracted from mice tails was used for genotyping PCR using specific primers (forward-ACCTCAGC AATGTGGAGAAGCC, reverse-TGCCCTCTGAGTTTAGC ATTG for wild-type, and reverse-GATGATCTGGACG AAGAGCATCA for $SELENOP1^{-/-}$). Products were run on a 1.5% DNA agarose gel with a SYBR Safe DNA gel stain (Invitrogen) and genotypes were confirmed under UV light.

Timm-Danscher Zn^{2+} Labeling

The Danscher modification of Timm's zinc stain ("neo-Timm's") was used to label intracellular chelatable zinc (i.e., not tightly bound to proteins or other molecules) (23). Deeply anesthetized mice received intraperitoneal (IP) injections of 20 mg/kg sodium selenite (2 mg/ml in normal saline). After 2 h, mice were perfused with saline followed by 4% paraformaldehyde (PFA) in saline. Brains were postfixed overnight in 4% PFA, dehydrated serially in 10% and 30% sucrose, and mounted in optimal cutting temperature compound (OCT) for cryostat sectioning. After thoroughly washing in PBS, sections were developed with the IntenSE M silver Enhancement kit (Amersham International) according to the manufacturer's protocol and as previously described (24).

6-Methoxy-8-P-Toluenesulfonamido-Quinoline (TSQ) Stain

TSQ is a Zn^{2+} fluorophore that binds intracellular chelatable Zn^{2+} in a 2:1 ligand-to-metal ratio that results in increased fluorescence emission at 490 nm in response to excitation at 360 nm (25). Serial sagittal cryosections (10 μm) of brain hemispheres were mounted on positively charged microscope slides. The slides were immersed with 4.5 μM TSQ (Enzo Lifesciences, UltraPure) in 140 mM sodium barbital and 140 mM sodium acetate buffer (pH 10) for 90 s, as previously described (26) and washed in 0.1% NaCl. TSQ-stained sections were imaged using DAPI filter settings (200 ms, monochrome with a 5x objective). The mean fluorescence intensity of the hippocampal CA1 stratum oriens and stratum radiatum, CA3 mossy fibers, and hilar region were measured with ImageJ software (NIH). The background was measured in unstained areas within lateral ventricles and subtracted from mean TSQ signals.

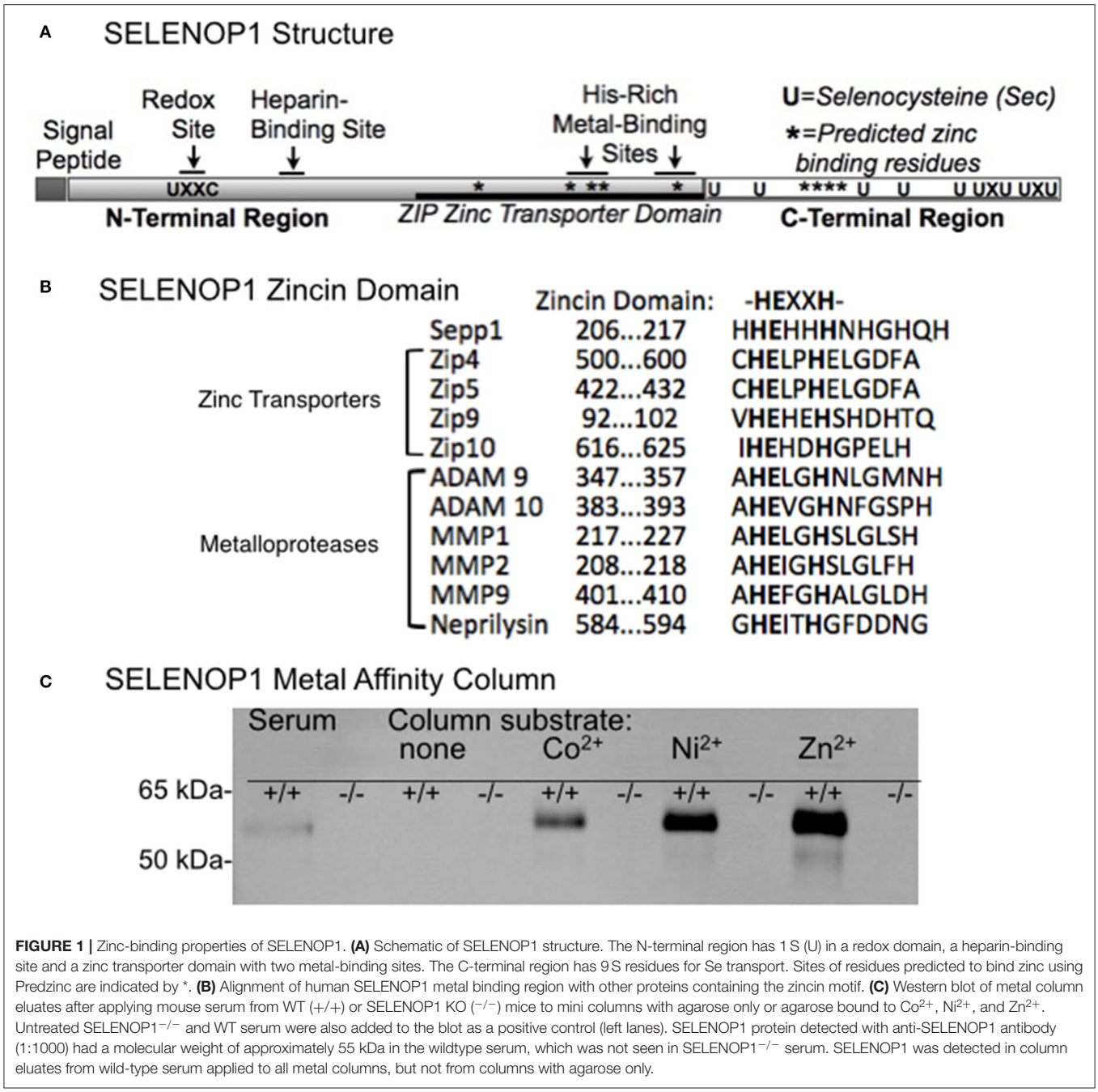

FIGURE 1 | Zinc-binding properties of SELENOP1. **(A)** Schematic of SELENOP1 structure. The N-terminal region has 1 S (U) in a redox domain, a heparin-binding site and a zinc transporter domain with two metal-binding sites. The C-terminal region has 9 S residues for Se transport. Sites of residues predicted to bind zinc using Predzinc are indicated by *. **(B)** Alignment of human SELENOP1 metal binding region with other proteins containing the zincin motif. **(C)** Western blot of metal column eluates after applying mouse serum from WT (+/+) or SELENOP1 KO ($^{-/-}$) mice to mini columns with agarose only or agarose bound to Co^{2+}, Ni^{2+}, and Zn^{2+}. Untreated SELENOP1$^{-/-}$ and WT serum were also added to the blot as a positive control (left lanes). SELENOP1 protein detected with anti-SELENOP1 antibody (1:1000) had a molecular weight of approximately 55 kDa in the wildtype serum, which was not seen in SELENOP1$^{-/-}$ serum. SELENOP1 was detected in column eluates from wild-type serum applied to all metal columns, but not from columns with agarose only.

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

The metal concentrations within frozen right brain hemispheres and liver samples were processed at the Agricultural Diagnostic Service Center (ADSC) run by the College of Tropical Agriculture and Human Resources (CTAHR), University of Hawaii. Dry ash sample preparations were subjected to acid digest before ICP-OES (0.01 ppm detection limit) to measure total brain and liver metal content (Zn^{2+}, Cu^{2+}, Fe^{2+}). Water blanks and solution standards were included with each run to calibrate results.

Protein Extraction and Western Blot

Proteins were extracted from frozen hippocampal tissue using CelLytic MT buffer (Sigma) per the manufacturer's instructions, denatured by heating in Laemmli sample buffer, resolved by SDS-PAGE on a 10–20% gradient Tris-HCl Criterion Precast gel (Bio-Rad Laboratories), and electrically transferred to polyvinylidene difluoride (PVDF) membranes. For detection of

Zn^{2+} regulating proteins, membranes were incubated in anti-metallothionein-3 (1:500, rabbit polyclonal, Biorbyt), and anti-ZnT1 and anti-ZnT3 (1:1000; 1:5000, rabbit polyclonal, Synaptic Systems). For detection of the SELENOP1 protein, membranes were incubated in anti-SELENOP1 (1:1000, rabbit monoclonal, Proteintech). For measurement of tau, antibodies recognizing tau phosphorylated at T231, S214 or S396 (1:1000, Invitrogen) or tau 5 (1:1000, Millipore). Membranes washed and then treated with corresponding secondary antibodies conjugated with infrared fluorophores (1:10,000; Licor). Blots were subsequently treated with anti α-tubulin (1:5000; Novus Biologicals) to control for loading. Membranes were imaged with the Odyssey infrared fluorescence system (LiCor), and densitometry analysis was performed on the Imagestudio software (LiCor).

Metal Agarose Column Purification

To observe SELENOP1 binding to biometals, we used mini spin-columns containing high-density agarose beads conjugated with Zn^{2+}, Ni^{2+}, and Co^{2+} (Agarose Bead Technologies) to isolate metal-binding proteins from wildtype and SELENOP1$^{-/-}$ mouse serum. A metal-free agarose column served as a negative control. Serum samples were diluted 1:100 in PBS and added to the column, gently shaken for 60 min at 4°C, then spun for 60 s at 800x g to collect flow-through. Columns were washed with increasing concentrations of imidazole (0, 10, and 20 mM) diluted in PBS, and then bound proteins were eluted with 250 mM imidazole diluted in PBS. Eluted proteins were determined with western blot using an anti-SELENOP1 antibody (27).

Live Hippocampal Slice Imaging

To measure stimulus-induced extracellular Zn^{2+}accumulation, hippocampal slices were prepared from 3 to 6 month old SELENOP1 KO and wild-type littermate mice as previously described (28). Following slice preparation, slices were acclimated to room temperature and superfused with oxygenated (95 O_2 and 5% CO_2 gas mix) artificial cerebral spinal fluid (ACSF, composition in mM: NaCl 130; KCl 3.5; glucose 10; $NaHCO_3$ 24; NaH_2PO_4 1.25; $MgSO_4$ 1.5; $CaCl_2$ 2.0) for at least 60 min. The CA1 stratum radiatum region of the slices was imaged with a Zeiss laser-scanning microscope using a 10X objective with the pinhole fully opened at 1 frame/s at 640 × 480 resolution. Cell-impermeant FluoZin-3 at 1.5 μM (Molecular Probes) was added to the ACSF to detect extracellular Zn^{2+} accumulation from hippocampal slices in response to the administration of a depolarizing (35 mM) KCl concentration for 60 s. In some experiments, a slow onset Zn^{2+} chelator (Ca^{2+}-EDTA) was added to remove contaminating Zn^{2+} in media and to reduce background fluorescence (29, 30). Fluorescence intensities of hippocampal slices in the CA1 stratum radiatum region upon addition of KCl for each slice were expressed as the fluorescence intensity over the fluorescence during baseline (F/F_0). The mean area under the curve during application of high K^+ for signals of SELENOP1$^{-/-}$ slices were compared to WT to determine if changes in Zn^{2+} release were altered.

FluoZin-3 Measurements in Cell Culture

SH-SY5Y cells were plated in 96-well plates and differentiated by exposure to Neurobasal media (Invitrogen) supplemented with B27 (Invitrogen) for 48 hrs. The media was then changed to Roth-Schweizer media (31) but with of 0, 10 or 100 nM. Alternatively, cultures in 10 nM Se were treated with 100 μM mercaptosuccinate (MCS), 0.1 μM RSL-3, 0.1 μM RSL-3 + 100 μM α-tocopherol, or 0.01 μM DMSO as a control for RSL-3. Cells were treated for either 5 days (DMSO, RSL-3, RSL-3 with α-tocopherol) or 7 days (Se concentrations and remaining conditions). Cells were rinsed twice in HEPES buffered saline [HBSS: (in mM) NaCl 146; KCl 3.5; glucose 10; 1.25; $MgSO_4$ 1.5; $CaCl_2$ 2.0, HEPES 10, NaOH 10]. Cells were loaded with 1 μM FluoZin-3 AM (Thermo Fisher Scientific) with 0.02% pluoronic F-127 for 30 min at 37°C in the dark, then rinsed twice with HBSS, and the fluorescence measured in HBSS. For additional controls, either 100 μM 2, 2′-dithiodipyridine (DTDP), 100 μM tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN), or 100 μM H_2O_2 were added to the HBSS 10 min before measuring fluorescence. Plates were scanned in a SpectraMax M3 fluorescent plate reader (Molecular Devices) with 494 nm excitation, 516 nm emission. After scanning, cells were fixed in 100% methanol at −20°C overnight, stained with 100 μg propidium iodide (PI), rinsed twice with HBSS, than scanned in HBSS at 536 nm excitation, 617 nm emission. FluoZin-3 fluorescence was normalized to PI fluorescence to correct for any differences in cell density.

Statistical Analysis

All statistical analyses were carried out with Graphpad Prism Software with measurements given as means ± SE. Comparisons between treatments, genotypes and sex were performed by student's unpaired *t*-test and one-way or two-way analysis of variance (ANOVA), with $p < 0.05$ considered significant. In general, we did not find sex differences in Zn^{2+} distribution or release unless shown; otherwise, males and females were averaged together by genotype.

RESULTS

Zn^{2+} Binding Properties of SELENOP1

Previous studies described the affinity of SELENOP1 for several metals, including Zn^{2+}. However, the biological interaction of SELENOP1 with Zn^{2+} is unclear. We investigated potential Zn^{2+}-binding domains of SELENOP1. Using the web-based software Predzinc (https://predzinc.bioshu.se/pred/), a web server that predicts zinc-binding proteins and zinc-binding sites from given sequences, we analyzed the SELENOP1 coding sequences for potential zinc-binding sites. We found that a His-residue within the SELENOP1 His-rich metal binding domain as well as two other His residues in the C-terminal region are predicted to be Zn^{2+} binding motifs, shown in **Figure 1A** by asterisks (*).

A domain search of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database indicates that the human SELENOP1 protein structure has a zinc-binding domain overlapping one of the His-rich regions, which has homology to the ZIP zinc

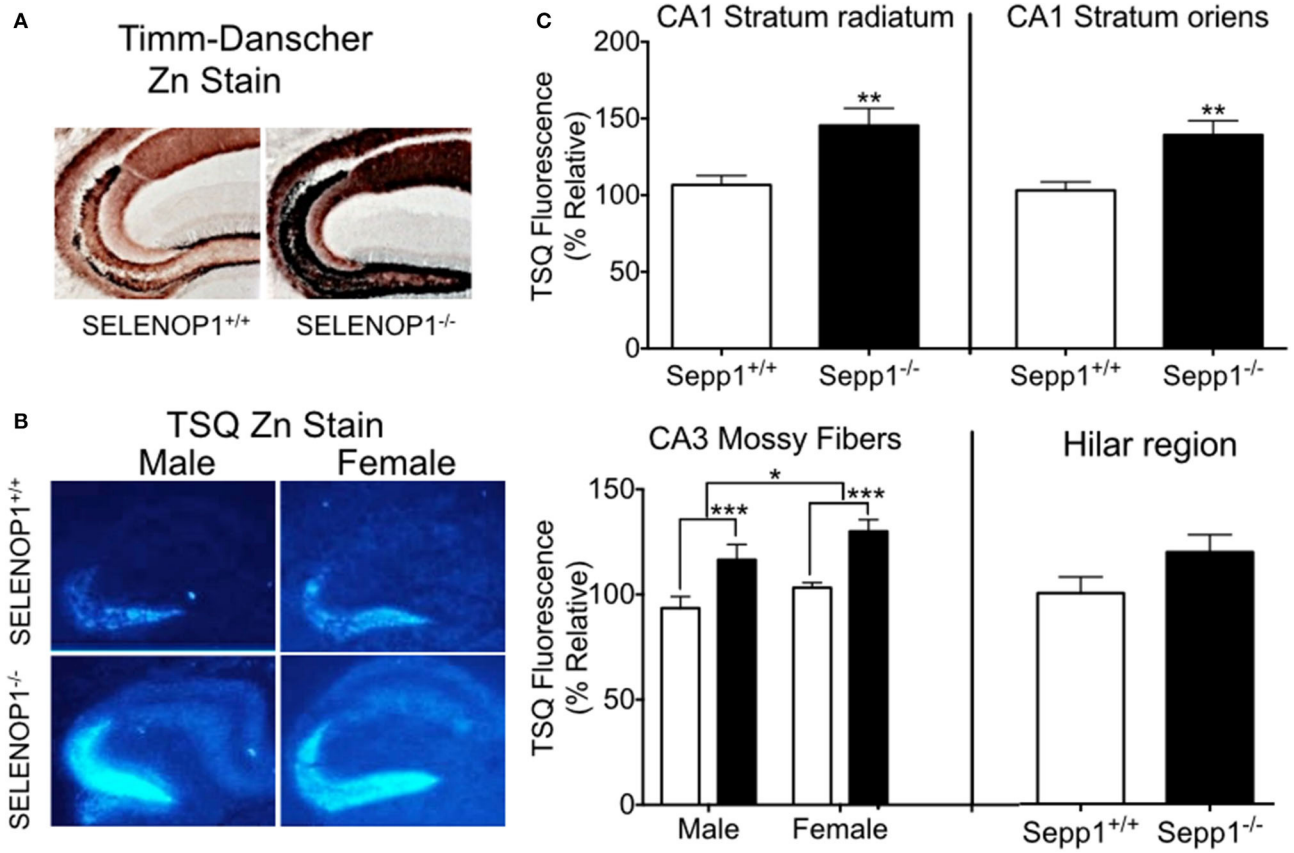

FIGURE 2 | Increased hippocampal zinc in SELENOP1 KO mice. **(A)** Neo-Timm's (Danscher) stain. Free Zn^{2+} in wildtype and SELENOP1$^{-/-}$ hippocampal neurons from mice injected with sodium selenite is revealed as a reddish-brown to dark brown color relative to the amount of total Zn^{2+}. **(B)** Representative images from male and female WT and SELENOP1$^{-/-}$ mouse hippocampus, labeled with the fluorescent Zn^{2+} indicator, TSQ. **(C)** Quantitation of TSQ fluorescent label indicates higher Zn^{2+} levels in SELENOP1$^{-/-}$ animals in all observed regions except for hilar region. The average values for each region were normalized to wild type males. $N = 6$ animals/group, 5–10 slices/animal. Statistical analysis is performed by Student's unpaired t- test or two-way ANOVA with Bonferroni's *post-hoc* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

transporter domain. Additionally, within this sequence is a zincin Zn^{2+}-binding motif, generally found in the metzincin family of metalloproteases (**Figure 1B**) (32).

Because of the predicted Zn^{2+}-binding region of SELENOP1, we tested SELENOP1's potential to bind Zn^{2+} ions by passing mouse serum through agarose columns bound to Co^{2+}, Ni^{2+}, or Zn^{2+}, or metal-free as a negative control. Western blot of sample elution from each column purification shows that SELENOP1 binds to Co^{2+}, Ni^{2+}, and Zn^{2+}, but not to the metal-free agarose column (**Figure 1C**). When serum from SELENOP1$^{-/-}$ mice was used, SELENOP1 immunoreactivity was not detected in eluent from any of the columns. This demonstrates that SELENOP1 is capable of binding different biometals, with a greater amount of binding to Zn^{2+} compared with Co^{2+} and Ni^{2+}.

Elevated Levels of Intracellular Zn^{2+} in SELENOP1$^{-/-}$ Hippocampus

To explore the effects of SELENOP1 on brain Zn^{2+} homeostasis, we first evaluated intracellular Zn^{2+} levels in SELENOP1$^{-/-}$ mice using histological methods. Our studies focused on the hippocampus as a zinc-rich region important for learning and memory (33). The Timm-Danscher method (34) revealed a pronounced increase in histologically-detectable Zn^{2+} in the hippocampus of SELENOP1$^{-/-}$ compared with SELENOP1$^{+/+}$ hippocampus (**Figure 2A**). The hilar and CA3 mossy fiber regions had the most intense Zn^{2+} labeling in both wild-type and SELENOP1$^{-/-}$ animals. However, all layers positive for Zn^{2+} were increased in KOs, including the stratum radiatum and stratum oriens of the CA1 and CA3 layers.

We further compared bioreactive Zn^{2+} levels in SELENOP1$^{-/-}$ and wildtype mouse hippocampi using TSQ labeling. Quantitation of TSQ fluorescence also revealed significantly higher levels of intracellular Zn^{2+} in the CA1 stratum oriens, stratum radiatum, and CA3 mossy fibers of the SELENOP1$^{-/-}$ animals compared to their control (**Figures 2B,C**). We also observed a sex difference in CA3, with increased Zn^{2+} in mossy fibers in female compared to male hippocampus regardless of genotype. Unstained hippocampal brain sections showed no fluorescence at TSQ wavelengths, indicating that differences were not due to autofluorescence signals. Hematoxylin staining

TABLE 1 | Metal content in brain and liver of Sepp1 wildtype (Sepp1$^{+/+}$) and knockout (Sepp1$^{-/-}$) mice at the age of 3 months (mg/kg of dry tissue, $n = 20$ each) measured by ICP-OES.

Tissue	Metal	Genotype		*P*-value*
		Sepp1$^{+/+}$	Sepp1$^{-/-}$	
Brain	Zn	12.04 ± 0.23[a]	12.34 ± 0.32	0.45
	Cu	4.480 ± 0.16	4.383 ± 0.29	0.77
	Fe	19.99 ± 1.31	18.25 ± 1.04	0.30
Liver	Zn	25.85 ± 1.04	25.20 ± 1.48	0.72
	Cu	6.048 ± 0.42	6.756 ± 0.61	0.35
	Fe	88.26 ± 4.34	90.91 ± 7.85	0.77

**Unpaired two-tailed t-test was used to determine the probability of differences between genotypes. [a]Mean ± SEM.*

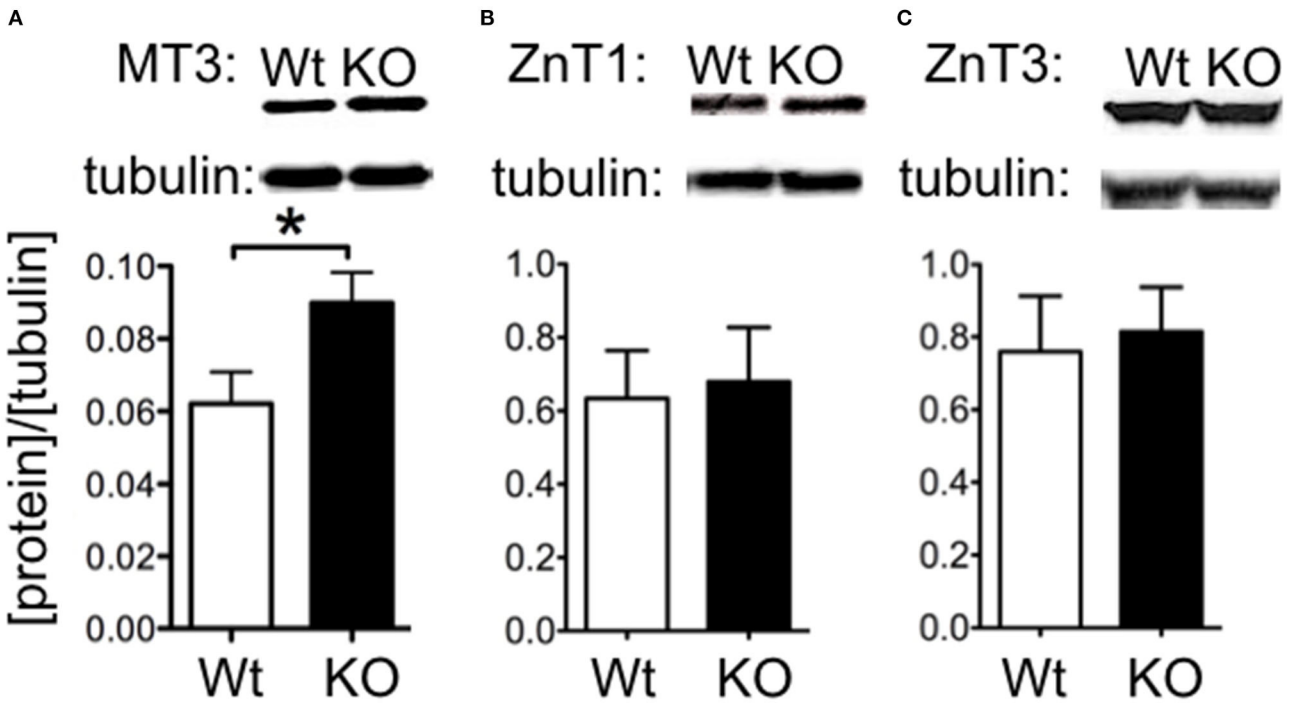

FIGURE 3 | Increased MT3 but not ZnT1 or ZnT3 protein levels in SELENOP1 KO hippocampus. Western blot showing expression levels of Zn^{2+} transporter proteins, ZnT1 and ZnT3, and Zn^{2+} storage pool, MT3. Protein densities were normalized to α-tubulin and averaged to male WT values. **(A)** ZnT1 protein levels were unchanged. **(B)** ZnT3 protein was also unchanged. **(C)** Increased MT3 expression. Representative bands are shown above each corresponding bar graph and were found to run at the predicted sizes: ZnT1 (45kDa), ZnT3 (42kDa), and MT3 (40kDa). Statistical analysis is performed by Student's unpaired *t*-test, $^*p < 0.05$.

of sections previously used for TSQ labeling showed no morphological differences between SELENOP1 wildtype and KO hippocampi. Based on our findings, SELENOP1 may be regulating Zn^{2+} levels directly or through one or more Zn^{2+} interacting proteins.

As we observed increased levels of intracellular Zn^{2+} within the hippocampus of SELENOP1$^{-/-}$ mice, we then investigated if the total brain Zn^{2+} levels are altered by subjecting whole brain hemispheres via ICP-OES to measure total metal content for Zn^{2+}, Cu^{2+}, and Fe^{2+}. There was no significant increase in total brain Zn^{2+} levels in SELENOP1$^{-/-}$ mice (**Table 1**). This may indicate that deletion of SELENOP1 results in changes to the distribution of Zn^{2+} within the brain rather than an increase in total brain Zn^{2+}.

Expression Levels of Zn^{2+}-Interacting Proteins

We investigated whether changes in Zn^{2+} regulating proteins could explain differences in Zn^{2+}. We investigated hippocampal expression of the Zn^{2+} transporters ZnT1 and the vesicle-associated ZnT3, as well as the metal storage protein MT3. Western blot indicated that ZnT1 and ZnT3 proteins were unchanged in SELENOP1$^{-/-}$ animals (**Figures 3A,B**). However, MT3 protein expression was increased (**Figure 3C**), suggesting

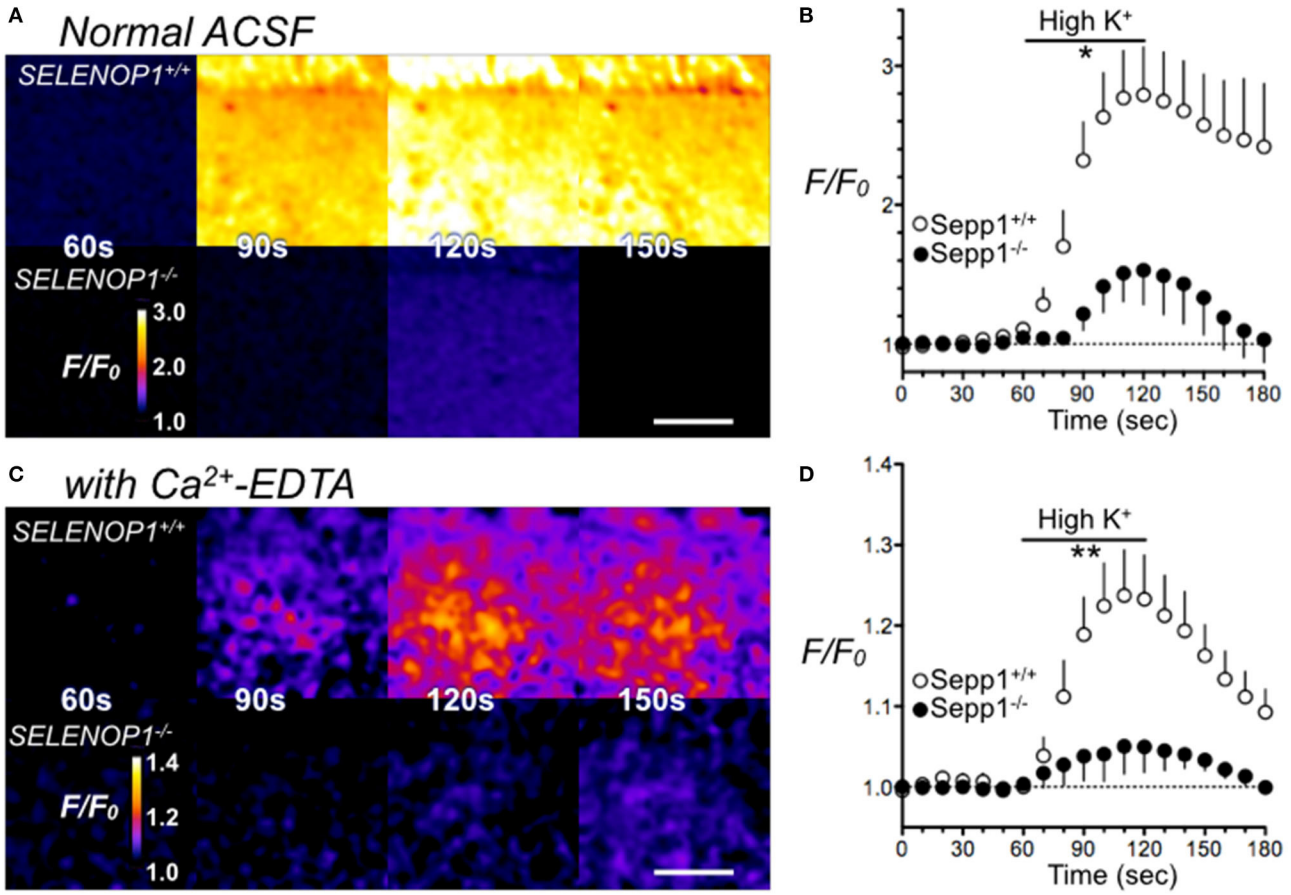

FIGURE 4 | Impaired Zn^{2+} release in SELENOP1 KO hippocampus. **(A)** Zn^{2+} release from hippocampal slices of SELENOP1$^{-/-}$ and WT mice visualized with Fluozin-3 following depolarization induced with ACSF containing high (35 mM) KCl solution. Fluorescence in SELENOP1$^{-/-}$ hippocampal slices was significantly reduced compared to WT animals. **(A)** Representative Fluozin-3 images from WT and SELENOP1$^{-/-}$ animals at 60s, 90s, 120s and 150s, after adding high K^+ from 60 to 120 s. **(B)** Data plotted as relative fluorescence (F/F_0) over time with measurements averaged every 10 s. WT, $n = 6$ slices, 3 mice; SELENOP1$^{-/-}$, $n = 5$ slices, 3 mice. **(C)** Zn^{2+} release visualized with Fluozin-3 in ACSF with the addition of Zn^{2+} chelator, Ca^{2+}-EDTA to reduce background fluorescence, following application of ACSF with high (35 mM) KCl. Representative Fluozin-3 images from WT and SELENOP1$^{-/-}$ animals at 60, 90, 120, and 150 s, after adding high K^+ from 60 to 120 s. **(D)** Data plotted as relative fluorescence (F/F_0) over time with measurements averaged every 5 s. WT, $n = 3$ slices, 3 mice; SELENOP1$^{-/-}$, $n = 4$ slices, 4 mice; Fluorescence in SELENOP1$^{-/-}$ hippocampal slices was significantly reduced compared to WT animals. Statistical analysis was performed by Student's unpaired *t*-test comparing the mean area under the curve for each genotype between 60 and 120 s, $^*p < 0.05$, $^{**}p < 0.01$.

that deletion of the SELENOP1 gene does not affect zinc transport through these major pathways, but rather upregulates expression of the Zn^{2+} storage protein. The enhanced expression of MT3 may be a result of a feedback mechanism in response to increased Zn^{2+} levels in the SELENOP1$^{-/-}$ hippocampus.

Zn^{2+} Release Is Impaired in SELENOP1$^{-/-}$ Hippocampus in Response to Neuron Depolarization

Most histologically-detectable Zn^{2+} in hippocampal neurons is vesicular (25). We investigated if the increased Zn^{2+} levels visualized by TSQ or Timm's staining in SELENOP1$^{-/-}$ mice results in increased extracellular accumulation following depolarizing stimuli designed to promote vesicular release. We imaged extracellular Zn^{2+} accumulation in hippocampal slices with a selective cell-impermeant fluorescent Zn^{2+} indicator, Fluozin-3, in the extracellular media. By depolarizing hippocampal cells with the addition of 35 mM KCl, we noticed an increase in fluorescence in slices from wild type mice, indicating release of Zn^{2+} into the extracellular space (**Figures 4A,B**). In contrast, we observed a minimal increase in fluorescence in hippocampal slices from SELENOP1$^{-/-}$ mice, indicating negligible Zn^{2+} release in the SELENOP1$^{-/-}$ hippocampus.

A high background fluorescence recording from Zn^{2+} contaminants in the reagents used could possibly mask Zn^{2+} release. To reduce background fluorescence, we also imaged slices with the Zn^{2+} chelator, Ca^{2+}-EDTA, added to the ACSF. Ca^{2+}-EDTA enables removal of basal levels of extracellular Zn^{2+}, but its slow kinetics do not prevent the detection of synaptically-evoked Zn^{2+} accumulation (35). Even in the presence of Ca^{2+}-EDTA, we still observed significantly larger

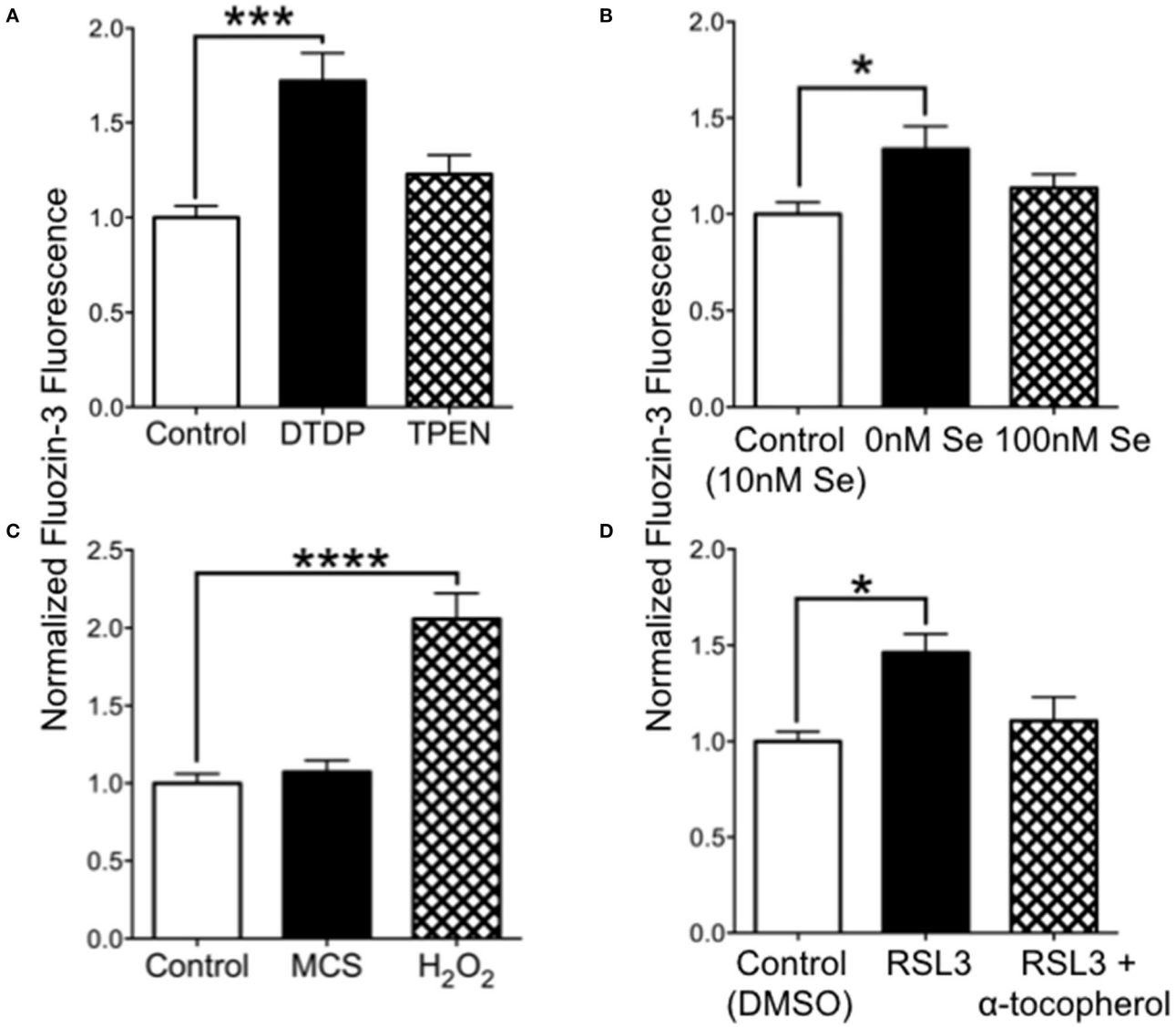

FIGURE 5 | Intracellular Zn^{2+} levels are increased by 0 Se and GPX4 inhibitor RSL-3. **(A)** Relative FluoZin-3 fluorescence of SH-SY5Y cells. Cells exposed to DTDP to release zinc showed increased fluorescence, while cells exposed to PTEN to reduce intracellular zinc had no change in fluorescence. **(B)** FluoZin-3 fluorescence was increased in cells grown in media with 0 nM Se relative to control media (10 nM), while 100 nM Se did not alter intracellular zinc. **(C)** Hydrogen peroxide but not GPX1 inhibitor mercaptosuccinate (MCS) increase intracellular zinc. **(D)** The GPX4 inhibitor RSL-3 increased intracellular zinc, which was reversed when RSL-3 was added with α-tocopherol. Statistical analysis is performed by one-way ANOVA followed by Dunnett's multiple comparison test. *$p < 0.05$, ***$p < 0.005$, ****$p < 0.0001$.

FluoZin-3 increases in wild-type hippocampus slices relative to SELENOP1$^{-/-}$ slices upon cell depolarization (**Figures 4C,D**). The baseline fluorescence (F_0) was not significantly different between SELENOP1$^{+/+}$ and SELENOP1$^{-/-}$ slices in either normal ACSF for Ca^{2+}-EDTA ACSF.

Selenium Deficiency Releases Intracellular Zn^{2+}

We hypothesized that increased oxidation from Se-deficient conditions could result in reduced intracellular chelation of Zn^{2+}. We tested this by measuring the fluorescence of cell-permeable FluoZin-3 in cultured SH-SY5Y cells. As shown in **Figure 5A**, release of intracellular zinc by DTDP, a thiol oxidizer which liberates intracellular Zn^{2+}, significantly increased FluoZin-3 fluorescence. However, the intracellular Zn^{2+} chelator TPEN did not reduce FluoZin-3 fluorescence, indicating that free non-chelated Zn^{2+} levels were too low to be detectable. We also found that cells grown in 0 Se culture media had significantly increased fluorescence compared with cells grown in our baseline media with 10 nM Se (**Figure 5B**). Oxidation with H_2O_2 greatly increased FluoZin-3 fluorescence,

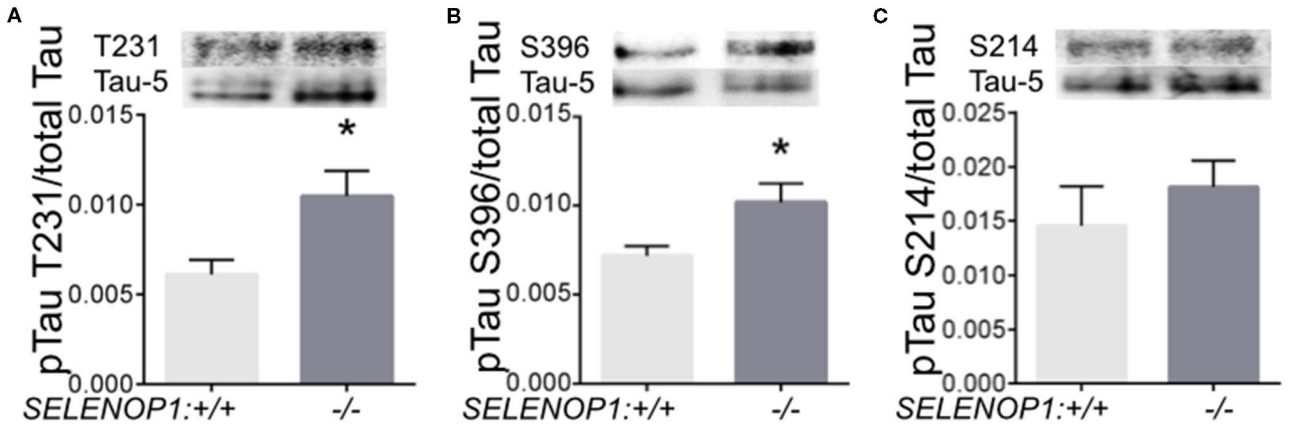

FIGURE 6 | Deletion of SELENOP1 increases tau phosphorylation in mouse hippocampus. Western blots of phospho-specific tau antibodies relative to the Tau-5 antibody, which recognizes total tau. Blot images are superimposed above graphs. **(A)** SELENOP1 deletion resulted in increased tau phosphorylation at threonine 231. **(B)** Serine 396 was also significantly reduced in SELENOP1 knockouts. **(C)** No change in phosphorylation at serine 214. $N = 4$ animals/group. Statistical analysis is performed by Student's unpaired *t*-test, $^*p < 0.05$.

demonstrating that oxidation could free Zn^{2+} from stores. To test if a reduction in activity of the H2O2-reducing selenoprotein GPX1, which depends on Se for synthesis, could lead to increased intracellular Zn^{2+}, we exposed cells to the GPX1 inhibitor mercaptosuccinate (MCS) (**Figure 5C**). MCS did not alter fluorescence, however, we found that RSL-3, an inhibitor of the phospholipid peroxidase selenoprotein GPX4, increased intracellular Zn^{2+} (**Figure 5D**). This increase was prevented by the vitamin E compound α-tocopherol, which reduces lipid peroxidation. These findings suggest that a deficiency in Se can increase free intracellular Zn^{2+} by causing a reduction in GPX4, which results in increased oxidation of lipids.

Elevated Tau Phosphorylation in SELENOP1 Knockout Hippocampus

Zn^{2+} promotes tau phosphorylation leading to neurofibrillary tangle formation (18). We questioned whether tau phosphorylation could be altered in the SELENOP1$^{-/-}$ mouse. We performed western blot analysis to compare specific pTau sites to total tau protein. We found that phosphorylation at threonine 231 and at serine 396 were significantly increased in SELENOP1$^{-/-}$ mice (**Figure 6A**). However, phosphorylation at the serine 214 site was unchanged (**Figures 6B,C**). Deletion of SELENOP1$^{-/-}$ thus results in a site-specific increase in tau phosphorylation.

DISCUSSION

Our findings show that deletion of SELENOP1 increased free (chelatable) intracellular Zn^{2+} levels. However, release of synaptic zinc was impaired. Although the Zn^{2+} storage protein MT3 was elevated without change to the zinc transporters ZnT1 or ZnT3, we showed that selenium deficiency could induce release of Zn^{2+} from stores, likely through a decrease of the selenoprotein GPX4 and a subsequent increase in lipid peroxidation. Interestingly, GPX4 helps to prevent neurodegeneration though ferroptosis (36). Lastly, we demonstrated an increase in site-specific phosphorylation of tau. These findings suggest that SELENOP1 plays a role in regulating storage of intracellular Zn^{2+}. This role may be important for preventing tau hyperphosphorylation in AD.

Dietary supplementation with selenium in the form of sodium selenate reduces tau phosphorylation to potentially reduce neurofibrillary tangle formation (37, 38). Selenate can act as an agonist for protein phosphatase 2A (PP2A), which targets tau phosphorylation (39). Interestingly, Zn^{2+} is an inhibitor of PP2A, and may promote neurofibrillary tangle formation (18). Hyper-phosphorylation of tau leads to neurofibrillary tangle formation, and de-phosphorylation by PP2A should reduce tangle formation. However, dietary selenate supplementation also upregulates the expression of selenoproteins (40, 41). We have previously reported that a reduction in selenoprotein S can promote tau phosphorylation (42). In a type 2 early clinical trial for selenate supplementation in Alzheimer's disease, selenate could increase brain selenium in some patients, and the increase in brain selenium correlated with lack of decline in performance on the Mini-Mental Status Examination (MMSE) (43). Thus, brain selenoproteins, including SELENOP1, may be important for preventing Alzheimer's pathology.

Zn^{2+} metabolism is altered in AD, resulting in abnormally enriched Zn^{2+} environments within the AD brain (44). Zinc-binding sites on the Aβ peptide result in Zn^{2+} mediated aggregation of Aβ and amyloid plaque formation. Furthermore, both the neuroprotective role of SELENOP1 against Aβ toxicity and the role of Zn^{2+} in protecting the cell against oxidative damage, could be working together to reduce the levels of Aβ stress observed in AD pathology, however more studies need to be done to further elucidate their contribution to alleviating oxidative stress.

We did not observe an increase in total brain Zn^{2+} levels with ICP-OES, suggesting only changes in local Zn^{2+} distribution.

The absence of SELENOP1 could result in increased oxidative stress in the brain and lead to Zn^{2+} release from MT3 (45), possibly inducing an upregulation of the Zn^{2+} storage protein. In the absence of SELENOP1, we observed a seemingly paradoxical impairment of Zn^{2+} release despite an overall increase in intracellular chelatable Zn^{2+}. This was surprising, since most chelatable Zn^{2+} is thought to be localized to synaptic vesicles (46). Overexpression of MT3 in SELENOP1$^{-/-}$ mice may affect the subcellular distribution of Zn^{2+} by limiting the amount of free Zn^{2+} available for loading into the synaptic vesicles. The protein Reelin can increase release of a subset of synaptic vesicles (47). This increase is dependent on Reelin binding to ApoER2. SELENOP1 is another ligand for ApoER2 (48, 49), and thus may also modulate vesicle release, possibly including zincergic vesicles. A decrease in release of Zn^{2+} vesicles could result in a "back-up" of these vesicles, which could contribute to the observed increase in chelatable Zn^{2+}. The APOE ε4 allele of the major ligand ApoE for ApoER2, increases risk of Alzheimer's disease (50). Interestingly, the APOE ε4 allele is also associated with decreased selenium in the brain (51), again suggesting a possible role for selenium and selenoproteins in preventing Alzheimer's disease.

We found an interesting sex difference in Zn^{2+} levels in the CA3 region of the hippocampus (**Figure 2**). Female mice generally had higher Zn^{2+} levels but with some variability. Previous studies have shown higher Zn^{2+} levels in female wild-type mice and AD mouse models (52, 53), although these studies did not agree on the age of sex differences. The differences suggest Zn^{2+} as a possible explanation for the increased risk of AD in women (54). Researchers recently discovered that estrogen increases hippocampal Zn^{2+}, which was cycle-dependent in female mice (55, 55), which may explain the increased Zn^{2+} in other studies as well as the variability of the current results.

SELENOP1 contains a putative metal-binding domain that can potentially bind Zn^{2+} with a high affinity. Our results demonstrate that SELENOP1 is capable of binding Zn^{2+} as well as Co^{2+} and Ni^{2+}. However, our finding that selenium deficiency and inhibition of GPX4 suggest that the absence of selenium and loss of antioxidant selenoprotein function in the SELENOP1 KO mice is responsible for the increased Zn^{2+} levels. The reason for the presence of the functional metal-binding domain of SELENOP1 remains unknown. It is possible that Zn^{2+} binding could alter the affinity of SELENOP1 for the ApoER2 receptor, allowing for regulation of Se by excess Zn^{2+}. Additionally, the homology of the Zn^{2+} binding domain with metalloproteases such as ADAM10 and neprilysin (**Figure 1**) opens the possibility of an enzymatic role in proteolysis. ADAM10 has a protective role in Alzheimer's disease as a putative α-secretase that promotes non-amyloidogenic cleavage of amyloid precursor protein, preventing amyloid beta formation (56).

The data presented here indicate that SELENOP1 may play a crucial role in the maintenance of brain Zn^{2+}. The SELENOP1 gene can affect Zn^{2+} metabolism and synaptic release from neuronal synapses. Zn^{2+} is increased in Alzheimer's disease and interacts with amyloid beta (17), and can also promote tau phosphorylation (18). Thus, Zn^{2+}-binding properties of SELENOP1 could contribute to the association of SELENOP1 with amyloid beta plaques in Alzheimer's disease (8). The SELENOP1-Zn^{2+} interaction has potentially important implications in neuronal function and synaptic physiology.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Hawaii IACUC.

AUTHOR CONTRIBUTIONS

FB, CS, DT, and AK designed the research. DT, AH, JP, and RR performed the research. FB analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Metabolism of Tracer ^{75}Se Selenium From Inorganic and Organic Selenocompounds Into Selenoproteins in Rats, and the Missing ^{75}Se Metabolites

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We now know much about selenium (Se) incorporation into selenoproteins, and there is considerable interest in the optimum form of Se for supplementation and prevention of cancer. To study the flux of ^{75}Se into selenoprotein, rats were fed 0 to 5 μg Se/g diet as selenite for 50–80 d and injected iv with 50 μCi of ^{75}Se-labeled selenite, selenate, selenodiglutathione, selenomethionine, or selenobetaine at tracer levels (~0.5 μg Se). The rats were killed at various times and ^{75}Se incorporation into selenoproteins was assessed by SDS/PAGE. These studies found that there is very rapid Se metabolism from this diverse set of selenocompounds to the common intermediate used for synthesis and incorporation of ^{75}Se into the major selenoproteins in a variety of tissues. No selenocompound was uniquely or preferentially metabolized to provide Se for selenoprotein incorporation. Examination of the SDS/PAGE selenoprotein profiles, however, reveals that synthesis of selenoproteins is only part of the full Se metabolism story. The ^{75}Se missing from the selenoprotein profiles, especially at early timepoints, is likely to be both low-MW and high-MW selenosugars and related precursors, as we recently found in livers of turkeys fed Se-adequate and high-Se diets. Differential metabolism of different selenocompounds into different selenosugar species may occur; these species may be involved in prevention of cancer or other diseases linked to Se status and may be associated with Se toxicity. Additional studies using HPLC-mass spectroscopy will likely be needed to fully flesh out the complete metabolism of selenium.

Keywords: glutathione peroxidase, SDS/PAGE, selenite, selenomethionine, selenosugar

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INTRODUCTION

We now know much about selenium (Se) incorporation into selenoproteins. Se at the selenide level is metabolized to selenophosphate, esterified to serine while attached to a novel selenocysteine tRNA, and incorporated into the selenoprotein backbone during translation at the position specified by a UGA codon and requiring a 3'UTR stem-loop selenocysteine (Sec) insertion sequence (1–6). At the time the experiments here were conducted, only five mammalian selenoproteins had been identified and characterized: glutathione peroxidase (GPX), plasma selenoprotein P (SELENOP),

phospholipid hydroperoxide glutathione peroxidase (GPX4), plasma GPX3, and thioredoxin reductase (TXNRD) (7). Cloning and expression of UGA-containing transcripts has now demonstrated that the mammalian selenoproteome consists of 24–25 selenoproteins (8, 9).

When these studies were conducted there was considerable interest in the optimum form of Se for supplementation and prevention of cancer (10–12). Both inorganic Se, like selenite, and organic Se, like selenomethionine (SeMet), had been shown to readily provide Se for GPX synthesis (13) and to prevent cancer in animal models (14). Some studies suggested that one form vs. another had differential bioavailability (15) or ability to prevent cancer (11). Dietary methionine (Met) had been shown to modulate Se incorporation from SeMet into GPX (13, 16, 17) and to prevent mammary tumors (18) because, unlike inorganic forms, intact SeMet is an excellent analog of Met for incorporation into general body proteins in place of Met, thus raising tissue Se content without biochemical activity (16, 17, 19). Selenobetaine (SeBetaine) as a methylselenol donor has high potency against DMBA-induced mammary tumors but it was postulated that anticarcinogenic effects of SeBetaine might be exerted without metabolism to selenoproteins (20). Se was also known to be toxic at higher levels (21–23), but it was not clear if there were additional selenoproteins that appear only under high Se status or that are associated just with Se toxicity (24, 25).

Thus, we developed a procedure using SDS slab gel gradient electrophoresis (SDS/PAGE) that separates and quantitates the various Se-containing protein subunits, including GPX (26). By sacrificing rats at various times after the iv injection of Se into rats, SDS/PAGE can monitor the flux of radioactive Se into and between the various detected selenoproteins. As reported previously only in abstract form, we used this procedure to examine the incorporation of ^{75}Se from selenite (27), selenodiglutathione (28), selenate (29), selenomethionine (30), and SeBetaine (31) in order to study Se metabolism leading to selenoprotein synthesis.

The prevailing thought at the time was that tissue Se is present as Sec in selenoproteins, as SeMet is incorporated into general body proteins, and as low molecular weight (MW) metabolites such as selenide, glutathione-Se intermediates, and methylated forms such as methylselenol (7). Low MW "selenosugar" species – seleno-N-acetyl galactose amine (SeGalNac) – first found in urine has also been found in liver as CH_3-SeGalNac and GS-SeGalNac (32). Note that the Se in these selenosugars is linked to galactose 1-carbon via a Se-C bond. Using HPLC coupled with Se-specific and molecule-specific mass spectroscopy, we recently found these low-MW species in livers of turkeys fed Se-adequate and high-Se diets, but we also found high-MW selenosugar species linked via selenodisulfide bonds (Se-S) to protein. Surprisingly, more Se was present as the selenosugar moiety in Se-adequate turkey liver, mostly decorating general proteins, than was present as Sec in selenoproteins; with high Se supplementation, these "selenosugar-decorated" proteins were further increased (33). This study on turkey liver shows the power of these approaches and more modern analytical techniques to uncover the full metabolism of Se.

Our hypotheses at the time were that ^{75}Se from injected ^{75}Se selenocompounds would be distributed differently in rats, would result in different ^{75}Se-labeling patterns of selenoproteins, and might lead to novel ^{75}Se-labeled selenoproteins under high Se status. We found, however, that there were no dramatic differences in ^{75}Se distribution between tissues, and that these selenocompounds were not differentially or preferentially metabolized to provide Se for selenoprotein incorporation. SDS/PAGE also did not detect ^{75}Se-labeling of novel selenoproteins under high Se status. What we did not recognize then was the importance of tissue ^{75}Se that was missing from the SDS/PAGE gels.

MATERIALS AND METHODS

Rat Procedures

The series of studies reported here were conducted in 1986–1991 and approved by the following Animal Care and Use Committees: University of Arizona (A3248 #86-0172 and #86-0357), and the University of Missouri (A3394 #1425). Male Holtzmann weanling rats were fed a basal 30% torula yeast-based diet that contained by analysis 0.005–0.018 μg Se/g diet (26, 34, 35). To prevent liver necrosis, the basal diet was supplemented with 100 IU/kg of all rac-α-tocopheryl acetate (Sigma Chemical Co., St. Louis, MO) at the expense of sucrose. Unless otherwise stated, the basal diet was supplemented with 0.4% D,L-methionine (U.S. Biochemical Corp., Cleveland, OH), and with 0, 0.2, 2.0, and/or 5.0 μg Se/g diet as selenite for 50–80 d, depending on the experiment. Rats were anesthetized with ether and injected iv in the femoral vein with 50 μCi of ^{75}Se-labeled selenocompounds at trace levels (∼0.5 μg Se), and killed 1, 3, 24, or 72 h (also 168 h for SeMet) after injection as described previously (26). Blood was sampled by cardiac puncture using a heparinized syringe; liver was perfused *in situ* with 0.15 M KCl to remove erythrocytes. Plasma was obtained by centrifugation (1,000 g × 30 min). Tissues were weighed, and portions of tissues were ^{75}Se-counted to calculate tissue ^{75}Se recovery. Liver and kidney were homogenized in 9 vol of 0.25 M sucrose, and the cytosolic fractions were prepared by subcellular fractionation. Heart, testes, and muscle (gastrocnemius from uninjected (right) leg) were homogenized in 9 vol of 10 mM Tris, 1% SDS, and 10 mM 2-mercaptoethanol buffer, pH 7.4, using a Brinkmann polytron, and the homogenates were centrifuged at 105,000 g x 60 min to obtain supernatants that were then subjected to SDS/PAGE (26).

SDS/PAGE Procedure

After preparation, 1,500 μg protein was mixed (1:1) with sample buffer (50 mM Tris, 1% SDS, 2% 2-mercaptoethanol), heated in a boiling water bath for 15 min, and loaded onto 3 mm slab gels with an acrylamide gradient from 7.5 to 20% (top to bottom) and electrophoresed at 60 mA per gel. The gels were fixed in methanol:acetic acid:water (5:1:4) containing 0.25% Coomassie brilliant blue R, and destained in methanol:acetic acid:water (75:50:875). Each lane was cut out, sliced into 2 mm slices, and counted. Protein standards of known MW were run to calibrate position with molecular weight (26).

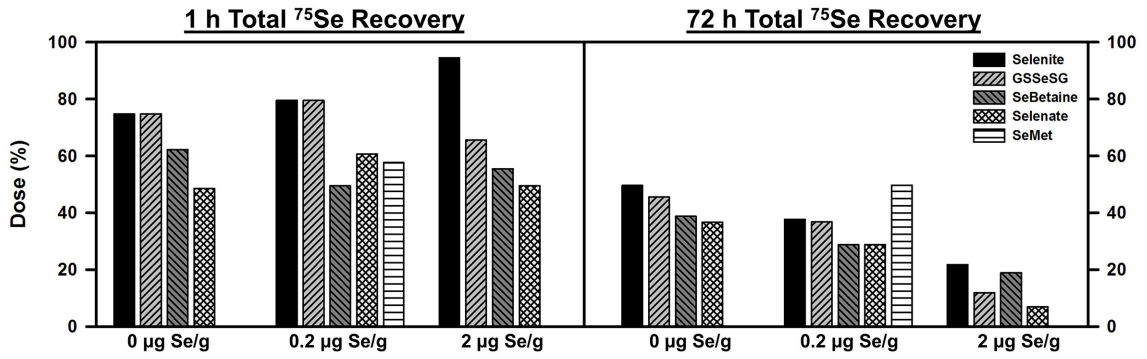

FIGURE 1 | Total ^{75}Se recovery at 1 and 72 h after injection ^{75}Se selenocompounds. Rats were fed the indicated dietary Se levels as selenite for 50–80 d, injected iv with 50 μCi of tracer ^{75}Se-labeled Selenite, GSSeSG, SeBetaine, Selenate, or SeMet, killed at 1 and 72 h afer dosing, and ^{75}Se retained in blood, liver, kidney, heart, testes, epididymus, and muscle was counted. Values are the percent of the administered ^{75}Se dose recovered (n = 1 per treatment at each time for each selenocompound, n = 26 total).

^{75}Se Compounds

[^{75}Se]selenite was obtained from commercial sources or produced at the Research Reactor at the University of Missouri. Individual rats were injected with 50 μCi of [^{75}Se]selenite (∼0.5 μg Se). L-[^{75}Se]SeMet (1.1 Ci/μmole) was obtained from Amersham. [^{75}Se]selenodiglutathione was synthesized from 2 mCi of [^{75}Se]selenite (63 μCi/μg Se), which were reduced with 5 mg ascorbate, oxidized with concentrated redistilled $HN0_3$ and then dried at 60oC. The resulting selenite (0.24 μmoles) was reduced with GSH on ice for 16 h at pH < 1.0, using a 4 GSH:1 Se stoichiometry. A sample was analyzed using a Dowex-Ni column, which showed that 97% of the applied ^{75}Se eluted after GSSG and thus was present as [^{75}Se]GSSeSG (36). [^{75}Se]selenobetaine (dimethylselenoacetate, SeBetaine) was synthesized from 2.5 mCi of [^{75}Se]selenite (70 μCi/μg Se) by borohydride reduction, and reacted with iodomethane to produce trimethylselenonium ion. After purification, the trimethylselenonium ion was pyrolyzed to form dimethylselenide, which was reacted with bromoacetic acid to form dimethylselenoacetate. Purification on SP-Sephadex resulted in >60% recovery as SeBetaine (37). [^{75}Se]selenate was prepared by oxidizing [^{75}Se]selenite with 30% H_2O_2. Following oxidation, complexation with 2, 3- diaminonaphthalene, a selenite-specific reaction, showed that <3% of the ^{75}Se remained as selenite. A Packard model 5650 refrigerated gamma counter with 3" KI crystal was used for ^{75}Se counting (60% efficiency for ^{75}Se).

RESULTS

Biomarkers of Se status of Holtzmann rats fed these diets have been reported thoroughly by our group and are not reported here. Plasma and liver glutathione peroxidase activities in rats fed the Se-deficient basal diet are typically 2% of levels found in rats supplemented with 0.2 μg Se/g diet (35) and are not further increased by 1–5 μg Se/g diet (35, 38). Liver Se concentrations for rats fed the basal Se-deficient diets for 4 wk are typically 0.25 nmol/g liver (0.02 μg Se/g) and 3% of levels in rats fed 0.2 μg Se/g diet. Liver Se concentration in rats fed 0.2, 2, and 5 μg Se/g are typically 0.66, 2.2, and 2.9 μg Se/g liver, respectively (38).

Se status did not have a large effect on ^{75}Se recovery, distribution, or retention for any of the compounds tested (**Figure 1**). At 1 h, 75 to 50% of the injected ^{75}Se was recovered in blood, liver, kidney, heart, muscle, and testes of these male rats, regardless of the form of Se. Selenite and GSSeSG recoveries were slightly higher than for SeBetaine, selenate, and SeMet at 1 h. By 72 h, total retention in these tissues ranged from 50 to 25% when injected into 0 and 0.2 μg Se/g diet rats, but <20% for rats fed 2 μg Se/g. At 72 h, total retention in rats fed 5 μg Se/g as selenite was 11%. ^{75}Se recovery from SeMet was only determined in rats fed 0.2 μg Se/g, but appeared higher than for the other forms. For the other four Se compounds, the recoveries at 72 h in rats fed 0.2 vs. 0 μg Se/g were only marginally reduced, as compared to the decrease in dietary Se concentration, suggesting that the relative flux of Se in rats fed 0.2 μg Se/g was little altered as compared to rats fed the Se-deficient diet.

Recovery of injected ^{75}Se in six tissues are shown in **Figure 2**. Plasma ^{75}Se retention was calculated based on a blood volume of 8% of total body weight and fraction of blood as plasma (26). At 1 h, 20% of the injected ^{75}Se was found in plasma in rats fed the Se-deficient diet. Supplemental dietary Se at 0.2 and 2 μg Se/g diet progressively deceased the recovered ^{75}Se in plasma. By 72 h, plasma retained ∼10% of the [^{75}Se]selenite in rats fed both 0 and 0.2 μg Se/g diet, but this was decreased to 5% with 2 μg Se/g diet. At 72 h, approximately half as much injected ^{75}Se was retained for the other selenocompounds as compared to selenite.

A different pattern was found for liver as compared to plasma. At 1 h, recovery of ^{75}Se from selenite doubled in rats fed 2 vs. 0 μg Se/g, and recoveries of ^{75}Se from GSSeSG, SeBetaine, and selenate were the same or higher in rats fed 0.2 and 2 μg Se/g diet as compared to rats fed the Se-deficient diet, suggesting a greater

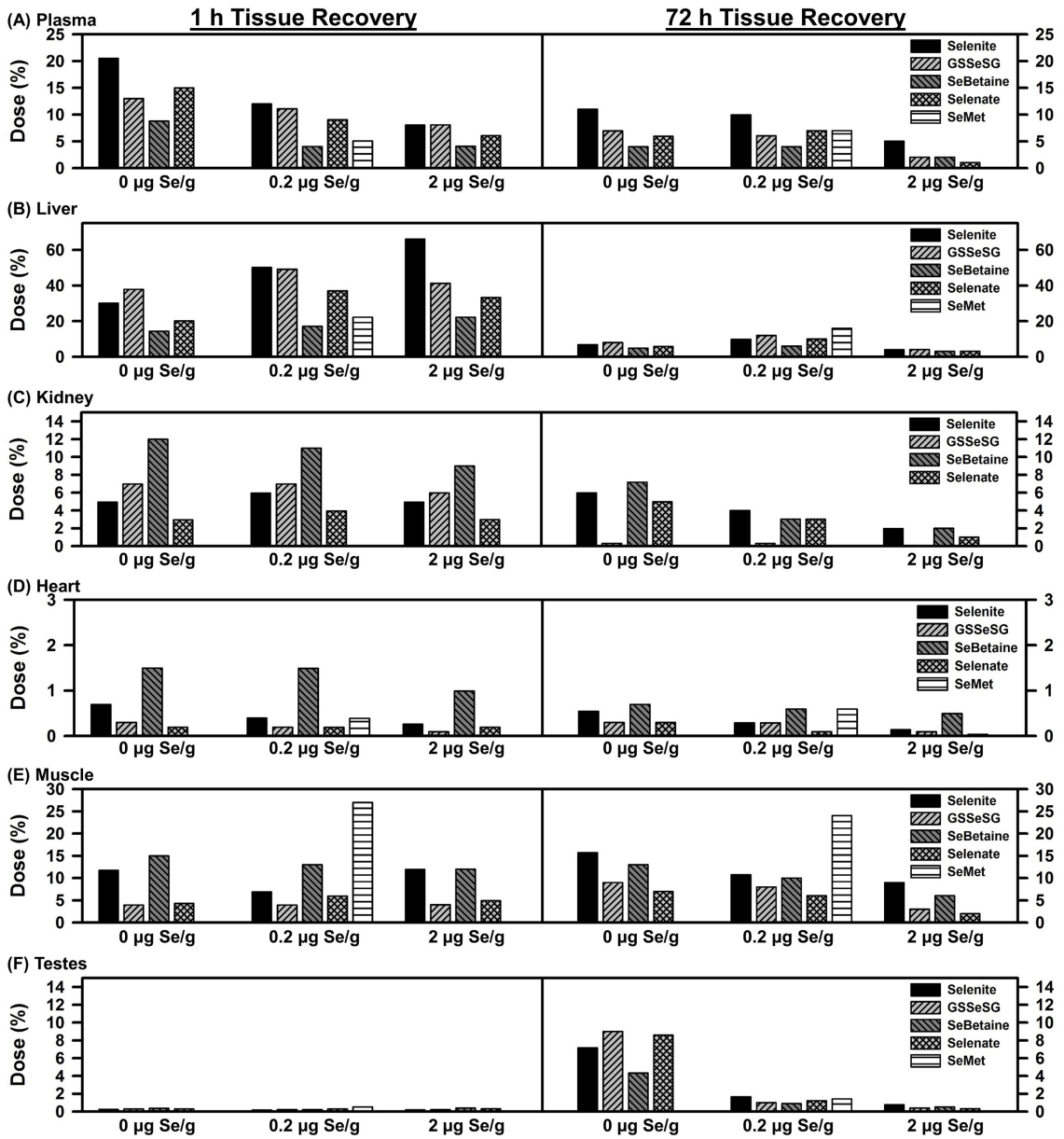

FIGURE 2 | Tissue ^{75}Se recovery at 1 and 72 h after injection ^{75}Se selenocompounds. Rats were treated as described for **Figure 1**. Values are the percent of the administered ^{75}Se dose recovered in each tissue ($n = 1$ per treatment at each time for each selenocompound, $n = 26$ total).

flux of ^{75}Se to other tissues in Se-deficient rats. By 72 h, recoveries were 4% or less in liver for all selenocompounds regardless of dietary Se; in rats fed 5 µg Se/g as selenite, liver ^{75}Se recoveries were 59 and 3% at 1 and 72 h, respectively, similar to levels in rats fed 2 µg Se/g (data not shown). Overall, there was little effect of Se status on the recovery of ^{75}Se in liver.

Kidney, however, provided a third pattern. The level of dietary Se supplementation had little effect on recovery of ^{75}Se at 1 h. Furthermore, the recovery of ^{75}Se at 1 h especially for SeBetaine but also GSSeSG was higher than for selenite, reflecting either targeted uptake by kidney, or reduced uptake/retention by liver and plasma. By 72 h, there was little ^{75}Se arising from GSSeSG found in kidney.

Heart also displayed higher retention of ^{75}Se from SeBetaine as compared to the other Se compounds at 1 h. And relative to plasma, liver, and kidney, retention of ^{75}Se at 72 h in heart remained more similar to retention levels at 1 h.

Recovery of ^{75}Se in muscle was calculated estimating that muscle was 40% of the total body weight of the rat (26). Recovery of ^{75}Se at 1 h and 72 h were almost identical for all Se compounds, and little affected by level of dietary Se. Even at 1 h, SeMet ^{75}Se retention was 4-times the level of selenite ^{75}Se retention

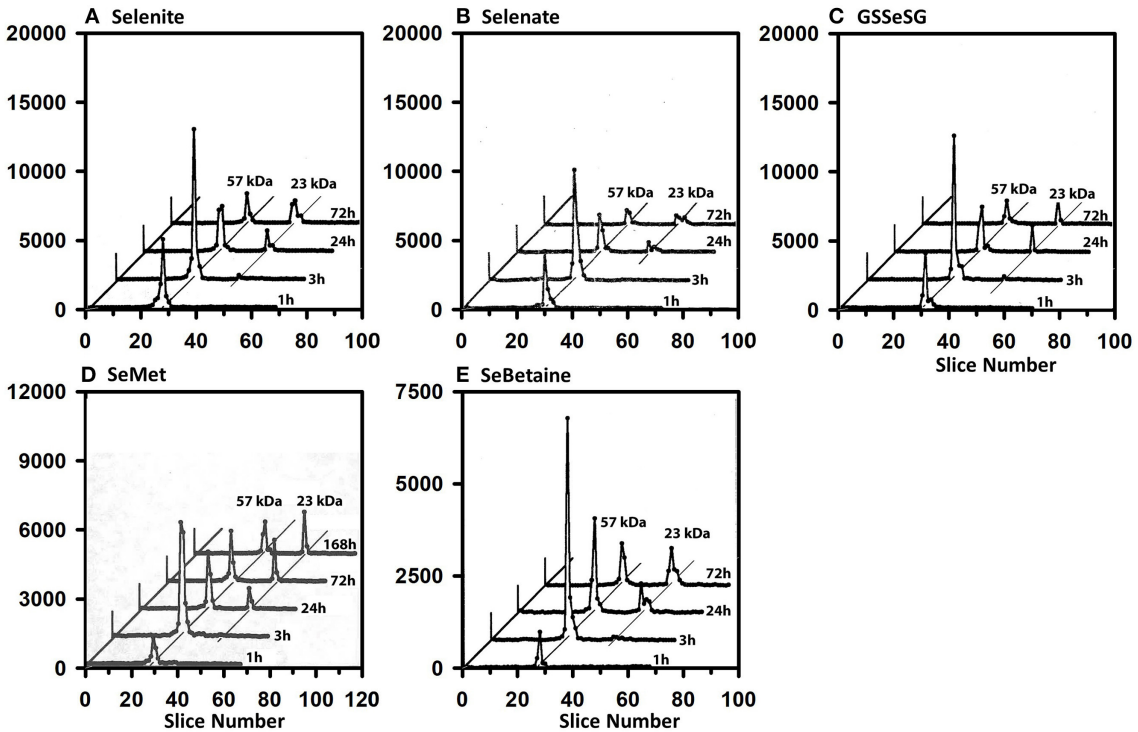

FIGURE 3 | Incorporation of ^{75}Se into plasma proteins. Rats were fed 0.2 μg Se/g diet as selenite for 50–80 d, injected iv with 50 μCi of tracer ^{75}Se-labeled Selenite **(A)**, Selenate **(B)**, GSSeSG **(C)**, SeMet **(D)**, and SeBetaine **(E)**, and killed at 1, 3, 24, or 72 h (or 168 h for SeMet) afer dosing. Plasma samples (1,500 μg protein) were separated using gradient SDS/PAGE. Sample lanes in each gel were cut into 2-mm slices and counted. The cpm for each slice are plotted to show ^{75}Se incorporation into selenoproteins of different subunit molecular weights. Slice 1 contains polypeptides of the highest molecular weight. Plots for 3, 24, and 72 h were staggered, and diagonal lines are drawn through the major ^{75}Se proteins to show the change in ^{75}Se incorporation in these species with time. Each time profile in each selenocompound panel is from one rat (n = 21 total rats). Plasma SELENOP is 57 kDa and GPX3 is 23 kDa.

in rats fed 0.2 μg Se/g. This distribution clearly shows the specific uptake and retention of SeMet relative to the other injected selenocompounds.

At 1 h, there was almost no ^{75}Se found in testes regardless of the form of Se administered. By 72 h, testes in Se-deficient rats retained 4–8% of the administered ^{75}Se. ^{75}Se retention was dramatically reduced in rats fed 0.2 μg Se/g diet, and further reduced in rats fed 2 μg Se/g. In contrast to the other five tissues, injected ^{75}Se was targeted to testes in Se deficiency, but this targeted flux was curtailed in Se-adequate male rats.

SDS/PAGE Analysis

The use of the SDS/PAGE analysis of ^{75}Se incorporation into selenoproteins used 2-mercaptoethanol treatment to separate protein subunits according to MW, and to reduce "loosely bound Se from proteins." Mercaptoethanol treatment will also reduce selenodisulfide linkages, thus releasing low-MW Se forms bound to proteins through these links. Subsequent SDS/PAGE eluted resulting low-MW species into the bottom buffer so that the resulting profiles only display high-MW protein subunits containing Sec. Potentially also retained on the gel might be other high-MW proteins with Se-C bonds, but this would not include Se species linked via selenodisulfide linkages such as selenosugars linked to protein cysteines. The result is the clean profiles of selenoproteins we reported in 1988 as compared to the gel filtration profiles, which showed 4 broad peaks, including >250 kDa species at the void volume, the ∼100 kDa peak containing tetrameric GPX1, the ∼20 kDa peak containing GPX4, and the largest peak containing low-MW species eluting at the column volume (26). Follow-up SDS/PAGE analysis of these individual peaks showed that the 100 kDa peak contained 23 kDa GPX1 subunits and the 20 kDa peak contained GPX4 polypeptide; the >250 kDa and low-MW peaks contained no ^{75}Se-labeled protein peaks after this 2-mercaptoethanol + SDS/PAGE analysis (39). These ^{75}Se species are the "missing" selenometabolites not detected in our use of SDS/PAGE to analyze for selenoproteins.

Full-length plasma SELENOP has a peptide MW of 43 kDa but is glycosylated to have an apparent MW of 57 kDa (40). **Figure 3** shows the SDS/PAGE ^{75}Se profile in plasma for the five selenocompounds at 1, 3, 24, and 72 h after iv ^{75}Se injection in rats fed 0.2 μg Se/g as selenite. The profiles are all remarkably the same. Maximum incorporation into SELENOP is observed at 3 h as reported previously (40). Notable ^{75}Se incorporation into plasma GPX3 is not observed until 24 h, and this level of incorporation remains at 72 h. These profiles clearly indicate that

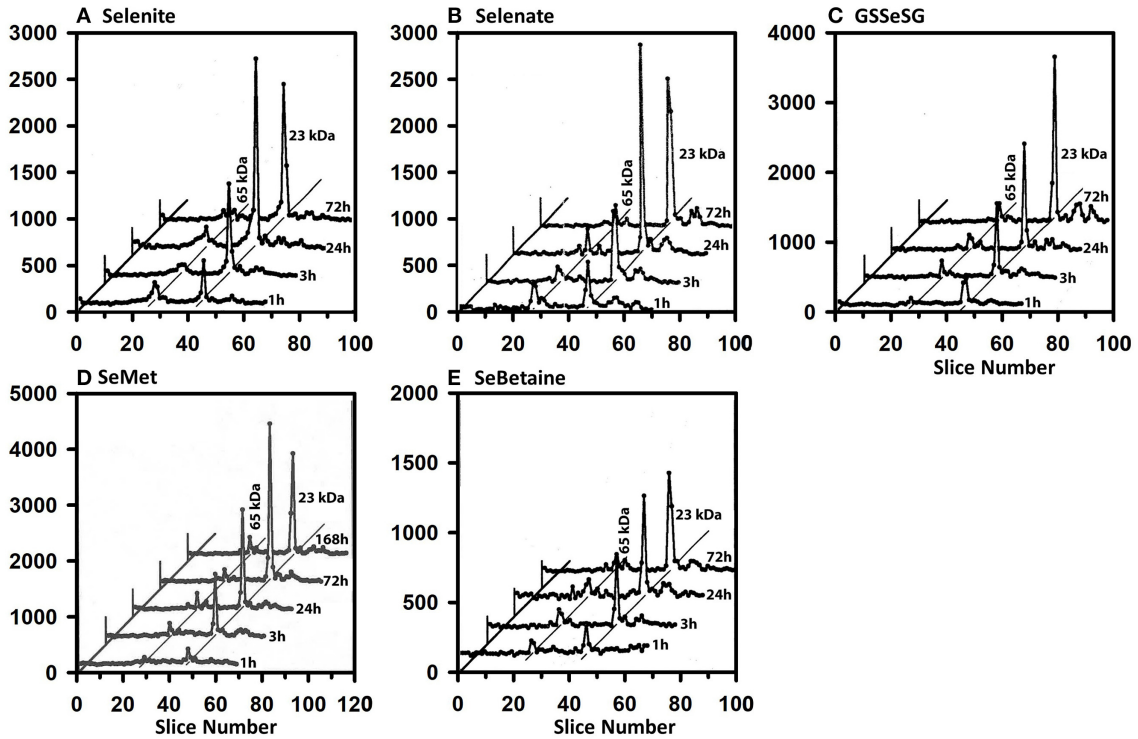

FIGURE 4 | Incorporation of ^{75}Se into liver cytosolic proteins from Selenite **(A)**, Selenate **(B)**, GSSeSG **(C)**, SeMet **(D)**, and SeBetaine **(E)**. Rats were treated as described for **Figure 3**. After homogenization and centrifugation at 105,000 g × 60 min, cytosols (1,500 μg protein) were separated using gradient SDS/PAGE as described for **Figure 3** (n = 21 total rats). Liver GPX1 is 23 kDa, and the 65 kDa species are likely to the isoenzymes of TXNRD1.

all five selenocompounds are rapidly metabolized to the common precursor used for incorporation into selenoproteins.

The ^{75}Se profiles in liver are also all remarkably the same for the five compounds (**Figure 4**). By 1 h, substantial injected ^{75}Se was rapidly incorporated into the 23 kDa GPX1 subunit, with maximal ^{75}Se labeling with selenite and selenate at 24 h. At 72 h, ^{75}Se incorporation into GPX1 from GSSeSG and SeMet was even higher than at 24 h, suggesting these species were more slowly metabolized into the Se precursor than for selenite and selenate. The reduced uptake of ^{75}Se from SeBetaine into liver resulted in slower labeling of GPX1. In addition, several additional selenoprotein subunits of 65 and 19 kDa were also labeled, but at far lower levels than for GPX1. These species are likely to be cytosolic thioredoxin reductase 1 (TXNRD1) with isoforms at 63 and 55 kDa, and GPX4 at 19 kDa.

The ^{75}Se selenoprotein profiles of heart supernatant (**Figure 5**) are also very similar for all four selenocompounds. At 1 and 3 h, the 65 kDa species contained more ^{75}Se than in GPX1 subunits for selenite, GSSeSG, and SeMet. This labeling diminished somewhat by 24 h when GPX1 subunit gained prominence, but both species retained ^{75}Se labeling at 72 h.

The ^{75}Se profiles of testes supernatant show a different story (**Figure 6**). Early on, the 65 kDa species were labeled at 3 h, but by 24 h the 17 kDa GPX4 is equally ^{75}Se-labeled from selenite, GSSeSG, SeMet, and SeBetaine. The GPX4 was the dominate ^{75}Se-labeled selenoprotein at 72 h.

Effect of Se Status on ^{75}Se-Selenoprotein Labeling

The same-sized 50 μCi tracer dose of ^{75}Se was injected at various times into rats fed Se-deficient (0 μg Se/g diet), Se-adequate (0.2 μg Se/g diet), and high Se (2 μg Se/g diet) to study the impact of Se status on flux of ^{75}Se into liver selenoproteins (**Figures 7A–C**). In Se-deficient liver, there was little effect of time after dosing on incorporation into selenoproteins, in contrast to what was observed in Se-adequate rats (**Figure 4**). Furthermore, in Se-deficient rats, the amount of ^{75}Se labeling of GPX1 was the same as the labeling of TXNRD at all times (**Figure 7A**), whereas ^{75}Se labeling of GPX1 increased dramatically in Se-adequate liver from 1 to 3 to 24 h after dosing **(Figure 7B)**. In high-Se rat liver, there was little incorporation of ^{75}Se into the 65 kDa species; ^{75}Se incorporation into GPX1 was considerably less as compared to Se-adequate liver, with the more modest incorporation doubling from 3 to 24 h, and doubling again from 24 to 72 h. The pattern of ^{75}Se incorporation from [^{75}Se] selenate (**Figures 7D–F**) was virtually the same as that observed with [^{75}Se]selenite, showing that both selenocompounds are metabolized in intact rats at similar rates into the precursor used for Se incorporation into selenoproteins.

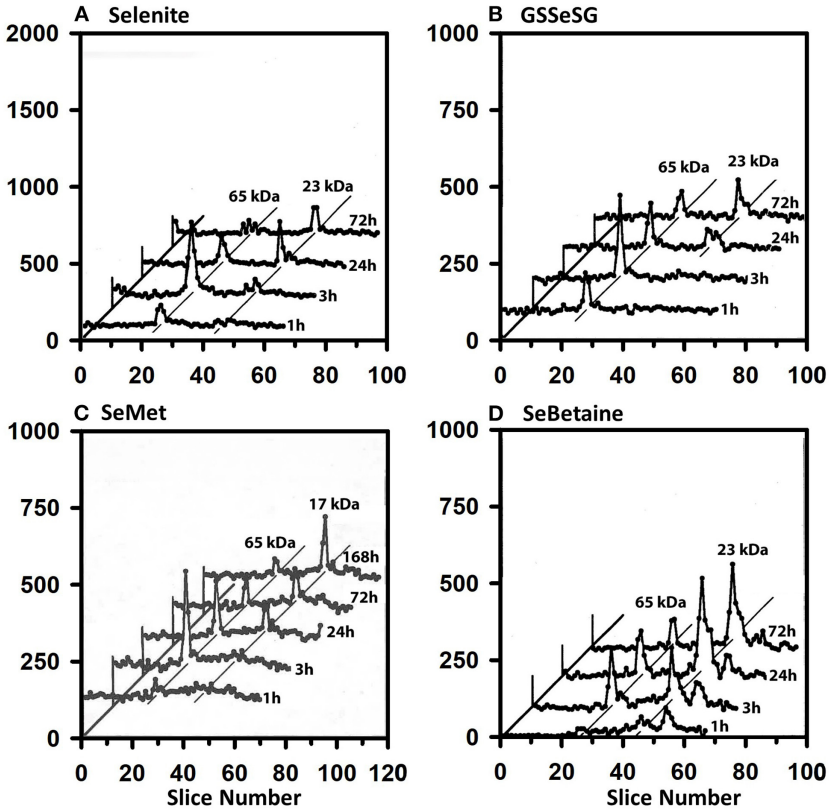

FIGURE 5 | Incorporation of ^{75}Se into heart supernatant proteins from Selenite **(A)**, GSSeSG **(B)**, SeMet **(C)**, and SeBetaine **(D)**. Rats were treated as described for **Figure 3**. Heart tissue was homogenized in Tris/SDS and centrifuged at 105,000 x g, and supernatants (1,500 μg protein) were separated using gradient SDS/PAGE as described for **Figure 3** ($n = 17$ total rats). GPX1 is 23 kDa, and the 65 kDa species are likely to the isoenzymes of TXNRD.

Effect of Met Status on ^{75}Se-Selenoprotein Labeling From [^{75}Se]SeMet

Because SeMet mixes with the Met pool and is incorporated non-specifically as a Met analog into general body proteins (16, 17, 19), we studied the effect of feeding three levels of dietary Met for 1 wk in the Se-adequate diet (0.2 μg Se/g diet as selenite) prior to injection of 50 μCi of tracer [^{75}Se]SeMet. Without Met supplementation, ^{75}Se incorporation into liver GPX1 from [^{75}Se] SeMet was approximately half the level of incorporation from [^{75}Se]selenite at all times in Se-adequate liver (**Figures 8A–C**). With 0.4% Met supplementation, the labeling of GPX1 from tracer [^{75}Se]SeMet was similar to that from [^{75}Se]selenite. Doubling dietary Met supplementation to 0.9% Met perhaps only slightly decreased the labeling of GPX1 relative to that observed with 0.4% dietary Met, suggesting that there was little enhanced release of ^{75}Se from [^{75}Se]SeMet to the precursor form of Se used for selenoprotein synthesis, at least in liver.

In contrast to liver, a different pattern of ^{75}Se incorporation from [^{75}Se]SeMet into plasma SELENOP was observed for the three levels of dietary Met (**Figures 8D–F**). The selenoprotein labeling patterns for SELENOP and GPX3 were virtually the same when Se-adequate rats were supplemented with 0 or 0.4% dietary Met for 1 week. Higher dietary Met supplementation at 0.9%, however, doubled the ^{75}Se labeling of plasma SELENOP at 3 and 24 h, as compared to labeling in 0.4% Met rats, indicating that there was increase SeMet catabolism releasing ^{75}Se for incorporation into SELENOP.

The "Missing" ^{75}Se

We used 2-mecaptoethanol treatment and SDS/PAGE analysis to focus on the flux of ^{75}Se into true selenoproteins, with the presumption that this would strip away low-MW selenometabolites and loosely bound selenospecies, including species linked by disulfide bonds. Our recent finding that low-MW and high-MW selenosugars are present in high quantities in Se-adequate and high-Se liver at least in turkeys (33), however, strongly suggests that the ^{75}Se we did not find in the SDS/PAGE ^{75}Se profiles is also important.

The recoveries of ^{75}Se in the gels following [^{75}Se]selenite injection are shown in **Figure 9** for rats fed 0 to 5 μg Se/g as selenite. The major plasma selenoprotein (**Figures 3**, **8**), SELENOP, is synthesized and secreted by the liver; the recovery of >70% of the applied ^{75}Se in plasma as SELENOP at 1 and 3 h after injection agrees other reports (40). Similarly, recovery of >50% of the injected ^{75}Se in the SDS/PAGE gels in testes supernatant, regardless of Se status, might be expected as

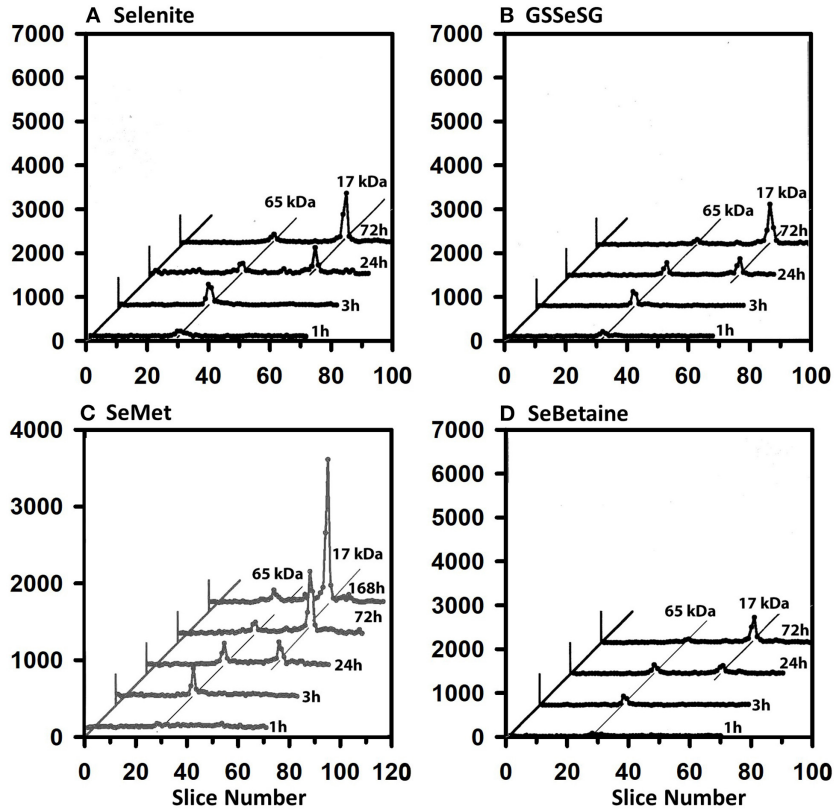

FIGURE 6 | Incorporation of ^{75}Se into testes supernatant proteins from Selenite **(A)**, GSSeSG **(B)**, SeMet **(C)**, and SeBetaine **(D)**. Rats were treated as described for **Figure 3**. Testes supernatants were prepared and analyzed by SDS/PAGE, as described for **Figure 5** (n = 17 total rats). GPX4 is 17 kDa, and the 65 kDa species are likely to the isoenzymes of TXNRD.

SELENOP is synthesized predominately by the liver, secreted, and then specifically targeted to the testes as mediated by the APOER2 receptor (LRP8) (41). In liver at 1 h, however, <15% of the applied ^{75}Se in liver cytosol was recovered on the gel as Sec-containing selenoproteins, regardless of Se status. At 3 h <40% was recovered in Se-deficient rat liver and <20% in Se-adequate rat liver; this low recovery matches with the lack of ^{75}Se labeling of liver GPX1 at 1 and 3 h (**Figures 4**, **7**). Increasing Se status decreased the recovery ^{75}Se at both 1 and 3 h, such that <5% of the applied Se was recovered as Sec selenoproteins in liver cytosol from rats fed 5 μg Se/g. A similar effect of Se status was observed in kidney at 1 and 3 h, although the ^{75}Se recovered in kidney was double the recoveries observed in liver. By 24 h in both liver and kidney, 30–50% of the applied ^{75}Se was recovered in the gels, consistent with the increased labeling of GPX1. Similar patterns were observed for tracer studies providing ^{75}Se as selenate or GSSeSG (data not shown). Clearly substantial cytosolic ^{75}Se was present as species other than Sec in selenoproteins. Especially in liver, even at 24 and 72 h, there was a progressive decline in ^{75}Se recovered as Sec selenoprotein as Se status increased from 0.2 to 2 to 5 μg Se/g diet.

When tracer ^{75}Se was injected as SeMet, the patterns were very different (**Figure 10**), showing that the early fate of SeMet is decidedly different than for inorganic Se. At least 30% of the applied ^{75}Se was recovered in the gel, regardless of tissue. With increasing time, there appears to be increased recovery of ^{75}Se in as Sec in selenoproteins in liver and heart, but not in plasma. Feeding a marginal Met diet or doubling the diet Met, however, had little effect on incorporation of ^{75}Se from SeMet into protein as assessed by recovery upon SDS/PAGE analysis. This matches with the selenoprotein profiles shown in **Figure 8**, with little effect of level of dietary methionine on the ^{75}Se labeling of GPX1 in liver and SELENOP in plasma.

DISCUSSION

These studies used only adult Holtzmann rats from our colony that were fed the basal Se-deficient diets supplemented with graded levels of Se as selenite for 50–80 days. The data for an individual selenocompound at each time in these figures was only collected for a single rat, so only the resulting patterns can be compared. No statistical analysis was conducted.

Collectively, the studies reported here present data from 80 individual rats. The SDS/PAGE profiles for these ^{75}Se tracer studies are very consistent and illustrate a constant time-driven pattern of ^{75}Se corporation into selenoproteins in four

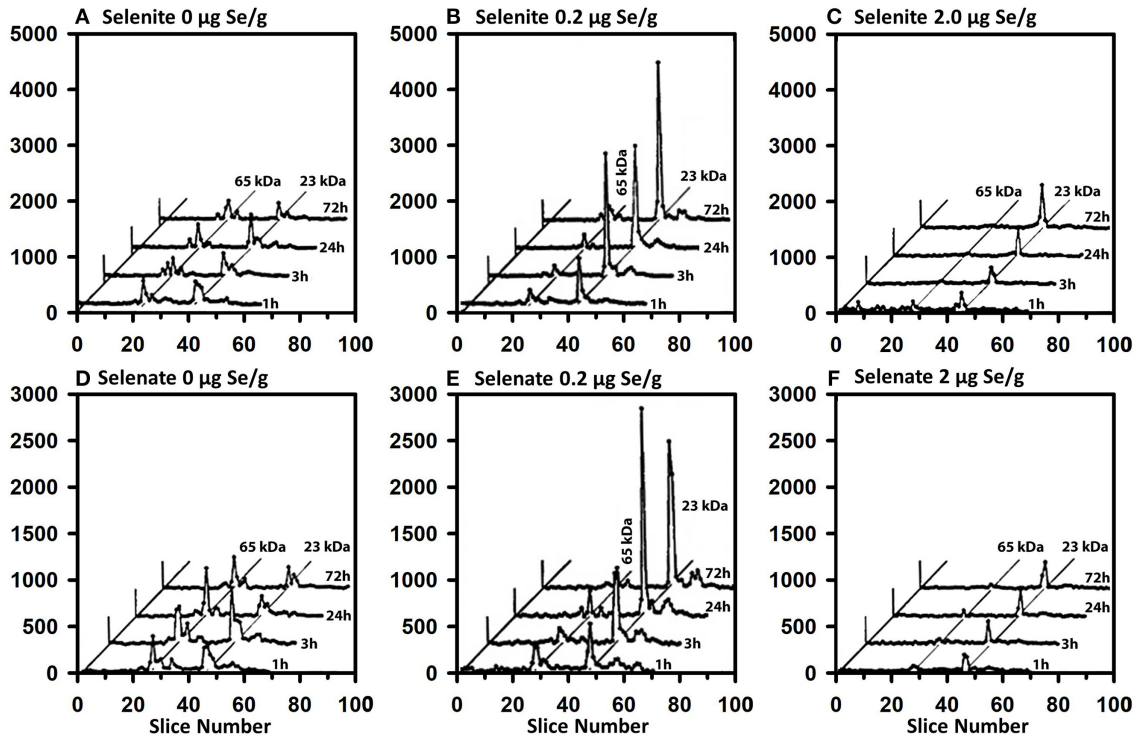

FIGURE 7 | Incorporation of ^{75}Se from [^{75}Se]selenite **(A–C)** and [^{75}Se]selenate **(D–F)** into liver cytosolic proteins. Rats were fed the indicated dietary Se levels as selenite for 50–80 d, injected iv with 50 µCi of tracer ^{75}Se-labeled selenite or selenate, and killed at 1, 3, 24, or 72 h afer dosing. After homogenization and centrifugation at 105,000 x g, cytosols (1,500 µg protein) were separated using gradient SDS/PAGE as described for **Figure 3** (n = 24 total rats). Liver GPX1 is 23 kDa, and the 65 kDa species are similar to the isoenzymes of TXNRD1.

tissues. The overall result is a clear pattern of very rapid Se metabolism from a diverse set of selenocompounds to a common intermediate used for synthesis and incorporation into well-defined ^{75}Se selenoprotein patterns for at least the major selenoproteins: plasma SELENOP and GPX3, liver and heart GPX1 and the 65 kDa species (most likely TXNRD1), and testes GPX4. No selenocompound resulted in incorporation into a profoundly different set of at least these major selenoproteins. Even SeBetaine, which had been identified as having distinct activity to prevent DMBA-induced mammary tumors, resulted in these same patterns. SeMet was similarly rapidly metabolized to the precursor used for selenoprotein synthesis. Collectively, these studies emphasize that this wide variety of selenocompounds are not uniquely or preferentially metabolized to provide Se for selenoprotein incorporation.

A schematic diagram of the metabolism of the five selenocompounds in these experiments is shown in **Figure 11**. All five ^{75}Se tracers were readily and rapidly metabolized to the selenide-level precursor used for co-translational incorporation of Se as Sec into selenoproteins (1–6). The various pathways shown in **Figure 11** have been discussed in detail previously (1, 3, 7, 20), with this same selenide-level selenospecies, the precursor for selenosugar synthesis (32, 33). The missing ^{75}Se metabolites, not detected by SDS/PAGE as selenoproteins, include low-MW selenosugars, high-MW "selenosugar-decorated" proteins, and other unknown metabolites (32, 33).

These studies used tracer levels of ^{75}Se. Estimates of total body burden of Se in an Se-adequate rat range from 48 to 61 µg total Se fed selenite (7, 42). Rats of this age consume ∼30 g diet/d, so feeding 0.2 µg Se/g diet would provide ∼6 µg of oral Se per day. A single injection of ∼0.5 µg Se in rats fed 0.2 µg Se/g diet represents ∼10% of the daily Se intake and ∼1% of the total body burden of Se, and thus can be considered a tracer. In Se-deficient rats fed the 0.02 µg Se/g diet or ∼0.6 µg per day, the 0.5 µg Se injection may represent an amount equivalent to that consumed in the diet. In a study with rats fed the Se-deficient diet for 60 d, injection of 15 µg Se as selenite failed to significantly raise GPX1 activity after 24 h (43). Here, the failure of the tracer ^{75}Se injections to increase ^{75}Se incorporation into GPX1 suggests the 0.5 µg Se dose was insufficient to substantially raise liver *Gpx*1 mRNA levels (**Figure 7**), further indicating that these were ^{75}Se tracer studies even in Se-deficient rats.

It is thought that selenide or a GSH-selenide intermediate are the precursor species used in the first step in Sec synthesis (3, 6). These studies show that both the inorganic and the organic selenocompounds were rapidly metabolized to the Sec-synthesis precursor. Furthermore, the pattern and timing of ^{75}Se labeling were almost identical in each tissue for all the selenocompounds. There was no apparent unique metabolism of

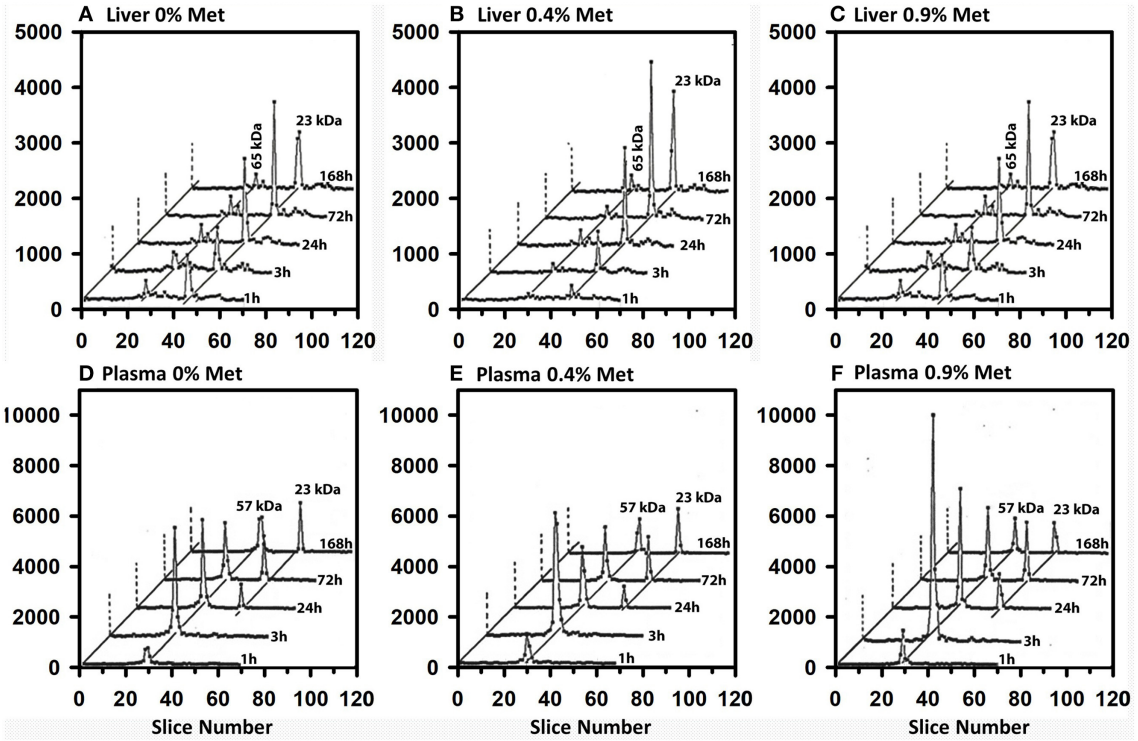

FIGURE 8 | Incorporation of ^{75}Se from [^{75}Se]SeMet into liver cytosolic **(A–C)** and plasma **(D–F)** proteins. Rats were supplemented with 0.2 μg Se/g diet as selenite in the basal diet containing 0.4% supplemental Met for 50–80 d. For 7 days prior to Se injection, supplemental Met was adjusted to 0, 0.4 or 0.9% D,L-Met; rats were then injected iv with 50 μCi of tracer ^{75}Se-labeled SeMet, and killed at 1, 3, 24, 72, or 168 h afer dosing. Liver cytosol and plasma samples were prepared as described in **Figures 3** and **4** ($n = 30$ total rats). Liver GPX1 is 23 kDa, and the 65 kDa species are similar to the isoenzymes of TXNRD1; plasma SELENOP is 57 kDa and GPX3 is 23 kDa.

one of these selenocompounds relative to the others, indicating that once internalized, the systemic Se metabolism of these species is the same. Furthermore, the similar timing for Se incorporation from these species indicates that the rate-limiting steps in selenoproteins synthesis occur after uptake and initial metabolism and are not associated with the differences in initial metabolism.

Today's understanding of selenoprotein expression and regulation can explain the observed ^{75}Se labeling patterns. When tracer [^{75}Se]selenite and [^{75}Se]selenate were injected into Se-deficient rats, Se deficiency dramatically decreased the labeling of GPX1 in liver relative to Se-adequate rats at 3 to 72 h after injection, but had little effect on labeling of the 65 kDa species. We now know that liver *Gpx*1 transcripts are dramatically reduced in liver by Se deficiency to 10% of Se-adequate levels (35, 38, 44), providing an explanation for the blunting of ^{75}Se incorporation into GPX1 in Se-deficient rats. Se repletion studies show that it takes 24 h to substantially raise liver GPX1 activity (43), further explaining the observed delay to 24 h in achieving the maximal ^{75}Se incorporation into liver GPX1 (**Figure 4**). The failure to see increased ^{75}Se incorporation into Se-deficient liver (**Figures 7A,D**) further shows that the administered ^{75}Se was as a tracer dose which did not substantially raise total Se status. High Se status (2 μg Se/g) markedly diminished the ^{75}Se labeling of both GPX1 and the 65 kDa species, illustrating additional dilution of the tracer ^{75}Se (**Figures 7C,F**).

Our studies in this rat model show that more than half of the selenoprotein transcripts are not significantly decreased by Se deficiency; *Txnrd*1 mRNAs are only decreased to 60% of Se-adequate levels (35), This can explain why sustained ^{75}Se incorporation into the 65 kDa species was observed starting at 1 h in Se-deficient rat liver. Similarly, transcripts for liver SELENOP liver are not decreased in Se deficiency, explaining the rapid labeling of plasma SELENOP by 3 h (**Figure 3**). *Gpx*3 transcripts in kidney are also non-significantly decreased only to 60% of Se-adequate levels (35), supporting the appearance of ^{75}Se-labeled GPX3 in plasma at 24 h. Thus, the subsequent research on selenoprotein expression and regulation of selenoprotein transcripts since these tracer studies were conducted provides supporting rationale and insight into observed patterns of ^{75}Se incorporation into selenoproteins.

Basic biochemical studies have shown that SeMet is readily acylated to Met-tRNA and is incorporated into proteins in place of Met (19). Nutritional studies have further shown that marginal dietary Met increases deposition of SeMet into body proteins and decreases release of Se for tissue GPX1 synthesis (17). In the present studies, feeding a marginal-Met vs. Met-adequate diet

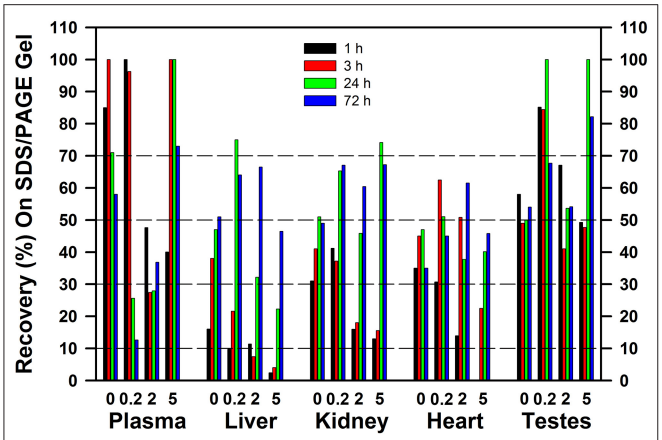

FIGURE 9 | Recovery of ^{75}Se from [^{75}Se]selenite as ^{75}Se-labeled selenoproteins on SDS/PAGE gels. Rats were supplemented with 0, 0.2, 2, or 5 μg Se/g diet as selenite for 50–80 d, injected iv with 50 μCi of [^{75}Se]selenite, and were tissues subjected to SDS/PAGE as described for **Figures 2–6** (n = 16 total rats). Values are the percent of the applied ^{75}Se recovered in the gel after SDS/PAGE in the indicated tissues at the indicated times.

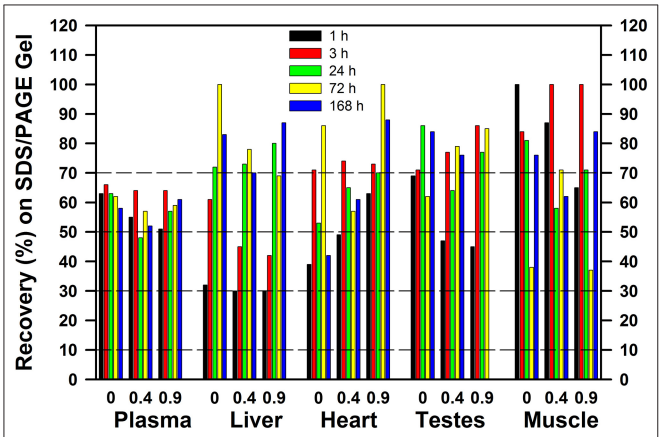

FIGURE 10 | Recovery of ^{75}Se from [^{75}Se]SeMet as ^{75}Se-labeled selenoproteins on SDS/PAGE gels. Rats were supplemented with 0.2 μg Se/g diet as selenite in the basal diet containing 0.4% supplemental Met for 50–80 d. For 7 days prior to Se injection, supplemental Met was adjusted to 0, 0.4, or 0.9% D,L-Met; rats were then injected iv with 50 μCi of [^{75}Se]SeMet,and tissues were subjected to SDS/PAGE as described for **Figures 2–6** (n = 15 total rats). Values are the percent of the applied ^{75}Se recovered in the gel after SDS/PAGE in the indicated tissues at the indicated times.

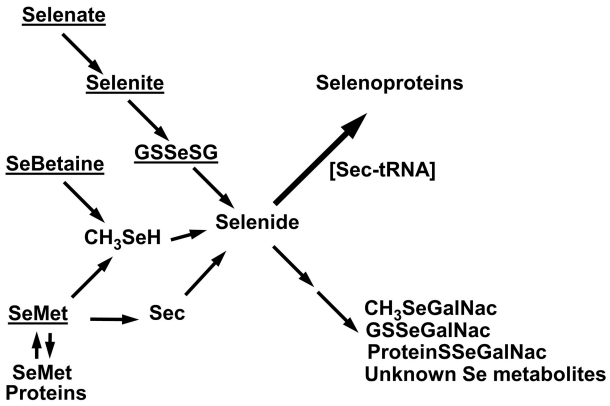

FIGURE 11 | Schematic diagram of Se metabolism to selenoproteins. Underlined are the five tracer selenocompounds administered in these experiments. Selenate is reduced to selenite; selenite can react with GSH to form GSSeSG, which is then further reduced to selenide. SeMet is degraded via transulfuration to Sec which can be metabolized to selenide, or SeMet is degraded via transamination to methaneselenol and then selenide. Alternatively, SeMet can be incorporated into general body proteins as a methionine analog. SeBetaine is degraded to release methaneselenol. Selenide is the precursor used for selenoprotein incorporation, as assessed by SDS/PAGE in these studies. Metabolites not detected by SDS/PAGE include the low MW selenosugars, high-MW "selenosugar-decorated" proteins, and other unknown metabolites.

for 1 wk prior to tracer [^{75}Se]SeMet injection had minimal effect of labeling of plasma SELENOP or GPX3, which indicated there was sufficient SeMet degradation to maintain the flux of Se into these species in Se-adequate rats. Similarly, high Met feeding for 1 wk also exerted at most small changes on SELENOP and GPX3 labeling. In liver in contrast, feeding a marginal Met diet for 1 wk prior to ^{75}Se injection increased labeling of liver GPX1, suggesting increased catabolism of SeMet to the Se precursor used for selenoprotein synthesis; high Met feeding for 1 wk had little effect on labeling of liver GPX1, perhaps because additional Se was incorporated into plasma SELENOP. Overall, feeding these varied Met diets to older rats for just 1 week did not have as dramatic effects as was found in longer non-tracer studies in young rats, or in studies on utilization of stored SeMet in general body tissues to provide Se for GPX1 synthesis (13, 16, 17).

The hidden story in these experiments is the extent of loss of ^{75}Se when tissue extracts were subjected to 2-mercaptoethanol treatment followed by SDS/PAGE. In liver and kidney at 1 and 3 h after ^{75}Se injection, especially in rats fed 0.2, 2, and 5 μg Se/g, there was an increasingly small amount of the cytosolic ^{75}Se detected as selenoprotein ^{75}Se; in rats fed 5 μg Se/g at 1 and 3 h, <5% of liver cytosolic ^{75}Se and < 20% of kidney cytosolic ^{75}Se was present in the selenoproteins retained in the SDS/PAGE gels. When these studies were conducted, we presumed that the missing ^{75}Se was low-MW intermediates on the pathways to selenoprotein incorporation or to formation of methylated excretion products (26). With our recent finding that more Se is present as selenosugars than is present as Sec even in Se-adequate turkey liver (33), the implication is that the missing ^{75}Se in these rat studies may have initially been selenosugars linked via selenodisulfide linkages nonspecifically to cysteine residues in high-MW general proteins. These species would be released by the 2-mercaptoethanol treatment and swept off at the bottom of the gel. Similarly, low MW selenosugar species such as CH_3-SeGalNac and GS-SeGalNac would be released as well. In rats fed 2 or 5 μg Se/g vs. 0.2 μg Se/g, there was even more missing ^{75}Se in rats, suggesting that increased quantities of these species are present in rat liver and kidney cytosols. The levels of these species in microsomal, mitochondrial, and nuclear fractions are completely unknown at present, as the turkey liver studies were

done on extracts of frozen tissue that would have included all subcellular organelles. Lastly, the increased retention of ^{75}Se in the gels at 24 and 72 h in liver and kidney, vs. 1 and 3 h, suggests that there may be rapid flux or turnover of Se within these missing, hypothetical, selenosugar pools of Se.

Low-MW selenosugars have been identified in animal tissues by multiple investigators, but they were always reported as being found in low-MW fractions. The discoverers of CH_3-SeGalNac in urine also reported separation of liver cytosol into a high-MW protein-containing fraction and a low-MW fraction by ultrafiltration, but reported CH_3-SeGalNac only in the low-MW fraction (32, 45). Other researchers used HPLC as the first step for plasma and tissue cytosol analysis and found late-eluting low-MW species that were identified as CH_3-SeGalNac and GSH-SeGalNac. These researchers also found broad early-eluting HPLC peaks that were described as containing high-MW selenoproteins/Se-binding proteins, but none of these reports recognized that the high-MW protein fractions could also contain selenosugars (46–49). Takahashi and colleagues (50) used stable isotope mass spectroscopy to identify GSH-SeGalNac and CH_3-SeGalNac in serum, liver, and kidney in Se-deficient rats given non-tracer doses of nine different selenocompounds, but they also showed uncharacterized broad high-MW Se-containing protein peaks in the HPLC profiles (50). Thus, the high-MW selenosugar-decorated proteins in turkey liver appears to be the first characterization of what might be missing in our SDS/PAGE gel profiles of rat selenoproteins.

CONCLUSIONS

In summary, these studies show that there is very rapid Se metabolism from a diverse set of selenocompounds to the common intermediate used for synthesis and incorporation of ^{75}Se into the major selenoproteins in a variety of tissues. Collectively, these studies emphasize that this wide variety of selenocompounds are not uniquely or preferentially metabolized to provide Se for selenoprotein incorporation. Furthermore, examination of the SDS/PAGE selenoprotein profiles shows that synthesis of selenoproteins is only part of the full Se metabolism story. The missing ^{75}Se species, especially at early timepoints, are likely to be low-MW and high-MW selenosugars and related precursors. Differential metabolism of various selenocompounds into different selenosugar species may occur; these species may be involved in the prevention of cancer or other diseases linked to Se status and may be associated with Se toxicity. Studies similar to these presented here, and characterization of the Se species in tissues by HPLC-MS, will be needed to more fully flesh out the complete metabolism of selenium.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee, University of Arizona (A3248 #86-0172 and #86-0357) Animal Care and Use Committee, University of Missouri (A3394 #1425).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Higher Dietary Se Intake Is Associated With the Risk of New-Onset Fracture: A National Longitudinal Study for 20 Years

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Background: The association between dietary selenium (Se) intake and osteoporosis-related fractures remains inconsistent. We aimed to examine the dose relationship between Se intake and incident fracture among Chinese adults.

Methods: The dietary data were retrieved from the China Health and Nutrition Survey conducted between 1991 and 2011, and 17,150 participants aged above 20 were included. A 3-day, 24-h recall of food intake was performed to assess cumulative average dietary Se intake. The fracture was based on self-report in each survey between 1997 and 2011. The association between Se intake and fracture was tested by Cox regression, and the non-linear association was examined by restricted cubic splines (RCS).

Results: There were 976 fracture cases during a mean of 10.2 years follow-up. In a fully adjusted Cox model, across the quartiles of Se intake, the hazard ratios (HRs) for fracture were 1.07 (95% CI .86–1.33), 1 (reference), 1.25 (95% CI 1.02–1.53), and 1.33 (95% CI 1.07–1.65). RCS showed a parabolic association (P non-linear = 0.037) between Se and fracture for men as well as a U-shape dose-response (P non-linear = 0.04) between Se and fracture for subjects living in highly urbanized areas.

Conclusion: In conclusion, there is a non-linear association between selenium intake and fracture, with higher intake associated with increased risk. The shape of the association varies by gender and urbanization level.

Keywords: Se, fracture, CHNS, China, adults

INTRODUCTION

Selenium, an essential trace element for human health, is involved in various physiological processes and function realization, such as immune response, anti-oxidation, anti-tumor, and thyroid hormone regulation (1). Previous studies have confirmed that in China, inadequate Se intake was linked to Keshan disease and Kashin-back disease (2). Meanwhile, other studies have demonstrated that excessive Se intake was also associated with chronic diseases, such as hypercholesterolemia, hypertension, and diabetes (3–6).

Grain, fish, and meat are major sources of Se intake for human beings (7). However, Se intake varies substantially by region. For example, the mean Se intake is 40 μg per day in Europe, while in the United States it is 93 μg per day for women and 134 μg per day for men (8, 9). According to the 2017 Reference Intake of Dietary Trace Elements issued by the National Health Commission of China, the estimated mean requirement (EAR), recommended nutrient intake (RNI), and tolerable upper limit intake (UL) of Se are 50, 60, and 400 μg, respectively (10). In addition, Se is unevenly distributed in the natural environment of China, about 72% of soil is Se-deficient (below.125 mg/kg), and the national average concentration of Se in soil is.239 mg/kg, ranging from.006 to 59.4 mg/kg (11, 12). The low-Se belt in the Chinese mainland (soil Se <0.2 mg/kg) sprawls from the northeast to the southwest, along with the Taihang and Qin Ling Mountains, Loess Plateau, and eastern part of the Tibetan Plateau (12). In contrast, the Se-rich belt (soil Se >20 mg/kg) spreads across Enshi in Hubei province, Ziyang in Shaanxi province, Shitai in Anhui province, and Yichun in Jiangxi province (13).

The relationship between Se intake and bone fracture has been explored in population-based studies (14–18). Se exists in the human body, mainly in the form of selenoprotein, for the maintenance of natural bone metabolism, proliferation, and differentiation; and the upkeep of homeostasis of extracellular matrix (ECM) (14) and bone mineral density (BMD) (15). Existing evidence suggests that the concentration of serum Se can affect the synthesis, fibril formation, and ultra-microstructure metabolism of cartilage collagen. Se deficiency is reported as an independent risk factor for abnormal cartilage collagen (16). Low Se in hair and plasma is related to BMD reduction (17, 18). However, inconsistent findings are reported on the association between Se intake and osteoporosis/fracture risk (17–22). A cross-sectional study of 7,407 middle-aged and older women in the United States, and a case-control study on 107 postmenopausal women in Turkey did not find any association between high Se intake and risk of osteoporosis and BMD (17, 19). A recent study on 1,365 Spanish adults (medium serum Se 84.9 μg/L at baseline) observed a U-shape dose-response relationship between serum Se and osteoporosis-related fracture (20). In contrast, a cross-sectional study in China argued that deficient dietary Se intake (<50 μg/day) is associated with higher prevalence of osteoporosis among 1,452 middle-aged and older people (21). The National Health and Nutrition Examination Survey (NHANES) among 2,983 American adults with relatively high Se intake (median Se intake of 101.5 μg/day), reports that higher Se intake was correlated with lower likelihood of bone fracture (22). The inconsistent findings of previous studies may be attributed to differences in study design, Se biomarkers, measuring instruments and methods for calculation of Se, and geographic regions and populations.

Few longitudinal studies have been conducted to examine the association between Se intake and risk of fracture. Although Se status is known for its antioxidant properties, the association between high Se intake and chronic disease remains unclear, probably attributed to its narrow safe window and other confounding factors (23, 24). In previous two national studies, higher Se intake decreased the risk of hypertension in north China but increased the risk of diabetes in the United States (23, 24). Furthermore, it has been pointed out that Se intake has a dose response to the incidence of diabetes and hypertension (4–6, 25–28). A cross-sectional study found that serum Se (referent point: 120 μg/L), Se hair (referent point: 8 μg/L), and Se nail (referent point: 6 μg/L) were related to blood pressure and hypertension in a U-shape trend (27). In a systematic review and meta-analysis, both low serum Se (<97.5 ug/L) and high serum Se (>132.5 ug/L) increased the risk of type 2 diabetes mellitus (T2DM) (26). Meanwhile, existing evidence suggests that patients with T2DM or hypertension have an increased risk of fracture, suggesting that chronic diseases may mediate the association between Se intake and risk of fracture (29, 30). Besides, Balvez-Fernandez et al. observed a U-shape curve for BMD and fracture associated with the increment of serum Se with a turning point of 105 μg/L (20).

Given this inconsistency, we hypothesized that a U-shape association exists between Se intake and fracture. Therefore, we aimed to investigate the association between dietary Se intake and fracture among Chinese adults aged 20 and above who participated in the China Health and Nutrition Survey (CHNS) between 1991 and 2011.

METHODS

Study Sample

The China Health and Nutrition Survey is an ongoing longitudinal follow-up project conducted by the Carolina Population Center at the University of North Carolina at Chapel Hill and the National Institute for Nutrition and Health (NINH) at the Chinese Center for Disease Control and Prevention (CCDC), aiming to study the health and nutritional status of residents from different aspects, such as socio-demographics, economic development, public resources, and health indicators. More than 30,000 individuals from 7,200 households in 15 provinces were sampled by the multi-stage stratified cluster random sampling process. CHNS began in 1989, and so far, there have been 10 waves of surveys conducted (in 1989, 1991, 1993, 1997, 2000, 2004, 2006, 2009, 2011, and 2015), and respondents in waves 1991–2011 were adapted to examine the association between Se intake and fracture in this study because dietary data were available.

Respondents who participated in at least two waves after 1997 were included in the study; 25,252 candidates aged 20 and above were identified in the preliminary screening. Subsequently, respondents without record of food intake ($n = 1123$), abnormal in daily energy intake (males: >6000 kcal or <800 kcal; females: >4000 or <600 kcal) ($n = 112$) (31), in pregnancy or lactation ($n = 168$), with only once completed survey record or no specific fracture information ($n = 6075$) and having fracture at baseline

Abbreviations: CHNS, china health and nutrition survey; BMI, body mass index; BMD, bone mass density; Se, selenium; RCS, restricted cubic splines; HR, hazard ratio; CI, confidence interval; NHANES, national health and nutrition examination surveys; MET, metabolic equivalent of task; CDC, centers for disease control.

($n = 714$) were excluded. A total of 17,150 participants were finally eligible for the analyses, and there were 976 new fracture cases (**Supplementary Figure 1**). The survey was approved by the University of North Carolina (United States) and the National Institute of Nutrition and Food Safety (China). A written informed consent was obtained from every participant.

Outcome Variable: Fracture

Self-reported history of fracture was collected by asking, "Have you ever had a fracture?" (32). In this study, the incident fracture was defined as a fracture that occurred in the follow-up period among those without a history of fracture at baseline.

Exposure Variables: Se Intake

In the China Health and Nutrition Survey, a dietary assessment is based on the food intake of three consecutive 24-h recall at the individual level. A food inventory at the household level, which included all available stored and purchased foods, was weighted during the same 3-day period. The dietary intake of each respondent was recorded by asking each family member to report all the foods they ate at home and away from home over the past 24 h for 3 consecutive days. Trained interviewers collected the details of the intake, such as amounts and types of food, types of meal, and places of having food using standard forms. The 3 consecutive days were randomly allocated to 2 week days and a week end day across the week for each sampling unit. Household food consumption was estimated by weighing and comparing the changes in the inventory at the beginning and the end of the survey. Condiment consumption for each member was estimated by the ratio of individual to the whole household energy intake. All family storage and leftovers purchased from a grocery or picked from their gardens were also weighed and recorded. Preparation waste was estimated when exact weighing was not available. At the end of the survey, all the remaining foods were weighed again and recorded. The amount of food in each dish was calculated through the household inventory, reporting the proportion of consumption for each family member (33). The assessment of the mean daily Se intake (μg) of each respondent for each food item was derived from the dietary data module, and the food code was decoded from the Chinese Food Composition Table (CFCT). Three versions of CFCT were used to evaluate the intake of foods and nutrition in Chinese residents. To be specific, the 1981 CFCT was adopted for the waves of 1989, 1991, and 1993; the 1991 CFCT was available for 1997 and 2000; the 2002/2004 CFCT was adopted for 2004, 2006, 2009, and 2011. The CFCT took the region into consideration for the nutrient content of individual food items. Thus, the Se intake in the analysis was region-specific.

Because of the repeated measure design, we used three indicators of Se intake: baseline Se intake, cumulative average Se intake, and most recent Se intake. The use of the cumulative mean of Se intake aimed to reduce the variation within individuals and reflect long-term intake levels from 1991 to 2011. For example, when Se intake was x, y, and z in 1991, 1993, and 1997, the cumulative mean intake was x in 1991, $(x + y)/2$ in 1993, and $(x + y + z)/3$ in 1997. The most recent Se intake was measured in a specific wave.

Covariates

Covariates, such as socio-demographics, health status, dietary patterns, and lifestyle factors, were collected with a structural questionnaire. Dietary foods were collapsed into 35 groups according to similar cooking styles and ingredients (34). Dietary patterns were constructed by factor analysis. The number of factors was determined according to the following rules: (1) eigenvalue >1; (2) scree plot; and (3) factor interpretability. Varimax rotation was used to help interpret the identified patterns. Two dietary patterns were identified and named "modern" and "traditional" dietary patterns (34). Physical activities were measured by consuming the metabolic equivalent of task (MET), which was calculated with the weighted mean of daily activities assessed with the Compendium of Physical Activities scale measuring the occupational, domestic, transportation, and leisure-time activities (35). An urbanization index was constructed and categorized into three levels (low, medium, and high), reflecting the living standards based on percentiles (32). Smoking status was categorized into non-smoker, ex-smokers, and current smokers. *Per capita*, annual household income was grouped as "low," "medium," and "high" based on tertiles. Body mass index (BMI) was used to assess obesity levels, and the cutoff point of overweight was 24 kg/m^2 (36). Geographically, we defined Heilongjiang, Liaoning, Shandong, and Henan as the north; and Jiangsu, Hubei, Hunan, Guizhou, and Guangxi as the south (24).

Hypertension is defined as systolic blood pressure above 140 mmHg or diastolic blood pressure above 90 mmHg, or having clinically confirmed hypertension. Self-reported diabetes was recorded according to the question "Did you have a clinical diagnosis of diabetes?"

Statistical Analyses

The continuous variables were described as mean ± standard deviation (SD), and the categorical variables were described as frequency and proportion (%). The categorical variables were analyzed by Chi-square test, and the continuous variables were tested by ANOVA or Kruskal–Wallis test. The multivariable Cox regression was used to assess the association between cumulative mean intake of dietary Se and incident fracture. The second quartile of Se intake (Q2) was set as the reference group. In sensitivity analyses, baseline and most recent dietary Se intakes were used in the Cox regression models instead of cumulative mean Se intake. A set of Cox regression models was built: Model 1- adjusting age, gender, and energy intake; Model 2- further adjusting smoking, alcohol drinking, income, urban, education, and physical activity; Model 3- further adjusting dietary patterns based on Model 2.

In the subgroup analyses, multiplicative interaction between dietary cumulative Se intake and covariates was tested by adding a cross-product term in the full multivariable Cox regression model, such as urbanization levels, sex, smoking, regions, and alcohol drinking. The RCS regression in the fully adjusted model was fitted with three knots (at 10, 50, and 90 percentiles) to examine the non-linear relationship between cumulative mean Se intake and incident fracture. We also conducted subgroup analyses by gender and urbanization levels.

All analyses were performed using Stata 16.1 (Stata Corporation, College Station, TX, United States). Statistical significance was considered when $P < 0.05$ (two-sided).

RESULTS

A total of 17,150 participants free of fracture at baseline were included in the analyses. Across the quartiles of Se intake, the mean (SD) of Se intake is 20 ± 5, 31.8 ± 2.9, 42.5 ± 3.6, and 71.2 ± 44.9, respectively, and the average Se intake for each evaluation wave is presented in **Supplementary Figure 3**. **Table 1** shows that individuals in the fourth quartile of Se intake are more likely to ($P < 0.001$) have a higher intake of macronutrients (proteins, carbohydrates, and fats) and energy, to follow a modern dietary pattern (characterized by animal-based diet), to be younger (41.4 ± 14.4). The prevalence of overweight (29.2%) and current smoking (34.4%) was also higher among those with high Se intake. Men had a higher Se intake than women.

A total of 976 incident fractures occurred during the study period, and the median follow-up time was 9.1 years (147,770 person-year). The incidence rates were 5.8, 6, 7, and 7.7 per 1,000 person-years across the quartiles of cumulative Se intake. The cumulative Se intake was positively associated with fracture after adjusting for age and gender. Compared with the second quartile of Se intake, the hazard ratios (HRs) and 95% confidence intervals (CIs) for the fracture were 1.22 (95% CI: 1–1.5) in the third quartile and 1.25 (95% CI: 1.01–1.54) in the fourth quartile when sociodemographic and lifestyle factors were adjusted (P-trend < 0.05). The association became slightly stronger when dietary patterns were adjusted, with HRs of 1.25 (95% CI: 1.02–1.53) in the third quartile and 1.33 (95% CI: 1.07–1.65) in the fourth quartile. Compared with model 3, the association stayed robust steadily when diabetes and hypertension were adjusted in model 4, with HR of 1.24 (95% CI:1.03–1.49) in the third quartile and 1.3 (95% CI: 1.07–1.58) in the fourth quartile (**Table 2**). A positive association between high Se intake and fracture was also found when we used baseline Se intake and most recent Se intake. **Supplementary Figure 2** shows the association between sociodemographic factors and fracture. Age, high income, urbanization levels, and overweight were positively associated but education was inversely associated with the risk of fracture.

In the subgroup analyses (**Table 3**), we did not observe any significant effects of sex, alcohol drinking, and smoking on the association between cumulative Se intake and fracture, while living at a high urbanization level was detected to exacerbate the association (P-interaction = 0.013). Among those living in a highly urbanized area, high Se intake was associated with an increased risk of fracture (HR: 1.76, 95% CI: 1.26–2.46) compared with the second quartile of Se intake.

A non-linear association between Se intake and fracture was found in restricted cubic splines analyses. Overall, no significant associations were determined in the RCS in the whole or in women (**Figures 1**, **2B**). However, there was a significant non-linear association for men (**Figure 2A**). In addition, a U-shape association was found between Se intake and fracture for participants living in highly urbanized areas (P-non-linearity = 0.004), with the lowest risk around the intake of 30 μg/day (**Figure 3A**). Additional complete information on restricted cubic splines analyses was performed in **Figure 2B** and **Figure 3B** as well as **Figure 3C**.

DISCUSSION

In this population-based study with 17,150 Chinese adults, there was a positive association between high Se intake and fracture, independent of sociodemographic and lifestyle factors, such as dietary patterns. The restricted cubic splines showed that the association was non-linear, particularly for men and subjects living in highly urbanized areas. In addition, the average Se intake ranges from 41.5 μg/day in 1991 to 41.9 μg/day in 2011, which has generally been rising since 1997 (**Supplementary Figure 3**). However, the overall Se intake is lower than China EAR (50 μg/day). Meanwhile, the Se intake of adults in China was relatively insufficient compared with that of adults in other countries (22, 37–39). In China, the median dietary intake is 40.8 μg/day for men and 39.5 μg/day for women (38). In the United States, the geometric means dietary Se intake was 101.5 μg/day according to a study (22), and the median dietary Se intake varied between 79–99 μg/day in another research (34). In Europe, the median of dietary Se intake was 94.3 μg/day (37).

The association between Se status and fracture is inconsistent. A longitudinal study in Spain showed that participants with higher Se intake had a significant risk of fracture (20). In contrast, a cross-sectional study in the United States found an inverse correlation between blood, serum, and dietary Se with the occurrence of fracture (22), which was subsequently confirmed by another two studies performed in the United States and China (38, 39). Nevertheless, no association was found between Serum Se and fracture subjects from five European cities (37). This study found that dietary Se intake was positively associated with fracture, and the association was strengthened after dietary patterns were adjusted, which was consistent with the previously published study in Spain (20). In the longitudinal study in Hortega, HRs for fracture across the tertiles of plasma Se were 1, 1.09 (95% CI: 55–2.16), 1.67 (95% CI: 91–3.04), and the HR of fracture for 80th percentiles of plasma Se distribution was 2.25 (95% CI: 1.13–4.49) compared with the 20th in a model that adjusted for age, sex, BMI, education, physical activity, urine cotinine, glomerular filtration, smoking, and alcohol drinking (20). However, negative associations between dietary Se intake and fracture were detected by the National Health and Nutrition Examination Surveys (NHANES) in the United States and a case-control study in China (22, 38). The possible reasons might be that the NHANES was a cross-sectional study and had a high baseline Se intake (mean 101.5 μg/day) and that several bone parameters were applied, such as total spine and femur BMD and Fracture Risk Assessment Tool (FRAX) scores and history of bone fractures (22). The limitations of the NHANES study included its cross-sectional study design and lack of adjustment for physical activity in the analysis. Similarly, with 726 pairs, the duration of exposure was difficult to confirm, and recall

TABLE 1 | Baseline sample characteristics by quartiles of selenium intake (n = 17,150).

Factors	Q1	Q2	Q3	Q4	*P*-value
	***N* = 4,331**	***N* = 4,315**	***N* = 4,255**	***N* = 4,249**	
Selenium intake (ug/d), mean (SD)	20.0 (5.0)	31.8 (2.9)	42.5 (3.6)	71.2 (44.9)	<0.001
Energy intake (kcal/d), mean (SD)	1,793.9 (558.2)	2,059.1 (561.2)	2,281.0 (601.0)	2,540.4 (687.6)	<0.001
Fat intake (g/d), mean (SD)	51.4 (30.9)	62.8 (31.2)	72.0 (33.2)	86.1 (40.7)	<0.001
Protein intake (g/d), mean (SD)	48.0 (14.4)	61.6 (14.9)	72.5 (16.8)	88.8 (23.8)	<0.001
Carbohydrate intake (g/d), mean (SD)	283.1 (115.1)	308.5 (116.3)	331.3 (126.6)	345.8 (131.7)	<0.001
Traditional southern dietary pattern score, mean (SD)	0.1 (0.7)	−0.0 (0.9)	−0.0 (1.0)	0.1 (1.3)	<0.001
Modern dietary pattern score, mean (SD)	−0.5 (0.6)	−0.3 (0.8)	0.0 (0.9)	0.6 (1.3)	<0.001
Age (years), mean (SD)	44.0 (16.3)	42.7 (15.4)	41.9 (14.8)	41.1 (14.4)	<0.001
BMI (kg/m2), mean (SD)	22.4 (3.3)	22.7 (3.3)	22.9 (3.3)	23.3 (3.4)	<0.001
Overweight (%)	19.1%	23.3%	24.9%	29.2%	<0.001
Sex (%)					<0.001
Men	37.3%	43.2%	52.5%	59.6%	
Women	62.7%	56.8%	47.5%	40.4%	
Income (%)					<0.001
Low	38.7%	28.7%	24.4%	20.0%	
Medium	33.5%	35.4%	34.2%	30.3%	
High	27.8%	35.8%	41.4%	49.7%	
Education (%)					<0.001
Low	52.8%	40.1%	33.7%	26.6%	
Medium	29.2%	33.6%	34.9%	35.1%	
High	18.1%	26.3%	31.5%	38.3%	
Diabetes (baseline in 2009 only, %) n = 1228; 214, 274, 409, 2038	7.5%	8.4%	8.2%	9.5%	0.83
Self-reported diabetes (%)	1.6%	1.8%	2.0%	2.9%	<0.001
Hypertension (%)	15.9%	16.3%	16.7%	17.5%	0.25
Urbanization (%)					<0.001
Low	43.5%	30.3%	24.4%	19.3%	
Medium	27.6%	29.9%	27.8%	25.7%	
High	28.9%	39.7%	47.7%	55.0%	
Smoking (%)					<0.001
Non-smoker	73.6%	70.6%	66.2%	63.2%	
Ex-smokers	2.0%	1.8%	1.9%	2.4%	
Current smokers	24.4%	27.6%	32.0%	34.4%	
7–9 h	76.1%	79.8%	81.7%	79.7%	
< = 6 h	11.5%	10.1%	8.8%	9.7%	
> = 10 h	12.4%	10.1%	9.5%	10.6%	
Physical activity (MET-hrs/week), mean (SD)	143.8 (118.2)	135.7 (113.0)	129.4 (108.0)	128.5 (108.7)	<0.001
Survey year (%)					<0.001
1997	49.1%	47.7%	46.1%	37.3%	
2000	14.3%	15.0%	13.3%	13.7%	
2004	9.1%	7.8%	8.3%	11.2%	
2006	3.9%	4.7%	5.1%	6.5%	
2009	6.6%	8.0%	9.5%	11.8%	
2011	16.9%	16.7%	17.8%	19.6%	

BMI, body mass index; MET, metabolic equivalent; h, hours.

bias might also be introduced in the Chinese case-control study. Moreover, measuring errors of antioxidants might also lead to over-or underestimates of Se intake (38). A 7-year multi-center study across five European cities indicated that high serum Se was not associated with hip fracture incidence among postmenopausal women (37), which was opposite to the findings of this study. It is probably because only few fracture cases (31 vertebral fractures, 80 non-vertebral fractures, seven hip fractures) were reported in the 2,374 healthy participants.

Several factors may explain the positive association between dietary Se intake and fracture. First, long-term high Se intake is associated with an increased prevalence of diabetes (26,

TABLE 2 | Hazard ratio (95%CI) for incident fracture by quartiles of selenium intake.

Quartiles of selenium intake	Q1	Q2	Q3	Q4	*P* for trend
A. Cumulative average selenium intake					
Cases	226	219	253	278	
Person-years	38,732	36,754	36,123	36,161	
Incident rate (per 1000)	5.8	6.0	7.0	7.7	
Model 1	0.99 (0.82–1.20)	1.00	1.18 (0.99–1.42)	1.36 (1.13–1.63)	0
Model 2	1.09 (0.88–1.36)	1.00	1.22 (1.00–1.50)	1.25 (1.01–1.54)	0.092
Model 3	1.07 (0.86–1.33)	1.00	1.25 (1.02–1.53)	1.33 (1.07–1.65)	0.021
Model 4	1.02 (0.83–1.24)	1.00	1.24 (1.03–1.49)	1.30 (1.07–1.58)	0.005
B. Baseline selenium intake					
Cases	217	257	258	244	
Person-years	40,567	38,197	35,992	33,014	
Incident rate (per 1000)	5.3	6.7	7.2	7.4	
Model 1	0.76 (0.64–0.91)	1.00	1.08 (0.91–1.29)	1.13 (0.95–1.35)	0
Model 2	0.84 (0.68–1.03)	1.00	1.09 (0.89–1.32)	1.06 (0.87–1.30)	0.023
Model 3	0.82 (0.67–1.01)	1.00	1.11 (0.91–1.34)	1.10 (0.89–1.35)	0.006
Model 4	0.79 (0.65–0.95)	1.00	1.05 (0.88–1.26)	1.10 (0.92–1.32)	0.001
C. Most recent selenium intake					
Cases	235	221	252	268	
Person-years	38,194	37,183	36,908	35,486	
Incident rate (per 1000)	6.2	5.9	6.8	7.6	
Model 1	0.99 (0.82–1.20)	1.00	1.17 (0.98–1.41)	1.29 (1.07–1.55)	0.003
Model 2	1.14 (0.92–1.42)	1.00	1.18 (0.96–1.46)	1.21 (0.98–1.50)	0.335
Model 3	1.11 (0.90–1.38)	1.00	1.21 (0.98–1.49)	1.29 (1.03–1.62)	0.112
Model 4	1.12 (0.92–1.36)	1.00	1.19 (0.99–1.44)	1.30 (1.06–1.60)	0.074

Model 1 adjusted age, gender, and energy intake.
Model 2 further adjusted intake of fat, smoking, alcohol drinking, income, urban, education, and physical activities.
Model 3 further adjusted dietary patterns [20](traditional south pattern represented high intake of rice, pork, and vegetables, and low intake of wheat; modern dietary pattern represented high intake of fruit, soy milk, egg, milk, and deep-fried food).
Model 4 further adjusted for self-reported diabetes and hypertension.
All the adjusted variables were treated as time-varying covariates.

TABLE 3 | Association between quartiles of cumulative selenium intake and fracture stratified by residence, gender, hypertension, region, and drinking.

Factors	Q1	Q2	Q3	Q4	*P* for interaction
Urbanization levels					0.013
Low	0.74 (0.50–1.09)	1.00	0.83 (0.54–1.30)	1.31 (0.84–2.05)	
Medium	1.00 (0.69–1.44)	1.00	1.40 (1.00–1.97)	0.96 (0.65–1.42)	
High	1.59 (1.09–2.31)	1.00	1.47 (1.05–2.04)	1.76 (1.26–2.46)	
Sex					0.644
Men	0.92 (0.66–1.28)	1.00	1.15 (0.87–1.51)	1.28 (0.97–1.70)	
Women	1.19 (0.88–1.60)	1.00	1.37 (1.01–1.86)	1.37 (0.98–1.92)	
Smoking					
No	1.23 (0.95–1.60)	1.00	1.35 (1.04–1.76)	1.51 (1.15–1.99)	0.338
Yes	0.74 (0.49–1.11)	1.00	1.09 (0.78–1.51)	1.07 (0.75–1.52)	
Alcohol drinking					
No	1.07 (0.83–1.39)	1.00	1.12 (0.86–1.46)	1.19 (0.89–1.58)	0.204
Yes	0.99 (0.65–1.48)	1.00	1.46 (1.05–2.04)	1.55 (1.10–2.18)	
Region					0.788
North	0.81 (0.56–1.16)	1.00	1.17 (0.85–1.61)	1.30 (0.94–1.80)	
South	1.03 (0.79–1.33)	1.00	1.22 (0.95–1.56)	1.07 (0.81–1.42)	

*Models adjusted for the same covariates as model 3 in **Table 2**. Stratification variables were not adjusted in the corresponding models.*

28). It has been shown that the use of hypoglycemic drug is associated with a fracture in patients with type 2 diabetes (40), and that among medications, the use of thiazolidinediones and insulin has a greater impact (41). Besides, hyperglycemia may also result in the decline of osteogenic differentiation and bone turnover, reducing the bone quality and muscle mass of patients (42). Second, hypertension may have an increasing arteriosclerosis index, positively related to BMD decrement and fracture occurrence (43). Thirdly, unlike the findings in Hortega, the association between Se intake and fracture risk in this study is found to be independent of dietary patterns, suggesting potential mechanisms other than food preference. Se is a well-known trace element with a narrow gap between the safe and toxic levels (44). It has been shown that a dietary Se intake of over 55 μg/day cannot improve selenoprotein synthesis or activity in Se-replete subjects (45, 46). In addition, a previous study has argued that oxidative stress biomarkers (GSSG/GSH and MDA) and serum Se concentration have an inverse effect when serum Se is below 110 μg/L, and that GPx1 reaches the saturation status (47). It has been verified that oxidative stress (OS) biomarkers might reach a plateau value at this point (48). The findings of this study added further evidence to the view that higher dietary Se intake, exceeding the threshold required for maximum antioxidant protection by GPx1, may reverse the effect of selenoprotein osteoblast proliferation, differentiation, and osteoclast activity (49). Nevertheless, there is no universal agreement on the optimal intake of Se with the saturation of selenoproteins, suggesting that further epidemiological and clinical evidence is needed.

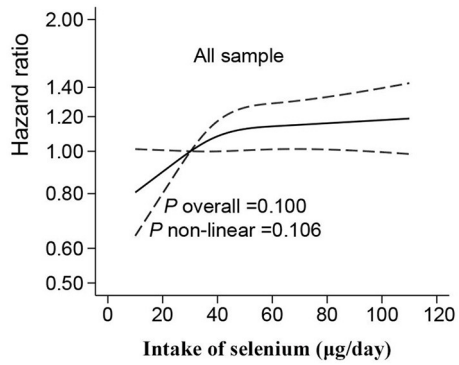

FIGURE 1 | Hazard ratios for fracture according to selenium intake in all the samples. Data were fitted by Cox proportional hazard regression. The solid line represents estimates (hazard ratio) using restricted cubic splines, and dash lines represent 95% CI. The models adjusted the same covariates with model 3 in **Table 2**.

In this study, the fracture risk showed a sharply increasing curve when cumulative Se intake was lower than 30 μg/day for men, and the increment of risk turned to slow down when it exceeded 30 μg/day. However, a dose-response relationship was not detected in women, which probably resulted from the sex difference in Se storage and metabolism (50–53). Laboratory experiments on rodent models also showed that a higher concentration of Se in serum and red blood cell (RBC) was found in female rats compared with male rats after being fed with Se supplementation (52, 53). In addition, a clinical trial was performed with healthy subjects at a high Se baseline, and four different doses of selenomethionine were provided (50). The overall serum Se of all the subjects was heightened after a year of intervention, but the concentration of selenoprotein remained invariant, which suggested that a high Se supplement might not enhance the activity of saturated selenoprotein (50). Furthermore, another study found that women had higher urinary Se excretion than men when receiving equal doses of selenomethionine, suggesting that sexual dimorphism exists in Se intake and metabolism in the human body (51). In addition, the hormonal status will also have an influence. A high estrogen level for premenopausal women may protect them from osteoporosis

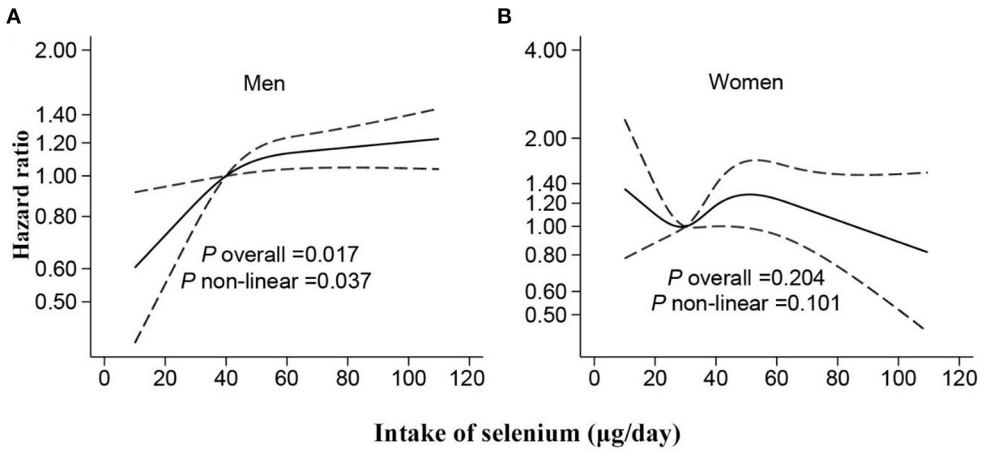

FIGURE 2 | Hazard ratios for fracture according to selenium intake stratified by gender [**(A)** for men; **(B)** for women]. Data were fitted by Cox proportional hazard regression. The solid line represents estimates (hazard ratio) by restricted cubic splines, and the dash lines represent 95% CI. The models adjusted the same covariates with model 3 in **Table 2**.

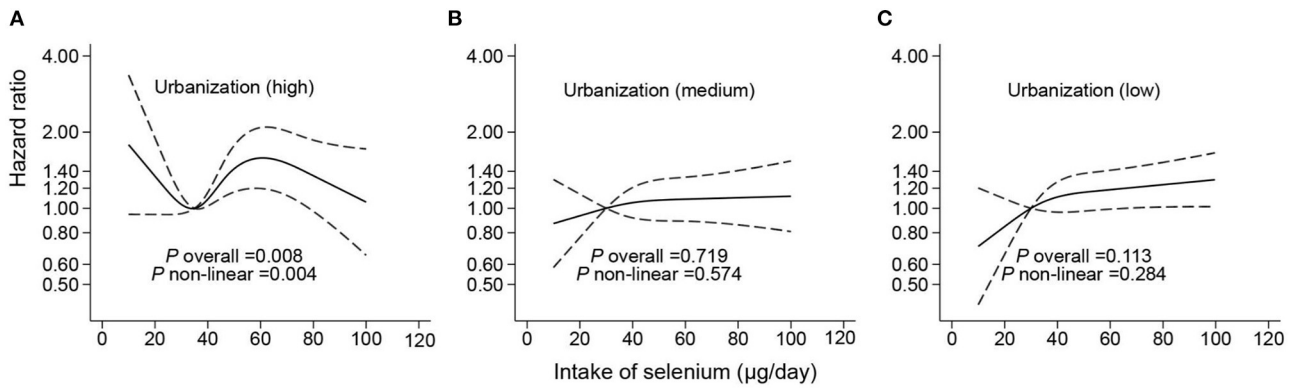

FIGURE 3 | Hazard ratios for fracture according to selenium intake stratified by urbanization level [**(A)** for high urbanization, **(B)** for medium urbanization, and **(C)** for low urbanization]. Data were fitted by Cox proportional hazard regression. The solid line represents estimates (hazard ratio) using restricted cubic splines, and the dash lines represent 95% CI. The models adjusted the same covariates with model 3 in **Table 2**.

and fracture, while a low testosterone level may cause loss of bone mass (54, 55).

A U-shape relationship between dietary Se intake and fracture risk was found among participants living in highly urbanized areas. It could be attributed to the interaction with air pollution. In highly urbanized regions, the environmental concentration of Arsenic (As), cadmium (Cd), lead (Pb), and black carbon (BC) is high (56). PM2.5 is the mass of particles per cubic meter in the air, with a size (diameter) generally <2.5 micrometers (μm), and is well-known as delicate particulate matter (2.5 micrometers is one 400th of a millimeter) (57). Studies have found that PM2.5 and BC were positively associated with an osteoporosis-related fracture (58). In addition, Se was also reported to have a strong affinity with heavy metals with which Se would combine to form non-toxic mental-bound selenoproteins and then excrete them from the body (59). In the second half of the curve, an exponential increment was found among those with high urbanization levels. The possible cause is that the western dietary pattern, containing high fat and protein from animal sources, is more prevalent among urban residents. Besides, residents in highly urbanized areas may have a higher prevalence of obesity and T2DM than the rural ones (60). Therefore, the combined effects of T2DM and obesity on fracture could be the mediator on the path from high Se intake to fracture occurrence in highly urbanized areas. Some studies found a pooled effect of diabetes and obesity on bone turnover and fracture in Finland and Canada (61, 62). Additionally, obesity may increase fracture risk independent from chronic disease, because increasing the bone burden and unbalanced buffer capacity of bone tissue provides an external impact (63).

Several strengths of this study need to be highlighted. First, most existing studies are focused on the association between most current dietary Se intake and fracture occurrence, so only few studies have explored the long-term effects. We included measures of 3-day dietary intake repetitively over 20 years, and used cumulative Se intake to reduce the error caused by the grand mean of single 3-day foods measures. Thus, Se intake may reflect long-term intake. Second, we examined the dose-response relationship between Se intake and fracture in various subgroups. Through this approach, we examined interactions and non-linear association. It may help to explore the threshold and saturation values of Se intake for the development of a precise nutrition intervention.

Several limitations of this study should also be considered. First, the use of a 3-day dietary intake survey may not adequately characterize long-term dietary Se intake. Second, the information collected from the respondents may suffer from recall bias on the history of fractures and other self-report variables. Although BMD and Fracture Risk Assessment Tool (FRAX) scores were both indicators of bone health, participants of CHNS were not examined for these indicators. Third, we did not have biomarkers of Se (e.g., hair Se, serum Se). Future studies will be expected to examine the association between the biomarkers of Se and fracture.

CONCLUSION

There is a non-linear association between Se intake and fracture. High Se intake increases the risk of fracture among Chinese adults. Along with the subsequent rise in Se intake, there is a parabolic increment of fracture risk in men and a U-shape fracture risk among people who live in highly urbanized areas. Further research is needed to make evidence-based policies and guidelines regarding Se intake for the promotion of bone health.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Institutional Review Board of the University of North Carolina at Chapel Hill and the National

Institute for Nutrition and Health, Chinese Center for Disease Control and Prevention. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YZhang and ZS contributed to the conception, analysis, and interpretation of data, drafted the report, and received the final version for publication. MY, YZhao, QY, SS, YL, and XL contributed to the analysis and interpretation of data, commented on the report, revised the manuscript, and approved the final version for submission. All authors read and approved the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.719147/full#supplementary-material

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Selenium Intake and Glycemic Control in Young Adults With Normal-Weight Obesity Syndrome

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Numerous endogenous functions related to antioxidant processes, reproduction, and thyroid metabolism, as well as actions related to glycemic control, have been attributed to selenium. This study aimed to evaluate whether dietary selenium consumption is associated with variables of glycemic control in a sample of young Brazilian adults with Normal-Weight Obesity (NWO) syndrome. This was a cross-sectional study that evaluated 270 individuals with adequate body weight and excess body fat, who had their body composition assessed by dual-energy X-ray absorptiometry. Socioeconomic, health, and lifestyle questionnaires and three 24-h food records were applied. Glycemic control markers were also evaluated. The prevalence of inadequate selenium intake was analyzed by the Estimated Average Requirement (EAR) cut-point method. The prevalence of disturbances in glycemic control markers according to selenium consumption was compared by either the chi-square or the Fisher's exact test, with individuals classified according to the EAR values for selenium. The associations were evaluated by multiple linear regressions, using the backward strategy. The mean ± standard deviation (SD) age was 23.7 ± 3.3 years, and the mean ± SD daily selenium intake was 59.2 ± 26.4 μg. The overall prevalence of inadequate selenium intake was 59.2%. Individuals with selenium intakes below the EAR (≤45 μg/day) showed higher concentrations of glycated hemoglobin (HbA_{1c}) ($P = 0.002$) and a higher prevalence of disturbances in HbA_{1c} than those with selenium intakes above the EAR (>45 μg/day) ($P = 0.001$). Dietary selenium intake was directly associated with female sex (β = 19.95, 95% CI 5.00 to 34.89; $P = 0.001$) and weight (β = 6.69, 95% CI 0.56 to 12.81; $P = 0.010$), and inversely associated with the percentage of total body fat (β = −0.80, 95% CI −1.56 to −0,04; $P = 0.010$) and HbA_{1c} (β = −7.41, 95% CI −13.06 to −1.75; $P = 0.010$). Considering the noticeable young age of the individuals evaluated and the high frequency of disturbances in HbA_{1c} concentrations in those with selenium consumption below the recommendation, it is suggested that adequate dietary intake or supplementation of this micronutrient should be guaranteed to prevent future possible complications associated with glycemic control disturbances.

Keywords: adults, adiposity, lifestyle, food consumption, glycated hemoglobin A

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INTRODUCTION

Selenium is a trace element that fulfills key roles related to human body homeostasis, thyroid gland function, and optimal functioning of the immune system (1, 2). Most of these actions are due to its participation as a component or cofactor of antioxidant enzymes, such as glutathione peroxidase and thioredoxin reductase, in addition to deiodinases (1). Inadequate serum selenium concentrations are associated with disorders of the thyroid gland (2), cancer (3), metabolic syndrome, cardiovascular disease, diabetes (4, 5), and obesity—albeit in a contradictory way (6–8).

Obesity in the classic sense, defined by the Body Mass Index (BMI), has been extensively studied in several aspects, including selenium metabolism. However, more recently, emphasis has been placed on the substantial role of excess body fat regardless of BMI classification. In 2006, the Normal-Weight Obesity (NWO) syndrome, a metabolic condition of excess body fat in individuals with normal BMI, was defined (9). It is known that individuals with NWO have a particular profile concerning the development of some harmful conditions, with emphasis on cardiometabolic risk factors, such as insulin resistance (IR) and dyslipidemias. These markers of cardiometabolic health are among the important aspects that should be evaluated and monitored in individuals with NWO because this condition seems to favor metabolic disorders in an intermediary way between individuals with normal BMI and body composition and those with obesity. Therefore, the role of dietary selenium intake in glycemic control in these individuals should also be investigated.

Although the most selenium-rich food is widely available in Brazil, there are few studies on the consumption of selenium in the Brazilian population and none in individuals with NWO. Analysis of the dietary intake of 34,003 Brazilians older than 10 years revealed a mean selenium intake of 107.6 μg/day, with no difference between income classes or urban and rural areas. Higher selenium intakes were found in women and residents of the northern region of Brazil. Among the elderly, mean selenium intake was lower than that in other age groups but still above the RDA (10), in contrast to the results of a study on elderly residents in the Rio Grande do Sul state, southern Brazil, which found an inadequate intake rate of 98% (11).

In general, studies investigating the relationship between selenium and glycemic control show contradictory results regarding glycemia and the prevalence of type 2 diabetes mellitus (DM2) but converge concerning serum insulin and the Homeostasis Model Assessment-Insulin Resistance (HOMA-IR) index and suggest that its action occurs through some mechanisms, including regulation of insulin signaling and glycolysis, pyruvate, and chromium metabolism (7, 12–15), as well as promoting changes in the expression of genes related to insulin and adiponectin receptors (*INSR* and *ADIPOR1*) and others that encode pyruvate metabolism enzymes (*LDH*, *PDHA*, *PDHB*) (16, 17).

In addition, it is believed that selenium could play a protective role against disorders of glucose metabolism by regulating oxidative stress and mimicking insulin action (18, 19). However, important results of a study with data from samples of American individuals (14) showed that selenium is associated with a higher risk of DM2. Therefore, it is currently believed that high serum selenium concentrations increase the risk of insulin resistance or DM2 (20). One explanation is that selenoprotein glutathione peroxidase 1 (GPX-1) is overexpressed under high selenium concentrations (21). Overexpression of GPX-I has been associated with insulin resistance in rats (22) and decreased expression of *PKM,* the gene encoding the glycolytic enzyme pyruvate kinase (23).

In a recent meta-analysis with studies including individuals from Europe and Asia with diseases such as DM2, gestational diabetes, polycystic ovary syndrome, heart disease, obesity, and others, selenium supplementation was shown to decrease the Homeostasis Model Assessment of Beta-Cell Function (HOMA-beta) and increase the Quantitative Insulin Sensitivity Check Index (QUICKI); however, it had no effect on blood glucose, HOMA-IR, glycated hemoglobin (HbA_{1c}), and adiponectin concentrations (8). In addition, after 16 years of monitoring more than 10,000 women in Italy, it was observed that those who developed diabetes, among other factors, had higher dietary selenium intake (60.9 vs. 56.8 μg/day) (24).

Considering that the association of dietary selenium intake with markers of glycemic control has produced variable results depending on the study population and that a similar investigation has not yet been carried out in adults with NWO, this study aimed to evaluate whether dietary selenium consumption is associated with glycemic control variables in a sample of young adults with NWO. The hypothesis is that selenium consumption is associated with serum concentrations of glucose; insulin; HbA_{1c}; and the HOMA-IR, HOMA-beta, and QUICKI indexes in these individuals.

MATERIALS AND METHODS

Design and Population

This study was carried out using two databases, applying the same inclusion and exclusion criteria. Both databases came from observational, analytical, and cross-sectional studies, collected from an academic community in Goiânia, Goiás, Brazil. The recruitment of volunteers was carried out through folders, social networks, and emails sent to students, employees, and professors at the Federal University of Goias (UFG), Brazil (**Figure 1**). The studies were approved by the UFG Research Ethics Committees (protocols n°. 2,772,022, Jul 16, 2018 and n° 865.062, Nov 10, 2014). All volunteers received information and clarification about all procedures and signed a consent form.

Both men and women aged between 20 and 59 years, with normal BMI (between 18.50 and 24.99 kg/m²) (25) and high body fat percentages (9, 25) were included. One hundred thirteen individuals were recruited in the first study and 157 in the second study, totaling 270 volunteers (**Figure 1**). The statistical power was estimated using R software, with an effect size of 0.21, a type I error probability of 0.05, and a sample size of 270 observations. The estimated power was 0.858.

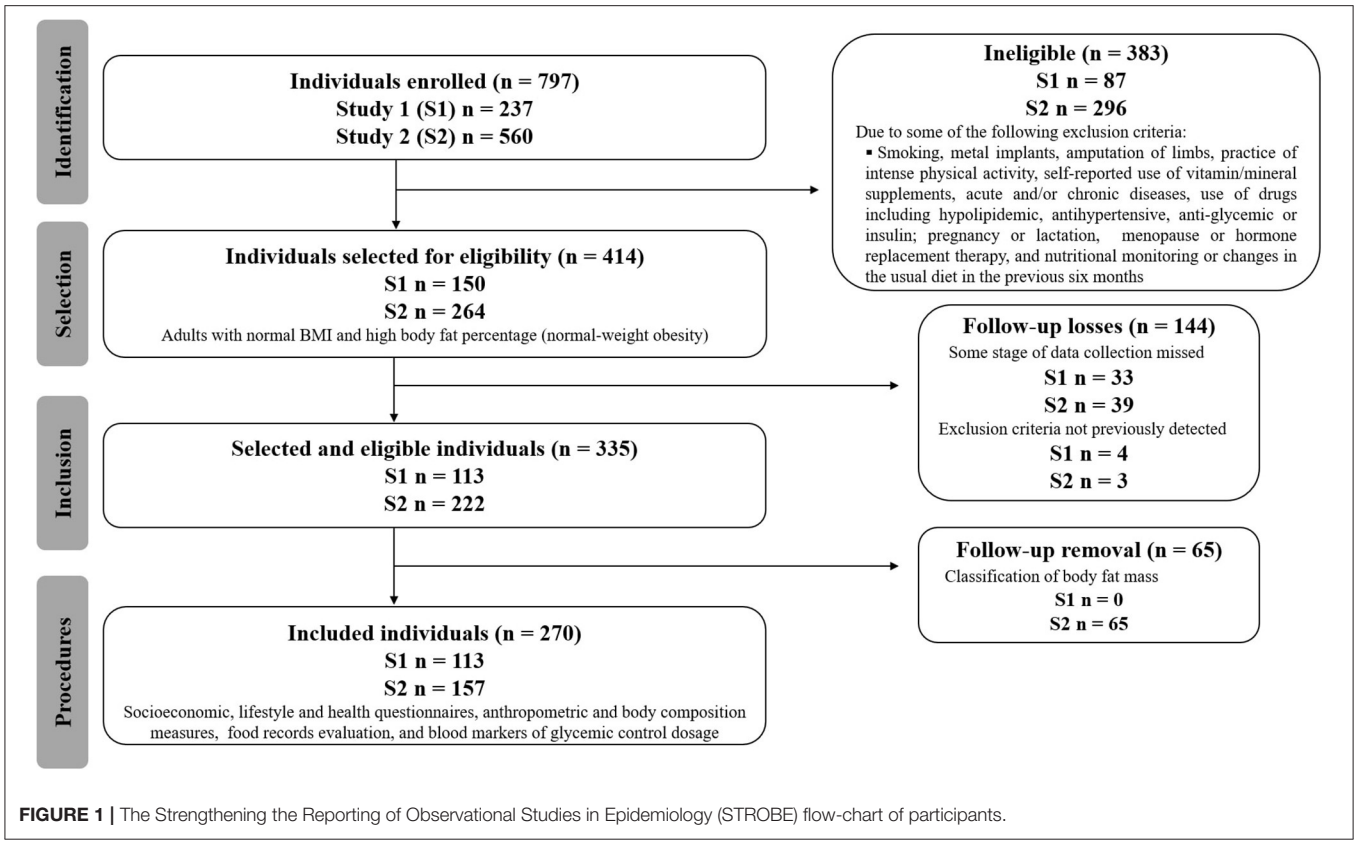

FIGURE 1 | The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) flow-chart of participants.

Study Population and Measurements

The first data collection was performed from May to September 2015, and the second one, from January to June 2019. Data collection took place in two meetings after participant recruitment. During the first appointment, individuals received all information about the research, and the informed consent form was presented. Individuals who met the inclusion criteria and agreed to participate answered the socioeconomic, demographic, lifestyle, and health questionnaires and were referred for anthropometric assessment, body composition, and food consumption analysis. The second appointment was previously scheduled, and the individuals were instructed to fast for 12 h to collect blood samples for biochemical tests.

All researchers on the team in both studies were trained and underwent standardization regarding the nutritional care protocol. In addition to this training, the researchers received training to measure anthropometric measures, according to the technique of anthropometry standardization recommended by Habicht (26).

Socioeconomic, Demographic, Lifestyle, and Blood Pressure Data

The application of a questionnaire referring to socioeconomic, demographic, and lifestyle data took place in an average of 30–40 min. During this stage, blood pressure was measured at three non-consecutive moments, and the mean values of systolic and diastolic pressures were used in the analyses (27).

Anthropometric Evaluation

Body mass was measured on a digital platform scale (Filizola Shop, São Paulo, Brazil), with a maximum load of 150 kg and an accuracy of 0.1 kg. Height was determined using a stadiometer (Seca Deutschland, Hamburg, Germany) with a maximum reach of 220 cm and a precision of 0.1 cm, according to the procedures described by Lohman et al. (28).

Body Composition Analysis

Individuals dressed in light clothing and without accessories were subjected to DEXA examination, using a Lunar DPX NT model DEXA scanner (General Eletric Medical Systems Lunar®; Madison, USA).

Determination of Glycemic Profile Markers

Blood was collected by a specialized technician, with disposable materials, in a strictly sanitized environment, from the median cubital vein for determination of the glycemic profile (fasting blood glucose, fasting insulin, and HbA_{1c}).

The colorimetric enzymatic method was used to determine the fasting blood glucose values, and the reference value for disturbances was ≥100 mg/dL (29). The electrochemiluminescence method was applied to determine the serum insulin concentration.

The immunoturbidimetric inhibition method was used to determine the concentration of HbA_{1c}, and the percentage was

calculated using the equation [(28.7 × HbA_{1c}) −46.7]. Results ≥5.7% were considered altered (29).

HOMA-IR was calculated according to the equation proposed by Matthews et al. (30): HOMA-IR = (FPI × FPG)/22.5, in which FPI refers to fasting plasma insulin and FPG, to fasting plasma glucose. This index assesses IR and was considered above normal when higher than 2.71 (29). The HOMA-beta index estimates the functional capacity of pancreatic beta cells and was calculated according to the equation proposed by Matthews et al. (30): HOMA-beta = (20 × FPI)/(FPG −3.5). Values above the 90th percentile of the sample were considered above normal. For the sample in this study, the cutoff point was >223.56.

The Quantitative Insulin sensitivity Check Index (QUICKI) was calculated from results of insulinemia and fasting glycemia using the equation: [1/(log FPI + log FPG)]. This index assesses insulin sensitivity, and the value adopted as a reference for altered values was less than the 10th percentile of the sample (29). For this study, the cutoff point was 0.3305.

Food Intake Analysis

Dietary food intake was assessed using three 24-h food records (R24H), on alternate days and different weeks, including a weekend day (31). Aiming at reducing possible collection errors, R24H were applied following the Multiple Pass Method (MPM) (32), which helps the interviewee to remember in detail the food and drinks consumed the day before the collection. In addition, strategies were used to assist in the measurement of portions, such as a photographic manual and standard tools for home measurements (33). The first R24H was applied at the first appointment, and the research team contacted the participants to collect the other R24h. These assessment instruments were applied by two nutritionists who underwent technical and practical training for the collection and evaluation of R24h.

The information collected was transformed into standard home measures, and the data evaluation was carried out with a Brazilian software (Avanutri®, Três Rios, Rio de Janeiro, Brazil). This software calculates the results of 21 micronutrients, including selenium, in micrograms. Selenium intake was adjusted to the energy value, according to the residual method (34). In summary, this method results in the estimation of the residual value of a regression model in which the independent variable is the total energy intake and the dependent variable is the raw consumption of the nutrient under analysis. Therefore, the residual value reflects an estimate of nutrient intake not correlated with total energy intake and directly related to general variation in food choice and composition.

Statistical Analysis

A double-entry database was developed to check the consistency of the results. Descriptive analysis, including mean ± SD or median (interquartile range – IQR), was performed for all quantitative variables. Shapiro–Wilk's W test was applied to evaluate the significance ($\alpha = 0.05$) of normality deviations observed in the residuals of mean comparisons tests and linear regression models.

Student's *t*-test or the Mann–Whitney test was applied to compare means. A comparison of the disturbances in glycemic control markers between individuals consuming selenium at levels below and above the EAR was performed using either the chi-square or Fisher's exact test, according to the number of individuals who presented disturbances. Associations between variables were assessed by multiple linear regression models, using the backward strategy. The variables that could associate with glycemic response and were added to the model included sex, weight, height, BMI, body fat percentage, and HbA_{1c}, age, systolic and diastolic blood pressure, waist circumference, android and gynoid fat, the android/gynoid ratio, fasting blood glucose, insulinemia, and the HOMA-IR, HOMA-beta, and QUICKI indexes. Although selenium intake was the main focus of the analysis, other dietary variables that also could interfere with glycemic response were added to the model, including carbohydrate, total fat; saturated, monounsaturated and polyunsaturated fatty acids; and protein intakes.

A value of $P < 0.05$ was considered statistically significant, and all analyses were performed in R software version 4.0.3 (35).

RESULTS

The total sample consisted of 270 adults (113 individuals from the study conducted in 2015 and 157 from the one conducted in 2019) (**Figure 1**), with NWO (adequate BMI and a high percentage of body fat). The mean ± SD age was 23.7 ± 3.3 years, and 70.4% of the participants were women.

For selenium, individuals showed a mean intake of 59.2 ± 26.4 μg/day. The overall prevalence of inadequate intake was 59.2% (54.7% for men and 63.7% for women). To analyze glycemic control marker disturbances, individuals were separated into two groups according to selenium consumption: (1) below the EAR (≤45 μg/day)—91 individuals (33.7%); and (2) above the EAR (>45 μg/day)—179 individuals (66.3%) (**Table 1**). When comparing the total sample according to sex, 30.5% ($n = 58$) of women and 41.2% ($n = 33$) of men showed selenium consumption below the EAR ($P = 0.089$).

There were no differences in weight, BMI, waist circumference, body fat percentage (% BF), android fat, and gynoid fat between the two groups (below and above the EAR). Individuals in the group below the EAR showed higher values of the ratio between android/gynoid body fat compared to those in the group above the EAR (**Table 1**).

Individuals in the group with selenium intake above the EAR had lower concentrations of HbA_{1c} than those in the group with intake below the EAR ($P = 0.002$) (**Table 2**). In addition, a higher prevalence of disturbances in HbA_{1c} concentrations was found in the group with selenium consumption below the EAR compared with the one with intake above EAR ($P = 0.001$) (**Table 3**). No differences were found in the other biomarkers of glycemic control between the two groups (**Table 3**).

Three individuals were excluded from the regression analysis due to missing data ($n = 267$). After all candidate variables were added into the multiple regression model using the backward strategy, the final model with the lowest Akaike criterion presented the variables sex, weight, height, BMI, body fat percentage, and HbA_{1c}. Sex, weight, body fat percentage,

TABLE 1 | Descriptive data of individuals with Normal-Weight Obesity Syndrome, according to the classification of dietary selenium intake.

	Below EAR (n = 91–33.7%)	Above EAR (n = 179–66.3%)	Total (n = 270–100.0%)	P-value
Sex				0.089
Male	33 (36.3)	47 (26.3)	80 (29.6)	
Female	58 (63.7)	132 (73.7)	190 (70.4)	
Age (years)	22.4 (21.1–26.3)	23.0 (21.2–25.0)	23.0 (21.2–25.0)	0.879
Selenium intake	33.4 ± 9.3	72.4 ± 22.2	59.2 ± 26.4	0.001
MSBP (mmHg)	108.4 ± 11.3	107.0 ± 10.8	107.5 ± 11.0	0.343
MDBP (mmHg)	65.5 (60.5–72.0)	65.0 (60.0–72.0)	65.0 (60.0–72.0)	0.606
Weight (kg)	61.2 (56.5–66.8)	60.6 (55.2–67.0)	61.0 (55.4–66.8)	0.649
Height (m)	1.7 (1.6–1.8)	1.7 (1.6–1.7)	1.7 (1.6–1.7)	0.481
BMI (kg/m²)	22.5 ± 1.6	21.8 (20.7–23.2)	22.0 (21.0–23.4)	0.476
MET (min/week)	400.0 (120.0–898.0)	297.0 (140.0–565.0)	330.0 (132.8–726.0)	0.062
%BF	34.7 (26.5–40.0)	35.2 (30.4–38.8)	35.0 (29.4–39.2)	0.672
%AF	37.5 (31.6–43.1)	35.2 (30.2–41.8)	35.8 (30.8–42.4)	0.308
%GF	46.2 (34.4–50.7)	47.1 (41.0–50.3)	46.7 (38.3–50.4)	0.475
A/G	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.005

Data are absolute numbers (percentages), mean ± standard deviation or median (interquartile range). MSBP, mean systolic blood pressure (mmHg); MDBP, mean diastolic blood pressure (mmHg); BMI, body mass index; MET, metabolic equivalent of task; %BF, body fat percentage; %AF, android body fat percentage; %GF, gynoid body fat percentage; A/G, ratio between %AF/%GF. Student's t-test or Mann-Whitney test.

TABLE 2 | Biochemical characterization of individuals with Normal-Weight Obesity Syndrome, according to dietary selenium intake.

	Below EAR (n = 91–33.7%)	Above EAR (n = 179–66.3%)	Total (n = 270–100.0%)	P-value
Blood glucose (mg/dL)	85.5 ± 7.3	85.2 ± 7.5	85.3 ± 7.4	0.800
Insulin (uU/mL)	7.2 (5.4–9.9)	7.2 (5.0–9.4)	7.2 (5.1–9.8)	0.443
HOMA-IR	1.6 (1.1–2.2)	1.5 (1.0–2.1)	1.5 (1.1–2.1)	0.384
HOMA-Beta	122.9 (83.8–164.5)	118.3 (88.3–171.6)	122.0 (86.5–169.0)	0.881
HbA_{1c}%	5.1 (4.7–5.6)	4.8 (4.6–5.1)	4.9 (4.6 –5.3)	0.002
QUICKI index	0.36 (0.34–0.38)	0.36 (0.34–0.38)	0.36 (0.34–0.38)	0.387

Data are mean ± standard deviation or median (interquartile range). HOMA-IR, homeostasis model assessment of insulin resistance; HOMA Beta, homeostatic model assessment of beta-cell function; HbA_{1C}, glycated hemoglobin. Student's t-test or Mann-Whitney test.

TABLE 3 | Disturbances in biomarkers of glycemic control of individuals with Normal-Weight Obesity Syndrome, according to dietary selenium intake.

	Below EAR (n = 91)	Above EAR (n = 179)	Total (n = 270)	P-value
Blood glucose	2 (2.2)	9 (5.0)	11 (4.1)	0.344
HOMA-IR	6 (6.6)	18 (10.1)	24 (8.9)	0.345
HOMA-beta	7 (7.7)	20 (11.2)	27 (10.0)	0.366
HbA_{1c}	20 (22.0)	15 (8.4)	35 (11.5)	0.001
Quicki index	8 (8.8)	19 (10.6)	27 (10.0)	0.637

Data are absolute numbers (percentage). HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-beta, homeostatic model assessment of beta-cell function; HbA_{1c}, glycated hemoglobin. Fisher's exact test or Chi-square test.

and HbA_{1c} were the variables most strongly associated with dietary selenium intake. The female sex was directly associated with a higher consumption of selenium (β = 19.95, 95% CI 5.00 to 34.89; P = 0.001), and a positive association was also observed for selenium consumption and weight (β = 6.69, 95% CI 0.56 to 12.81; P = 0.010). However, negative associations of body fat percentage and HbA_{1c} with selenium consumption were observed (β = −0.80, 95% CI −1.56 to −0,04; P = 0.010, and β = −7.41, 95% CI −13.06 to −1.75; P = 0.010, respectively). The final regression model is shown in **Table 4**. The variables that sequentially left the model during the backward procedure to derive the final regression model were age; systolic blood pressure; diastolic blood pressure; waist circumference; android fat; gynoid fat; the android/gynoid ratio; fasting blood glucose; insulinemia; and the HOMA-IR, HOMA-beta, and QUICKI indexes. It is important to highlight that the regression model was also tested only for females, considering the highest percentage of women in the sample; however, as the results were not different from those observed in the analysis of both sexes combined (data not shown), it was decided to present the results of the total sample. In addition, adding macronutrients, saturated, monounsaturated, and polyunsaturated fatty acids to the regression model did

TABLE 4 | Associations among selenium intake and explanatory variables, determined by multiple regression model adjusted by the backward strategy ($n = 267$).

	Coefficient (β)	SE	95% CI	*t* value	pr (>|t|)	*P* value
Intercept	785.2937	389.01	19.28–1551.31	2.019	0.044*	0.01
Variables						
Female sex	19.9459	7.59	5.00 to 34.89	2.628	0.009**	0.001
Weight	6.6873	3.11	0.56 to 12.81	2.150	0.032*	0.010
Height	−450.6025	231.24	−905.95 to 4.75	−1.949	0.052·	0.050
BMI	−15.2345	8.77	−32.50 to 2.03	−1.737	0.084·	0.050
%BF	−0.8027	0.39	−1.56 to −0.04	−2.072	0.039*	0.010
HbA_{1c}	−7.4051	2.87	−13.06 to −1.75	−2.579	0.010*	0.010

*Significance codes: *0.01, **0.001, ·0.05.*
Residual standard error: 25.59 on 260 degrees of freedom (DF).
Multiple R-squared: 0.08447.
Adjusted R-squared: 0.06334.
F-statistic: 3.998 on 6 and 260 DF.
***P-value: 0.000757**.*
BMI, body mass index; %BF, body fat percentage; HbA_{1c}, glycated hemoglobin.

not change the association between dietary selenium intake and HbA_{1c}.

DISCUSSION

The assessment of risk markers for metabolic diseases in individuals with NWO is important to reduce the medium and long-term risks of cardiovascular diseases and DM2. In a quick search of the scientific literature, it is possible to identify many studies that investigate the relationship between selenium supplementation and glycemic control markers; however, few report this association for food consumption, and none has been carried out with individuals with NWO.

Our results show that mean dietary selenium consumption was above that recommended by the DRIs (59.2 ± 26.4 μg/day); however, approximately one third of participants did not reach the expected minimum values. We also observed that dietary selenium intake was inversely associated with serum concentrations of HbA_{1c} and body fat percentage. Individuals with intake above the EAR for selenium had lower concentrations of HbA_{1c}, which may suggest better glycemic control. Although HbA_{1c} should not be used alone for DM2 diagnosis or glycemic profile monitoring, it is extremely relevant in clinical practice, as it allows for screening of mean blood glucose in the last 90 days (36). The association with body fat percentage draws attention to the need of monitoring this parameter in addition to the BMI.

Studies about the action of selenium on glycemic control have produced divergent results. A recent meta-analysis of randomized clinical trials (RCTs), including 1,441 individuals with ages ranging from 10 to 85 years, verified that selenium supplementation reduced insulin secretion as assessed by the HOMA-beta index and increased the QUICKI index; however, significant results were not observed for disturbances in HOMA-IR and HbA_{1c} concentrations (8). On the other hand, in a study with 2,420 non-diabetic Canadians (mean age of 42 years), dietary selenium intake was positively associated with glycemic control, so that insulin resistance, measured by HOMA-IR and HOMA-beta, decreased with increasing selenium consumption among women but not among men ($P < 0.001$); lower daily selenium consumption was observed among those with the highest HOMA-IR indexes ($P < 0.001$) and a negative correlation between selenium and insulin resistance was observed up to a consumption limit of 1.6 μg/kg/day (37). Although this study shows relevant data, the mean dietary consumption of selenium (109.22 ± 1.18 μg/day) was 1.84 times higher than that observed in our study, which makes comparisons of results difficult.

Two RCTs on selenium supplementation carried out in Danish elderly and Polish adults reinforce our main result that higher intakes of selenium are associated with lower concentrations of HbA_{1c}, even within different populations and with different doses of selenium (16, 38). By contrast, some studies have failed to demonstrate a decrease in HbA_{1c} concentrations (12, 39) or found high serum selenium concentrations associated with increased concentrations of glucose and HbA_{1c}, as in 41,474 American adults with a mean dietary selenium intake of 98.0 ± 55.0 μg/day (40) and in 8,824 Chinese with a mean consumption of 52.4 μg/day (41).

Some mechanisms to justify the controversial action of selenium in relation to the processes associated with glucose metabolism are speculated. An *in vivo* study with diabetic mice showed that the use of sodium selenate increased the expression of peroxisome proliferator-activated receptor gamma (PPAR-γ), which acts on insulin resistance and increases lipid metabolism (42). The selenium compounds of the oxidation state $^{+}$IV inhibited the activity of protein tyrosine phosphatases (PTP) (42), responsible for dephosphorylating the insulin receptor, attenuating its action (43). In this context, selenium as sodium selenite would antagonize and compromise glycemic control.

Robertson and Harmon (44) and Campbell et al. (45) suggest that selenium could provide protection against DM2 due to its association with oxidative stress control by increasing the activity of GPx in pancreatic beta cells and decreasing the damage caused by reactive oxygen species (ROS). This was verified in a mouse beta-cell line (Min6) in which beta-cell sensitivity increased

in response to sodium selenate, expressed via increased GPx activity (5-fold), and a notable increase in the activity of insulin promoter factor 1 (*Ipf1*). A response to sodium selenite was also observed via increased mRNA levels of *Ipf1* and *Ins*. The increased antioxidant activity of GPx culminates in beneficial effects on the level of pancreatic beta-cell mass and insulin synthesis; however, long-term increased insulin production and secretion can result in chronic hyperinsulinemia (46).

In our study, in addition to the results observed in relation to HbA_{1c} concentrations, positive associations were found between dietary selenium consumption with female sex and weight, and an inverse association between selenium intake and body fat percentage was observed. The Canadian CODING study with 3,054 participants (adults from the province of Newfoundland, in eastern Canada, mean age of 42 years), observed a mean dietary selenium consumption of 108.10 μg/day, and decreasing consumption in μg/kg/day along the BMI ranges. The authors found a significant and dose-dependent association between tertiles of selenium consumption and weight, BMI, waist circumference, total body fat percentage, and gynoid and android fat percentages and demonstrated that dietary selenium can account, regardless of other factors, for 9–27% of the variation in body fat percentage (47).

Thereafter, a study published by the same group of CODING (n = 2,420), showed divergent results regarding the type of association between dietary selenium consumption and body weight but also observed an inverse association with body fat percentage, BMI, and body water (37). A similar result was also found in a study with a representative national sample of Chinese individuals, in which the body fat percentages were also lower in individuals consuming selenium in the highest quintiles compared with those with intake in the lowest quintiles (41).

Possible explanations for the role of selenium in adipogenesis are also related to PPAR-γ, which is responsible for adipocyte differentiation and distribution of adipose tissue, and is thus related to increased tissue insulin sensitivity and weight control. In animals receiving sodium selenate, hepatic PPAR-γ expression was 2.5 times higher than in those with selenium deficiency or treated with sodium selenite (42). On the other hand, a recent study with rat and human cell cultures of pre-adipocytes showed inhibition of adipogenesis due to a decrease in the expression of PPAR-γ and fatty acid synthase mRNAs, while there was an increase in growth factor-β (48). The mechanisms involved in the association of selenium with adiposity are not yet fully elucidated, but there is evidence suggesting that serum selenium concentrations are negatively associated with adiposity, especially visceral fat. However, this information needs to be carefully considered because it involves numerous other processes and not only dietary selenium intake as a modifier of serum concentration (49).

Among the limitations of our study, the cross-sectional design can be considered, as it does not allow us to make inferences about cause and effect between dietary selenium consumption and the other markers. However, it suggests that there may be interactions between the consumption of this nutrient and glycemic control markers and, for this reason, may guide future investigations on dietary consumption and the effect of selenium on biochemical and metabolic pathways and the expression of genes related to variation in HbA_{1c} concentrations. We were not able to measure blood selenium levels, which can also be considered a limitation. On the other hand, this is the first study to assess associations between dietary selenium intake and markers of glycemic control in individuals with NWO.

In conclusion, dietary consumption of selenium above the minimum values recommended for groups by the dietary guidelines was associated with lower serum HbA_{1c} concentrations, a long-term marker of disturbances in blood glucose, and body fat percentage. Considering that the individuals evaluated in our study had NWO, and the disturbances in HbA_{1c} found in the group consuming selenium below the EAR, we can observe that despite their noticeably young age, these individuals showed cardiometabolic markers that are more commonly seen in older people. Therefore, further studies with individuals with NWO are strongly recommended, as one of the basic principles of nutrition is to reduce or mitigate the risk of non-communicable chronic diseases throughout life.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, upon a reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by UFG Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS and AP: acquisition, interpretation, and analysis of data. LH: data interpretation and writing the manuscript. CC: conceptualization, experimental design, obtaining resources, interpretation of data, and writing the manuscript. All authors read and approved the final manuscript.

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Selenotranscriptome Network in Non-alcoholic Fatty Liver Disease

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Observational studies indicate that selenium may contribute to the pathogenesis of non-alcoholic fatty liver disease (NAFLD). Transcriptomic exploration of the aetiology and progression of NAFLD may offer insight into the role selenium plays in this disease. This study compared gene expression levels of known selenoprotein pathways between individuals with a healthy liver to those with NAFLD. Publicly available gene expression databases were searched for studies that measured global gene expression in liver samples from patients with steatosis and non-alcoholic steatohepatitis (NASH) and healthy controls (with [HOC] or without [HC] obesity). A subset of five selenoprotein-related pathways (164 genes) were assessed in the four datasets included in this analysis. The gene *TXNRD3* was less expressed in both disease groups when compared with HOC. *SCLY* and *SELENOO* were less expressed in NASH when compared with HC. *SELENOM*, *DIO1*, *GPX2,* and *GPX3* were highly expressed in NASH when compared to HOC. Disease groups had lower expression of iron-associated transporters and higher expression of ferritin-encoding sub-units, consistent with dysregulation of iron metabolism often observed in NAFLD. Our bioinformatics analysis suggests that the NAFLD liver may have lower selenium levels than a disease-free liver, which may be associated with a disrupted iron metabolism. Our findings indicate that gene expression variation may be associated with the progressive risk of NAFLD.

Keywords: selenium, non-alcoholic steatohepatitis, selenoproteins, selenocysteine lyase, ferroptosis

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is identified as a spectrum of progressive conditions that range from simple accumulation of fat in >5% of hepatocytes (steatosis) to non-alcoholic steatohepatitis (NASH), which is marked by the coexistence of steatosis, liver-cell injury and inflammation (steatohepatitis) (1) and, ultimately, cirrhosis (2). NAFLD is closely linked to obesity and is commonly referred to as the hepatic manifestation of the metabolic syndrome, given that it often coexists with dyslipidaemia, hypertension, insulin resistance and abdominal obesity (2). The prevalence of NAFLD increases proportionally with body mass index (BMI). While the prevalence of NAFLD is about 25% in the general population, it may increase to over 90% amongst individuals with BMI $\geq$ 30 kg/m^2 (3).

Oxidative stress plays a pivotal role in the pathogenesis of NAFLD (4). The increase in hepatic iron observed in approximately one-third of NAFLD patients is often considered to play a pathogenic role given iron's ability to donate electrons and catalyse the production of reactive oxygen species (ROS) (5). Thus, it has been proposed that therapies designed to reduce

cellular oxidative load may have therapeutic potential for reducing the risk of NAFLD (6, 7). The essential micronutrient selenium was demonstrated to play a pivotal role in redox homeostasis, thus curbing ROS (8). Disturbances in selenium bioavailability or metabolism have been linked with liver diseases such as NAFLD (9). The redox functions of selenium are carried out by selenocysteine (Sec) residues, a cysteine (Cys) cognate with selenium in the place of sulphur, in proteins (10). The human selenoproteome is small, with only 25 selenoproteins identified in the genome (11). Several selenoproteins are designated according to their function: three proteins comprise the iodothyronine deiodinase (DIO) family (DIO1, DIO2, and DIO3) which control thyroid hormone activation; five proteins comprise the glutathione peroxidase (GPX) family (GPX1, GPX2, GPX3, GPX4, and GPX6), involved in detoxification of ROS using glutathione; three proteins comprise the thioredoxin reductase (TRXR) family (expressed from three separate genes: *TXNRD1*, *TXNRD2*, and *TXNRD3*), which reduces thioredoxin to prevent oxidative damage; methionine sulphoxide reductase B1 (MSRB1), which plays a role in innate immunity by reducing methionine sulphoxide; selenophosphate synthetase 2 (SEPHS2), that provides selenophosphate to the selenoprotein synthesis apparatus; and selenoprotein P (SELENOP), a plasmatic transporter of selenium to other tissues produced in the liver. Other selenoproteins in the human proteome are SELENOF, SELENOH, SELENOI, SELENOK, SELENOM, SELENON, SELENOO, SELENOS, SELENOT, SELENOV, and SELENOW. Most of the selenoproteins act in reactions requiring strong redox capacity, and are deemed critical for several functions in the body, such as immune response, metabolism of thyroid hormones, and detoxification of heavy metals and toxins (12).

Little information is available regarding the link between circulating selenium and NAFLD progression (9). Selenium status has been demonstrated to be lower in individuals with chronic liver diseases such as alcoholic fatty liver (13) and cirrhosis (14). Findings from animal studies suggest a beneficial effect of selenium on NAFLD. Selenium supplementation recovered abnormal liver function tests (15), decreased the number of stellate cells and reduced fibrosis (16) in rodents subjected to liver injury induced by carbon tetrachloride. However, available literature investigating the role of selenium in human NAFLD is limited to observational studies, which suggest that selenium, particularly *via* the selenium transporter, SELENOP, may play a detrimental role in NAFLD pathogenesis (9). High selenium dietary intake was associated with increased risk of NAFLD in a Chinese population (17). A cross-sectional epidemiological study in Chinese adults reported that those in the highest quartile for plasma selenium (>247.4 μg/L) had a 54% higher prevalence of NAFLD compared with those in the lowest quartile (<181.6 μg/L) (18). Choi et al. (19) reported that adults in the highest tertile for plasma SELENOP had a 7.5-fold greater risk of NAFLD than those in the lowest tertile. Similarly, a cross-sectional study demonstrated that serum SELENOP was 6.8-fold higher in adults with NAFLD when compared with healthy controls (20). Furthermore, SELENOP has been associated with severity of NAFLD in two cross-sectional studies involving patients in China and Italy (21, 22), however it should be noted that in some of these studies additional biomarkers of total selenium were not assessed, lacking thus a more comprehensive profile of selenium in NAFLD. As only observational studies have been conducted to investigate the association between selenium and NAFLD beyond SELENOP, studies that provide mechanistic insights are necessary to elucidate the role of selenium in the pathogenesis of NAFLD.

Recently, particular attention has been directed to exploring a gene expression signature of NAFLD to understand the molecular pathways that underpin NAFLD aetiology and disease progression (23–26). Due to the global and exploratory nature of these studies, particular pivotal pathways and their associated genes have not been explored. Notably, pathways involving selenoproteins and selenium metabolism have not been specifically investigated. Hepatic selenium and selenoprotein-related pathways comprise genes involved in the metabolism of selenium and selenoproteins, interaction of selenoproteins with other micronutrients, antioxidant response and ferroptosis. These pathways are of great interest as they can elucidate how selenium underlies chronic pathological mechanisms in NAFLD. Using publically available global gene expression datasets, this study aimed to compare gene expression of known selenoprotein and selenium-containing pathways between individuals with healthy liver to those with steatosis and NASH. Given that individuals with obesity present with a higher risk of NAFLD than lean individuals, we further stratified healthy individuals into obese and non-obese for a more informative assessment.

Abbreviations: BMI, body mass index; CBS, cystathionine beta-synthase; CP, caeruloplasmin; CTH, cystathionine gamma-lyase; Cys, cysteine; DHA, docosahexaenoic acid; DIO, iodothyronine deiodinase; GEO, Gene Expression Omnibus; GO, gene ontology; GPX, glutathione peroxidase; HC, normal weight healthy control; HOC, healthy obese control; INS, insulin; KEGG, Kyoto Encyclopaedia of Genes and Genomes; MS, methionine synthase; MSRB1, methionine sulphoxide reductase B1; MTHFR, methylenetetrahydrofolate reductase; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; PAI-1, plasminogen activator inhibitor-1; PCA, principle component analysis; PLG, plasminogen; RMA, robust multi-array averaging; ROS, reactive oxygen species; SAF, Steatosis-Activity-Fibrosis; SCLY, selenocysteine lyase; Sec, selenocysteine; SELENOP, selenoprotein P; SEPHS2, selenophosphate synthetase 2; TRXR, thioredoxin reductase; TSTD1, Thiosulfate Sulfurtransferase Like Domain Containing 1; UGT1A6, UDP Glucuronosyltransferase Family 1 Member A6; VLDL, very-low-density lipoprotein.

MATERIALS AND METHODS

Figure 1 provides an overview of the methods used in this study.

Dataset Selection

Gene expression datasets from online repositories (GEO and ArrayExpress) were selected based on the following criteria: (i) contained liver biopsy samples from healthy individuals (with or without obesity) and from individuals with a clinical diagnosis of steatosis or NASH; and (ii) raw gene expression data was available for download and re-analysis for all samples. Both microarray and RNA sequencing data were included. Demographics (age, sex, BMI, and disease state) were gathered from the papers associated with each dataset. Where there were discrepancies in

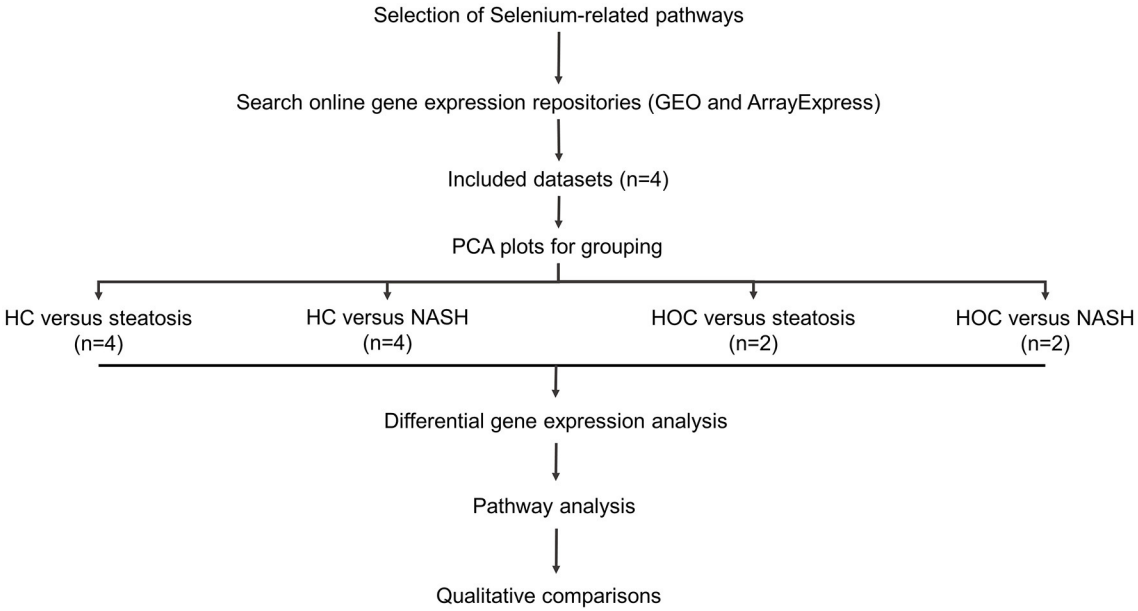

FIGURE 1 | Study workflow. Included datasets were downloaded from GEO or ArrayExpress. PCA plots were used to determine whether normal-weight healthy control (HC) and healthy obese controls (HOC) could be grouped together and whether steatosis and NASH patients could be grouped. All groups were analysed individually. Differentially expressed genes were defined as genes with an adjusted *P*-value < 0.05. For pathway analysis, genes of interest were defined as genes with a log fold change in the same direction in the majority of datasets (HC: 3/4 datasets, HOC: 2/2 datasets) and where the difference was significant in at least 50% of these datasets. HC, normal-weight healthy controls; HOC, healthy obese controls; NAFLD, non-alcoholic fatty liver disease. n, number of studies used for the analysis.

numbers between datasets and their corresponding published papers, the demographics reported in the dataset description files were used, if available. When this information was not available, the differences in numbers are reported. Subjects from all the datasets provided written informed consent before tissue sample donation and inclusion in the respective studies.

Pathway Selection

Five pathways from the Wikipathways database (27) were identified as having selenium or selenoproteins as key elements. The pathways were: Selenium metabolism and selenoproteins (WP28); Selenium micronutrient network (WP15); Ferroptosis (WP4313); Glutathione metabolism (WP100); and Oxidative stress (WP408). The ferroptosis pathway was included because the selenoprotein GPX4 is a key regulator of this cell death pathway. These pathways contained a total of 164 unique genes. Gene identifiers were extracted from chosen pathways and used to create a subset of the normalised gene expression data for each dataset. These subsets were used for downstream analysis and data visualisation.

Pre-processing and Normalisation

All pre-processing and statistical analysis was performed in R (version 3.6.3). Pre-processing and normalisation of microarray data was performed using the ArrayAnalysis pipeline that has been previously described (28). ArrayAnalysis utilises the functions within the affy package (version 1.62) for background correction, and robust multi-array normalisation was applied to all microarray datasets. Raw gene counts were downloaded from GEO and were used for all downstream analysis of RNA sequencing data.

Statistical Analysis

All differential gene expression analysis was conducted within studies. Principle component analysis (PCA) plots were used to assess the degree of overlap in expression data between control (normal weight and healthy obese) and disease (steatosis and NASH) groups. For microarray data, these plots were generated using the "prcomp" functions in the R base stats package (version 3.6.3), and graphs generated using the "factoextra" (version 1.0.7) and "ggplot2" packages (version 3.3.3) in R. For RNA sequencing data, the "plotPCA" function within the DESeq2 package (version 1.24.0) was used to generate PCA plots. Linear mixed modelling was utilised to assess significantly differentially expressed genes in each comparison. For microarray data, linear mixed modelling was completed using the "limma" package (version 3.40.6) in R and for RNA sequencing data, the "DESeq2" package was used. Log fold changes were calculated as log base 2 of the fold change.

Benjamini-Hochberg adjustment for multiple testing was applied at the gene level to determine significance for genes differentially expressed between groups. Significance of differential gene expression was inferred at an adjusted *P*-value < 0.05.

Comparisons across studies were made at the pathway level. In this way, genes of interest were defined as those in which (i) the log fold change between groups was in the same direction in the majority of studies and (ii) this reached significance (adjusted *P*-value < 0.05) in at least 50% of these studies.

Data Visualisation

Differential gene expression data (log fold changes and adjusted *P*-values) for all the included datasets were visualised on chosen pathways using PathVisio (Version 3.3.0). Overlap of significant differentially expressed genes between studies was visualised using Euler or Venn diagrams as appropriate.

RESULTS

Study Characteristics

Out of 11 originally identified datasets, five met the inclusion criteria (GSE126848, E-MEXP-3291, GSE61260, GSE63067, and GSE48452) (25, 29–32). The remaining six were excluded as they either did not have a healthy control group or raw data was not available. Two of the included datasets (31, 32) pertained to the same cohort (GSE61260 and GSE48452) and contained overlapping samples, hence only the dataset which contained the highest number of samples (GSE61260) (31) was included in the analysis, to prevent over-sampling. All four datasets included in this analysis contained samples from subjects diagnosed with steatosis and NASH. Two datasets (29, 31) contained samples from HC and HOC, and the other two datasets contained only HC samples (25, 30). Control samples from the included studies were obtained from: (i) cholecystectomy surgery (25), (ii) healthy individuals recruited at the hospital (no further details on tissue collection were provided) (29), (iii) major oncological surgery where liver malignancy was excluded (31), or (iv) liver tissue bank (30). Subjects from all the datasets provided written informed consent before tissue sample donation and inclusion in the respective studies.

Table 1 describes the study characteristics of the four public datasets included in our analysis. Suppli et al. (29) have only included samples from males without diabetes, while the other studies did not have such a restrictive selection criterion. However, Frades et al. (25) and Lake et al. (30) did not provide information about diabetes status. BMI, reported by all the studies except Lake et al. (30), was notably lower in the population included in the study by Suppli et al. (29) than the other studies. Where BMI was reported, it was adequate (18.5–24.99 kg/m^2) in all HC groups. Histopathological evaluation of samples was performed using different scoring systems among the studies: Suppli et al. (29) used the Steatosis-Activity-Fibrosis (SAF) algorithm (33); Horvath et al. (31) and Lake et al. (30) used the NAFLD activity score (NAS) (34); and Frades et al. (25) used the semiquantitative evaluation method proposed by Brunt et al. (35).

Functional analysis was reported in two of the original studies: out of the 16 gene ontology (GO) terms identified by Ahrens et al. (32) [which contains a large subset of gene expression data reported by Horvath et al. (31)], 12 terms were also identified when we used our list containing the 164 selected genes. Furthermore, our list of genes resulted in nine KEGG (Kyoto Encyclopaedia of Genes and Genomes) pathways similar to those identified by Frades et al. (25). See **Supplementary Table 5** for this full list.

PCA Plots

Groups within studies were compared to assess whether control [Normal weight healthy control (HC) and Healthy obese control (HOC) groups] and/or disease (steatosis and NASH groups) samples within studies could be pooled for differential analysis. Overall, PCA plots revealed that controls were different within studies and, as such, these groups were kept separate. Regarding steatosis and NASH samples, PCA plots for Suppli et al. (29) and Horvath et al. (31) revealed that steatosis and NASH samples were not different, while the plots for Lake et al. (30) indicated that the two groups were different. Inconclusive information could be extracted from Frades et al. (25) given the limited sample size ($n = 2$) for steatosis. Despite the similarities observed for two studies, steatosis and NASH were kept separate for analysis as they have clearly distinct clinical phenotypes (**Supplementary Figure 1**).

Differentially Expressed Genes Within Studies

Of the 164 genes included in this analysis, four genes (*UGT1A6*, *MAPK10*, *INS*, and *TSTD1*) had no gene expression data available in any of the included studies.

Normal-Weight Healthy Controls vs. Steatosis

Suppli et al. (29) had the most differentially expressed genes between HC and steatosis groups with 72 genes presenting statistically significant differences, followed by Horvath et al. (31) with 6, Lake et al. (30) with 1, and Frades et al. (25) had no genes significantly different between groups. A total of two genes were common in two out of four datasets (*MTR* and *GSTO1*), but no gene was differentially expressed between HC and steatosis in all datasets (**Figure 2A**). A list with all the genes included in the comparison between HC and steatosis across the datasets is available in **Supplementary Table 1**.

Suppli et al. (29) was the only study that demonstrated differentially expressed selenoprotein genes between HC and steatosis groups. Amongst the 13 genes, eight (*DIO1, GPX2, SEPHS2, SELENOK, SELENOS, SELENOT, SELENOF*, and *SELENOW*) had higher, and five (*DIO3, GPX1, SELENON, SELENOO,* and *TXNRD3*) had lower expression in steatosis.

Normal-Weight Healthy Controls vs. NASH

Overall, there were more genes significantly different between HC and NASH than in the HC vs. steatosis comparison. In HC vs. NASH comparison, Lake et al. (30) had the most genes significantly differentially expressed with 86 genes, followed by Suppli et al. (29) with 80 genes, Horvath et al. (31) with 27 and Frades et al. (25) with 1 gene. A total of eight genes were common in three out of four datasets (*PLG, JUN, ACSL4, MTR, CBS, GGT5, SLC11A2,* and *SOD1*), but no gene was differentially expressed between HC and NASH in all datasets (**Figure 2B**; **Supplementary Table 2**).

In Lake et al. (30) all the 14 selenoprotein genes differentially expressed between the two groups (*SELENOF, SELENOI, SELENOK, SELENOO, SELENOS, SELENOV, SELENOT, SEPHS2, TXNRD2, TXNRD3, GPX1, GPX2, MRSB1,* and *DIO2*) had a lower expression in NASH. In Suppli et al. (29), all the genes

TABLE 1 | Study characteristics.

Study, country	Study population	Dataset	NAFLD diagnosis	Study groups	Groups characteristics	Method
Suppli et al. (29), Denmark	Adults without diabetes and absence of excessive alcohol intake (>20/12 g/day for male/female)	GSE126848	Ultrasonographic evidence of hepatic steatosis, elevated liver enzymes, and compatible liver histology[a] in the absence of other (viral, alcohol, metabolic) causes of steatohepatitis.	Normal-weight healthy controls (BMI: 18.5–25) (n = 14/14)	Age: 39.5 ± 12.0 Male: 100% BMI: 23.1 ± 1.6 No lobular inflammation, hepatocyte, ballooning, or fibrosis 1 individual showed steatosis (stage 1)	RNA-Seq
				Healthy obese controls (BMI: 30–40 kg/m^2) (n = 12/12)	Age: 36.6 ± 10.2 Male: 100% BMI: 33.2 ± 1.3 No lobular inflammation, hepatocyte, ballooning, or fibrosis 1 individual showed steatosis (stage 1)	
				Steatosis: hepatic steatosis, elevated liver enzymes, compatible liver histology; presence of steatosis in >5% of hepatocytes; SAF < 2[a] (n = 15/15)	Age: 39.4 ± 10.6 Male: 60% BMI: 32.8 ± 5.1 Steatosis (mostly severe) Mild-grade lobular inflammation	
				NASH: presence of steatosis in > 5% of hepatocytes; SAF ≥ 2[a]; hepatocyte ballooning morphology (n = X16/16)	Age: 38.9 ± 17.0 Male: 75% BMI: 33.9 ± 6.2 Fibrosis stage: 1–2	
Horvath et al. (31), Germany	Liver samples obtained from adults during major oncological surgery where liver malignancy was excluded (control group); liver samples from adults with suspected NAFLD undergoing liver biopsy or assessment of liver histology (NAFLD groups)	GSE61260	Histological evidence according to NAFLD activity score (NAS)[b] in the absence of other (viral, alcohol, metabolic) causes of steatohepatitis	Normal-weight healthy controls (n = 38/38)	Age: 58.1 ± 18.3 Male: 42% BMI: 23.9 ± 3.0	Microarray: Affymetrix Human Gene 1.1 ST Array
				Healthy obese controls (n = 24/24)	Age: 49.1 ± 11.4 Male: 21% BMI: 42.9 ± 8.0	
				Steatosis (n = 23/23)	Age: 41.3 ± 6.2 Male: 52% BMI: 51.1 ±9.3	
				NASH (n = 24/24)	Age: 45.3 ±12.3 Male: 50% BMI: 51.2 ±10.8	

(Continued)

TABLE 1 | Continued

Study, country	Study population	Dataset	NAFLD diagnosis	Study groups	Groups characteristics	Method
Frades et al. (25), Spain	Liver samples from adults obtained during laparoscopic cholecystectomy (control group) or laparoscopic bariatric surgery (NAFLD groups).	GSE63067	Histological evidence (macrovesicular steatosis, lobular and portal inflammation)[c] in the absence of other (viral, alcohol, metabolic) causes of steatohepatitis.	Normal-weight healthy controls (n = 7/6)	Age: 57.6 (23–79 y) Male: 50% BMI: 24.6 Diabetes: NA	Microarray: Affymetrix Human Genome U133 Plus 2.0 Array
				Steatosis (n = 2/6)	Age: 43.3 (23–72 y) Male: 67% BMI: 48.2 Diabetes: NA	
				NASH: steatohepatitis grade 1 (macrovesicular steatosis, lobular and portal inflammation) (n = 9/9)	Age: 41.1 (24–61 y) Male: 78% BMI: 44.4 Diabetes: NA	
Lake et al. (30), United States	Liver samples acquired from liver tissue bank	E_MEXP_3291	Histological evidence (macrovesicular steatosis, lobular and portal inflammation)[b] in the absence of other (viral, alcohol, metabolic) causes of steatohepatitis.	Normal-weight healthy controls (n = 19/19)	Age: 42.15 (16–70 y) Male: 55% BMI: NA Diabetes: NA	Microarray:Affymetrix GeneChip Human 1.0ST Arrays
				Steatosis: >10% fat deposition without inflammation or fibrosis (n = 10/10)	Age: 46.7 (16–66 y) Male: 40% BMI: NA Diabetes: NA	
				NASH with fatty liver: >5% fat deposition, marked inflammation, fibrosis (n = 9/9)	Age: 56.8 (41–68 y) Male: 11% Diabetes: NA	
				NASH without fatty liver: <5% fat deposition, increased inflammation, fibrosis (n = 7/7)	Age: 52.7 (33–66 y) Male: 11% BMI: NA Diabetes: NA	

Data presented as mean ± SD or mean (range). n = samples included in this analysis/samples included in the original study.
[a]According to Steatosis-Activity-Fibrosis (SAF) (33).
[b]According to NAFLD activity score (NAS) (34).
[c]According to Brunt et al. (35).
BMI, Body Mass Index; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

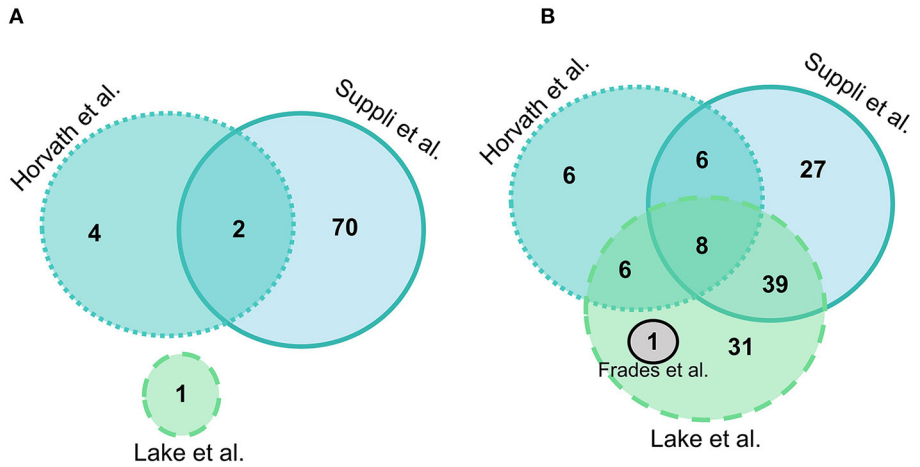

FIGURE 2 | Euler diagram of the number of significantly differentially expressed genes (adjusted *P*-value < 0.05) in liver samples: **(A)** Normal-weight controls vs. steatosis; **(B)** Normal-weight controls vs. NASH. Grey indicates Frades et al. (25), light blue indicates Suppli et al. (29), green indicates Lake et al. (30), aqua indicates Horvath et al. (31).

with higher expression in steatosis were also more expressed in NASH when compared to HC (*SELENOF, SELENOK, SELENOS, SELENOT, SELENOW, GPX2, GPX3, DIO1,* and *SEPHS2*), in addition to the *TXNRD1* gene. Similarly, four out the five genes with lower expression in steatosis also had lower expression in NASH (*SELENOO, SELENON, DIO3,* and *TXNRD3*) compared to HC. Finally, Horvath et al. (31) had four selenoprotein genes differentially expressed between HC and NASH, one with lower expression (*SELENOP*) and three with higher expression (*SELENON, GPX1,* and *GPX4*) in NASH. No significant differences were observed for selenoprotein genes between HC and NASH groups in Frades et al. (**Supplementary Table 2**). The *SCLY* gene, which encodes a key enzyme in selenium metabolism, was less expressed in NASH in Lake et al. (30) and Frades et al. (25).

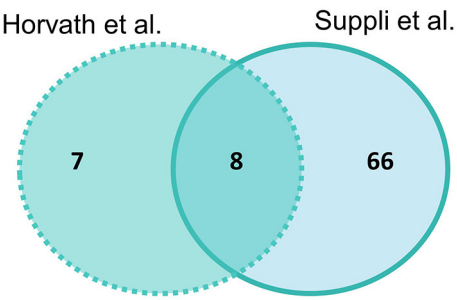

FIGURE 3 | Venn diagram of number of significantly differentially expressed genes (adjusted *P*-value < 0.05) in liver samples of healthy obese controls vs. NASH. Light blue indicates Suppli et al. (29), aqua indicates Horvath et al. (31).

Healthy Obese Controls vs. Steatosis

Two studies had HOC data available for comparison with steatosis and NASH. Overall, there were fewer genes significantly different between HOC and both steatosis and NASH samples. Suppli et al. (29) was the only study out of the two that demonstrated differentially expressed genes between HOC and steatosis with 62 genes (30 with lower expression and 32 with higher expression in steatosis). Of these genes, 11 coded for selenoproteins of which four genes had lower expression (*SELENON, SELENOO, GPX1,* and *TXNRD3*) and seven genes had higher expression (*SELENOK, SELENOS, SELENOW, GPX2, GXP3, DIO1,* and *SEPHS2*) in steatosis. The log fold changes of all genes were in the same direction as the comparison between HC vs. steatosis and NASH (**Supplementary Table 3**).

Healthy Obese Controls vs. NASH

Suppli et al. (29) had the most differentially expressed genes for HOC vs. NASH with 74 genes and Horvath et al. (31) had 15 genes, with eight genes overlapping between studies (*SELENON, TNXRD3, PLG, MAOA, SAT2, ALOX5, GGT5,* and *ACSL4*) (**Figure 3**). Of these overlapping genes, three genes were less expressed (*PLG, TXNRD3,* and *MAOA*) and two (*GGT5* and *ACSL4*) more expressed in NASH in both studies. The log-fold changes of the other three genes were in opposite directions between studies: *SAT2* had higher expression in NASH in Suppli et al. (29) and lower expression in Horvath et al. (31); and *ALOX5* and *SELENON* had lower expression in NASH in Suppli et al. (29) and higher expression in Horvath et al. (31) (**Supplementary Table 4**).

Pathway Analysis

Normal-Weight Healthy Controls vs. Disease Groups (Steatosis and NASH)

Within the explored pathways, genes were considered of interest if the log fold change between HC and disease groups were in the same direction in three out of four included datasets, and the difference was significant (adj. *P*-value < 0.05) in at least two of the three datasets. There were no genes that met these

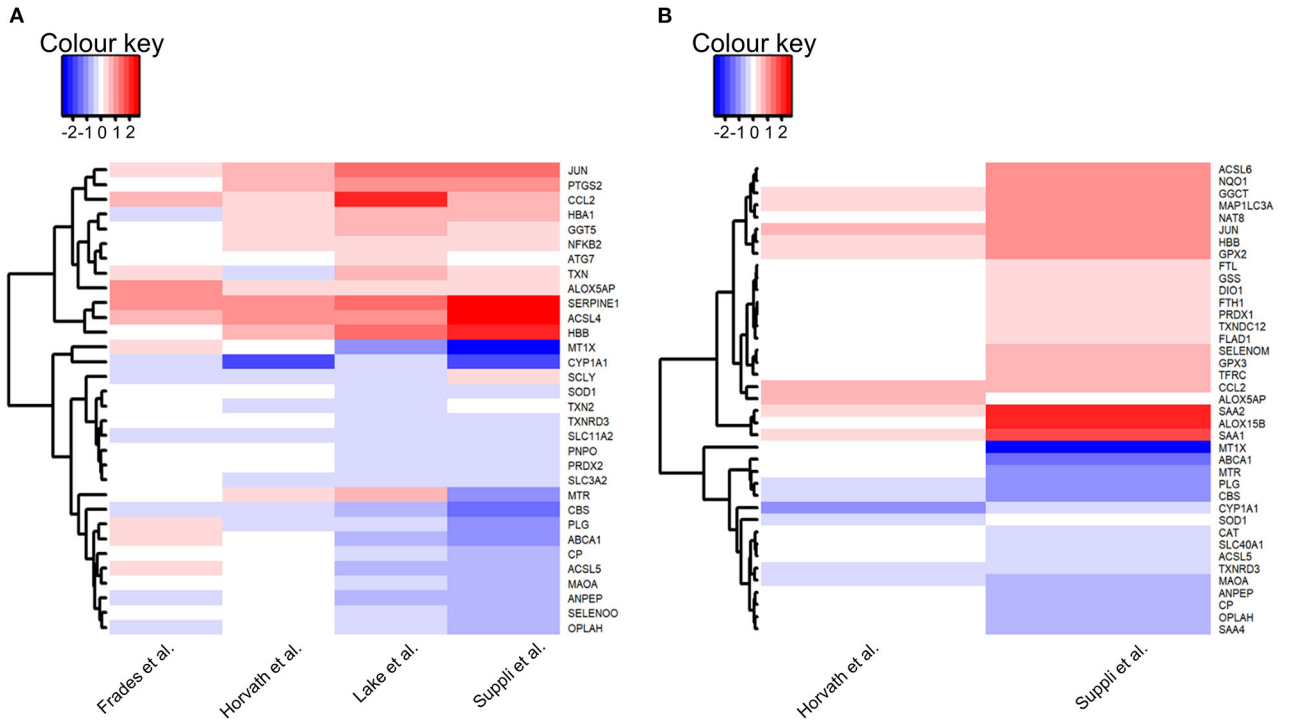

FIGURE 4 | Heatmaps of genes of interest in liver samples: **(A)** Normal-weight healthy controls vs. NASH; **(B)** Healthy obese controls vs. NASH. Genes of interest were defined as genes for which the log fold change was in the same direction in the majority of datasets (HC: 3/4 datasets, HOC: 2/2 datasets) and with an adjusted *P*-value < 0.05 in at least 50% of these datasets. Red depicts increased expression in NASH compared to controls and blue depicts decreased expression in NASH compared to controls. Heatmaps were generated using the heatmap.2 function in gplots (version 3.0.3) in R (version 3.6.3).

criteria when HC was compared with steatosis, and therefore no pathways were explored. A total of 32 genes were of interest in the comparison HC vs. NASH (**Figure 4A**).

Of the five pathways explored in this analysis, no pathway was consistently more highly or lowly expressed in NASH compared to HC. There were no consistent differences in expression for selenoprotein genes between groups in the "glutathione metabolism" and "ferroptosis" pathways. In the "glutathione metabolism" pathway, expression of two genes was lower in NASH (*OPLAH* and *ANPEP*) and expression of *GGTA1* was higher. In the "ferroptosis" pathway, four genes (*CP, SLC3A2, SLC11A2,* and *ACSL5*) had lower expression in NASH and two genes (*ATG7* and *ACSL4*) had higher expression.

There were no consistent differences in gene expression of selenoproteins across studies between NASH and HC samples in the "oxidative stress" pathway. However, there was a significantly lower expression of repression of ROS producing systems, notably, a decrease in *CYP1A1* expression in NASH.

In the "selenium metabolism and selenoproteins" pathway, the selenoproteins *TXNRD3* and *SELENOO*, as well as the gene *SCLY*, were less expressed in NASH while the gene *JUN* was more expressed in the disease group. There were no differences in selenoprotein genes in the "selenium micronutrient network" pathway, which encapsulates several genes encompassed in the other pathways. Nonetheless, nine genes (*TXN*, *ALOX5AP*, *HBA1*, *HBB*, *COX2*, *NFκB*, *MTR*, *CCL2,* and *SERPINE1*) had higher expression in NASH whereas six genes, including the already mentioned *TXNRD3* and *SELENOO* as well as *SOD1*, *PNPO*, *CBS,* and *PLG,* had lower expression in NASH.

A summary of all the genes of interest differently expressed between HC vs. disease groups according to the examined pathways is presented in **Table 2**.

Healthy Obese Controls vs. Disease Groups (Steatosis and NASH)

HOC vs. Steatosis

Genes were considered of interest in the assessed pathways if the log fold change in the comparison of HOC vs. steatosis or NASH was in the same direction in both included datasets and the difference was significant (adj. *P*-value < 0.05) in at least one dataset. Twenty-one genes met these criteria in HOC vs. steatosis and 39 in HOC vs. NASH comparison (**Figure 4B**).

For the "ferroptosis" pathway three genes were higher (*FTL*, *ATG5,* and *MAP1C3A*) and two were lower in steatosis (*SLC40A1* and *MAP1LC3C*). Two selenoprotein genes (*GPX2* and *GPX3*) encompassed in the "glutathione metabolism" pathway had higher expression, and one gene had lower expression (*OPLAH*) in steatosis, suggesting an increase in glutathione utilisation. For the "oxidative stress" pathway, two genes (*NFκB1* and *MTX1*) were lower in steatosis compared to HOC. Besides the selenoproteins *GPX2, DIO1,* and *SEPHS2,* the gene for *TRNAU1AP* also had higher expression in steatosis

TABLE 2 | Genes of interest in the comparisons normal-weight healthy controls (HC) × steatosis, normal-weight healthy controls (HC) × NASH, healthy obese controls (HOC) × steatosis, healthy obese controls (HOC) × NASH.

Pathways	HC × steatosis	HC × NASH	HOC × steatosis	HOC × NASH
Glutathione metabolism	○	↓ *OPLAH* and *ANPEP* ↑ *GGTA1*	↓ *OPLAH* ↑ *GPX2* and *GPX3*	↓ *OPLAH* and *ANPEP* ↑*GSS* and *GGTLA1* ↑ *GPX2* and *GPX3*
Ferroptosis	○	↓ *CP*, *SLC3A2*, *SLC11A2,* and *ACSL5* ↑ *ATG7* and *ACSL4*	↓*SLC40A1* and *MAP1LC3C* ↑ *FTL*, *ATG5,* and *MAP1LC3A*	↓ *SLC40A1, CP,* and *MAP1LC3C* ↑ *FTH1*, *FTL*, *ACSL6*, *ACSL4*, *MAP1LC3A, TFRC,* and *GSS*
Oxidative stress	○	↓*CYP1A1*	↓ *NFκB* and *MXT1*	↓ *MAOA*, *CYP1A1,* and *MT1X*
Selenium metabolism and selenoproteins	○	↓ *TXNRD3* and *SELENOO* ↓ *SCLY* ↑ *JUN*	↓ *TXNRD3* ↑ *GPX2* and *DIO1* ↑ *SEPHS2* and *TRNAU1AP*	↓*TXNRD3* ↑*GPX2, GPX3,* and *DIO1* ↑*JUN*
Selenium micronutrient network	○	↓ *TXNRD3* and *SELENOO* ↓*SOD1*, *PNPO*, *CBS,* and *PLG* ↑*TXN*, *ALOX5AP*, *HBA1*, *HBB*, *COX2*, *NFκB*, *MTR*, *CCL2,* and *SERPINE1*	↓ *ABCA1*, *MTR*, *MTHFR,* and *PLG* ↑ *DIO1* ↑ *HBB*, *SAA1, SAA2,* and *SEPHS2*	↓*SOD1*, *SAA4*, *ABAC1*, *MTR*, *CBS,* and *PLG* ↓*TXNRD3* ↑*SAA1*, *SAA2*, *CCL2*, *HBB,* and *SERPINE1* ↑*SELENOM*, *DIO1*, *GPX2*, and *GPX3*

↓ Lower expression in the disease group (either steatosis or NASH); ↑ higher expression in the disease group (either steatosis or NASH). HC, Normal-weight Healthy Controls; HOC, Healthy Obese Controls. Full gene names are available in ***Supplementary Tables 1–4****.* ○ *Means no genes of interest were found in this comparison (HC vs steatosis).*

in the "selenium metabolism and selenoproteins" pathway, and *TXNRD3* had lower expression in steatosis. Four genes had lower expression in steatosis (*ABCA1*, *MTR*, *MTHFR,* and *PLG*) and five genes had higher expression (*SEPHS2*, *DIO1*, *HBB*, *SAA1,* and *SAA2*) in the "selenoprotein micronutrient network" pathway.

HOC vs. NASH

For the "ferroptosis" pathway, expression of seven genes was higher (*FTH1*, *FTL*, *ASCL6*, *ACSL4*, *MAP1LC3A, TFRC,* and *GSS*) and expression of three genes was lower (*SLC40A1, CP,* and *MAP1LC3C*) in NASH. Expression of *SLC40A1*, the gene encoding the iron exporter ferroportin, was lower in NASH. Interestingly, expression of *MAP1LC3C*, a paralogue of *MAP1LC3A*, was also lower in the disease group. However, the expression of *MAP1LC3C* is not high in the liver (36), so this observation may not be biologically significant. In addition to *GSS*, three genes encompassed in the "glutathione metabolism" pathway had higher expression (*GGTLA1*, *GPX2,* and *GPX3*), and two genes had lower expression (*OPLAH* and *ANPEP*) in NASH, suggesting an increase in glutathione utilisation. For the "oxidative stress" pathway three genes (*MAOA*, *CYP1A1,* and *MT1X*) had lower expression in NASH.

For the "selenium metabolism and selenoproteins" pathway, in addition to the genes already mentioned (*GPX2* and *GPX3*), expression of *DIO1* and *JUN* was higher, and *TXNRD3* was lower in NASH. Finally, in the "selenium micronutrient network" pathway, 11 genes (*SELENOM*, *DIO1*, *GPX2*, *GPX3*, *ALOX15B*, *FLAP*, *SAA1*, *SAA2*, *CCL2*, *HBB,* and *SERPINE1*) had higher expression in NASH and seven genes (*TXNRD3*, *SOD1*, *SAA4*, *ABCA1*, *MTR*, *CBS,* and *PLG*) had lower expression in NASH compared to HOC.

A summary of all the genes of interest differentially expressed between HC vs. disease groups according to the assessed pathways is presented in **Table 2**.

Comparisons Between HC vs. NASH and HOC vs. NASH

There were 17 genes of interest commonly different in both the HC vs. NASH and HOC vs. NASH comparisons. Of these 17 genes, 12 had lower expression in NASH compared to both HC and HOC and four had higher expression in NASH. One gene, *MTR,* had higher expression in NASH compared to HC but lower expression in NASH compared to HOC (**Table 2**).

DISCUSSION

This study used publicly available human global gene expression datasets to uncover pathways involving selenium and selenoproteins that may participate in chronic pathological mechanisms of NAFLD (**Figure 5**). Previous observational studies have suggested an association between selenium and SELENOP levels in plasma with increased risk and severity of NAFLD (18–22). Nonetheless, the bioinformatics approach undertaken in this study revealed no differences at the gene level for *SELENOP*. Despite this result, it is possible that SELENOP quantities, as well as of other selenoproteins, are still elevated in NAFLD due to regulation of their translation at the ribosome level (37, 38), downstream of transcriptional mechanisms evaluated herein. Furthermore, we observed that the gene encoding the selenoprotein *TXNRD3* was less expressed in both disease groups (NASH and in steatosis) when compared with HOC, while no significant differences between HC and steatosis samples were observed. Also, *SCLY,* which encodes the enzyme responsible for catalysing removal of selenium from selenocysteine, was less expressed in NASH when compared with HC, as was the selenoprotein *SELENOO*. In contrast, gene expression of the selenoproteins *SELENOM*, *DIO1*, *GPX2,* and *GPX3* was higher in the NASH group when compared to HOC. Moreover, lower expression of iron-associated transporters,

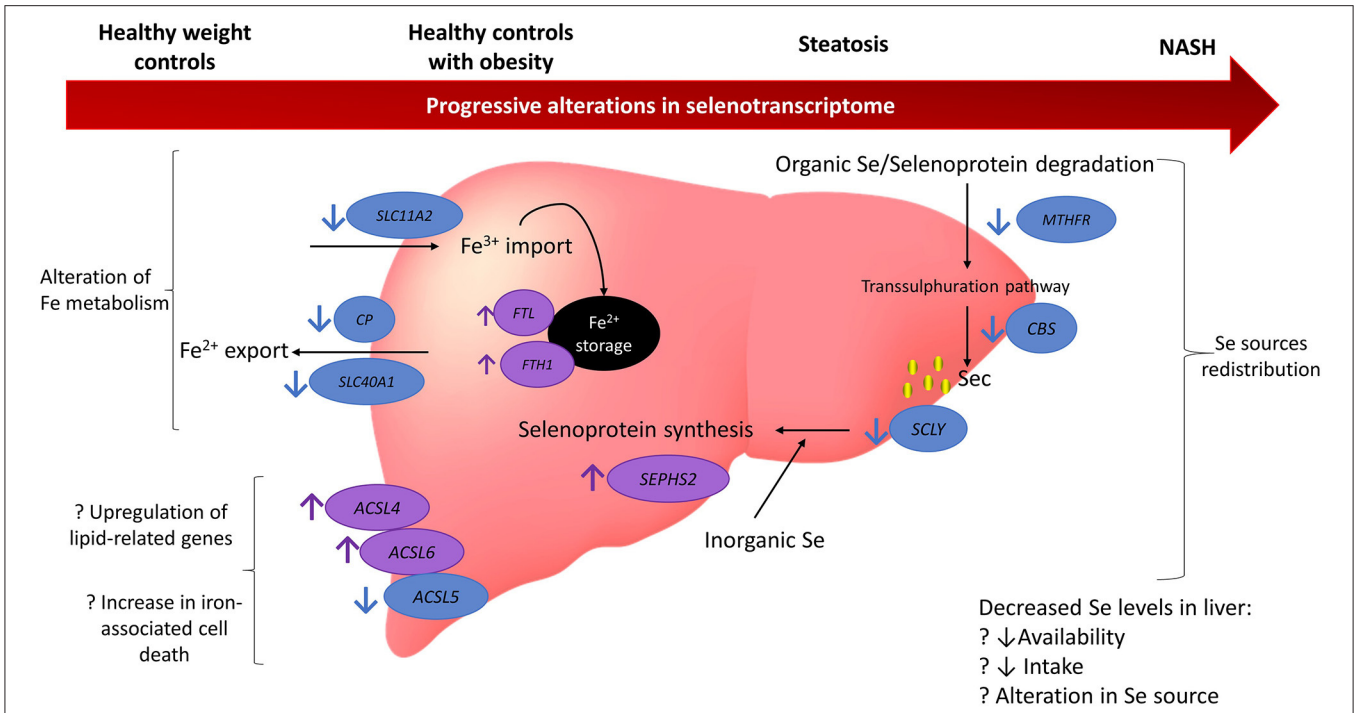

FIGURE 5 | Model summarising the findings from gene expression analysis of 164 genes related to selenium and selenoproteins. Findings suggest there may be a redistribution in selenium sources with a reduction in organic selenium degradation *via* the transsulphuration pathway and an increase in the metabolism of inorganic selenium. Iron metabolism may also be disturbed with an increase in iron storage and promotion of factors related to iron-associated cell death. There may be several reasons for these alterations and they appear to be progressively altered as NAFLD deteriorates. Blue circles and arrows indicate genes down-regulated in NAFLD compared to controls (either healthy weight or obesity) and the purple circles and arrows indicate genes up-regulated in NAFLD compared to controls. Question marks indicate hypothesised reasons for alterations that cannot be fully tested in the current study. MTHFR, methylenetetrahydrofolate reductase; CBS, cystathionine beta-synthase; SCLY, selenocysteine lyase; ACSL5, acyl-CoA synthetase long chain family member 5; ACSL6, acyl-CoA synthetase long chain family member 6; ACSL4, acyl-CoA synthetase long chain family member 4; SCL40A1, solute carrier family 40 member 1; CP, ceruloplasmin; SLC11A2, solute carrier family 11 member 2; Fe, iron; NASH, non-alchoholic steatohepatitis; Se, selenium.

higher expression of genes encoding the ferritin subunits, as well as differential expression of members of the *ACSL* gene family, point to a disturbed iron metabolism which may enhance ferroptosis under conditions of NAFLD. We also observed that for several genes, the difference between NASH and HC was larger than when NASH was compared with HOC or steatosis, supporting the notion that gene expression variation may be associated with the progressive risk of NAFLD.

Although we here present a novel approach that targeted specific molecular pathways, we note that some of these pathways have been previously described as to be perturbed across increasing NAFLD severity in previous studies. For instance, Hoang et al. (39) reported that pathways for glutathione metabolism ("glutathione synthesis and recycling") and oxidative stress ("oxidative stress induced senescence") were upregulated in liver samples as the NAS and fibrosis stage increased, although no associations were observed for selenium or selenoprotein pathways. Further, Zhu et al. (40) identified several upregulated selenoproteins (*SELENON*, *SELENOP*, *SELENOT*, *SELENOW*, *DIO2*, *DIO3*, *GPX4,* and *GPX5*) in mild NAFLD liver samples when compared to healthy controls. This therefore suggests that selenium related processes are progressively perturbed in NAFLD development and that a targeted approach as taken by the current study can be used to uncover the specific pivotal pathways and genes involved.

The essential role of selenoproteins in hepatic function was highlighted after mice genetically devoid of hepatic selenoproteins developed hepatocellular degeneration and necrosis, leading to early death (41). Selective loss of housekeeping selenoproteins, such as DIO1, SELENOP, TXNRD1, and SELENOF, led to upregulation of genes involved in cholesterol biosynthesis, and downregulation of genes involved in cholesterol metabolism and transport, suggesting an overall effect of this subset of selenoproteins in hepatic lipoprotein metabolism that favours hypercholesterolaemia (42). Amongst the selenoproteins associated with the metabolism of cholesterol, our findings revealed a higher expression of *DIO1* in the disease groups (NASH and steatosis) when compared to healthy obese controls. Lower expression of the selenoprotein *TXNRD3* was observed in the disease groups when compared with both control groups (HC and HOC). The isoform 3 for TRXR, encoded by *TXNRD3*, has an atypical kinetic behaviour when compared to the other two TRXR isoforms as it can reduce both thioredoxin and glutathione, and thus can function as a thioredoxin reductase, glutathione reductase, and glutaredoxin (43). Despite its low concentration in the liver, TRXR3 plays

an essential role in the antioxidant system along with TRXR1 and TRXR2, besides their involvement in cell proliferation and redox-regulated signal cascades (44). Higher expression of *GPX2* and *GPX3* was observed in both disease groups when compared to HOC. GPX2 protein is present in the gastrointestinal tract, including the liver (45) and, therefore, may play a role in disease pathogenesis. However, GPX3 is secreted into the circulation (45), so any role it plays in NAFLD progression will be indirect and remote from the liver. Combined, molecular studies utilising mouse models of selenoprotein deficiency indicate that the effects on liver function cannot be generalised to all selenoproteins, as some are crucial while others can be less needed in hepatic physiology, and the specific role of each one should be considered. Absence of a clear pattern for the regulation of human hepatic selenoprotein expression between disease and control groups, as we herein report, reinforces this variability.

Other genes highly involved in the antioxidant response are *OPLAH*, which encodes 5-oxoprolinase, and *SOD1,* which encodes for Cu/Zn-superoxide dismutase 1. OPLAH is part of the glutathione cycle, responsible for converting 5-oxoproline, a degradation product of glutathione, into glutamate. Low expression of *OPLAH*, as seen in both steatosis and NASH, is associated with accumulation of 5-oxiproline and increased oxidative stress in *in vivo* and *in vitro* studies (46–48). Likewise, SOD1 is a key component of the cellular responses to oxidative stress, dismutating superoxides into hydrogen peroxide and molecular oxygen. The downregulation of *SOD1* in NASH that we uncovered in our analysis is consonant with animal model results, and suggests a profound implication of this gene in disease pathogenesis. Mice lacking the gene for SOD1 are considered an animal model to study hepatocellular carcinoma development following NASH, as they spontaneously present in their hepatocytes high oxidative stress, impaired very-low-density lipoprotein (VLDL) secretion that leads to lipid accumulation, necroptosis and inflammation (49). Consistent with this is the finding that *CCL2*, a proinflammatory chemokine, was increased in NASH compared with both control groups. CCL2 is known to be associated with development of inflammation and recruitment of monocytes in NASH (50). It is also compelling that, as NASH and NAFLD are risk factors for the development of hepatocellular carcinoma, the *JUN* proto-oncogene was also found to be upregulated in our analysis of NASH samples. *JUN* encodes for the c-Jun transcription factor subunit of the AP-1 complex (51) that participates in inflammatory responses to several types of cancer (52).

We found that *SCLY* expression was lower in NASH samples when compared to HC. *SCLY* encodes the enzyme Sec lyase (SCLY), that de-selenates organic Sec from dietary sources, the transsulphuration pathway or selenoprotein degradation (53) to provide selenide for selenoprotein synthesis. Hence, SCLY is at the intersection between degradation and biosynthesis of selenoproteins, allowing for recycling of selenium and thus may control selenium distribution (54). Interestingly, mice lacking SCLY develop hepatic steatosis (55), which is aggravated by selenium deficiency (55) and not restored by selenium supplementation (56); it is unknown if this mouse model develops NASH as it ages. The hepatic steatosis presented by the homozygous *SCLY* knockout mouse model informs that downregulation of *SCLY* may also be implicated in NAFLD development in humans. In addition, we found higher expression of *SEPHS2* in steatotic livers compared to HOC. *SEPHS2* is involved in inorganic selenite assimilation for selenoprotein synthesis. This gene was reported to be highly expressed in the rodent liver (57, 58). As both *SCLY* and *SEPHS2* play a role in providing selenium from different sources to selenoprotein synthesis, it is possible that either a shift in or redistribution of selenium sources for selenoprotein synthesis occurs in NAFLD livers.

The role of SCLY in selenoprotein degradation has been understudied, however its participation in the trans-selenation pathway is better understood. The trans-selenation pathway sequentially uses the methionine cycle enzymes and the transsulphuration pathway acting upon methionine to metabolise organic selenocompounds, particularly selenomethionine. The reactions of the methionine cycle are catalysed by methionine synthase (MS, encoded by the gene *MTR*) and methylenetetrahydrofolate reductase (MTHFR), while cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (CTH) participate in the trans-selenation pathway to generate Sec as the final product, which serves as a specific substrate for SCLY (59). In addition to a lower expression of *SCLY*, our analysis uncovered that, overall, *MTHFR* and *CBS* were also less expressed in NAFLD, suggesting that methionine/selenomethionine metabolic capacity may be reduced in the disease. In alignment with our findings, a mouse model of fatty liver disease also presented with diminished CBS and transsulphuration metabolites, which negatively impacts glutathione metabolism (60), thus corroborating the critical participation of these selenium-related pathways in NAFLD pathogenesis. We speculate that the reduction in trans-selenation activity is either caused by diminished selenomethionine availability to be metabolised in NAFLD hepatocytes or a consequence of dysfunctional methionine metabolism that impacts selenium metabolism as collateral damage. Nevertheless, the observed lower expression of *SCLY*, *MTHFR,* and *CBS* in the disease, is possibly indicative of a reduced transsulphuration capacity, which is required for the metabolisation of methionine and selenomethionine. This, combined with the higher expression of *SEPHS2*, strengthens the hypothesis of a shift in selenocompound utilisation during NAFLD pathogenesis to be tested in the future. Moreover, it suggests that a role for selenium metabolism beyond selenoproteins may persist in NAFLD's pathophysiology.

Ferroptosis is a unique non-apoptotic-programmed cell death pathway dependent on iron and lipid hydroperoxides. This pathway is characterised by the inhibition of the xc– system, responsible for Cys import, causing limited glutathione biosynthesis. Additionally, disturbed intracellular iron storage and PUFA-enriched phospholipids form the scenario required for ferroptosis (61). Given that GPX4 can reduce lipid peroxides when glutathione levels are decreased, this selenoprotein is a key negative regulator of this cell death pathway (62). Ferroptosis was reported as the initial cell death mechanism that triggers

steatohepatitis (63). Changes in iron parameters, including increased serum ferritin and hepatic iron, have often been reported in association with NAFLD, although the evidence is conflicting (64), suggesting that any role for iron in NAFLD is likely to be the target of multiple regulatory processes, hence the importance of exploring this pathway in this study. Our analysis demonstrated that, compared to HC, *SLC11A2*, the gene encoding the importer, divalent metal transporter 1, was less expressed in NAFLD. Although not statistically significant, *SLC11A2* was also lower compared to HOC. Similarly, *SLC40A1*, the gene encoding the iron exporter ferroportin, was lower in steatosis and NASH than in HOC, and caeruloplasmin (*CP*) was decreased in NASH compared to both control groups. Ferroportin and CP act together to export cellular iron, ferroportin transporting ferrous iron through the membrane and CP catalysing oxidation to ferric iron, which is necessary for release (65). These findings suggest a contraction of hepatic iron metabolism in NAFLD, whereby iron uptake is being decreased, and the release of iron already within the cell is curtailed. This is consistent with the observed higher expression of genes encoding the ferritin subunits (*FTL* and *FTH1*) in the disease, suggesting an increased capacity for storage of iron already in the cell. Ferritin is regulated post-transcriptionally (66), with protein levels being more responsive to iron than mRNA levels, so the decrease in transcript observed in these datasets suggests a chronic response.

Expression of *ACSL4*, an acyl-CoA synthetase, is higher in NASH compared both to HC and HOC. *ACSL4* is a known stimulator of iron-associated cell death (67), being involved in the synthesis of polyunsaturated fatty acids, which are conducive to peroxidation. In contrast, *ACSL5* was less expressed in NASH compared to HC. ACSL5 has been associated with apoptotic cell death in the gut and steatotic liver, and decreased ACSL5 with reduced sensitivity to cell death in HepG2 cells (68, 69). *ACSL6* is more highly expressed in NASH than HOC, although it has previously been reported in several cell lines, including the hepatoma cell line HepG2, not to be associated with ferroptosis (67). Increases in acyl-CoA synthetases are consistent with our previous findings in AML12 mouse hepatocytes that lipid accumulation occurs within 16 hours of iron loading and continues to increase over the subsequent 8 hours (70). However, the mechanism behind differential regulation of three *ACSL* isoforms, all of which are associated with cell death pathways, remains unclear. ACSL4, ACSL5, and ACSL6 have substrate preferences for long-chain polyunsaturated fatty acids (C20 and C22), although ACSL6 is more specific for docosahexaenoic acid (DHA; C22:6) (71–73) and its hepatic expression is lower (36). However, we cannot rule out that higher expression of *ACSL4* and *ACSL6* may simply be associated with a general upregulation of lipid-related gene expression in NAFLD (74).

Gene expression of autophagy-like proteins was higher in the disease states: *ATG7* was higher in NASH in comparison to HC, and ATG5 was higher in steatosis when compared to HOC. These proteins participate in a ubiquitin-like pathway which is a precursor to phagophore formation (75). Maturation of phagophores into autophagosomes requires *MAP1LC3* (76), of which the alpha form is more highly expressed and the gamma form less expressed in NAFLD than HOC. Together, these observations suggest induction of autophagy to remove damaged cell components, although potentially not induction of ferroptosis. Whilst ferritin degradation may occur *via* autophagy (so-called ferritinophagy) to release iron that then induces ferroptosis (77), it is unclear why ferritin transcripts would be more highly expressed under a ferroptotic phenotype. Autophagic pathways are a component of many degradation pathways and may not necessarily lead to cell death; hence, the upregulation of ferritin may allow the cell to sequester iron and repair oxidative or inflammatory damage without committing to ferroptotic cell death. Confirmation of this hypothesis would require further investigation.

The lower expression of plasminogen (*PLG*) and higher expression of *SERPINE1*, which encodes plasminogen activator inhibitor-1 (PAI-1) in NAFLD may be an indicator of the presence of a prothrombotic state in the disease. PAI-1 inhibits the tissue plasminogen activator, resulting in a decreased conversion of plasminogen into active plasmin, which is crucial for degradation of fibrin clots (78). Animal models show that PAI-1 is associated with fat accumulation in the liver (79) and progression of inflammation and hepatic fibrosis (80). Given the association between PAI-1 and endothelial dysfunction (81, 82), Ciavarella et al. (83) emphasised that PAI-1 plays a critical role in the pathogenesis of a prothrombotic state in NAFLD, which links the disease with an increased risk for cardiovascular events.

Overall, our findings indicate a larger difference between NASH and HC than when NASH was compared to either HOC or steatosis. Although this does not apply to all the genes of interest, this observation supports the idea of a progressive change in gene expression across the different stages of the disease. Surprisingly, no differences between HC and steatosis were observed, while steatosis presented some significant differences when compared with HOC. Although this should be further investigated, we hypothesise that this is a product of the criteria we applied, given that only two datasets had data for the comparison between HOC and steatosis, and therefore the log fold changes are more consistent in these two than in the four datasets used for the comparisons with HC.

Limitations of whole -omics analysis include the potential to miss subtle, yet biologically relevant, pathway alterations. This is particularly pertinent in trace element homeostasis, where slight alterations in transcript, protein or metabolite levels may have large downstream effects. A key strength of this study relies on the investigation of specific pivotal pathways, thus enabling the exploration of molecular mechanisms that are commonly an oversight in global exploratory studies. Given that this study concerns a bioinformatic analysis of a limited number of genes and is of an exploratory nature, a stringent adjustment method (Benjamini-Hochberg) was chosen to minimise false-positive over false-negative results. Comparing liver samples between steatosis, NASH, and controls with and without obesity advances the understanding of gene expression changes associated with the progressive risk of NAFLD, even though this comparison has been limited by the inclusion of only two studies that reported data for HC and HOC. Small sample sizes in omics studies often hinder the exploration of modest variations in gene expression in complex diseases such as NAFLD. Thus, the

comparison of multiple studies as performed in this analysis allows for larger sample size and increased utility of these data, ultimately supporting translation of research findings into practise. Nonetheless, this approach imposes some limitations that should be acknowledged. As our bioinformatics analysis used publicly available data, we were limited by the lack of information on the subjects' individual characteristics, including demographics (age and ethnicity) and clinical history which may have shed some additional insight into the variability between datasets. The inclusion of this additional phenotypic information for the datasets available in the repositories would allow more complex analysis to facilitate data interpretation and increase the utility of these data more broadly. The lack of dietary data precludes identifying potential differences in selenium intake, which could explain some differences seen across different studies. Furthermore, studies included in our analysis used different scoring systems for histopathologic classification of liver lesions used by the selected studies. Although this can lead to changes in the discrimination between steatosis and NASH, the scoring methods applied can appropriately separate controls and disease. Also, the included datasets obtained control samples from different situations, such as major surgeries or liver tissue bank (which commonly receives samples from subjects involved in motor vehicle accidents), which may be implicated in different underlying physiological processes. This study brings insight into the role selenium plays in NAFLD. Still, given its exploratory nature, future research is needed to validate our findings, for example, through qPCR or Nanostring technology. A combined proteomics approach would add further information as to whether our findings persist at the protein level.

CONCLUSIONS

Our study brings findings from a preliminary analysis that shed light on how selenium may be involved in the regulation of pathological mechanisms in NAFLD. Our findings suggest that the NAFLD liver may have lower selenium levels than a disease-free liver, and further studies targeting *TXNRD3* and *SCLY* along with enzymes involved in the transsulphuration pathway (particularly *MTHFR* and *CBS*) may elucidate the pathophysiological role of selenium metabolism in NAFLD. Furthermore, our study provides evidence for a link between selenium and iron metabolism, which is known to be disrupted in NAFLD, and may contribute to cell death *via* ferroptosis, ultimately leading to cirrhosis.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analysed in this study. This data can be found here: Repositories: GEO and ArrayExpress. Accession Numbers: GSE126848, E-MEXP-3291, GSE61260, GSE63067, and GSE48452.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KD and BC: study conceptualisation and writing—original draft. KD: data curation and formal analysis. LS, RG, and BC: writing—review and editing. BC: supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2021.744825/full#supplementary-material

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