MICRONUTRIENTS AND FATTY ACIDS IN PRECISION NUTRITION STRATEGIES

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MICRONUTRIENTS AND FATTY ACIDS IN PRECISION NUTRITION STRATEGIES

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EDITORIAL
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Editorial: Micronutrients and Fatty Acids in Precision Nutrition Strategies

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Editorial on the Research Topic

Micronutrients and Fatty Acids in Precision Nutrition Strategies

PRECISION NUTRITION

The importance of precision nutrition in health management strategies is gaining attention. Precision nutrition aims to translate individual molecular signatures into applicable personalized dietary and nutritional recommendations and ultimately a personalized dietary plan. Some endeavors have been made to customize macronutrient intake for individual predispositions, through the implementation of different dietary regimes, such as the Mediterranean diet with optimized fat intake (1), or a keto diet that favors fat intake (2). Huge interest, however, remains in supplying adequate amounts of essential microelements, individual nutrients, and bioactives, based on personal predispositions which include genetics, microbiota features, and molecular nutrition biomarker profiles. Having said that, this Research Topic reflects recent works dealing with nutrigenetic and gene *x* dietary/environment interactions, micronutrients, and microbiota responsiveness to bioactive supplementation, altogether evaluated in various health contexts.

MICROELEMENTS AND BIOACTIVES IN EARLY LIFE DEVELOPMENT AND HEALTHY AGING

Early development largely depends on maternal nutritional competence, which primarily relies on intake but could also be modulated by endogenous factors such as the genetic background of a mother and even the developing fetus, both factors influencing the adequate nutritional supply of an offspring. The first 1,000 days concept requires appropriate delivery of essential nutrients, otherwise, a set of metabolic derangements may occur in malnourished children (3). In addition to the nutritional intake itself, a genetic make-up might modulate mother-offspring nutritional transfer. Thus, it has been suggested that a genetic variation in *SLC5A5* (rs775249401) gene locus might modulate transportation of iodine into breast milk, with A-allele carrier mothers being more efficient with iodine transportation to breast milk when exposed to inadequate iodine intake (Siro et al.). The results have been demonstrated in women of African descent and warrant more research in the race- and ethnic-specific context. On the other hand, a genome-wide association study across 7 million single nucleotide polymorphisms in Asian populations of Malay, Indian, and Chinese women, revealed that genetic variation (rs4588) in *GC* gene encoding for vitamin D binding protein was associated with low vitamin D status in mothers and cord blood, while the variation in *CYP2J2*

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(rs10789082) was associated with low vitamin D in the antenatal analysis only (Sampathkumar et al.). Precision nutrition tailored strategies based on adequate vitamin D supplementation during pre- and peri-conception periods could largely improve vitamin D deficiencies in both women and babies, potentially associated with immunological benefits and musculoskeletal health.

On the other side of the lifespan continuum, adequate supplementation of bioactives such as polyphenols and microelements from fruits and medicinal plants might improve healthy aging and prevent neurodegenerative disease through a panel of nutri(epi)genetic mechanisms, as well as gut microbiota-brain axis, as reviewed in Milošević et al.

POLYPHENOLS AND MICRONUTRIENTS IN IMMUNITY AND HEMOSTASIS

A body of research has recently been directed toward gut microbiota different organ axes, with gut microbiota eubiosis playing an essential role in immunological balance and prevention of a leaky gut through sustained intestinal integrity and low permeability. Fine modulation of gut microbiota profiles remains an outstanding goal for future personalized strategies, and a recent paper has shown that polyphenols from medicinal plants might have growth-stimulating effects on probiotic bacterial and yeast strains (Milutinović et al.).

The importance of microelements in immunological response has also been demonstrated in the burning topic of COVID-19 disease, with unprecedented effects on public health globally. In adult Serbian subjects, but not in a pediatric population, variations in genes involved in vitamin D metabolism (*DHCR7/NADSYN* rs12785878 and *CYP2R1* rs10741657 variants) were linked with severe COVID-19 disease (Kotur et al.), implicating the potential of vitamin D-based precision supplementation strategies in boosting immunological response toward more efficient COVID-19 prevention and suppression.

Precision nutrition supplementation strategies of iron might also depend on the genetic variation coding for fibrinogen and FXIII molecules, as indicated in the recent epidemiological cohort of black African descent (Rautenbach et al.). In this study, biomarkers of iron status, such as ferritin and transferrin, were shown to be linked with fibrinogen, fibrin formation, and fibrinogen lysis, implicating a role of circulating iron in hemostasis.

BIOACTIVES AND NUTRIENTS IN CHRONIC DISEASES

Due to the increased incidence of cardiovascular disease worldwide, associated with a striking increase in obesity, related to nutritional transitions toward Westernized dietary patterns, novel personalized approaches in cardiometabolic conditions are highly desirable.

Zinc is involved in the inflammatory response and disturbed zinc homeostasis is an early feature of cardiovascular disease. Zinc status is essential for optimal cellular and tissue proliferation, hormonal and immunological balance, and randomized controlled zinc interventions to alleviate cardiovascular disease, as well as nutrigenetic implications of zinc transporter proteins and metallothionein, are much needed (Knez and Glibetic). The necessity of zinc supplementation has also been established in reportedly zinc-deficient hemodialysis patients, who also presented with an inadequate long-chain omega-3 fatty acid intake (Takic et al.), indicating potential interaction between fatty acid and micronutrient metabolism. Furthermore, the interaction between dietary bioactives i.e., polyphenols from Aronia berry and polyunsaturated fatty acid metabolism, has also been reported in dyslipidemic women, with those consuming polyphenol-rich berry juice having a lower inflammatory arachidonic acid/eicosapentaenoic acid ratio (Stojković et al.). The same study demonstrated that habitual polyphenol intake also induced decreases in Long Interspersed Nucleotide Element-1 methylation levels in peripheral blood leukocytes, which was dependent on *MTHFR* variant allele (C677T), indicating nutri(epi)genetic forces elicited by polyphenol consumption. Aside from microelements and bioactives, fatty acid profiles are influenced by the quality of a diet. In obese subjects, *FADS2* rs174583 variant, involved in dietary fatty acid conversion, interacted with the level of adherence to either Dietary Approach to Stop Hypertension or Mediterranean diet, finally affecting triglyceride concentration/diastolic blood pressure in women/men and glucose serum levels, respectively (Khodarahmi et al.).

Finally, precision nutrition strategies are of special importance in populations with specific nutritional and dietary needs, which was demonstrated in a recent study in underground coal miners, where unexpected direct correlations between serum vitamin D levels and anthropometric and biochemical indicators were demonstrated, the latter commonly indicative of obesity, metabolic syndrome, and fatty liver disease (Šarac et al.).

FOUNDATION FOR FUTURE STUDIES

In order to implement precision nutrition postulates in practice, further race-, ethnicity-, and population-specific randomized controlled studies are needed to validate responsiveness to a particular nutrient and dietary intervention depending on the holistic interpretation of individual genetic and molecular biomarker profiles. Further research should put additional attention on population groups with specific nutritional and dietary needs, such as occupational groups and athletes.

AUTHOR CONTRIBUTIONS

MMZ wrote the manuscript and IK, LZ, and CMS reviewed the manuscript. All authors approved the final version of the manuscript.

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Association of Vitamin D, Zinc and Selenium Related Genetic Variants With COVID-19 Disease Severity

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Background: COVID-19 pandemic has proved to be an unrelenting health threat for more than a year now. The emerging amount of data indicates that vitamin D, zinc and selenium could be important for clinical presentation of COVID-19. Here, we investigated association of genetic variants related to the altered level and bioavailability of vitamin D, zinc and selenium with clinical severity of COVID-19.

Methods: We analyzed variants in genes significant for the status of vitamin D (*DHCR7/NADSYN1* rs12785878, GC rs2282679, *CYP2R1* rs10741657, and *VDR* rs2228570), zinc (*PPCDC* rs2120019) and selenium (*DMGDH* rs17823744) in 120 Serbian adult and pediatric COVID-19 patients using allelic discrimination. Furthermore, we carried out comparative population genetic analysis among European and other worldwide populations to investigate variation in allelic frequencies of selected variants.

Results: Study showed that *DHCR7/NADSYN* rs12785878 and *CYP2R1* rs10741657 variants were associated with severe COVID-19 in adults ($p = 0.03$, $p = 0.017$, respectively); carriers of *DHCR7/NADSYN* TG+GG and *CYP2R1* GG genotypes had 0.21 and 5.9 the odds for developing severe disease, OR 0.21 (0.05–0.9) and OR 5.9 (1.4–25.2), respectively. There were no associations between selected genetic variants and disease severity in pediatric patients. Comparative population genetic analysis revealed that Serbian population had the lowest frequency of *CYP2R1* rs10741657 G allele compared to other non-Finish Europeans (0.58 compared to 0.69 and 0.66 in Spanish and Italian population, respectively), suggesting that other populations should also investigate the relationship of *CYP2R1* variant and the COVID-19 disease course.

Conclusion: The results of the study indicated that vitamin D related genetic variants were implicated in severe COVID-19 in adults. This could direct prevention strategies based on population specific nutrigenetic profiles.

Keywords: DHCR7, CYP2R1, nutrigenetics, COVID-19, population genetics

INTRODUCTION

COVID-19 pandemic has proved to be an unrelenting health threat for more than a year now. More contagious and more deadly SARS-CoV-2 variants are a major concern, especially because effective, causal therapy is still unavailable, and vaccination coverage rates are lower than anticipated. Moreover, protection from vaccination is likely to last only for a season or two. One of the strategies to save lives is to identify groups at risk of severe COVID-19 disease and to implement measures aimed at those groups. The elderly, male, obese, chronic disease patients, patients with malignancies, immunocompromised, dark-skinned, socioeconomically disadvantaged and tobacco users suffered the most death toll from COVID-19 (1, 2). Also, interindividual genetic variations might be implicated in more severe COVID-19 (3, 4).

Another strategy directed toward reducing severe COVID-19 symptoms relies on high dose vitamins and trace elements (micronutrients) supplementation. Among micronutrients important for adequate immune function, vitamin D, zinc and selenium are of particular importance for coping with viral, respiratory infections, such as COVID-19 (5). Adequate status of these micronutrients is not only important for immune function and viral clearance, but also might mitigate life-threatening complications of SARS-CoV-2 infection, such as thrombosis and uncontrolled inflammation which leads to cytokine storm. Namely, vitamin D via vitamin D receptor (VDR) mediates immune function and regulation, strengthening of epithelial barriers and antioxidant defense, and also regulates expression of SARS-CoV-2 receptor, ACE2 (6–8). Zinc exerts direct anti-viral effects and also serves as a cofactor of dozens of proteins important for immune function and regulation, and antioxidative defense (9). Selenium via selenoproteins regulates immune function and provides antioxidant defense and vaso-protection (10).

Although important for various processes in our body, a lot of people worldwide have inadequate status of vitamin D, zinc or selenium. There is a pandemic of vitamin D deficiency, especially in higher latitudes due to inadequate sun exposure necessary for the synthesis of this vitamin (11). Selenium deficiency is prevalent in Europe and other parts of the world where soil is poor of this element, while zinc status is compromised predominantly in underdeveloped regions (12). Vitamin D, zinc and selenium deficiency is associated with factors of COVID-19 severity, such as old age, obesity, diabetes, dyslipidemia and chronic and acute inflammation. COVID-19 patients have lower status of vitamin D, zinc and selenium than healthy individuals (13). Also, patients with severe COVID-19 disease have lower status of vitamin D, zinc and selenium than mild and moderate disease patients (14–16). Preliminary evidence from intervention studies suggests that supplementation with these micronutrients protects from severe COVID-19 disease (17, 18).

Apart from chronic and acute health conditions, lifestyle and nutrition, genetic factors also play a role in bioavailability and status of vitamin D, zinc and selenium. Several large genome-wide association studies (GWAS) identified variants located near genes involved in synthesis, transport and metabolism of vitamin D, namely *DHCR7/NADSYN1*, *GC* and *CYP2R1*, respectively (19–21). Variants in these genes are associated with serum level of 25OHD, which is a measure of vitamin D status. Also, genetic variants in *VDR* gene, which codes for vitamin D receptor that regulates expression of more than 200 human genes (22), have been shown to increase susceptibility to acute lower respiratory infections in children, as well as several inflammatory and autoimmune diseases (23–26). GWAS studies comprising three large independent cohorts associated selenium status measured in whole blood and erythrocytes (27), as well as nails (reflects longer duration of selenium exposure) (28) with a locus near *DMGDH* gene. The same locus was implicated in a response to selenium supplementation in another GWAS (29). Zinc nutrigenetics is less studied, but a few candidate variants associated with status of this trace elements are identified in a large GWAS, and the most significant association was noted for rs2120019 variant near *PPCDC* gene (27).

Building on this premise, we hypothesize that genetic variants linked to suboptimal level or decreased bioavailability of vitamin D, zinc and selenium, may also serve as markers of disease course in COVID-19 patients, influenced by the level of these micronutrients. Therefore, we evaluated genetic determinants of vitamin D (*DHCR7/NADSYN1* rs12785878, *GC* rs2282679, *CYP2R1* rs10741657, *VDR* rs2228570), zinc (*PPCDC* rs2120019) and selenium (*DMGDH* rs17823744) as risk factors for severe forms of COVID-19. Furthermore, we attempted to investigate the inter-population diversity of the selected genetic variants. Inter-population nutrigenetic variability could suggest different relevance of nutrigenetic factors in populations exposed to different nutritional habits and specific environmental factors. These data should be taken into account when evaluating the possible relationship of nutrigenetic variants with severe COVID-19 forms in other populations struck by the pandemic.

MATERIALS AND METHODS

Patients

Present study included 120 patients with diagnosis of COVID-19 (adult and pediatric) who were treated in tertiary healthcare institutions, the Clinic of Pulmonology, Clinical Center of Serbia and Children's Hospital for Lung Diseases and Tuberculosis, Medical Center "Dr Dragiša Mišović," Belgrade, Serbia, between April and June of 2020. For all patients, a COVID-19 infection was defined as a positive SARS-CoV-2 laboratory test (viral RNA RT-PCR test from nasopharyngeal swabs). From each patient, peripheral blood sample was collected for DNA isolation and further genetic analyses.

Clinical data were assessed using electronic health records. From a total of 120 COVID-19 patients included in the study, clinical data were available for 115 patients, 73 adults and 42 pediatric.

Adult COVID-19 patients were categorized into mild, moderate or severe clinical course according to the National Institutes of Health (NIH) Definition of COVIID-19 Disease Severity (COVID-19 Treatment Guidelines Panel. *Coronavirus Disease 2019 (COVID-19) Treatment Guidelines*. 2020. Available at: *http://www.covid19treatmentguidelines.nih.gov)*, as follows:

mild – patients with symptoms such as fever, fatigue, cough, myalgia, and headache, but without dyspnea or pneumonia; *moderate* - those with evidence of pneumonia based on imaging showing up to 50% of lung involvement, who had blood oxygen saturation >93% on room air; *severe* - patients who demonstrated pneumonia with >50% of lung involvement on imaging or had blood oxygen saturation level equal to or <93% on room air and required supportive oxygen therapy.

Pediatric patients were enrolled during the first peak of the COVID-19. At that time, SARS-CoV-2 positive pediatric patients were admitted to the hospital if they had COVID-19 symptoms or for observation due to previous health issues. Most of the recruited pediatric patients (90.5%) demonstrated mild form of the disease. Therefore, for the purpose of this study, we classified pediatric patients into 2 groups – *symptomatic,* who were diagnosed with pneumonia or any typical COVID-19 symptoms such as cough, fever, sore throat, fatigue, and headache or *asymptomatic,* patients tested positive for SARS-CoV-2 with medical history of recurrent respiratory diseases.

This study was approved by the Ethics Committee of the Institute of Molecular Genetics and Genetic Engineering University of Belgrade (approval for sample collection and biobank formation O-EO-016/2020, 06.05.2020.; approval for the genetic study O-EO-016/2020/1, 03.09.2020). Informed consent was obtained from each participant or their parents/legal guardians. The study was performed in accordance with the Declaration of Helsinki.

Genotyping

Genomic DNA was isolated from the whole blood samples using QIAamp DNA Blood Mini Kit (Qiagen) and stored at −20°C until analysis.

Genetic variants *DHCR7/NADSYN1* rs12785878, *GC* rs2282679, *CYP2R1* rs10741657, *VDR* rs2228570, *PPCDC* rs2120019 and *DMGDH* rs17823744 were analyzed using TaqMan SNP Genotyping Assays (Thermo Fisher Scientific) (C__32063037_10, C__26407519_10, C__2958430_10, C__12060045_20, C__304992_10, C__32728760_10), according to the manufacturer's instructions with modifications. A total volume of 8 μl PCR reaction contained 36 ng of DNA, 1x Taqman Genotyping Master Mix and 1x TaqMan SNP Genotyping Assays (Thermo Fisher Scientific). Genotyping was performed on a real-time PCR system (Applied Biosystems 7500, Thermo Fisher Scientific). Genotype calling was implemented with built-in 7500 System Software v1.3.1 (Thermo Fisher Scientific).

Statistical Analysis

Statistical analyses were performed in software SPSS v23.0 and R v.3.6.2.

Differences in demographic and clinical data between COVID-19 mild/moderate/severe subgroups were examined using adequate statistical tests for continuous and discrete data (Mann-Whitney U test, Chi-square or Fisher exact test).

For genetic data, Hardy-Weinberg equilibrium was examined to assess the performance of the genotyping assays. Differences in genotype and allele counts distribution for each variant between COVID-19 subgroups were tested using the Chi-squared test. Impact of each genetic variable on severity of the COVID-19 was estimated by odds ratio with 95% confidence interval using a logistic regression model in order to control for confounding factors, such as age and gender.

Frequencies of the analyzed variants were compared between group of Serbian COVID-19 patients and other populations using two openly available variant databases, 1,000 Genomes Project (1KGP) and Genome Aggregation Database (gnomAD) v.3.0 (30, 31). Chi square or Fisher exact tests were used to measure significant differences in allelic distributions between Serbian and European populations (Finish and non-Finish, particularly Italian, Spanish, Britain and USA with European ancestry), and other worldwide populations (African, East and South Asian, American Latino/Admixture and Ashkenazi Jewish), applying Bonferroni correction for multiple testing.

We examined the level of population genetic variability at each genetic loci using the maximal global difference in risk allele frequency (delta AF), which was calculated by subtracting the maximum and the minimum allele frequency across analyzed populations.

All tests were bi-directional. The threshold for statistical significance was defined using appropriate multiple comparisons correction methods, such as Bonferroni or Benjamini-Hochberg false discovery rate (FDR).

RESULTS

Description of COVID-19 Patient Groups

In the selected study group, 73 were adults while 42 were pediatric patients. Among adults 65.8% were females and the median age was 43 (range 20–82); among pediatric patients 38.1% were females and median age was 9 (range 0.3–17). Demographic and clinical descriptions of COVID-19 patients are presented in **Table 1**.

In the group of adult COVID-19 patients, 10 (13.7%) required supportive oxygen therapy and 3 (4%) had COVID-19 related death outcomes. Examining available clinical data, patients were categorized into mild ($n = 35$), moderate ($n = 21$) or severe ($n = 17$) disease groups. Age distribution of the patients significantly varied between groups; gradual increase in participants' age has been observed across mild, moderate and severe groups ($p < 0.001$). In the severe group, a significantly higher number of patients suffered from hypertension, diabetes and were using ACE inhibitors, compared to mild and moderate groups ($p < 0.001$, $p < 0.001$, $p < 0.001$, respectively). The highest percent of lymphopenia and thrombocytopenia events were observed in the severe group, 93.8 and 56.3%, respectively. Also, patients with severe COVID-19 had higher levels of CRP, compared to patients with moderate and mild disease ($p < 0.001$).

Pediatric COVD-19 patients were categorized into two groups – asymptomatic ($n = 20$) and symptomatic ($n = 22$). There was no significant difference in distribution of age, gender, nor in the presence of comorbidity between the two groups, except past respiratory diseases. The level of CRP was significantly higher in COVID-19 symptomatic compared to asymptomatic pediatric patients ($p = 0.003$).

TABLE 1 | Demographic and clinical characteristic of COVID-19 patients.

	Adult COVID-19 ($n = 73$)			
	Mild	**Moderate**	**Severe**	***p***
N (%)	35 (47.9)	21 (28.8)	17 (23.3)	
Age, median [IQR]	37 [29–49]	41 [33.7–52]	61 [49.3–67]	**<0.001**
Gender, Female n (%)	28 (80)	11 (52.4)	9 (52.9)	0.05
Obesity, n/available (%)	5/33 (14)	7/19 (36.8)	6/16 (37.5)	0.1
Hypertension, n/available (%)	7/35 (20)	5/21 (23.8)	10/16 (62.5)	0.01
Diabetes, n/available (%)	1/35 (3)	2/21 (9.5)	4/16 (25)	0.05
ACE inhibitors, n/available (%)	5/35 (14.3)	2/21 (9.5)	8/15 (53.3)	**0.006**
% $SatO_2$, median [IQR]	98 [98–99]	97.5 [97–99]	95.5 [87–97]	**<0.001**
CRP, median [IQR]	0.6 [0.2–4.3]	17.2 [4.7–91.7]	135.6 [96.7–260.4]	**<0.001**
Febrile, n/available (%)	7/35 (20)	11/21 (52.4)	14/16 (87.5)	**<0.001**
Lymphopenia (<0.8 x109/L), n/available (%)	7/34 (20.6)	9/21 (42.9)	15/16 (93.8)	**<0.001**
Thrombocytopenia (<150,000/mm^3), n/available (%)	5/33 (15.2)	3/21 (14.3)	9/16 (56.3)	**0.005**
	Pediatric COVID-19 ($n = 42$)			
	Asymptomatic	**Symptomatic**	***p***	
N (%)	20 (47.6)	22 (52.4)		
Age, median [IQR]	9.0 [7.3–13.5]	10.0 [1.7–15.0]	0.9	
Gender, Female %	8/20 [40]	8/22 (36.4)	0.8	
Obesity, n/available (%)	0/20 (0)	1/22 (4.5)	1	
Hypertension, n/available (%)	0/20 (0)	1/22 (4.5)	1	
Diabetes, n/available (%)	0/20 (0)	0/22 (0)	NA	
% $SatO_2$	98.0 [97.0–99.0]	98.0 [96.7–98.3]	0.3	
CRP, median [IQR]	0.45 [0.2–0.97]	2.7 [0.53–11.3]	**0.002**	
Lymphopenia (<0.8 x109/L), n/available (%)	0/20 (0)	4/22 (18.2)	0.1	
Thrombocytopenia (<150,000/mm^3), n/available (%)	0/20 (0)	0/22 (0)	NA	

To deal with missing data, each variable count was presented along with the total available data for that category (n/available). Differences between groups were tested using Mann-Whitney U test for continuous data, or Chi-square/Fisher exact test for discrete data. Benjamini-Hochberg correction for multiple testing was applied (FDR of 0.05); significant probabilities after correction were presented in bold. IQR, interquartile range; $SatO_2$, Blood oxygen saturation; CRP, C-reactive protein.

Association of Genetic Variants With the Risk of Severe COVID-19

COVID-19 patients were genotyped for variants in *DHCR7/NADSYN1*, *GC*, *CYP2R1*, *VDR*, *PPCDC* and *DMGDH* genes (**Table 2**). Distributions of genotypes of all analyzed variants were in accordance with Hardy-Weinberg equilibrium.

We compared the distribution of analyzed genotypes between different COVID-19 severity groups. In the group of adult patients, two outcomes were considered in the logistic regression model – first included both mild and moderate disease, and second was severe disease (**Table 3**). Logistic regression used genotypes of analyzed variants as variables, controlled for age and gender in multivariate models. To increase the statistical power of the study, a dominant genetic model has been applied, in particular, a group of homozygous carriers of more frequent allele were compared to heterozygous and homozygous carriers of the less frequent, minor allele. Analysis showed that carriers of *DHCR7/NADSYN1* G allele (TG+GG), associated with lower levels of 25OHD were 4.9 times less likely (or had 0.21 the odds) to develop a severe form of COVID-19, compared with carriers of TT genotype. When adjusted for age and gender, observed association was still significant (OR 0.21, 95% CI 0.05–0.9, $p = 0.03$). Association has also been observed between severe COVID-19 and *CYP2R1* GG genotype, previously related to decreased levels of 25OHD. In the logistic regression model, *CYP2R1* GG carriers had 5.9 times higher odds to develop severe disease, adjusted for age and gender (OR 5.9, 95% CI 1.4–25.2, $p = 0.017$). Additionally, we applied adjustment for obesity in multivariate logistic regression models, showing that *DHCR7/NADSYN1* rs12785878 and *CYP2R1* rs10741657 variants remained significant predictors of severe COVID-19 in adults (OR 0.22, 95% CI 0.06-0.93, $p = 0.02$; OR 4.9, 95% CI 1.5-16.4, $p = 0.009$, respectively). Our study did not show association of *GC* and *VDR* variants with higher risk of severe COVID-19 in adults.

Homozygous carriers of *DMGDH* A allele, which has been related to lower levels of selenium, in our study were less likely to present severe form of COVID-19 (OR 0.2, 95% CI 0.09–0.9, $p = 0.03$). However, this association was not significant after correction for age and gender. Regarding zinc-related

TABLE 2 | Genotype and allele frequencies of COVID-19 patients ($n = 120$).

Genetic variant	Genotype	*n* (freq)	HW	Allele	freq
DHCR7/NADSYN1 rs12785878	TT	56 (0.47)	0.2	T	0.70
	TG	56 (0.47)		G	0.30
	GG	8 (0.07)			
GC rs2282679	TT	59 (0.50)	0.8	T	0.70
	TG	49 (0.41)		G	0.30
	GG	11 (0.09)			
CYP2R1 rs10741657	GG	36 (0.3)	0.1	G	0.58
	GA	67 (0.56)		A	0.42
	AA	17 (0.14)			
VDR rs2228570	GG	53 (0.44)	0.5	G	0.65
	GA	51 (0.43)		A	0.35
	AA	16 (0.13)			
PPCDC rs2120019	TT	65 (0.54)	0.4	T	0.75
	TC	49 (0.41)		C	0.25
	CC	6 (0.05)			
DMGDH rs17823744	AA	87 (0.72)	0.7	A	0.85
	AG	31 (0.26)		G	0.15
	GG	2 (0.02)			

HW, hardy weinberg equilibrium; freq, frequency (number of observations/total number).

PPCDC variant, we observed no significant association with severe COVID-19 outcome in adult patients.

In pediatric COVID-19 cases, we applied a logistic regression model to predict two outcomes – asymptomatic and symptomatic disease, using genotype data (**Table 4**). No significant associations with increased risk for symptomatic pediatric COVID-19 disease were found with any of the analyzed genetic variants.

Comparative Population Genetics

Finally, we aimed to compare allele frequencies of selected genetic variants in Serbian COVID-19 study group with their frequencies in other populations. Therefore, we retrieved variant site data from the two genetic databases, 1KGP and gnomAD. Distributions of alleles frequencies (AF) associated with decreased levels of selected micronutrients in European and worldwide populations have been presented in **Figure 1** (data used for calculations are provided in **Supplementary Table 1**). For all analyzed genetic variants, observed AF in Serbian study group did not diverge from overall AF in European non-Finish populations. When comparing Serbian study group with populations of non-Finish Europeans, we noticed a difference in the frequency of *DHCR7/NADSYN1* allele G between Italian compared to Serbian population (0.22 and 0.3, respectively, $p = 0.07$). Difference was also observed in the frequency of *CYP2R1* rs10741657 allele G between Serbian (0.58) and Spanish (0.69) as well as Italian population (0.66) ($p = 0.02$, $p = 0.08$, respectively). Serbian population had the lowest frequency of the *CYP2R1* rs10741657 G variant among European non-Finish populations, while Spanish and Italians demonstrated the highest. However, noted differences were not statistically significant. As expected, significant differences were mostly seen between Serbian and African, South and East Asian, American Latino/Admixed, and Ashkenazi populations.

The level of population genetic variability at each selected loci was assessed using delta AF (dAF), representing the difference between maximum and minimum AF value across analyzed populations. The highest dAF was observed for the *DHCR7/NADSYN1* rs12785878 variant (dAF 0.56). The lowest dAF was detected for *CYP2R1* rs10741657 and *DMGDH* rs17823744 variants (dAF 0.15 and 0.15, respectively).

DISCUSSION

In this study, we focused our attention to genetic variants related to altered level, bioavailability or mechanism of action of vitamin D, zinc and selenium, aiming to investigate their association with different COVID-19 presentation in children and adults. Serbia, like much of Europe and the world, faces micronutrient deficiency/insufficiency which is in part influenced by genetic variability. The prevalence of suboptimal levels of vitamin D and selenium in Serbian population is estimated to be around 50% (32, 33), while zinc insufficiency is around 7% (12). Bearing in mind that even a mild insufficiency of these micronutrients can cause suboptimal immune function, implications for COVID-19 pandemic can be formidable.

Results of our study showed association of *DHCR7/NADSYN1* rs12785878 and *CYP2R1* rs10741657 variants with severe form of COVID-19 in adult patients. In the pediatric COVID-19 group, we did not detect any associations between analyzed genetic factors and disease severity.

Variant rs12785878 is located 8 kilobases upstream of the *DHCR7* gene on chromosome 11q12. *DHCR7* codes for 7-dehydrocholesterol reductase, a key enzyme in cholesterol

TABLE 3 | Association of the analyzed genotypes with the risk of severe COVID-19 disease in adults.

		Adult COVID-19 ($n = 73$)			Mild+moderate vs. severe disease				
Genetic variant	**Genotype**	**Mild ($n = 35$)**	**Moderate ($n = 21$)**	**Severe ($n = 17$)**	**Genetic model***	**OR [CI]**	***P***	**OR^{adj} [CI]**	**p^{adj}**
DHCR7/NADSYN1 rs12785878	TT	12 (34.3%)	9 (42.8%)	12 (70.6%)	TT^{R} vs. TG+GG	**0.25 [0.08–0.81]**	**0.02**	**0.21 [0.05–0.9]**	**0.03**
	TG	20 (57.1%)	9 (42.8%)	5 (29.4%)					
	GG	3 (8.6%)	3 (14.3%)	0 (0%)					
GC rs2282679	TT	16 (45.7%)	8 (40.0%)	8 (47.1%)	TT^{R} vs. TG+GG	0.8 [0.3–2.6]	0.8	1.3 [0.3–5.0]	0.7
	TG	17 (48.6%)	9 (45.0%)	8 (47.1%)					
	GG	2 (5.7%)	3 (15.0%)	1 (5.8%)					
CYP2R1 rs10741657	GG	11 (31.4%)	4 (19.0%)	10 (58.8%)	GA+AA^{R} vs. GG	**3.9 [1.3–12.1]**	**0.018**	**5.9 [1.4–25.2]**	**0.017**
	GA	18 (51.4%)	14 (66.7%)	4 (23.5%)					
	AA	6 (17.1%)	3 (14.3%)	3 (17.6%)					
VDR rs2228570	GG	15 (42.9%)	8 (38.1%)	7 (41.2%)	GG^{R} vs. GA+AA	1.0 [0.3–3.0)	1	0.8 [0.2–3.2]	0.7
	GA	15 (42.9%)	10 (47.6%)	8 (47.1%)					
	AA	5 (14.3%)	3 (14.3%)	2 (11.7%)					
PPCDC rs2120019	TT	20 (57.1%)	11 (52.4%)	7 (41.2%)	TT^{R} vs. TC+CC	1.8 [0.6–5.3]	0.3	1.4 [0.4–5.2]	0.6
	TC	14 (40.0%)	9 (42.8%)	8 (47.0%)					
	CC	1 (2.9%)	1 (4.8%)	2 (11.8)					
DMGDH rs17823744	AA	28 (80.0%)	17 (81.0%)	9 (53.0%)	AG+GG^{R} vs. AA	0.27 [0.09–0.9]	0.03	0.4 [0.1–1.8]	0.2
	AG	7 (20.0%)	2 (9.5%)	8 (47.0%)					
	GG	0 (0%)	2 (9.5%)	0 (0%)					

**To increase statistical power of the study, a dominant genetic model was applied. Homozygous carriers of more frequent allele were compared to heterozygous and homozygous carriers of less frequent, minor allele. ^{R}Group that included homozygous carriers of allele associated with higher levels of analyzed micronutrient was set to be a referent group in the logistic regression model.*

OR, odds ratio; CI, confidence interval; adj, adjusted for age and gender. Bolded p-values remained significant after Benjamini-Hochberg correction for multiple testing ($p < 0.033$), if FDR was set to 0.1.

biosynthesis and vitamin D cutaneous production. Particularly, the activity of DHCR7 enzyme decreases the level of vitamin D precursor availability, 7-dehydorcholesterol, shunting it in the direction of cholesterol biosynthesis (34). *DHCR7* rs12785878 variant was firstly noted in a large GWAS of 25OHD concentrations in 33 996 individuals of European descent by (21), and subsequently confirmed in other numerous studies, where the G allele was shown to be associated with lower 25OHD serum levels. In our comparative genetic population analysis, we showed that frequency of G allele markedly varied across different populations; it ranged from 0.75 in African and Asian populations to 0.22 in European populations. Considerable differences in allele frequencies between populations may indicate a locus that has undergone positive selection in a specific geographical area. It has been suggested that positive selection influenced the *DHCR7* gene, increasing the frequency of reduced activity variant (allele T) in European populations (35). Reduced activity of DHCR7 leads to increased availability of 7-dehydrocholesterol for vitamin D synthesis allowing Europeans to avoid deficiency in northern latitudes. Our results suggested an association of the rs12785878 variant with the severe form of COVID-19. However, in our study, carriers of the TT genotype which has been linked to higher circulating level of 25OHD, were more likely to develop a severe form of COVID-19. Given the immune-enhancing aspects of vitamin D, one might expect the opposite. However, it should be noted that the roll of *DHCR7* variants to vitamin D status is constrained to vitamin D synthesis in the skin exposed to UV light and probably does not influence contribution of dietary sources and supplementation to the level of this vitamin. COVID-19 patients enrolled in our study were hospitalized with this disease in the spring of 2020 during or right after lockdown measures were in place. That means that vitamin D was scarcely synthetized in skin during that time and, therefore, *DHCR7* variants had limited influence on vitamin D level. Regardless of vitamin D production, *DHCR7* variants could influence immune function mediated through cholesterol metabolites. Namely, reduced activity of DHCR7 leads to increased 7-dehydrocholesterol levels which influences interferon production and viral clearance (36).

After initial UV-light mediated production of vitamin D3 in the skin, the second step in the synthesis of active vitamin D is catalyzed in the liver by cytochrome P450 (CYP) enzyme which hydroxylates carbon 25, producing the intermediate 25-hydroxyvitamin D3, or 25OHD, the major circulatory form of the vitamin D (37). This enzyme is encoded by the *CYP2R1* (cytochrome P450, family 2, subfamily R, polypeptide 1) gene. Variant rs10741657 located near the *CYP2R1* gene was linked by several studies to 25OHD serum concentrations (21, 38), with the allele G shown to be associated with lower 25OHD serum levels, and with the GG homozygotes having the lowest levels. Our results indicated an association between severe COVID-19 and *CYP2R1* GG genotype. In the logistic regression model, *CYP2R1* rs10741657 GG carriers had 5.9 times higher odds to develop severe disease. We noticed low level of variability of allele G frequency for *CYP2R1* rs10741657 variant across analyzed populations (dAF 0.15); G allele frequencies ranged from 0.58

TABLE 4 | Association of the analyzed genotypes with the risk of symptomatic COVID-19 disease in children.

		Pediatric COVID-19 ($n = 42$)		Asymptomatic vs. symptomatic disease				
Enetic variant	**Genotype**	**Asymptomatic ($n = 20$)**	**Symptomatic ($n = 22$)**	**Genetic model***	**OR [CI]**	***p***	**OR^{adj} [CI]**	***p*^{adj}**
DHCR7/NADSYN1 rs12785878	TT	11 (55%)	7 (31.8%)	TT^{R} vs. TG+GG	2.6 [0.7–9.2]	0.1	3.1 [0.8–11.8]	0.1
	TG	8 (40.0%)	14 (63.6%)					
	GG	1 (5.0%)	1 (4.5%)					
GC rs2282679	TT	10 (50.0%)	13 (59.1%)	TT^{R} vs. TG+GG	0.7 [0.2–2.3]	0.6	0.7 [0.2–2.5]	0.6
	TG	6 (30.0%)	8 (36.4%)					
	GG	4 (20.0%)	1 (4.5%)					
CYP2R1 rs10741657	GG	4 (20.0%)	5 (22.7%)	GA+AA^{R} vs. GG	1.2 [0.3–5.2]	0.8	1.2 [0.3–5.2]	0.8
	GA	13 (65.0%)	15 (68.2%)					
	AA	3 (15.0%)	2 (9.1%)					
VDR rs2228570	GG	9 (45.0%)	11 (50.0%)	GG^{R} vs. GA+AA	0.8 [0.2–2.7]	0.7	0.8 [0.2–2.8]	0.7
	GA	8 (40.0%)	8 (36.4%)					
	AA	3 (15.0%)	3 (13.6%)					
PPCDC rs2120019	TT	14 (70.0%)	11 (50.0%)	TT^{R} vs. TC+CC	2.3 [0.7–8.3]	0.2	2.6 [0.7–9.9]	0.2
	TC	6 (30.0%)	9 (40.9%)					
	CC	0 (0%)	2 (9.1%)					
DMGDH rs17823744	AA	14 (70.0%)	17 (77.3%)	AG+GG^{R} vs. AA	1.5 [0.4–5.8]	0.6	1.5 [0.4–5.8]	0.6
	AG	6 (30.0%)	5 (22.7%)					
	GG	0 (0%)	0 (0%)					

**To increase statistical power of the study, a dominant genetic model was applied. Homozygous carriers of more frequent allele were compared to heterozygous and homozygous carriers of less frequent, minor allele. ^{R}Group that included homozygous carriers of allele associated with higher levels of analyzed micronutrient was set to be a referent group in the logistic regression model. OR, odds ratio; CI, confidence interval; adj, adjusted for age and gender.*

in Serbian, which was the lowest among European populations, to 0.73 in African population. The highest frequency of the G allele among Europeans was observed in Italian and Spanish populations (0.66 and 0.69, respectively). Recent study that reported negative correlations between mean levels of vitamin D in various European countries with COVID-19 cases per million people, suggested that Spain, Italy and Switzerland are the most vulnerable countries since they have very low levels of vitamin D in the aging population (39). Our results could be of particular interest for these populations. Relatively high occurrence of the *CYP2R1* rs10741657 G variant in different populations, reproducible association with decreased 25OHD levels and observed relationship with severe COVID-19 in our group of patients, gives a solid ground for future studies to examine relationship of this *CYP2R1* variant with the clinical course of COVID-19 in other populations.

A role of vitamin D in the pathogenesis of COVID-19 has been extensively studied since the beginning of the pandemic. Calcitriol (1, 25-dihydroxyvitamin D3) has an important role in regulating renin angiotensin system by enhancing the expression of ACE2, which is the main target of SARS-CoV2 cell entry (7, 8). Also, vitamin D has an immunomodulatory effect and it can prevent macrophages to release excessive proinflammatory cytokines and chemokines (6, 40). A recent study showed that the intake levels of relevant micronutrients such as vitamin D are inversely associated with higher COVID-19 incidence and/or mortality (41).

Furthermore, other studies sought for more detailed nutrigenetic markers as factors that might contribute to the bioavailability of vitamin D and therefore influence COVID-19 susceptibility and/or clinical course. A study which aimed to assess the association between variants in the *GC* gene that encodes vitamin D binding/transport protein (DBP), and the prevalence and mortality rates of COVID-19, pointed out to rs7041 variant (42). Another recent study (not peer-reviewed yet) performed in Portuguese population of COVID-19 patients found an association of *GC* rs2282679 variant with COVID-19 disease severity (43). Our study indicated an association between *DHCR7/NADSYN1* rs12785878 and *CYP2R1* rs10741657 variants and the severity of COVID-19, but the variants *GC* rs2282679 and *VDR* rs2228570 were not linked to a higher risk of severe COVID-19 in adults. In a recent Mendelian randomization study (not peer-reviewed yet) on vitamin D and COVID-19 in individuals of European ancestry, genetically increased 25OHD concentrations did not protect against COVID-19 susceptibility, but increased the odds for hospitalization and severe disease (44). Another Mendelian randomization study failed to find evidence of a linkage between vitamin D deficiency and COVID-19 infection rates or severe disease (45). However, described studies did not consider true vitamin D deficiency in participants, and it can be possible that vitamin D supplementation could benefit insufficient/deficient patients (44, 45).

Nutrigenetics of zinc and selenium were discussed in the context of COVID-19 as a promising strategy to implement personalized approach to strengthening antioxidant and antiviral defense (41). However, the present study is the first study on genetic determinants of zinc and selenium that included COVID-19 patients. Our results did not show association of these

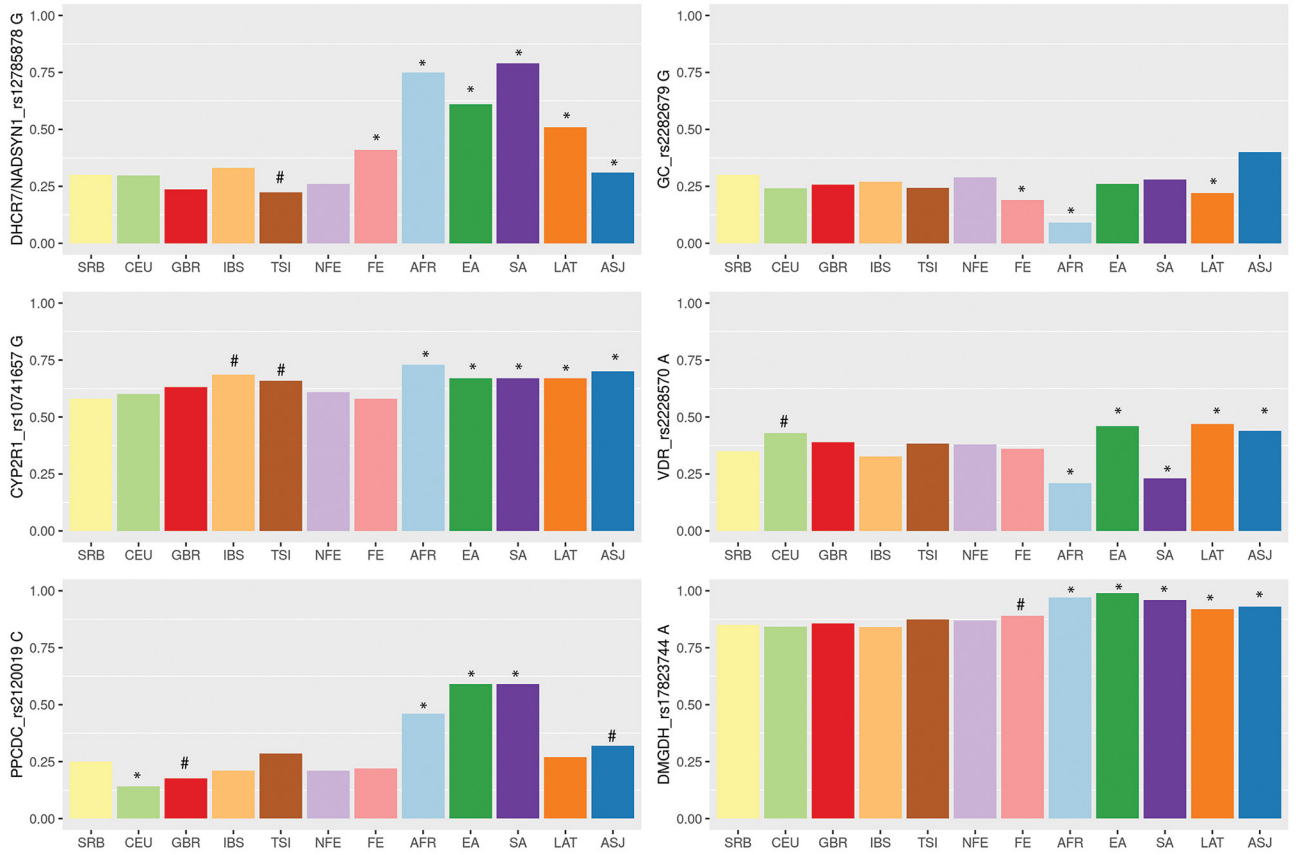

FIGURE 1 | Variability of vitamin D, zinc and selenium related genetic loci among wordwide populations based on the 1,000 Genome project (1KGP), Genome Aggregation Database (gnomAD) and Serbian COVID-19 study group data. Populations: SRB, Serbian; CEU, Utah residents with Northern and Western European ancestry (1KGP); GBR, British in England and Scotland (1KGP); TSI, Tuscany in Italy (1KGP); IBS, Iberian populations in Spain (1KGP); FIN-Finnish European (gnomAD); NFE-non-Finnish European, including Northwestern European, Bulgarian, Estonian, Swedish, Southern European, and Other non-Finnish European (gnomAD); AFR, African (gnomAD); EA, East Asian (gnomAD); SA, South Asian (gnomAD); LAT, Latino/Admixed American (gnomAD); ASJ, Ashkenazi Jewish (gnomAD). Barplots are showing frequencies of the alleles associated with lower levels of selected micronutrients. Differences in allelic distributions were analyzed between Serbian and other populations using Chi square test followed by Bonferroni correction for multiple testing. Statistical significance was marked with *($p = <$ 0.008), while statistical trend with #($0.1 < p < 0.008$).

variants with severe COVID-19, however, our univariate analysis pointed out to *DMGDH* rs17823744 variant, which warrants further investigation. DMGDH is implicated in homocysteine metabolism and it is postulated that there is a connection between selenium exposure and the homocysteine metabolic pathways (28). Apart from locus near *DMGDH* gene, which was noted in several GWAS cohorts focused on selenium status, other studies analyzed genes coding for selenoproteins. Candidate gene approach studies or studies that analyzed protein activity, associated glutathione peroxidase 1 (*GPX1*), *GPX4* and selenoprotein P (*SELENOP*) with response to selenium supplementation (46–48). Although important for selenium action, these genes were not associated with selenium status in GWAS studies.

Apart from nutrigenetics, other studies also dealt with genetic factors associated with COVID-19 severity, but the results were not consistent. A GWAS study of almost 4,000 healthy volunteers and severely ill Italian and Spanish COVID-19 patients associated loci on chromosomes 3 and chromosome 9 (near ABO locus) (3), while another GWAS study of 2,244 critically ill UK patients associated variants located on chromosomes 12, 19 and 21 with more severe disease or poor outcome (4). None of these loci are located near nutrigenetic variants analyzed in the present study.

Results of this study should be evaluated in larger cohorts of COVID-19 patients. Small number of patients is the main limitation of this study, although, both mild and moderate, as well as severe groups are well-represented among adult patients. Among pediatric patients, the majority of patients had mild symptoms so we could not investigate risk factors of severe disease in children. Instead, genetic variants were analyzed as risk factors of symptomatic disease.

Another limitation of this study is lack of micronutrient measurement needed to assess actual status of vitamin D, zinc and selenium. We focused solely on the genetic variants associated with altered levels of vitamin D, zinc and selenium. Status of the micronutrient is highly confounded variable

that depends on numerous factors. Beside genetics, nutrition habits, individual lifestyle and presence of comorbidities are also important. Therefore, comprehensive nutrigenetic studies complemented with measures of nutritional intake, micronutrient's serum level and detailed lifestyle evaluations are needed in the future.

This study highlights the importance of personalized as well as population based strategies directed toward reduction of COVID-19 burden. These strategies can be implemented through employment of established nutrigenetic markers, such as genetic variants involved in vitamin D disposition. Our results pointed out to *DHCR7/NADSYN1* rs12785878 and *CYP2R1* rs10741657 variants, both involved in vitamin D synthesis, as potential risk factors of severe COVID-19. Holistic approach comprising nutrigenetics, micronutrient, status, lifestyle and clinical parameters could effectively combat micronutrient deficiency, and potentially improve anti-viral defense. This approach is especially important in populations at risk of micronutrient deficiency.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study was approved by the Ethics Committee of the Institute of Molecular Genetics and Genetic Engineering University of Belgrade (approval for sample collection and biobank formation O-EO-016/2020, 06.05.2020.; approval for the genetic study O-EO-016/2020/1, 03.09.2020).

AUTHOR CONTRIBUTIONS

NK: conceptualization, investigation, statistical analysis, writing - draft preparation, and editing. AS: methodology, investigation, and writing – draft preparation. KK: investigation, methodology, and writing – draft preparation. VG: data analysis and interpretation, investigation, and writing – draft preparation. BZ: methodology, investigation, writing — review, and editing, VS-T, MS, ZZ, OO, GS, and LL: methodology, sample collection, and clinical data analysis. SP: concept and design of the study, writing –review, and editing, BS: concept and design of the study, statistical analysis, results interpretation, drafting, and review of final manuscript. 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.2021.689419/full#supplementary-material

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The Interaction Between Fatty Acid Desaturase-2 (FADS2) rs174583 Genetic Variant and Dietary Quality Indices (DASH and MDS) Constructs Different Metabolic Phenotypes Among Obese Individuals

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Background and Aim: Genetic variation in fatty acid desaturases (FADS) has previously been linked to several diet-related diseases. We aimed to determine whether the FADS2 rs174583 variant interacts with the Dietary Approach to Stop Hypertension (DASH) score and Mediterranean dietary score (MDS) to influence cardio-metabolic risk factors among obese adults.

Methods: This cross-sectional study was performed among 347 apparently healthy obese adults (aged 20–50 years). Dietary quality indicator scores (DASH and MDS) were generated using a validated 147-item Food Frequency Questionnaire (FFQ). The FADS2 rs174583 variant was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The gene–diet interaction was analyzed by the ANCOVA multivariate interaction model.

Results: A significant interaction was observed between rs174583 and adherence to the DASH score in relation to serum triglyceride (TG) concentration among the female group ($P_{\text{Interaction}} = 0.046$); CT-genotype carriers who were assigned to the second tertile of DASH compared with those in the first tertile had a lower TG level ($P < 0.05$). Another significant interaction was revealed between adherence to MDS score and rs174583 polymorphism on serum glucose levels ($P_{Interaction} = 0.044$); the lowest mean of glucose level was observed in homozygous minor subjects (TT) in the third tertile of MDS, in comparison with other tertiles of this dietary index ($P < 0.05$). There was a similar significant interaction between DASH and rs174583 in relation to diastolic blood pressure ($P_{\text{Interaction}} = 0.038$) among the male group. Additionally, a significant positive association was found between TT genotype and odds of having high TG both in the crude (OR, 3.21; 95% CI, 1.02–10.14) and adjusted (OR, 3.58; 95% CI, 1.07–11.97) models, taking into account different confounders.

Conclusion: Adherence to the dietary quality indicators (DASH and MDS) modified the relationship between FADS2 rs174583 polymorphism and cardio-metabolic risk factors in obese subjects. Prospective cohort studies are needed to confirm the results of our study.

Keywords: diet quality, obesity, gene-diet interaction, fatty acid desaturase (FADS) gene, cardio-metabolic risk

INTRODUCTION

Obesity, as a consequence of nutrition transition, has emerged as a major global health problem and is now a worldwide epidemic (1). Based on World Health Organization (WHO) estimation in 2016, over 650 million adults were obese. Iran, like many other developing countries, is joining the global obesity epidemic; the national prevalence rate of obesity among Iranian adults was 22.7% in 2016 (2). Obesity is known to be a complex and preventable disorder which is highly associated with a high risk of chronic diseases such as metabolic syndrome, type 2 diabetes mellitus (DM2), cardiovascular disease (CVD), and certain cancers (3). Obesity is also a cause of cardio-metabolic risk factors which are related to the risk of several abovementioned health problems (4). On the other hand, epidemiological studies have demonstrated that plasma fatty acid composition plays an important role in the development of chronic diseases, such as obesity and its related consequences (5). Recently, CVD has been considered as the leading cause of death in Iran (6), and it has been estimated that 50% of all deaths per year and 79% of deaths associated with chronic diseases are attributed to CVD (7, 8). Obesity, as a multifactorial condition, is caused by genetic makeup, environmental factors, and the synergistic interaction between them (9). In other words, major modifiable environmental factors such as diet can modify the genetic predisposition to various disorders. Over the last two decades, approximately 140 obesity susceptibility genes have been found to be related to weight gain or obesity (10) and generally 40–60% of the variations in obesity-related phenotypes have been explained by hereditary factors (11). Considering the broad roles that polyunsaturated fatty acids (PUFAs) have in the development of non-communicable diseases (12), recent candidate gene studies have concentrated on the contribution of genetic variants in fatty acid desaturases (FADS) to changes in the profile of endogenous fatty acids (13). The FADS1 and FADS2 genes which are localized on chromosome 11 (11q12–q13.1) encode delta-5 (D5D) and delta-6 fatty acid desaturases (D6D), respectively (14, 15). Altered activities of D5D and D6D, key enzymes in PUFA metabolism, were described in obesity and a number of chronic diseases such as metabolic syndrome, type 2 diabetes, cardiovascular diseases, and some malignancies (16). Recently, single-nucleotide polymorphisms (SNPs) in FADS1 and FADS2 genes have been associated with altered activity of D5D and D6D enzymes and also risk of obesity (17) and its-related conditions, such as elevated level of triglyceride, decreased high-density lipoprotein cholesterol (HDL-C) concentrations (18), and a higher risk of coronary disease (19) and type 2 diabetes (20). Nevertheless, there are some inconsistencies in the outcomes of SNP association studies which may, in part, be explained by interactions with environment factors, such as dietary influences.

Several socio-demographic factors such as sex, age, and socioeconomic status (SES) (21) as well as psychological factors (22) have indicated to play a main role in the development of obesity and its related health outcomes. Among psychosocial stressors, depression and anxiety have received special attention in the past years and a growing number of studies have reported a strong and independent relationship between these psychological disorders and the development of chronic diseases such as CVD (23). It has been suggested that chronic diseases are influenced by the interaction between mental health and various unhealthy behaviors such as poorer dietary quality and physical inactivity (24). Diet, as an important lifestyle factor, plays a crucial role in the prevention and treatment of obesity and its complications (25). In recent years, a dietary pattern approach that represents a broader picture of food and nutrient intake and accounts for the complexity of the diet has gained much attention in disease prevention, compared to their single dietary components (26). Among a large number of priori-defined diet quality indices that have been proposed in the nutrition literature, the Dietary Approach to Stop Hypertension (DASH) score and Mediterranean dietary score (MDS) have been widely used (27). These diet quality indices (DASH and MDS) have been frequently associated with a reduced risk of diseases, such as diabetes, CVD, and several forms of cancer (27, 28). Although a growing body of research has confirmed the significant and consistent protection provided by adherence to these healthy dietary patterns in relation to various health outcomes (29, 30), other studies have not provided clear evidence of the beneficial effects of high adherence to these diet indices (31, 32). Moreover, there is a relative shortage of knowledge on these health promotion indices in developing countries (33–35). On the other hand, responses to dietary interventions for the prevention and treatment of obesity and its related health conditions differ among various ethnicities with different genetic structures (36), suggesting the existence of gene–diet interactions which may play a main role in these interindividual inequalities. Thus, understanding the nature of gene–diet interactions is crucial in the treatment and management of obesity and its related chronic conditions and it can even be used to develop individualized effective dietary strategies. To the best of our knowledge, the interactions between adherence to the dietary quality indices (DASH score and MDS) and rs174583 polymorphism of FADS2 gene in relation to cardio-metabolic factors have not been examined. Therefore, we examined whether rs174583 polymorphism of the FADS2 gene has an association with cardio-metabolic risk factors in an

obese Iranian population and whether those influences might be modulated by dietary quality indicators (DASH score, MDS).

MATERIALS AND METHODS

Participants

This study was carried out as a cross-sectional study among 347 apparently healthy obese adults (58.2 % male, 41.8 % female) within the age range of 20–50 years. This study was performed from November 2017 to October 2018 in Tabriz city, one of the major cities in the northwest part of Iran. Study participants were recruited using the convenience sampling method through announcements and posters placed in healthcare facilities and public places in the city of Tabriz. These announcements contained information on the inclusion criteria (good health, obesity (BMI ≥30), and ages of 20–50 years). Initially, 400 participants were willing to take part in the research. After screening for eligibility based on the inclusion and exclusion criteria, 48 individuals were excluded from the study. The exclusion criteria for the present study included pregnant, lactating, and menopausal women; medical history of chronic diseases such as cardio-vascular diseases, hypertension, hyperlipidemia, diabetes, renal diseases, hepatic disorders, and cancer; and having any recent surgery such as bariatric surgery. Additionally, people receiving any medications and supplements which had effects on weight and variables studied such as loop diuretics, corticosteroids, antidepressants, statins, and antihypertensive agents were also excluded from the study. Before the commencement of the study, the objective of the study was described for eligible participants and they had time to ask their questions to the research team. During the study, two subjects dropped out of the study because their dietary questionnaires were incomplete. We also excluded participants ($n = 3$) whose total energy intake was outside the range of 800–4,200 kcal/day as under-reports and over-reports of energy intakes (37, 38). The required sample size for the present study was calculated by considering the association between dietary quality indices and obesity as a key dependent variable. With regard to the correlation coefficient (r) of 0.25 (39), $\alpha = 0.05$, and power of 80%, using G*Power software, the minimum sample size was calculated to be 225, and taking into account 20–25% dropout, the final sample size of 340 was considered for the study. Finally, a total of 347 available subjects who agreed to participate were enrolled in the current study and written consent was obtained from each subject before enrollment to the study. The Ethical Committee of the Tabriz University of Medical Sciences approved the study protocol (registration codes IR.TBZMED.REC.1399.874 and IR.TBZMED.REC.1396.768). Metabolic syndrome (MetS) was defined using criteria established by The National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP) III (40). Individuals were identified to have MetS if they had three or more of the following criteria: waist circumference > 102 cm (men) or 88 cm (women), blood pressure ≥ 130/85 mmHg, fasting triglyceride (TG) level ≥ 150 mg/dl, fasting high-density lipoprotein cholesterol (HDL-C) level <40 mg/dl or 50 mg/dl (women), and fasting blood sugar ≥ 100 mg/dl. The cardio-metabolic risk factors were also defined based on Adult Treatment Panel III (40).

Dietary Assessment and Dietary Score Calculation

The habitual dietary intakes of the participants were assessed with the use of a 147-item semiquantitative food frequency questionnaire (FFQ) with prior evidence of validity and reliability (41, 42). All information was gathered by expert dietitians in a face-to-face interview with each participant. The study subjects were asked to report their frequency and amount of intake of each food item during the previous year based on daily, weekly, monthly, and yearly bases. After that, portion sizes of foods were converted to daily intakes (grams) using household measurements. Since the Iranian food composition table (FCT) (43) is incomplete, USDA FCT was also used to provide information missing from the Iranian FCT (44).

DASH Score

The level of conformity of the individuals' diet to the DASH diet was determined by a scale proposed by Fung et al. (45). This indicator was constructed based on eight food-group or nutrient components which were emphasized or minimized in the DASH diet. This approach strongly encourages the intake of fruits, vegetables, low-fat dairy products, nuts, legumes, and whole grains while discouraging the consumption of red and processed meats, sodium, and sweetened beverages. Sex-specific quintile intakes of the dietary components were calculated within the study population. Participants with the highest quintile of intakes of emphasized components (fruits, vegetables, whole grains, low-fat dairy, nuts, and legumes) received a score of five, while those with the lowest quintile of these intakes were given a score of one. For the remaining components in which their low intakes were desirable (sodium, red and processed meats, and sweetened beverages), a reverse-scoring method was used. Then, the scores for all components were summed up to obtain an overall DASH score (ranged 8–40). A higher total score represents a higher conformity to the DASH diet and a better nutrition quality.

MDS

The level of conformity of the subjects' diet to the Mediterranean dietary pattern was assessed using the MDS proposed by Trichopoulou et al. (46). The sex-specific median intakes of its components (nine items) were calculated. The scoring system was as follows: score one was given for intakes at or above the median for protective components such as vegetables, legumes, fruits and nuts, cereals, fish, and seafood, and a high ratio of monounsaturated fatty acid (MUFA) to saturated fatty acid (SFA) as well, otherwise a value of 0 was assigned. For non-protective components (like meats and dairy products), a value of 1 was assigned for intakes less than the median, otherwise a value of zero was given. As there was no reliable data on alcohol consumption in the current study, this component was omitted and the scoring system was modified with a total score of eight points. Finally, the overall score was reported as a summation of all component scores ranging between 0 (minimal adherence to the Mediterranean diet) and 8 (maximum adherence).

Socio-Demographic, Anthropometric, and Blood Pressure Assessments

Information of socio-demographic variables such as age, gender, marital status, smoking, medical history, and socioeconomic status (SES) was obtained by a trained interviewer. To determine the SES, questions on occupational position, educational status, family size, and house ownership were considered as individual indicators. The total score determined by summing the three scale scores was categorized into three groups: low, middle, and high. The level of physical activity was estimated using a short version of the International Physical Activity Questionnaire (47). Body weight and height were directly measured using a Seca scale (Seca, Germany) and a tape measure with a precision of 100 g and 0.1 cm, respectively, while the subjects were in light clothing without shoes. Bioelectrical impedance analysis (BIA) technology (Tanita, BC-418 MA, Tokyo, Japan) was applied to assess the body composition. Waist circumference (WC) was measured using a flexible inelastic tape to the nearest 0.1 cm, at the narrowest level without applying any pressure to the body. Blood pressure (BP) measurements were conducted using a standard mercury sphygmomanometer twice after a 15-min rest in a sitting position. The average of the two measurements was reported as the participants' BP.

Mental Health and Appetite Assessment

To assess the severity of the emotional disturbance of participants, they were asked to complete the Depression, Anxiety and Stress Scale-21 Items (DASS-21) questionnaire which has been previously validated in Iran (48, 49). For example, Sahebi et al. reported the Cronbach's alpha of the Iranian version of DASS-21 to be 0.77 for depression, 0.79 for anxiety, and 0.78 for stress (48). DASS-21 consists of three categories of seven-item self-report scales which measure the negative states of depression, anxiety, and stress. Items in this questionnaire were scored based on a Likert scale from zero ("did not apply to me at all") to 3 ("applied to me very much or most of the time"). A total score for every scale was computed by summing up the scores for the relevant items and multiplying them by two which could range from 0 to 42. Based on cutoff scores which have been proposed by Lovibond and Lovibond, all the participants were divided into five groups: normal, mild, moderate, severe, and extremely severe depression, anxiety, and stress (50). Higher scores of each subcategory indicated a greater degree of mood disruption.

A 100-mm Visual Analog Scale (VAS) questionnaire was applied to assess appetite sensation. This questionnaire, which has been validated in literature, includes questions about feelings of hunger, satiation, fullness, prospective food consumption, thirst, and the desire to eat something sweet, salty, or fat (51). The participants completed this scale by placing a slash on the 100-mm horizontal line corresponding to their feelings, and subsequently quantification of the measurement was performed by measuring the distance from the left side of the line to the mark.

Biochemical Assessments

Fasting venous blood samples were obtained from all participants and then were centrifuged (10 min at 4,500 rpm, 4°C) to

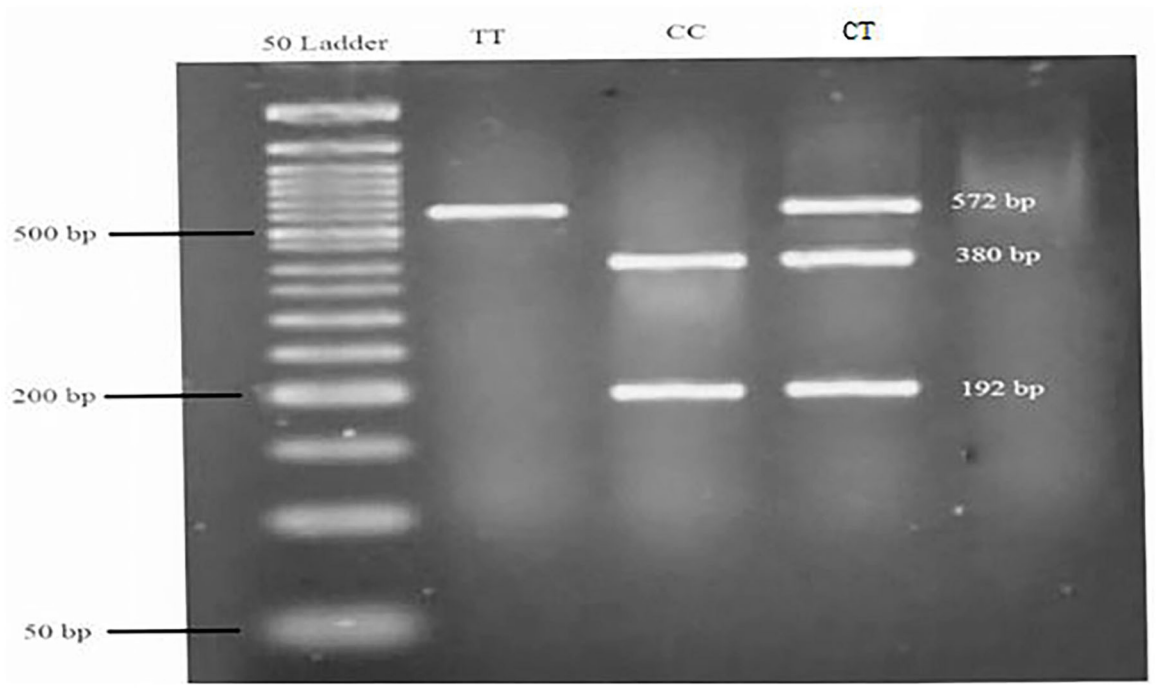

FIGURE 1 | Genotyping of the FADS2 rs174583 variant by TaqI PCR-RFLP analysis. A 50-bp ladder was used to determine the length of the digested products. TT = homozygous mutated (572 bp), CC = homozygous wild-type (380 and 192 bp), CT = heterozygous mutated (572, 380, and 192 bp).

TABLE 1 | Characteristics of the study participants.

	%	Mean (SD) or Median (25 and 75 percentiles)
Age (y)		38.08 (7.49)
Weight (kg)		96.10 (12.87)
FM (Kg)		33.83 (9.16)
FFM (kg)		62.29 (12.38)
WC (cm)		108.89 (9.90)
Sex		
Male	58.20	
Female	41.80	
Physical activity level, (%)		
Low	47.80	
Moderate	27.70	
High	24.50	
Marital status, (%)		
Married	86.00	
Single	14.00	
SES, *n* (%)		
Low	2.70	
Middle	52.90	
High	44.40	
Stress, *n* (%)		
Normal	40.4	
Mild	17.0	
Moderate	23.4	
Severe	14.4	
Extremely severe	4.8	
Anxiety, (%)		
Normal	36.2	
Mild	8.5	
Moderate	25.0	
Severe	13.8	
Extremely-severe	16.5	
Depression, (%)		
Normal	45.7	
Mild	12.8	
Moderate	23.5	
Severe	9.0	
Extremely severe	9.0	
Appetite		33.58 (8.96)
LDL-C, (mg/dl)		119.47 (30.91)
HDL-C, (mg/dl)		44.97 (8.85)
Cholesterol, (mg/dl)		188.43 (33.73)
TG, (mg/dl)		119.97 (58.46)
AIP		0.03 (0.24)
Glucose, (mg/dl)		91.00 (85.00, 98.00)
Insulin, U/mL		13.10 (9.10, 23.30)
HOMA-IR		3.20 (1.95, 5.28)
QUICKI		0.33 (0.03)
SBP (mmHg)		115.64(16.44)
DBP (mmHg)		76.33 (12.32)
Mets (%)	56.1	
FADS2 (%)		
CC	37.8	
CT	51.9	
TT	10.3	

WC, waist circumference; SES, socio-economic status; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-C, low density lipoprotein cholesterol; HDL, high-density lipoprotein-cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; QUICKI, quantitative insulin sensitivity check index; AIP, athrogenic index of plasma; MetS, metabolic syndrome; FM, fat mass; FFM, fat free mass; FADS, fatty acid desaturase.

TABLE 2 | Daily dietary intake of macronutrients, energy and several food groups in relation to MDS and DASH score among study participants.

Variables	Mean (SD) or Median (25 and 75 percentiles)
Macronutrients	
Carbohydrate (g/day)	440.02 (164.76)
Protein (g/day)	97.86 (34.67)
Fats (g/day)	108.04 (46.85)
Energy intake (kcal/day)	3042.91 (1077.89)
Fiber (g/day)	58.03 (42.30, 92.44)
DASH score [total score (8–40)]	23.97 (4.27)
MDS [total score (0–8)]	4.01 (1.43)
Fruits (g/day)	444.03 (289.52, 751.85)
Vegetables (g/day)	320.53 (198.02, 439.48)
Nuts (g/day)	10.98 (5.01, 23.72)
Legumes (g/day)	49.13 (28.70, 73.33)
Red and processed meat (g/day)	49.05 (27.53, 78.91)
Cereals (g/day)	523.44 (355.91, 701.92)
Fish and seafood (g/day)	6.37 (1.91, 13.767)
Dairy products (g/day)	260.62 (140.26, 435.56)
MUFA/SFA	1.21 (0.31)

MUFA, monounsaturated fatty acid; SFA, saturated fatty acid, MDS, Mediterranean diet score; DASH, dietary approach to stop hypertension.

separate serum. Aliquots were frozen at−80 C until analysis. Serum triglyceride (TG), total cholesterol (TC), glucose, and high-density lipoprotein cholesterol (HDL-C) were assayed using commercial kits (Pars Azmoon, Tehran, Iran). Serum insulin was also analyzed with commercially available enzyme-linked immunosorbent assay kits (Bioassay Technology Laboratory, Shanghai Korean Biotech, Shanghai City, China) according to the manufacturer's instructions. The concentration of low-density lipoprotein cholesterol (LDL) was calculated using the Friedewald formula (52). Atherogenic index of plasma (AIP) was determined as the base 10 logarithm of TG to HDL-C ratio (53). Homeostasis model assessment-insulin resistance index (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI) as indicators of insulin resistance were calculated based on the standard formula (54, 55).

TABLE 3 | Socio-demographic and anthropometric characteristics and cardio-metabolic risk factors according to the tertiles of dietary quality indices in men.

	DASH				MDS			
	T1	T2	T3	P*	T1	T2	T3	P*
Age (y)	36.41 (5.91)	38.11 (6.90)	41.67 (6.00)	**0.008**	37.91 (6.14)	38.50 (6.59)	38.63 (9.04)	0.969
Weight (kg)	103.60 (10.39)	102.52 (9.34)	99.65 (11.47)	0.617	102.06 (9.84)	102.53 (10.82)	101.48 (9.25)	0.960
FM (Kg)	30.35 (7.27)	28.71 (6.68)	28.42 (8.01)	0.644	28.93 (7.83)	29.43 (6.76)	29.56 (7.86)	0.952
FFM (kg)	73.26 (5.24)	73.86 (7.57)	71.24 (5.36)	0.477	73.14 (6.58)	73.15 (6.49)	71.93 (4.46)	0.961
WC (cm)	114.31 (8.05)	112.47 (6.29)	112.57 (8.30)	0.644	113.49 (8.01)	112.90 (7.29)	113.50 (6.41)	0.926
Physical activity level, (%)				0.808				0.763
Low	35.3	29.4	35.3		41.2	44.1	14.7	
Moderate	46.9	40.6	12.5		34.4	62.5	3.1	
High	26.7	50.0	23.3		30	60	10	
Marital status, (%)				0.682				0.620
Married	35.8	39.5	24.7		34.6	55.6	9.8	
Single	40.0	40.0	20.0		40.0	53.3	6.7	
SES, *n* (%)								
Low	0.0	0.0	0.0	0.371	0.0	0.0	0.0	**0.037**
Middle	30.0	43.3	26.7		20.0	66.7	13.3	
High	40.0	38.5	21.5		43.1	49.2	7.7	
Stress, *n* (%)				**0.004**				0.481
Normal	21.7	45.7	32.6		26.1	65.2	8.7	
Mild	41.2	47.1	11.8		52.9	35.3	11.8	
Moderate	47.1	29.4	23.5		47.1	47.1	5.9	
Severe	60.0	20.0	20.0		20.0	60.0	20.0	
Extremely severe	66.7	33.3	0.0		50.0	50.0	0.0	
Anxiety, (%)				0.788				0.541
Normal	35.7	42.9	21.4		33.3	57.1	9.5	
Mild	66.7	16.7	16.7		50.0	33.3	16.7	
Moderate	22.7	36.4	40.9		36.4	54.5	9.1	
Severe	35.7	42.9	21.4		14.3	71.4	14.3	
Extremely-severe	50.0	41.7	8.3		58.3	41.7	0.0	
Depression, (%)				**0.049**				0.647
Normal	27.8	40.7	31.5		31.5	59.3	9.3	
Mild	41.7	41.7	16.7		41.7	50.0	8.3	
Moderate	55.6	33.3	11.1		38.9	55.6	5.6	
Severe	33.3	50.0	16.7		33.3	50.0	16.7	
Extremely severe	50.0	33.3	16.7		50.0	33.3	16.7	
Appetite	35.09 (11.31)	34.74 (8.87)	33.96 (7.83)	0.908	33.12 (9.77)	35.43 (9.31)	36.11 (10.23)	0.490
LDL-C, (mg/dl)	122.61 (27.08)	122.69 (29.83)	113.79 (22.70)	0.201	119.14 (24.18)	122.70 (29.51)	113.95 (27.22)	0.471
HDL-C, (mg/dl)	41.88 (6.75)	44.46 (9.28)	40.62 (6.24)	0.220	41.71 (6.41)	43.24 (8.93)	42.75 (6.58)	0.675
Cholesterol, (mg/dl)	192.09 (28.44)	191.30 (35.83)	183.14 (25.87)	0.404	188.18 (27.86)	192.18 (33.83)	181.00 (26.24)	0.601
TG, (mg/dl)	134.00 (106.00, 194.00)	104.50 (76.25, 138.50)	121.00 (85.00, 168.00)	**0.050**	119.00 (86.50, 174.50)	116.00 (83.00, 164.50)	129.00 (85.50, 177.00)	0.814
AIP	0.16 (0.22)	0.02 (0.27)	0.12 (0.22)	**0.032**	0.11 (0.23)	0.07 (0.26)	0.07 (0.22)	0.753
Glucose, (mg/dl)	90.00 (84.00, 101.00)	91.00 (86.00, 99.25)	96.00 (85.00, 108.00)	0.345	91.00 (86.00, 107.00)	91.00 (85.00, 97.00)	94.00 (83.00, 101.50)	0.813
Insulin, U/mL	13.30 (9.00, 23.30)	12.00 (9.37, 20.48)	9.40 (8.40, 16.90)	0.275	12.15 (8.85, 23.63)	12.00 (9.00, 19.00)	9.40 (8.35, 21.85)	0.825
HOMA-IR	3.22 (1.94, 5.03)	2.87(2.25, 4.57)	2.04 (1.78, 4.79)	0.451	3.06 (1.98, 5.26)	2.84 (1.95, 4.64)	2.51 (1.70, 5.09)	0.771
QUICKI	0.33 (0.03)	0.32 (0.02)	0.34 (0.03)	0.401	0.33 (0.03)	0.33 (0.03)	0.34 (0.03)	0.796
SBP (mmHg)	120.00 (105.00, 130.00)	120.00 (110.00, 130.00)	110.00 (110.00–120.00)	0.480	120.00 (110.00, 130.00)	120.00 (107.50, 130.00)	110.00 (110.00, 125.00)	0.857

(Continued)

TABLE 3 | Continued

	DASH				MDS			
	T1	T2	T3	P*	T1	T2	T3	P*
DBP (mmHg)	77.50 (12.57)	77.30 (10.04)	70.43 (16.61)	0.143	76.76 (11.67)	75.38 (14.34)	74.38 (8.63)	0.816
Mets (%)	41.5	31.7	26.8	0.815	43.9	48.8	7.3	0.150
FADS2 (%)				0.756				0.365
CC	37.1	28.6	34.3		28.6	54.3	17.1	
CT	41.0	48.7	10.3		48.7	46.2	5.1	
TT	30.0	30.0	40.0		10.0	90.0	0.0	

*Data are presented as mean (SD) or median (25 and 75 percentiles). *Analysis of variance for continuous variables and χ^2 test for categorical variables. WC, waist circumference; SES, socio-economic status HOMA-IR, homeostasis model assessment of insulin resistance; LDL-C, low density lipoprotein cholesterol; HDL, high-density lipoprotein-cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; QUICKI, quantitative insulin sensitivity check index; AIP, athrogenic index of plasma; MetS, metabolic syndrome; FM, fat mass; FFM, fat free mass; FADS, fatty acid desaturase.*

Genotyping

Genomic DNA was extracted from whole blood using the chloroform technique, and a NanoDrop ND-1000 spectrophotometer was used to investigate the concentration and purity of extracted DNA. All available DNAs were expected to be genotyped for rs174583. SNP rs174583, which is located in the 61842278 position of chromosome 11 in the intron of FADS2, was genotyped by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The PCR was completed using primers with the following sequence: forward, 5′ AGGAAGCAGACCACAGAGTC 3′; reverse, 5′ TC CTTCGTCTGGTGTCTCAG 3′. PCR reactions were performed in a final volume of 10 μl which contains 5 μl Master Mix (Ampliqon, Denmark), 2 μl extracted DNA, 1 μl primers, and 2 μl distilled water. The PCR cycles in a DNA thermocycler (Bio-Rad T100 Thermal Cycler) were optimized to 95 °C for 10 min of initial denaturation; amplification consisted of 35 cycles at 95 °C for 15 s (denaturation), and annealing at 60 °C for 20 s and 50 s of extension at 72 °C and final extension occurred at 74 °C for 10 min. According to the restriction sites on the sequence of the amplified DNA, TauI (cat. num. ER1652, USA) as a restriction enzyme was used to digest amplified DNA. Afterward, all digested PCR products were visualized by green staining gel electrophoresis on 1.5% agarose gel in a Gel Doc system (U.V.P Company, Cambridge, UK). As shown in **Figure 1**, after electrophoresis, the T allele appeared as a 572-bp fragment whereas the C allele was cleaved by the restriction enzyme and appeared as fragments with lengths of 192 and 380 bp.

Statistical Analysis

Sample distributions were tested for normality according to descriptive measures such as coefficients of skewness and kurtosis, mean, and standard deviation (56). The data were presented as means ± standard deviations (SD) for normally distributed continuous variables, frequencies or percentages for categorical variables, and the median (25 and 75 percentiles) for skewed continuous variables. Sex-stratified one-way analysis of variance (ANOVA) and chi-square tests were used to compare continuous and discrete variables across different tertile categories of dietary quality indices. Multinomial logistic regression in different models was utilized to estimate the associations between diet quality indices and rs174583 genotypes, as well as the association of this variant with odds of MetS and cardio-metabolic risk factors. Gene–diet interactions were tested by analyses of covariance using the General Linear Model procedures, after adjusting for confounding factors (age, physical activity, SES and WC). All of these gene–diet interaction analyses were conducted based on sex categories, and then significant interactions were depicted as plot to help their illustration. The P-value < 0.05 was considered as statistically significant. Statistical Package for Social Sciences (SPSS, Inc., Chicago, IL, version 21) was used for all statistical analyses.

RESULTS

The demographic, anthropometric, clinical, biochemical, and genetic characteristics of all participants are summarized in **Table 1**. The mean (± SD) age of participants was 38.08 ± 7.49 years, and the mean (± SD) WC was 108.89 ± 9.90 cm. More than one half of the individuals were male (58.2 %), and 86.0 % were married. In relation to physical activity, 47.8 % of the subjects had low physical activity and more had middle SES (52.90%). It was found that 56.1 % of participants had MetS and the overall prevalence of rs174583 genotypes was 37.8, 51.9, and 10.3 for CC, CT, and TT, respectively (**Table 1**). Daily intakes (g/day) of macronutrients, micronutrients, and several food groups in relation to diet quality indices (MDS and DASH score) among study participants are presented in **Table 2**. The mean (± SD) energy intake was 3049.91 ± 1077 kcal/d. For the total sample, the mean (± SD) DASH score was 23.97 ± 4.27 points and the MDS score was 4.01± 1.43 points. The distributions of the study population regarding general characteristics and clinical, genetic, and biochemical parameters across the tertiles of dietary quality indices among men and women are presented in **Tables 3**, **4**. Among men, in comparison with the lowest tertile, those who were assigned to the top tertile of the DASH score were older ($P = 0.008$) and were less likely to have higher severity of mental health disorders such as depression ($P = 0.049$) and stress

TABLE 4 | Socio-demographic and anthropometric characteristics and cardio-metabolic risk factors according to the tertiles of dietary quality indices in women.

	DASH				MDS			
	T1	T2	T3	P*	T1	T2	T3	P*
Age (y)	36.94 (8.77)	38.51 (7.93)	38.78 (8.00)	0.669	38.23 (8.78)	37.29 (8.14)	39.33 (7.60)	0.677
Weight (kg)	87.73 (8.84)	91.77 (16.56)	89.66 (8.13)	0.418	88.57 (13.92)	89.01 (10.27)	93.62 (13.47)	0.322
FM (Kg)	37.75 (6.09)	39.93 (11.06)	38.49 (5.98)	0.633	38.71 (9.37)	37.81 (6.76)	41.15 (9.62)	0.358
FFM (kg)	50.26 (3.75)	51.62 (6.52)	51.18 (3.77)	0.466	49.58 (4.99)	51.44 (4.85)	52.47 (4.94)	0.154
WC (cm)	104.45 (9.37)	104.32 (11.03)	104.70 (10.18)	0.966	103.09 (10.03)	103.95 (9.25)	108.00 (11.90)	0.207
Physical activity level, *n* (%)				0.814				0.853
Low	33.9	41.1	25.0		33.9	42.9	23.2	
Moderate	45.0	25.0	30.0		45.0	45.0	10.0	
High	31.3	50.0	18.8		25.0	56.3	18.8	
Marital status, *n* (%)				0.413				0.827
Married	35.8	42.0	22.2		35.8	44.4	19.8	
Single	36.4	18.2	45.5		27.3	54.5	18.2	
SES, *n* (%)				0.264				0.995
Low	60.0	20.0	20.0		20.0	40.0	40.0	
Middle	34.8	43.5	21.7		36.2	47.8	15.9	
High	33.3	27.8	38.9		33.3	38.9	27.8	
Stress, *n* (%)				0.377				0.723
Normal	46.7	33.3	20.0		36.7	43.3	20.0	
Mild	40.0	33.3	26.7		40.0	46.7	13.3	
Moderate	22.2	40.7	37.0		33.3	44.4	22.2	
Severe	41.2	41.2	17.6		29.4	47.1	23.5	
Extremely severe	0.0	100.0	0.0		33.3	66.7	0.0	
Anxiety, *n* (%)				0.557				0.286
Normal	34.6	38.5	26.9		50.0	34.6	15.4	
Mild	40.0	40.0	20.0		10.0	60.0	30.0	
Moderate	44.0	44.0	12.0		40.0	44.0	16.0	
Severe	33.3	16.7	50.0		25.0	41.7	33.3	
Extremely-severe	26.3	47.4	26.3		26.3	57.9	15.8	
Depression (%)				0.699				0.959
Normal	40.6	28.1	31.3		31.3	50.0	18.8	
Mild	41.7	41.7	16.7		33.3	33.3	33.3	
Moderate	34.6	38.5	26.9		42.3	46.2	11.5	
Severe	36.4	63.6	0.0		45.5	27.3	27.3	
Extremely severe	18.2	45.5	36.4		18.2	63.6	18.2	
Appetite	34.18 (7.06)	30.97 (8.56)	32.26 (8.87)	0.265	32.72 (8.56)	32.33 (8.79)	32.22 (6.08)	0.972
LDL-C, (mg/dl)	109.66 (26.43)	127.14 (35.24)	123.07 (38.40)	0.088	117.38 (35.47)	119.20 (33.80)	125.21 (31.84)	0.732
HDL-C, (mg/dl)	48.58 (8.27)	46.23 (10.33)	47.52 (9.44)	0.590	48.68 (9.90)	47.29 (9.49)	45.50 (8.14)	0.520
Cholesterol, (mg/dl)	179.27 (30.28)	194.51 (37.54)	191.87 (40.42)	0.191	186.39 (37.50)	188.33 (37.10)	191.61 (33.10)	0.890
TG, (mg/dl)	105.18 (37.71)	105.74 (42.17)	106.39 (45.88)	0.994	101.65 (49.15)	109.21 (33.22)	104.50 (44.42)	0.737
AIP	−0.04 (0.18)	−0.02 (0.23)	−0.04 (0.26)	0.890	−0.08 (0.26)	−0.01 (0.18)	−0.03 (0.24)	0.399
Glucose, (mg/dl)	91.76 (9.23)	92.94 (13.38)	89.13 (9.55)	0.440	92.32 (10.90)	91.11 (11.61)	91.22 (10.60)	0.893
Insulin, U/mL	14.50 (9.45, 26.85)	14.50 (9.10, 25.50)	16.20 (8.90, 24.20)	0.937	15.40 (8.70, 22.70)	14.80 (9.65, 25.95)	14.25 (5.95, 25.90)	0.898
HOMA-IR	3.26 (1.99, 6.24)	3.91 (1.95, 5.70)	3.44 (1.96, 5.44)	0.874	3.68 (1.95, 5.32)	3.34 (2.09, 5.62)	4.12 (1.38, 6.28)	0.943
QUICKI	0.33 (0.03)	0.32 (0.03)	0.33 (0.03)	0.724	0.33 (0.03)	0.32 (0.03)	0.33 (0.04)	0.561
SBP(mmHg)	112.85 (17.49)	114.31 (14.82)	117.09 (13.79)	0.596	114.06 (15.88)	113.79 (14.99)	116.83 (16.78)	0.750
DBP(mmHg)	75.45 (13.90)	77.09 (9.31)	78.57 (12.40)	0.629	75.77 (11.40)	75.69 (10.78)	81.50 (14.36)	0.170
MetS (%)	28.0	40.0	32.0	0.260	44.0	32.0	24.0	0.643

(Continued)

TABLE 4 | Continued

	DASH				MDS			
	T1	T2	T3	P*	T1	T2	T3	P*
FADS2 (%)				0.537				0.897
CC	33.3	50.0	16.7		37.5	37.5	25.0	
CT	42.9	26.2	31.0		33.3	45.2	21.4	
TT	16.7	50.0	33.3		33.3	33.3	33.3	

*Data are presented as mean (SD) or median (25 and 75 percentiles). *Analysis of variance for continuous variables and χ^2 test for categorical variables. WC, waist circumference; SES, socio-economic status; HOMA-IR, homeostasis model assessment of insulin resistance; LDL-C, low density lipoprotein cholesterol; HDL, high-density lipoprotein-cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; TG, triglyceride; QUICKI, quantitative insulin sensitivity check index; AIP, athrogenic index of plasma; MetS, metabolic syndrome; FM, fat mass; FFM, fat free mass; FADS, fatty acid desaturase.*

TABLE 5 | Odds ratio (OR) and confidence interval (CI) for the association between dietary quality indices and FADS2 rs174583 genotypes.

	Men			Women		
	CC	CT	TT	CC	CT	TT
DASH score (total score)						
Crude	1(Ref.)	0.93 (0.83–1.04)	1.05 (0.88–1.25)	1(Ref.)	0.99 (0.90–1.12)	1.12 (0.91–1.37)
Model 1*	1(Ref.)	0.94 (0.84–1.06)	1.03 (0.86–1.23)	1(Ref.)	1.00 (0.89–1.12)	1.12 (0.90–1.38)
Model 2**	1(Ref.)	0.94 (0.84–1.06)	1.03 (0.86–1.23)	1(Ref.)	1.00 (0.89–1.12)	1.12 (0.90–1.38)
MDS (total score)						
Crude	1(Ref.)	0.72 (0.49–1.06)	1.09 (0.61–1.93)	1(Ref.)	0.93 (0.69–1.26)	1.06 (0.62–1.83)
Model 1	1(Ref.)	0.75 (0.50–1.11)	1.06 (0.59–1.91)	1(Ref.)	0.93 (0.68–1.26)	0.97 (0.56–1.67)
Model 2	1(Ref.)	0.75 (0.50–1.11)	1.06 (0.59–1.92)	1(Ref.)	1.00 (0.72–1.40)	1.00 (0.57–1.76)

*The multivariate multinomial logistic regression was used for estimation of ORs and confidence interval (CI). *Adjusted for age, physical activity and socio-economic status. **Additionally adjusted for waist circumference.*

($P = 0.004$). The distribution of SES for men was significantly different among tertile of MDS ($P = 0.037$); those who had a higher level of SES were assigned to the second tertile of this index. Additionally, men in the third tertile of the DASH score compared with the first tertile had lower AIP ($P = 0.032$) and TG ($P = 0.050$) levels. No significant differences were observed in the frequencies of genotypes across different tertiles of diet quality indices (MDS and DASH) ($P > 0.05$). Among women, no statistical significant difference was found regarding qualitative and quantitative variables across tertile categories of dietary quality indices (**Table 4**). Also, we did not observe statistically significant differences in genotype frequencies of the FADS2 rs174583 polymorphism across MDS and DASH score tertiles ($P > 0.05$) in this group.

A sex-stratified analysis for the association between dietary quality indices (DASH and MDS scores) and FADS2 rs174583 genotypes is shown in **Table 5**, and as summarized in this table, no significant association was revealed between dietary quality indices and the rs174583 variant in either crude or multivariate-adjusted models, among men and women. Crude and multivariable-adjusted odds ratios and 95% confidence intervals (95% CI) for MetS and cardio-metabolic factors (high cholesterol, high LDL-C, low HDL-C, high TG, elevated blood pressure, hyperglycemia, high HOMA-IR, low QUIKI) across FADS2 rs174583 genotypes are presented in **Table 6**. There was a significant positive association between the TT genotype and the odds of having high TG when compared to the CC genotype, either in the crude (OR, 3.21; 95% CI, 1.02–10.14) or in the adjusted model 1 (OR, 3.32; 95% CI, 1.00–11.01) and model 2 (OR, 3.58; 95% CI, 1.07–11.97), taking into account various potential confounders.

To confirm whether FADS2 rs174583 polymorphism interacts with dietary quality indices to modulate cardio-metabolic risk factors, we carried out covariance analyses and illustrated the significant interactions, as shown in **Figure 2**. Among the female participants, a significant interaction was observed between rs174583 and adherence to the DASH score in relation to serum TG concentration, and this result remained significant even after controlling for confounding variables ($P_{\text{Interaction}} = 0.046$). Female CT-genotype carriers who were assigned to the second tertile of DASH compared with those in the first tertile had the lower TG level ($P < 0.05$) (**Figure 2**). Accordingly, in the female group, another significant interaction was revealed between adherence to the MDS score and rs174583 polymorphism on serum glucose levels ($P_{\text{Interaction}} = 0.044$), such that the lowest mean of glucose level was observed in homozygous minor subjects (TT) in the third tertile of MDS, in comparison with other tertiles of this dietary index ($P < 0.05$). Among the male group, no FADS–diet interaction was found except for DASH and rs174583 in relation to diastolic blood pressure (DBP) (P

TABLE 6 | Odds ratio (OR) and confidence interval (CI) for FADS2 rs174583 genotypes and cardio-metabolic risk factors[a] of participants.

	FADS2		
	CC	**CT**	**TT**
MetS[b]			
Crude	1 (Ref.)	0.68 (0.33–1.39)	1.81 (0.59–5.52)
Model 1	1 (Ref.)	0.76 (0.36–1.61)	1.64 (0.52–5.23)
Model 2	1 (Ref.)	0.88 (0.40–1.93)	1.74 (0.53–5.70)
High Cholesterol			
Crude	1 (Ref.)	1.50 (0.72–3.12)	2.69 (0.86–8.37)
Model 1	1 (Ref.)	1.52 (0.72–3.23)	2.61 (0.82–8.32)
Model 2	1 (Ref.)	1.41 (0.65–3.02)	2.55 (0.79–8.17)
High LDL-C			
Crude	1 (Ref.)	1.06 (0.05–2.27)	0.82 (0.25–2.73)
Model 1	1 (Ref.)	1.19 (0.54–2.61)	0.73 (0.22–2.51)
Model 2	1 (Ref.)	1.13 (0.51–2.53)	0.68 (0.20–2.36)
Low HDL-C			
Crude	1 (Ref.)	1.03 (0.53–2.01)	3.32 (0.96–11.50)
Model 1	1 (Ref.)	1.00 (0.51–1.99)	3.24 (0.92–11.38)
Model 2	1 (Ref.)	1.07 (0.53–2.15)	3.37 (0.95–12.00)
High TG			
Crude	1 (Ref.)	0.61 (0.26–1.43)	**3.21 (1.02–10.14)[c]**
Model 1	1 (Ref.)	0.69 (0.29–1.66)	**3.32 (1.00–11.01)[c]**
Model 2	1 (Ref.)	0.77 (0.32–1.87)	**3.58 (1.07–11.97)[c]**
High blood pressure			
Crude	1 (Ref.)	0.73 (0.35–1.51)	0.65 (0.19–2.28)
Model 1	1 (Ref.)	0.85 (0.40–1.82)	0.58 (0.16–2.10)
Model 2	1 (Ref.)	0.98 (0.44–2.19)	0.53 (0.14–2.08)
Hyperglycemia			
Crude	1 (Ref.)	1.21 (0.50–2.89)	2.94 (0.87–9.95)
Model 1	1 (Ref.)	1.32 (0.54–3.22)	2.72 (0.78–9.41)
Model 2	1 (Ref.)	1.37 (0.55–3.44)	2.67 (0.75–9.55)
High HOMA-IR			
Crude	1 (Ref.)	1.05 (0.53–2.08)	0.88 (0.29–2.69)
Model 1	1 (Ref.)	1.06 (0.53–2.12)	0.83 (0.27–2.58)
Model 2	1 (Ref.)	1.10 (0.54–2.24)	0.81 (0.26–2.54)
Low QUIKI			
Crude	1 (Ref.)	1.42 (0.43–4.63)	0.79 (0.14–4.36)
Model 1	1 (Ref.)	1.53 (0.46–5.12)	0.81 (0.15–4.55)
Model 2	1 (Ref.)	1.69 (0.50–5.75)	0.80 (0.14–4.53)

The multivariate multinomial logistic regression across Tertiles of dietary DASH score and MDS was used for estimation of ORs and confidence interval (CI). Model1, Adjusted for age, sex and physical activity. Model 2, Further adjusted for waist circumference and socio-economic status. [a]Defined as having high cholesterol (total cholesterol ≥ 220 mg/dl), high LDL-C (≥160 mg/dl), low HDL-C (<40 mg/dl for men and < 50 mg/dl for women), high TG (≥ 150 mg/dl), high blood pressure (systolic/diastolic blood pressure ≥130/85 mmHg), hyperglycemia (fasting blood sugar ≥100 mg/dl), high HOMA-IR (> 2.6) and low QUIKI (<38). [b]Defined as the presence of 3 of the following components: (1) abdominal adiposity (waist circumference > 88 cm); (2) low serum HDL cholesterol (< 50 mg/dL); (57) high serum triacylglycerol (≥150 mg/dL); (3) elevated blood pressure (≥130/85 mm Hg); (4) abnormal glucose homeostasis (fasting plasma glucose ≥110 mg/dL). HOMA-IR, homeostasis model assessment of insulin resistance; LDL-C, low density lipoprotein cholesterol; HDL-C, high-density lipoprotein-cholesterol; TG, triglyceride; QUICKI, quantitative insulin sensitivity check index; MetS, metabolic syndrome. [c]Indicates statistically significant values as P < 0.05.

interaction = 0.038), where homozygous carriers of the major allele who were assigned to the highest tertile of DASH had significantly lower DBP levels than those in the second tertile ($P < 0.05$).

DISCUSSION

The current study, based on our knowledge, is the first attempt to study the interactions between FADS2 gene polymorphism (rs174583) and dietary quality indicators in relation to cardio-metabolic risk factors. We found consistently significant interactions between adherence to DASH and rs174583 polymorphism of the FADS2 gene in relation to serum TG level among female subjects; the CT heterozygote which was assigned to the second tertile of DASH had the lower TG level than those who were in the first tertile. Additionally, adherence to the MDS modified the effects of FADS2 rs174583 polymorphisms on levels of glucose in women; minor homozygote carriers with the highest adherence to MDS had the lowest glucose concentrations. On the other hand, being in the highest tertiles of DASH could indicate favorable effects in decreasing DBP in male homozygous carriers of the major allele. Another main finding in this study was that FADS2 rs174583 was associated with higher odds of having high serum TG in multivariate-adjusted models. The minor allele frequency of FADS2 rs174583 in our research (36 %) was lower than reported in a sample of the Taiwanese (58) and European (HapMap database) population. The reason for the differences in allele frequencies by population or ethnic group is not fully understood but could be, in part, explained by differences in sample size, study design, lifestyle, and other demographic characteristics among studies.

In the present study, we revealed novel evidence from an aspect of gene–diet interaction that the associations between the FADS2 rs174583 polymorphism and cardio-metabolic risk factors depend on the diet consumed. Therefore, it seems that improving adherence to dietary quality indices can attenuate the genetic association with metabolic risk factors. As elucidated above, a good adherence to both DASH and MDS could show favorable effects in reducing some of the metabolic risk factors including TG and glucose in female minor allele carriers and DBP in male major allele carriers. Mechanisms involved in these gender-dependent differences are not clear; however, these discrepancies can be attributed to heterogeneities in regional depots of adipose tissue and hormonal status (59). Although there is no relevant published study regarding the FADS2 rs174583–diet interaction to compare our results with, a cross-sectional genome-wide association study (GWAS) in the Korean population reported significant interactions between total fat intake and the FADS1 and haplotype of FADS1 and FADS2 in relation to the MetS risk; carriers of the FADS1 rs174547 and FADS2 rs2845573 minor alleles have a greater predisposition to MetS and its components such as dyslipidemia, and, on the other hand, intermediate intake of dietary fat protected carriers of the FADS1 major alleles against the risk of MetS (60). Another investigation in Swedish adults indicated that minor allele carriers of FADS1 had lower LDL-cholesterol

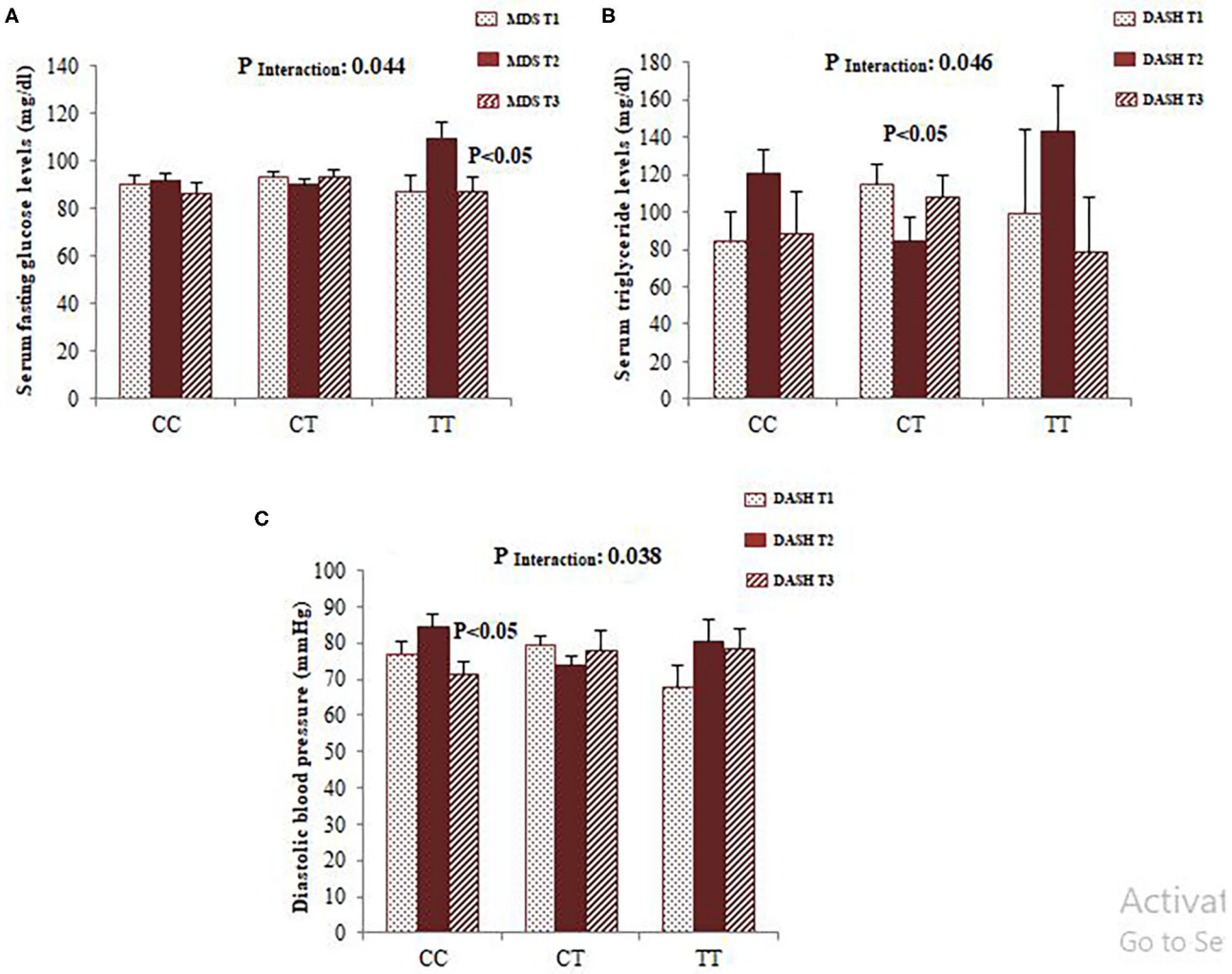

FIGURE 2 | Interaction between FADS2 rs174583 and MDS on the serum concentration of glucose **(A)** and interaction between FADS2 rs174583 and DASH score on the serum concentration of triglyceride **(B)** among women. Interaction between FADS2 rs174583 and DASH score on DBP among men **(C)**. The bars indicate mean. Error bars: SE of means. *P*-values of interactions were adjusted for age, WC, physical activity, and socio-economic status.

concentrations, following the consumption of a low n-3 long-chain polyunsaturated fatty acid diet (61). Evidence emerging from human-based studies has reported that genetic variation in FADS genes through changes in enzyme activity, which is usually estimated using product-to-substrate ratios, and plasma and erythrocyte fatty acid composition affect disease risk factors (62). Accordingly, several previous studies have reported a lower risk of mortality, insulin resistance, and obesity with reduction of the D6D activity (63, 64). Likewise, the associations have been observed between polymorphisms in FADS genes and high D6D activity (65), as well as between these variants and increased levels of inflammatory markers and fasting insulin and a higher risk of coronary artery disease (66). Thus, our results regarding the association between rs174583 polymorphism and greater odds of having TG concentrations are consistent with the results of other previous studies, where the FADS2 rs174583 polymorphism was associated with an increased risk of overweight and its related consequences (17). The biological mechanisms underlying how FADS genetic variations contribute to disease development have not yet been elucidated. However, it has hypothesized that these variants may impair desaturase activity and lead to a decrease in n-3 LC-PUFA in the red blood cell (RBC) and consequently deleterious metabolic effects (58).

Altogether, our results suggest that adherence to the healthy dietary pattern could modify the genetic association with some of the cardio-metabolic parameters. Substantial evidence supports a protective effect of improved adherence to the diet quality scores, which represent a broader picture of dietary intake, on health outcomes (26). For instance, Saraf-Bank et al. in a sample of Iranian subjects revealed that higher healthy eating

index (HEI) 2010 scores were inversely associated with lower risk of MetS and its components (67). Likewise, similar results were observed in our previous study in which high adherence to HEI was found to be inversely associated with MetS risk (44). Therefore, it is not surprising that high compliance with these healthy dietary patterns suppresses the adverse influence of greater genetic susceptibility to cardio-metabolic risk factors in carriers of the FADS2 risk allele; in other words, people appear to be more prone to the beneficial effects of improving diet quality. The precise underlying mechanisms behind these interactions are not fully understood. Nevertheless, the favorable modulating effects of these indices could be explained by their components which are accompanied with high intake of fruits, vegetables, whole grains, seafood and plant proteins, nuts, and fiber and low intake of saturated fatty acids (68). In this regard, extensive evidence from epidemiologic studies has revealed the inverse associations of fruit, vegetables, and unsaturated fats with nutrition-related health conditions (69). In the present study, we found that greater adherence to the DASH score was associated with lower AIP and TG levels in men. These findings were in concordance with previous studies that reported a protective role for DASH diet against cardio-metabolic risk factors. A number of meta-analyses of RCT have revealed inverse relationships between DASH and CVD risk factors (70, 71). Additionally, Maddock et al. confirmed that long-term adherence to a DASH-type diet is related to a favorable cardiovascular-risk profile in adulthood (72). As mentioned above, since this diet is characterized by a higher consumption of healthy food items such as whole grain, fruits and vegetables, low-fat dairy products, lean meat, poultry and fish, nuts, and legumes which are rich dietary sources of potassium, magnesium, calcium, and dietary fiber, health benefits of excellent adherence with the DASH score are of no surprise (73).

A major strength of this study is that we, for the first time, found significant interactions between healthy dietary patterns and genetic predisposition in relation to cardio-metabolic risk factors, which is highly novel; therefore, our findings emphasize the importance of considering the influence of gene–diet interactions in relation to health outcomes. Thoroughly, the identification and better understanding of gene–diet interactions may open new opportunities for developing precision nutrition strategies based on subjects' genotype for the prevention and control of diseases. Moreover, diet quality scores provide comprehensive measures of diet than the single-component approach. Another strength of the present study was that we applied a reliable and validated FFQ for dietary assessment. Nonetheless, some limitations of this investigation need to be highlighted. First, although the cross-sectional design of the study makes causal inferences impossible, this research helps to generate the hypothesis to be studied more rigorously using cohort studies or other types of study. Second, under-reporting of dietary intake which is significant among obese people may cause misclassifications in dietary variables and ultimately this phenomenon can result in bias in the diet–disease relationship (74). Nevertheless, we removed the lower and higher extreme values of dietary intake. Third, despite that we have carefully adjusted for multiple confounders in the analyses, residual confounding by other unmeasured or unknown variables could not be fully eliminated. Fourth, it might be limited by the relatively small sample size. Thus, our finding should be interpreted with caution and replication in large epidemiological cohort studies is needed. Fifth, we have to mention that only one variant from a single gene was analyzed in this study while a number of other candidate genes have been implicated in the pathogenesis of the obesity phenotype and its dependent complications. Last, our conclusions may not be generalized to the general population as the present study was performed in Tabriz with different cultures, dietary intakes, and other lifestyle factors.

CONCLUSION

In conclusion, we found for the first time the statistically significant interactions of FADS2 rs174583 with adherence to the dietary quality indicators (DASH and MDS) in relation to cardio-metabolic risk factors in both women and men. Female carriers of the risk allele with higher adherence to DASH and MDS had lower adjusted means of TG and glucose concentrations, respectively. Likewise, a similar interaction was observed for DBP in male homozygous carriers of the major allele. Additionally FADS2 rs174583 polymorphism was associated with higher odds of having high serum TG. Therefore, our findings highlight the importance of improving adherence to a healthy diet in prevention of obesity-dependent complications especially in subjects with greater genetic susceptibility. Subsequently, prospective cohort studies are needed to confirm the results of our study.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IR.TBZMED.REC.1399.062. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MK contributed to the data collection, manuscript writing, performed the statistical analysis, and data interpretation. MA conceptualized and designed the study. Moreover, MA revised the manuscript and approved the final manuscript as submitted. LN was involved in lab works. All authors contributed to the article and approved the submitted version.

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Polyphenol-Rich *Aronia melanocarpa* Juice Consumption Affects *LINE-1* DNA Methylation in Peripheral Blood Leukocytes in Dyslipidemic Women

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Cardiovascular disease (CVD) is associated with alterations in DNA methylation and polyunsaturated fatty acid (PUFA) profile, both modulated by dietary polyphenols. The present parallel, placebo-controlled study (part of the original clinical study registered as NCT02800967 at www.clinicaltrials.gov) aimed to determine the impact of 4-week daily consumption of polyphenol-rich *Aronia melanocarpa* juice (AMJ) treatment on *Long Interspersed Nucleotide Element-1* (*LINE-1*) methylation in peripheral blood leukocytes and on plasma PUFAs, in subjects ($n = 54$, age range of 40.2 ± 6.7 years) at moderate CVD risk, including an increased body mass index, central obesity, high normal blood pressure, and/or dyslipidemia. The goal was also to examine whether factors known to affect DNA methylation (folate intake levels, *MTHFR* C677T gene variant, anthropometric and metabolic parameters) modulated the *LINE-1* methylation levels upon the consumption of polyphenol-rich aronia juice. Experimental analysis of *LINE-1* methylation was done by MethyLight method. *MTHFR* C677T genotypes were determined by the polymerase chain reaction–restriction fragment length polymorphism method, and folate intake was assessed by processing the data from the food frequency questionnaire. PUFAs were measured by gas–liquid chromatography, and serum lipid profile was determined by using Roche Diagnostics kits. The statistical analyses were performed using Statistica software package. In the comparison after vs. before the treatment period, in dyslipidemic women ($n = 22$), we observed significant decreases in *LINE-1* methylation levels (97.54 ± 1.50 vs. 98.39 ± 0.86%, respectively; $P = 0.01$) and arachidonic acid/eicosapentaenoic acid ratio [29.17 ± 15.21 vs. 38.42 (25.96–89.58), respectively; $P = 0.02$]. The change (after vs. before treatment) in *LINE-1* methylation directly correlated with the presence of *MTHFR* 677T allele, average daily folate intake, and the change in serum low-density lipoprotein cholesterol but inversely correlated with the change in serum triacylglycerols ($R = 0.72$, $R^2 = 0.52$, adjusted $R^2 = 0.36$, $P = 0.03$).

The current results imply potential cardioprotective effects of habitual polyphenol-rich aronia juice consumption achieved through the modifications of DNA methylation pattern and PUFAs in subjects at CVD risk, which should be further confirmed. Hence, the precision nutrition-driven modulations of both DNA methylation and PUFA profile may become targets for new approaches in the prevention of CVD.

Keywords: *Aronia melanocarpa*, polyphenols, polyunsaturated fatty acids, *LINE-1,* methylation, peripheral blood leukocytes, cardiovascular risk

INTRODUCTION

Pathogenesis of human chronic diseases, such as cancer and cardiovascular disease (CVD), is related to aberrant global and locus-specific DNA methylation patterns (1, 2). Methylation of DNA, catalyzed by DNA methyltransferases (DNMTs), is one of the main epigenetic processes, which most commonly occurs at cytosine-guanine (CpG) dinucleotide clusters and results in downregulation of gene expression (3).

Various exogenous and endogenous factors modulate DNA methylation. Folate (vitamin B9) is found in a variety of plant foods and participates in one-carbon metabolism, resulting in the formation of S-adenosyl-methionine (SAM) that acts as a methyl group donor (4). Methylenetetrahydrofolate reductase (MTHFR) activation catalyzes the conversion of homocysteine to methionine, which is a direct precursor of SAM. The presence of T allele at a common C677T (Ala222Val) *MTHFR* gene polymorphic site is associated with a decreased activity of the enzyme, thus reducing the methyl group bioavailability and subsequently inhibiting the methylation of DNA (5).

Methylation of *Long Interspersed Nucleotide Element-1* (*LINE-1*), the largest member of the *LINE* retrotransposon family of DNA repeat elements (comprising about 17% of the human genome) (6), is considered a surrogate marker of global DNA methylation (7, 8). Methylation status of *LINE-1* in peripheral blood leukocytes is associated with metabolic parameters, such as blood glucose and lipid profiles (9, 10), and global DNA methylation in these cells has been found to depend on demographic and lifestyle factors: age, gender, blood pressure, body mass index (BMI), and dietary habits, including polyunsaturated fatty acid (PUFA) supplementation (9, 11–14). In addition, the studies have reported that the inhibition of DNA methylation may prevent the progression of cancer and CVD (1, 2, 15). Hence, the methylation status of *LINE-1* in peripheral blood leukocytes represents a potential CVD biomarker, and certain lifestyle factors, like diet, may modulate cardiovascular risk by influencing alterations in DNA methylation patterns, thus emphasizing the importance of precision nutrition strategies in the prevention of CVD.

Regular consumption of *Aronia melanocarpa* juice is associated with CVD beneficial effects in human studies (16–18) and animal models (19). Aronia is rich in bioactive polyphenols (20) and, compared with other berry fruits, contains higher levels of polyphenolic compounds (21). Of note, dietary polyphenols are reported to favorably modulate DNA methylation status by inhibiting DNMT activity (22). In addition, aronia polyphenols affected the composition of plasma PUFAs in individuals at CVD risk (23). To the best of our knowledge, no study has examined the relation between polyphenols contained in aronia berry and DNA methylation status. Therefore, the present parallel, placebo-controlled, 4-week study aimed to investigate the interconnection between daily consumption of polyphenol-rich aronia juice and *LINE-1* methylation in peripheral blood leukocytes and plasma fatty acids, in subjects at moderate CVD risk. We also examined whether folate intake and *MTHFR* C677T gene variant, as well as the anthropometric and metabolic parameters, modulated the *LINE-1* methylation levels upon the consumption of polyphenol-rich aronia juice.

MATERIALS AND METHODS

Study Design, Study Subjects, and Intervention Treatments

The current research represents a parallel, placebo-controlled, 4-week nutritional intervention. The research is designed as a substudy, forming part of the original clinical study that lasted 6 months, and is registered at ClinicalTrials.gov as NCT02800967. The original study included nonsmoking adults at moderate CVD risk, defined as the presence of at least one of the following: increased BMI (25–30 kg/m^2), central obesity (waist circumference $\geq$ 80 cm for women and $\geq$ 94 cm for men), and high normal blood pressure [systolic/diastolic blood pressure (SBP/DBP) $>$ 120/80, $\leq$ 139/89 mm Hg]. Exclusion criteria were the presence of chronic disease, self-reported allergy to polyphenols, pregnancy, lactation, blood donation 16 weeks before the start of the study, and parallel participation in another clinical trial. During the course of the study, the participants were asked to follow their habitual diet, including study treatments as part of it, and to do their usual physical activity. They were also asked to strictly refrain from berries and berry products and to avoid excess amounts of polyphenol-rich food, inclusive of olive oil, green tea, and nuts.

Abbreviations: AA, arachidonic acid; BMI, body mass index; COMT, catechol-O-methyltransferase; CVD, cardiovascular disease; DBP, diastolic blood pressure; DNMTs, DNA methyltransferases; EDTA, ethylenediaminetetraacetic acid; EPA, eicosapentaenoic acid; Glu, glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; *LINE-1*, *Long Interspersed Nucleotide Element-1*; MTHFR, methylenetetrahydrofolate reductase; MUFA, monounsaturated fatty acid; PCR, polymerase chain reaction; PUFA, polyunsaturated fatty acid; SAH, S-adenosyl-L-homocysteine; SAM, S-adenosyl-methionine; SBP, systolic blood pressure; SFA, saturated fatty acid; TAG, triacylglycerols; TC, total cholesterol; WC, waist circumference.

For purposes of the current substudy, additional *a posteriori* inclusion criterion was the presence of dyslipidemia defined as either elevated serum total cholesterol (≥5.2 mmol/l), elevated low-density lipoprotein cholesterol (LDL-C) (≥3.4 mmol/l), or elevated serum triacylglycerols (≥1.7 mmol/l). In order to address the objectives of the substudy, 54 subjects were included, whose *LINE-1* methylation status in peripheral blood leukocytes and *MTHFR* C677T gene variant were additionally analyzed. They received either original polyphenol-rich *A. melanocarpa* juice (assigned as AMJ treatment, $N = 34$ subjects) or polyphenol-free placebo beverage (assigned as PLB treatment, $N = 20$ subjects). The research flow diagram is shown in **Figure 1**.

The original polyphenol-rich aronia juice used in the study was registered at the Serbian Ministry of Health as a dietary supplement and was donated from "Nutrika" LTD (Belgrade, Serbia). The placebo drink was made to match the appearance, taste, and nutritional composition of the original aronia juice, but without bioactive polyphenols. It was previously reported that the daily amount of 100 ml of the placebo was safe for human consumption (24). Total polyphenols in the original aronia juice were determined using a modified Folin-Ciocalteu method (25), and it was found that the consumed daily amount of 100 ml of the juice contained 1,177.11 mg of gallic acid equivalents of polyphenols. Proanthocyanidins were highly represented among the contained polyphenols, as demonstrated in the analysis of the composition of herein used aronia juice (24). The study compliance was assessed according to returned empty bottles of intervention drinks and self-reports.

The study protocol adhered to the regulations of the 1975 Declaration of Helsinki and was approved by Clinical Hospital Centre Zemun, Belgrade, Serbia, Ethics Committee Approval, No: 2125, 2013. The written informed consent was given by all participants before the commencement of the study.

Sample Collection

Study participants were instructed for overnight fasting, and venous blood was collected the next morning between 8 and 9 AM into the sample tubes for serum and ethylenediaminetetraacetic acid (EDTA)-evacuated tubes. The sample collection was done at two time points, before and after the corresponding 4-week treatments (AMJ and PLB).

Assessment of Study Variables

For the assessment of baseline dietary intake, trained staff conducted structured interviews with study subjects and collected data by using the food frequency questionnaire and repeated 24-h dietary recalls. The subjects were assisted with 125-item photo-booklet containing simple foods and composite dishes (26). Data from dietary recalls were analyzed using the nutritional platform for comprehensive diet evaluation (26, 27).

Bio-impedance analyzer TANITA UM072 balance (TANITA Health Equipment H.K. Ltd, Hong Kong, China) was used for the determination of body weight. Total cholesterol, high-density lipoprotein cholesterol (HDL-C), LDL-C, triacylglycerols, and glucose from serum were determined by Roche Diagnostics Kits, using the chemistry analyzer (Cobas c111, Roche Diagnostics, Basel, Switzerland).

The procedure of determination of plasma phospholipid fatty acids composition was previously described in detail by Pokimica et al. (23). Briefly, plasma lipids were extracted using a 2:1 chloroform–methanol mixture with 2,6-di-tert-butyl-4-methylphenol (10 mg/100 ml) added as an antioxidant. Phospholipids were separated from other lipid subclasses on a thin-layer chromatography silica plate using a mixture of petroleum ether, diethyl ether, and acetic acid (87:12:1). The methyl esters of fatty acids were obtained by transmethylation with 2M sodium hydroxide in methanol, at 85°C for 1 h, and with 1M sulfuric acid in methanol, at 85°C for 2 h. The mixture was cooled down to room temperature and centrifuged at 1,860 × g for 15 min, and the upper phase was dried up using a stream of nitrogen. The fatty acid methyl esters were recovered in hexane and separated by using RTX 2330 capillary column (60 m × 0.25 mm × 0.2 μm; Restek, Bellefonte, PA, United States), on Shimadzu GC-2014 gas chromatograph (Kyoto, Japan) with flame ionization detector. The flow of air, hydrogen, and helium (carrier gas) was 320, 30, and 5 ml/min, respectively. The temperature of the detector was 260°C and of the injection port 220°C. The initial column temperature of 140°C was maintained for 5 min and then increased to 220°C at a rate of 3°C/min, and 220°C was kept for 20 min. Fatty acids were identified by comparing peak retention times with calibration mixtures (PUFA-2, Supelco, Bellefonte, PA, United States, and 37 FAMEs mix, Sigma Chemical Co., St. Louis, MO, United States). The amounts of individual fatty acids in plasma phospholipids were presented as the relative area percentage of the total pool of detected fatty acids.

Analysis of *LINE-1* Methylation

The fasting peripheral blood samples of each participant, collected with EDTA before and after the corresponding treatments, were used for the total leukocyte genomic DNA isolation, by a phenol–chloroform extraction-based method (28). The quantity of DNA was estimated with BioSpecnano spectrophotometer (Shimadzu Biotech, Kyoto, Japan).

Five hundred (500) ng of each sample genomic DNA was used for sodium bisulfite conversion with EpiTect® Bisulfite kit (Qiagen, Hilden, Germany). Bisulfite-converted DNA was then used for the quantification of *LINE-1* methylation by MethyLight real-time PCR. To normalize DNA input, an Alu sequence-based real-time PCR control reaction was performed in parallel with each *LINE-1* reaction. The method was previously developed and validated, its precision and reproducibility were confirmed (8, 29), and the currently used protocol was described in detail by Božović et al. (29).

MTHFR Genotyping

The genotypes of *MTHFR* C677T variant were determined from the total leukocyte genomic DNA samples, by PCR–restriction fragment length polymorphism method reported in Coppedè et al. (30), in all subjects included in the methylation analysis.

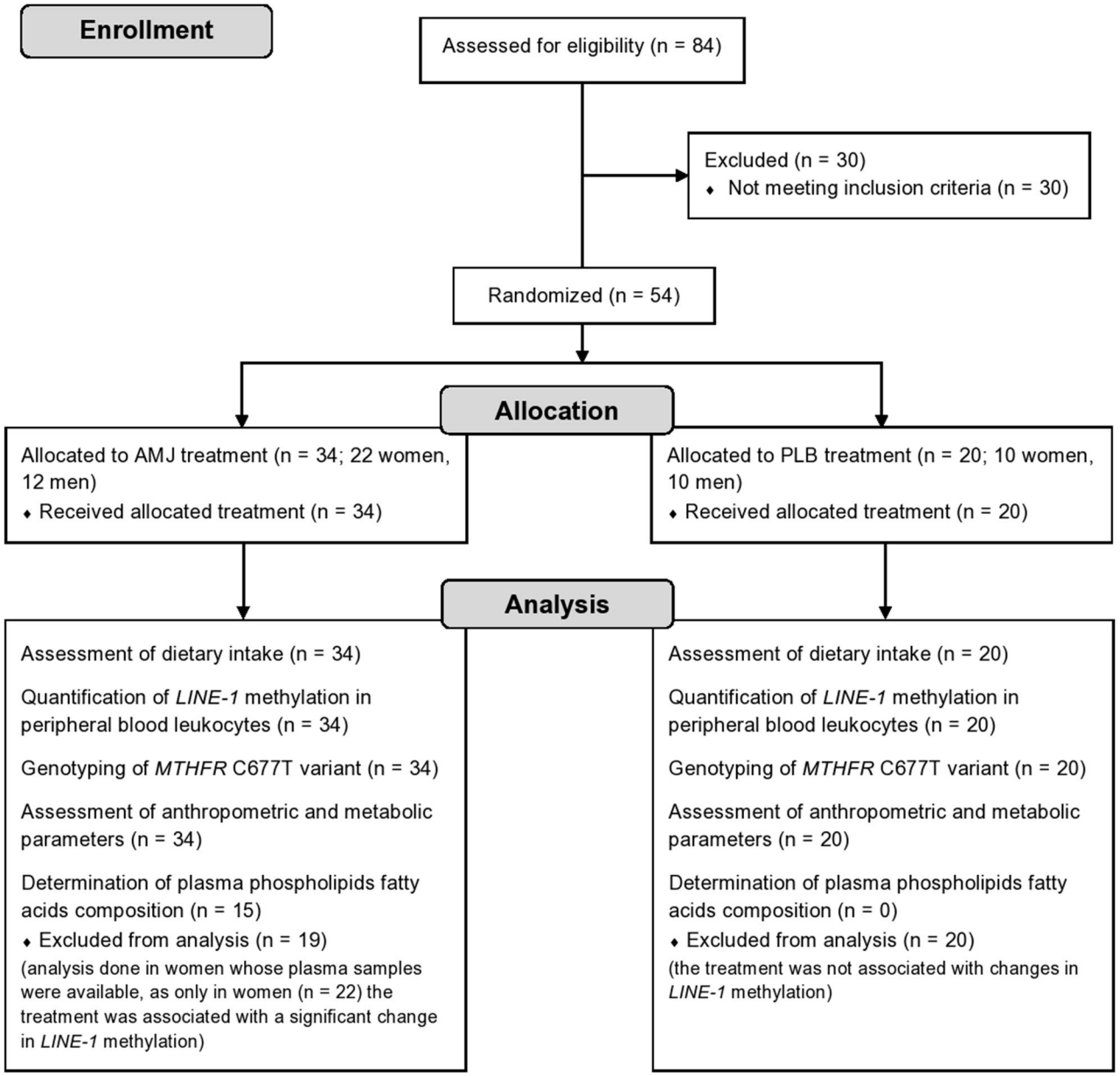

FIGURE 1 | Study flow diagram. *N*, number of subjects; AMJ, polyphenol-rich *Aronia melanocarpa* juice treatment; PLB, polyphenol-free beverage; placebo, treatment.

Statistical Analyses

The comparisons of *MTHFR* C677T genotype frequencies between groups and estimation of deviations from Hardy–Weinberg equilibrium were done with Fisher's exact test and the χ^2 test. Due to a small number of the TT genotype carriers ($N < 3$) in at least one of the analyzed groups, we applied the dominant genotype model, CT + TT vs. CC, instead of the additive. Depending on the distribution of continuous variables (age, average daily energy intake, average daily folate intake, SBP, DBP, waist circumference, BMI, serum glucose, triacylglycerols, total cholesterol, HDL-C, LDL-C, plasma fatty acids, and *LINE-1* methylation levels), tested by the Shapiro–Wilks test, the appropriate parametric or non-parametric tests were performed. Between-group comparisons were done using a *t*-test or the Mann–Whitney U-test, while within-group comparisons (after vs. before each treatment) were done by *t*-test for dependent samples or the Wilcoxon matched-pairs test, for normally and non-normally distributed data, respectively. The treatment effects were investigated in the complete sample and in women and men, separately. The relationship between *LINE-1* methylation and the anthropometric and metabolic parameters (age, average daily folate intake, average daily energy intake, SBP,

TABLE 1 | Baseline characteristics of the study participants.

	AMJ	PLB	*P*
No. of subjects	34	20	
Age (years)	41.1 ± 6.6	38.5 ± 6.8	0.17
Average daily energy intake (kCal)	2075 ± 555	1745 (1168–3385)[#]	0.29
Average daily folate intake (μg)	239.7 ± 75.2	203.3 (98.2–461.5)[#]	0.54
SBP (mmHg)	118.3 ± 13.4	120.6 ± 16.2	0.57
DBP (mmHg)	73.2 ± 10.0	74.0 ± 13.7	0.82
Waist circumference (cm)	89.8 ± 10.9	92.2 ± 15.7	0.51
BMI (kg/m^2)	27.4 ± 3.5	27.8 ± 6.2	0.78
Glucose (mmol/l)	4.8 (3.8–7.2)[#]	5.1 ± 0.8	0.27
TAG (mmol/l)	0.9 (0.4–4.1)[#]	0.9 (0.5–5.0)[#]	0.72
TC (mmol/l)	5.5 ± 1.1	5.2 ± 1.0	0.27
HDL-C (mmol/l)	1.5 (0.8–2.9) [#]	1.6 ± 0.4	0.54
LDL-C (mmol/l)	3.5 ± 0.9	3.3 ± 1.0	0.56
[†]***MTHFR* C677T genotype, No. (%)**			
CC	15 (44.1)	7 (35.0)	
CT + TT	19 (55.9)	13 (65.0)	0.58

Continuous variables with a normal distribution are presented as mean ± standard deviation; [#]continuous variables with a non-normal distribution are presented as median (minimum–maximum); P-values related to the between-treatment difference in the variable distribution, significant difference at $P < 0.05$.
[†]The observed MTHFR C677T genotype frequencies are consistent with Hardy–Weinberg equilibrium, in each analyzed group (χ^2 test, $P > 0.05$).
AMJ, polyphenol-rich Aronia melanocarpa juice treatment; PLB, polyphenol-free beverage, placebo treatment; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; TAG, triacylglycerols; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

DBP, waist circumference, BMI, serum glucose, triacylglycerols, total cholesterol, HDL-C, LDL-C, and plasma fatty acids) was investigated by regression analyses. T-test/Mann–Whitney *U*-test was used to examine the association between *LINE-1* methylation and *MTHFR* genotypes, by a genotype model, CT+TT vs. CC. In the statistical tests, $P < 0.05$ were considered statistically significant. The statistical analyses were performed using Statistica 8.0 software package (StatSoft, Inc. 1984-2007).

RESULTS

Baseline Characteristics of the Study Subjects and Effects of Polyphenol-Rich Aronia Juice Consumption on Cardiometabolic Parameters

Baseline characteristics, determined before each of the two treatments, with polyphenol-rich aronia juice (AMJ) and polyphenol-free beverage (placebo, PLB), for the whole treated study groups are shown in **Table 1**. For the treated groups separated by gender, the baseline characteristics are shown in **Supplementary Table 1**. There were no significant differences in the baseline parameters (AMJ vs. PLB) within any of the groups (whole sample, women and men), except for serum total cholesterol in men [AMJ vs. PLB = 5.9 ± 1.0 vs. 4.9 ± 1.2 mmol/l; P (*t*-test) = 0.04] (**Table 1** and **Supplementary Table 1**).

Within-group comparisons of treatment effects (after vs. before treatment) toward anthropometric and metabolic parameters are presented in **Supplementary Table 2**. Even though significant differences were found for DBP, waist circumference, glucose, and HDL-C [P (*t*-test for dependent samples/Wilcoxon matched pairs test) < 0.05] (**Supplementary Table 2**), only values for waist circumference dropped out of the clinical ranges.

Effects of Polyphenol-Rich Aronia Juice Consumption on *LINE-1* Methylation

The comparisons of *LINE-1* methylation levels after vs. before polyphenol-rich AMJ treatment and polyphenol-free beverage (placebo) treatment (PLB) are presented in **Figure 2**. Although the AMJ treatment tended to decrease *LINE-1* methylation levels, there was no significant difference in the whole study group [after vs. before treatment = 97.93 (93.58–99.84)% vs. 98.16 (93.58–99.91)%; P (Wilcoxon matched pairs test) = 0.14] (**Figure 2A**). We found a significant decrease in *LINE-1* methylation after the AMJ treatment in women (after vs. before treatment = 97.54 ± 1.50 vs. 98.39 ± 0.86%; P (*t*-test for dependent samples) = 0.01) (**Figure 2C**).

In women, where we demonstrated that polyphenol-rich AMJ treatment significantly changed *LINE-1* methylation, the multiple regression model showed significant effects of average daily folate intake, *MTHFR* C677T genotypes (model CT+TT vs. CC), change (Δ, after vs. before treatment) in triacylglycerols, and change (Δ) in LDL-C on the change (Δ) in *LINE-1* methylation levels, while controlling for age ($R = 0.72$, $R^2 = 0.52$, adjusted $R^2 = 0.36$, $P = 0.03$) (**Table 2**).

Impact of Polyphenol-Rich AMJ Treatment on the Profile of Plasma Fatty Acids

Composition of plasma phospholipids fatty acids was analyzed in women who consumed polyphenol-rich aronia juice, as,

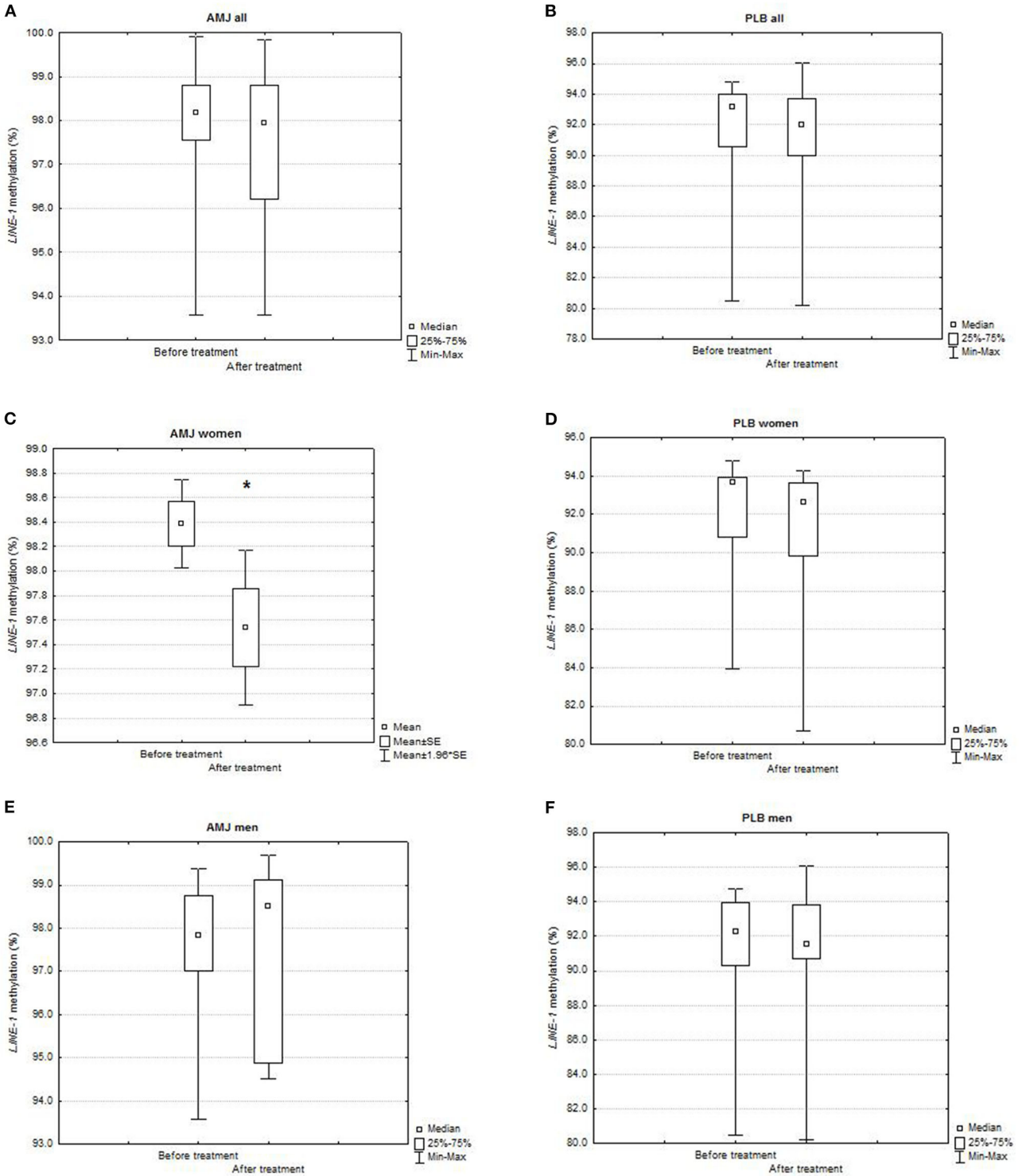

FIGURE 2 | Comparison of *LINE-1* methylation levels (%) before and after treatment in: **(A)** all subjects on AMJ treatment ($N = 34$; Wilcoxon matched pairs test, $P = 0.14$), **(B)** all subjects on PLB treatment ($N = 20$; Wilcoxon matched pairs test, $P = 0.10$), **(C)** women on AMJ treatment ($N = 22$; *t*-test for dependent samples, $P = 0.01$), **(D)** women on PLB treatment ($N = 10$; Wilcoxon matched pairs test, $P = 0.11$), **(E)** men on AMJ treatment ($N = 12$; Wilcoxon matched pairs test, $P = 0.53$), **(F)** men on PLB treatment ($N = 10$; Wilcoxon matched pairs test, $P = 0.65$). AMJ, polyphenol-rich *Aronia melanocarpa* juice treatment; PLB, polyphenol-free beverage, placebo treatment; mean represents normally distributed *LINE-1* methylation levels (%); median represents non-normally distributed *LINE-1* methylation levels (%); SE, standard error; statistical significance at $P < 0.05$ (*); *N*, number of subjects.

TABLE 2 | Multiple regression analysis for the change (Δ) in *LINE-1* methylation levels (%) in women who consumed polyphenol-rich *Aronia melanocarpa* juice (AMJ treatment, $N = 22$).

Predictor variable	β	Std. error of β	*P*
Age (years)	−0.40	0.21	0.08
Average daily folate intake (μg)	0.49	0.19	**0.02**
MTHFR C677T genotypes (model CT + TT vs. CC)	0.50	0.19	**0.02**
Δ Triacylglycerols (mmol/l)	−0.53	0.20	**0.02**
Δ Low-density lipoprotein cholesterol (mmol/l)	0.46	0.19	**0.03**

Regression summary: $R = 0.72$, $R^2 = 0.52$, adjusted $R^2 = 0.36$, $P = 0.03$.
Δ: change, after treatment value—before treatment value; significant difference at $P < 0.05$ (bolded text).

only in these subjects, aronia juice consumption was associated with a significant change in *LINE-1* methylation levels. Profiled fatty acids are shown for 15 women treated with aronia juice (**Supplementary Table 3**), whose plasma samples were available for the analysis, representing a part of previously published data (23). Overall baseline plasma phospholipid fatty acids comprised ∼48% SFAs, ∼11% MUFAs, and ∼41% PUFAs. Among the most abundant fatty acids were palmitic acid (∼30.5%) > linoleic acid (∼23%) > stearic acid (∼17.5%) > arachidonic acid (AA) (∼11%), while least quantitatively represented fatty acids were eicosapentaenoic acid (EPA) and adrenic acid (∼0.3–0.4%). Within PUFAs, the proportion of omega-6 was ∼10-fold higher than that of omega-3 (∼37.4 vs. ∼3.6%). Comparisons of fatty acid levels after vs. before the AMJ treatment in female subjects revealed a significant decrease in AA/EPA ratio (29.17 ± 15.21 vs. 38.42 (25.96–89.58), respectively; Wilcoxon matched pairs test, $P = 0.02$), as AA levels significantly decreased (10.18 ± 2.16 vs. 11.16 ± 2.61 %, respectively; *t*-test for dependent samples, $P = 0.01$) and EPA levels significantly increased (0.43 ± 0.20 vs. 0.28 ± 0.12 %, respectively; *t*-test for dependent samples, $P = 0.04$) (**Supplementary Table 3**). Along with AA, dihomo-γ linolenic acid also decreased following the treatment in women [2.34 (1.59–4.33)% vs. 2.89 ± 1.04%, respectively; Wilcoxon matched pairs test, $P = 0.02$]. The same type of change was observed for adrenic acid [0.34 ± 0.12% vs. 0.40 (0.23–0.98)%, respectively; Wilcoxon matched pairs test, $P = 0.003$] (**Supplementary Table 3**).

The changes (Δ, after vs. before treatment) in levels of individual fatty acids did not significantly correlate with a change (Δ) in *LINE-1* methylation levels, with the exception of adrenic acid ($R = 0.60$, $R^2 = 0.36$, adjusted $R^2 = 0.31$, $P = 0.02$). Multiple regression models revealed no significant relations between a change (Δ) in *LINE-1* methylation levels and changes (Δ) in levels of each class of fatty acids: SFAs ($R = 0.53$, $R^2 = 0.28$, adjusted $R^2 = 0.17$, $P = 0.13$), MUFAs ($R = 0.43$, $R^2 = 0.18$, adjusted $R^2 = -0.04$, P = 0.51), and PUFAs ($R = 0.86$, $R^2 = 0.74$, adjusted $R^2 = 0.49$, $P = 0.09$).

DISCUSSION

The main finding of this study is that the 4-week daily consumption of *A. melanocarpa* juice decreased the *LINE-1* methylation levels in peripheral blood leukocytes in women with CVD risk factors, including overweight and dyslipidemia. Daily treatment with Aronia melanocarpa juice, assigned as AMJ treatment, contained 1.18 g of total polyphenols. This amount corresponds to an estimated average daily intake of about 1 g of total polyphenols from the dietary sources (31), provided that, in the composition of aronia polyphenols, the most abundant are anthocyanins, proanthocyanidins, hydroxycinnamic acids, and flavonols (32), which is in compliance with the findings of a previous study that characterized the composition of aronia juice used in the current research (24). Confirmation that the currently demonstrated significant reduction in *LINE-1* methylation is due to the impact of polyphenols, rather than of other bioactive components of aronia juice, lies in the fact that placebo treatment exerted no significant effects on *LINE-1* methylation. In line with this study, global DNA methylation levels in peripheral leukocytes were significantly reduced after 2-week-long consumption of polyphenol-rich cocoa product, in individuals at cardiovascular risk (13).

Herein observed decreased DNA methylation levels after the consumption of polyphenol-rich aronia juice may be attributed to the role of polyphenols as natural DNMT inhibitors (22). Namely, catechins and their metabolites, which belong to one of the main aronia polyphenol classes—proanthocyanidins (32), can increase the production of S-adenosyl-L-homocysteine (SAH), a potent inhibitor of DNMTs and a participant of the folate-methionine cycle, through the inhibition of catechol-O-methyltransferase (COMT)-mediated O-methylation of catechol substrates (33, 34). Among the main endogenous substrates for both liver and leukocyte, COMT is a catechol estrogen (33, 35), potentially explaining the finding of the present methylation analysis exclusively in women. In addition, catechins suppress MTHFR activity, hence blocking the folate-methionine cycle and reducing the methyl group bioavailability (36). The antioxidant and anti-inflammatory activities of polyphenol-rich aronia products, by which they contribute to cardiovascular protection (17, 37), might be attributed to the role of polyphenols as DNMT inhibitors. By reducing the activity of DNMTs, the prevalent aronia flavonol, quercetin, decreases the promoter methylation levels and activates the expression of target genes, such as a transcription regulator nuclear factor erythroid 2-related factor 2 (38), which is involved in the anti-inflammatory and antioxidant mechanisms in vascular cells and macrophages (38–40). Moreover, aronia polyphenols may be involved in the

inflammatory response by affecting the activity/production of DNMTs *via* cytokines, since it has been demonstrated that aronia anthocyanins affected the production of inflammatory mediators (37), and under the influence of IL-1, there have been changes in DNMT expression and genomic methylation in human cells *in vitro* (41).

With regard to our results, the proposed mechanisms of polyphenol-induced inhibition of DNA methylation (33, 34, 36) would depend on daily folate intake and *MTHFR* C677T gene variant. We found that the change (after vs. before the AMJ treatment) in *LINE-1* methylation levels in women correlated directly with average daily folate intake, and this is consistent with previously defined effects of folate on the modulation of DNA methylation (4). Concerning the effects of *MTHFR* C677T gene variant, our finding is in line with the study of Nojima et al. (42), which showed that the global methylation levels in peripheral blood leukocytes were significantly increased in carriers of the 677T allele. This was obtained when the *MTHFR* variant was analyzed in interaction with another factor that affected the methylation of DNA—an inflammatory marker blood concentration (42). Similarly, except with folate intake, our regression model denoted an interaction of the *MTHFR* variant with circulating LDL-C and triacylglycerol levels, as metabolic factors have been associated with *LINE-1* methylation (9, 10, 43). Namely, the current change in *LINE-1* methylation in women correlated positively with the change in LDL-C and negatively with the change in triacylglycerols, which should be pointed out along with the fact that our female subjects are dyslipidemic. So far, few studies have investigated the relationship between *LINE-1* methylation and lipid profile and yielded conflicting results (9, 10, 43), emphasizing the need for further research. The present positive correlation of peripheral blood leukocyte *LINE-1* methylation with circulating LDL-C levels is in line with two studies, which have investigated middle-aged subjects with cardiovascular risk factors and no evidence of CVD (10, 43). Since we have established this positive correlation between changes in *LINE-1* methylation and circulating LDL-C levels in subjects whose *LINE-1* methylation was significantly decreased, following the consumption of aronia juice, our finding may support the currently proposed cardioprotective action of polyphenol-rich aronia products attributed to the role of polyphenols as DNMT inhibitors. Accordingly, the same type of correlation would be expected between changes in *LINE-1* methylation and serum triacylglycerols levels, but we found them to correlate inversely. Still, findings regarding the link of *LINE-1* methylation with serum triacylglycerols are rather inconsistent, as one of the mentioned studies reported a positive correlation (10), while in the other there was no significant relation (43). With respect to pathogenesis of CVD and the proposed explanation, LDL-C was shown to cause vascular endothelial dysfunction in part by changing the DNMT activity and thus altering the DNA methylation, given that, in endothelial cells, LDL-C inhibited the transcription of a gene important in maintaining the endothelial function, *KLF2*, through the activation of DNMT1 (44).

Along with the investigated classical lipid parameters (circulating cholesterol and triacylglycerols), we examined plasma fatty acids profile in women who consumed polyphenol-rich aronia juice, since the treatment significantly changed the *LINE-1* methylation levels only in these individuals, and this change in *LINE-1* methylation was found to correlate with the changes in lipid parameters. We observed a significant reduction of the AA/EPA ratio due to a significant decrease in plasma AA and a significant increase in EPA levels, in target dyslipidemic female subjects who underwent AMJ treatment. Similarly, in a larger group of study participants at cardiovascular risk, from which our substudy group was sorted out, there had also been a significant AA/EPA reduction in aronia polyphenols consumers, noting that this reduction had been due to a significant decrease in AA, while EPA had not increased significantly (23). Furthermore, in a recent study that has investigated the link between *FADS2* gene variants, fatty acid metabolism, and aronia polyphenol intake in the overweight, there was a trend of AA/EPA reduction in individuals who consumed aronia juice, compared to placebo consumers (45). There has been evidence for an association between total plasma AA and ischemic stroke (46), and the increase in circulating EPA levels has been shown to have beneficial effects on cardiovascular health (47). Both findings (46, 47) are expected because the ratio of AA to EPA reflects regulatory mechanisms of the inflammatory process, as these two PUFAs compete for the conversion to two classes of bioactive eicosanoids: pro- and anti-inflammatory (48, 49). Hence, in addition to the well-known markers, such as circulating total cholesterol, LDL-C, and triacylglycerols, the AA/EPA ratio has recently been identified as a sensitive marker of cardiovascular risk, given that a lower AA/EPA ratio was associated with a decreased risk of coronary artery disease, acute coronary syndrome, myocardial infarction, stroke, chronic heart failure, and peripheral artery disease [reviewed in Davinelli et al. (50)]. Our female study subjects who consumed aronia juice had high baseline AA/EPA levels, indicating a chronic low-grade inflammation and an increased risk of cardiovascular events. Thus, a significant decrease in AA/EPA ratio following the AMJ treatment is in line with proposed anti-inflammatory and cardioprotective effects of aronia juice polyphenols, also supporting a suggested relation of these effects with the currently observed decrease in *LINE-1* methylation levels in women.

The findings of the present study should be interpreted in light of limiting factors, primarily a sample size. Nevertheless, the study design has strength regarding the measurement of *LINE-1* methylation levels both before and after each applied treatment, together with the corresponding anthropometric and metabolic parameters, allowing the accurate determination of treatment-dependent changes in parameter values. Another strength of the study is the use of a reliable food frequency questionnaire for the baseline dietary intake assessment with the subsequent analysis of data collected from 24-h dietary recalls, performed by using a validated nutritional platform for comprehensive diet evaluation. This allowed balancing of baseline dietary intake of the study subjects across each of the two interventional treatments, assuming that dietary intake did not change within the 4-week treatment period. Still, the possible confounding effects of temporal changes in dietary habits may not be excluded and represent a limiting factor of the study design. Another

limiting factor is a possible interindividual variability in response to polyphenol treatment, related to polyphenol bioavailability and other factors that fell beyond the scope of this study.

In conclusion, the main novelty brought by the present study is a change in *LINE-1* methylation levels after the 4-week habitual consumption of polyphenol-rich aronia juice, which indicates an impact of aronia polyphenols on DNA methylation. The current results suggest cardioprotective effects of aronia polyphenols in subjects at CVD risk, achieved through the modifications of *LINE-1* DNA methylation pattern and AA/EPA ratio. Our findings merit further investigation, particularly in view of the fact that the precision nutrition-driven modulations of both DNA methylation and PUFA profile may become pertinent targets for new approaches in the prevention and treatment of CVD.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Clinical Hospital Centre Zemun, Belgrade, Serbia. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LS performed the epigenetic, genetic and statistical analyses, interpreted the results, and wrote the manuscript. MZe was involved in the assessments of dietary intake, anthropometric and metabolic parameters, and the analysis of plasma fatty acids composition. AS, MZi, MZe, and LS designed the study. MZe, MZi, and AS revised the manuscript. MBu assisted with the epigenetic analysis. MBo was involved in laboratory work and sample collection. MG contributed to the conceptualization of the study. All authors read and agreed to the final 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.2021.689055/full#supplementary-material

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Characterization of Genetic Variants in the SLC5A5 Gene and Associations With Breast Milk Iodine Concentration in Lactating Women of African Descent: The NUPED Study

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Background: The sodium iodide symporter is responsible for the transfer of iodine into breast milk and is encoded for by the *SLC5A5* gene. The role of genetic variants in the *SLC5A5* gene locus in relation to the transfer of iodine from plasma into breast milk in healthy lactating individuals has, to our knowledge, not been explored.

Objective: To identify and characterize possible genetic variants of the SLC5A5 gene in women of African descent living in urban South Africa, and to study associations with breast milk iodine concentrations (BMIC) in lactating women.

Methods: This study is affiliated to the Nutrition during Pregnancy and Early Development (NuPED) cohort study ($n = 250$ enrolled pregnant women). In a randomly selected sub-sample of 32 women, the *SLC5A5* gene was sequenced to identify known and novel variants. Of the identified variants, genotyping of selected variants was performed in all pregnant women who gave consent for genetic analyses ($n = 246$), to determine the frequency of the variants in the study sample. Urinary iodine concentration (UIC) in spot urine samples and BMIC were measured to determine iodine status. Associations of SLC5A5 genetic variants with BMIC were studied in lactating women ($n = 55$).

Results: We identified 27 variants from sequencing of gene exomes and 10 variants were selected for further study. There was a significant difference in BMIC between the genotypes of the rs775249401 variant ($P = 0.042$), with the homozygous GG group having lower BMIC [86.8 (54.9–167.9) μg/L] compared to the (A) allele carriers rs775249401$_{(AG+AA)}$ [143.9 (122.4–169.3) μg/L] ($P = 0.042$). Of the rs775249401$_{(GG)}$, 49% had UIC <100 μg/L and 61% had BMIC <100 μg/L. On the other hand, 60% of the rs775249401$_{(AG+AA)}$ carriers had UIC <100 μg/L, and none had a BMIC <100 μg/L.

Conclusion: Our results suggest that A-allele carriers of rs775249401$_{(AG+AA)}$ are likely to have higher iodine transfer into breast milk compared to the homozygous GG counterparts. Thus, genetic variations in the SLC5A5 gene may play an important role in the transfer of iodine from plasma into breast milk and may partially explain inter-individual variability in BMIC.

Keywords: breast milk, iodine concentration, lactating women, *SLC5A5* gene, sodium iodide symporter, urinary iodine concentration

INTRODUCTION

The sodium iodide symporter (NIS) is an intrinsic plasma membrane glycoprotein mediating iodide uptake into thyroid follicular cells. The NIS protein consisting of 643 amino acids is encoded by the *SLC5A5* gene, which is located on the forward strand of chromosome 19 (19: 17,982,754–18,005,983, GRCh37.p12) with an open reading frame consisting of 1929 nucleotides arranged as 14 introns and 15 exons (1). The NIS plays a crucial role in iodine metabolism and thyroid regulation (2). Besides thyroid hormone synthesis, the NIS is expressed in breast tissue during late pregnancy and lactation and is responsible for the transfer of iodine from plasma into mammary epithelial cells of lactating breasts (3).

Breast milk is a crucial source of iodine for the breastfeeding infant (4). Thus, adequate breast milk iodine concentration (BMIC) is important for meeting the iodine requirements of infants. Maternal iodine intake, estimated by measuring urinary iodine concentration (UIC), is known to greatly influence BMIC. Therefore, lactating women with insufficient iodine intake reportedly excrete insufficient breast milk iodine to meet the infants' needs (4–6). BMIC of 92 to 150 μg/L have been suggested to provide sufficient iodine to infants (6–8), but consensus on a BMIC threshold to define adequate iodine nutrition has not yet been reached. However, mean and median BMIC values were shown to vary widely between areas, with values typically ranging from <50 μg/L in iodine-deficient areas (4) to 100–150 μg/L in areas of iodine sufficiency (7) but being as high as 150–180 μg/L in areas of adequate iodine supply (9, 10). In a previous cross-sectional study in a convenience sample of 100 lactating women living in a semi-urban area of South Africa, we observed a median BMIC of 179 (126–269) μg/L with large inter-individual variations (11).

Previous research has shown that there is a preferential fractional excretion of iodine in breast milk rather than urine among participants with poor iodine status living in iodine sufficient regions (12). Additionally, women from iodine-deficient regions showed a constant partitioning of iodine into breastmilk. As such, participants with suboptimal iodine status have been shown to present with adequate BMIC. This could be explained by a protective mechanism that allows for a steady supply of iodine to breastfed infants of lactating women with suboptimal iodine status (12, 13), and the NIS is likely to play a major role.

Since there are limited data available on the role of genetic variants in the *SLC5A5* gene in relation to breast milk iodine concentration, we aimed to characterize genetic variants in the *SLC5A5* gene of women of African descent living in urban South Africa. Further, we investigated the relationship between selected variants with breast milk iodine concentration in lactating women.

MATERIALS AND METHODS

Study Design and Site

The NuPED study was a prospective cohort study conducted in Johannesburg, South Africa from March 2016 to July 2018. The study protocol has be previously published (14). In brief, pregnant women ($n = 250$) were enrolled if they were between 18 and 39 years of age, <18 weeks gestational age, born in South Africa or a neighboring country, have lived in Johannesburg for at least 12 months, were able to communicate effectively in one of the local languages, non-smoking, and expecting a singleton. Pregnant women were excluded from participation if they reported use of illicit drugs, had a known non-communicable disease such as diabetes, renal disease, history of high blood cholesterol and hypertension, and had a known infectious disease such as tuberculosis or hepatitis, or known serious illness such as cancer, lupus or psychosis. HIV positive women were included in the study. Pregnant women were assessed at <18, 22, and 36 weeks gestation. Follow-up assessments in the women and their infants were performed at 6, 7.5, and 12 months after birth. Of the 250 enrolled women, a total of 98 mother-infant pairs participated in the 6-month follow-up.

In a randomly selected sub-sample of 32 women, the *SLC5A5* gene was sequenced to identify and characterize genetic variants. Of the identified variants, genotyping of selected variants was performed in all pregnant women enrolled in the NuPED study and who gave consent for genetic analyses ($n = 246$), to determine the frequency of the variants in the study sample.

This study was conducted according to the guidelines of the Declaration of Helsinki and all procedures involving human participants were approved by the Human Research Ethics Committees (HREC) of the North-West University (NWU-00186-15-A1 for the NuPED pregnancy phase, NWU-00049-16-A1 for the postnatal phase) and the University of the Witwatersrand, Johannesburg (M150968 and M161045). These studies were also reviewed by the Rahima Moosa Mother and Child Hospital (RMMCH) research review committee, the Gauteng Department of Health, and the Johannesburg Health

District's District Research Committee. Further ethical approval was granted for this sub-study by the North-West University HREC (NWU-00455-19-S1 for this study). All participants gave written informed consent.

Blood Sample Collection and Genetic Analysis

Genomic DNA Isolation and Next-Generation Sequencing

A venous blood sample was collected into trace element-free ethylenediaminetetraacetic acid (EDTA)-coated vacutainer tubes via venepuncture of the antecubital area of the arm. Blood samples were processed by centrifugation (at 2,000 rpm for 15 min) within 1 h after blood collection to separate plasma, red blood cells and buffy coat. The buffy coat was aliquoted and stored in a 1:1 vol: vol RNA*later* (Ambion, Thermo Fischer Scientific) on-site at −20°C for a maximum of seven days. The samples were then transported on dry ice to the Centre of Excellence for Nutrition (CEN) laboratories in Potchefstroom, South Africa and stored at −80°C for genomic DNA (gDNA) isolation.

Genomic DNA (gDNA) was isolated from buffy coat using the Maxwell® 16 instrument and Maxwell® 16 DNA Purification Kit (AS1010) (Promega Corporation) following the manufacturer's instructions. Quantification of gDNA was done using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fischer Scientific).

Massively parallel next-generation sequencing (NGS) was performed using the Ion Torrent platform on the subset of 32 randomly selected samples. A custom Ion Ampliseq panel was designed with the online Ampliseq designer (https://ampliseq.com/, last accessed July 2020). This customized panel included the *SLC5A5* gene locus spanning a target region size of 20.83 kbp with 20 amplicons covering 100% of the targeted sequence. Library preparation was performed on the Ion Chef® as per the manufactures specifications. A total of 10 ng gDNA (0.67 ng/μl) was used as input volume for library preparation. Library and template preparation were done with the Ion AmpliSeq™ Kit for Chef DL8 for 32 reactions (Cat number: A29024,) and the Ion 510™ and Ion 520™ and Ion 530™ Kit—Chef (Cat number A34019), respectively. The entire coding region of each selected gene, including flanking regions of introns-exons was sequenced according to 200bp chemistry, using an Ion 530™ Chip Kit-4 Reactions (Cat number: A27763) and the Ion S5™ System (Cat number: A27212, ThermoFisher, MA, USA).

Data analysis of raw reads obtained from the Ion S5™ System was done with the Torrent Suite (v.5.8). The fastq files were uploaded to the sequence reads archive Bioproject PRJNA735618. The primary analysis included signal calling and base-calling. Quality control of the bases was filtered according to a Phred quality score of 20, depth > 10 and quality score of >500 frets were met. The sequence files were aligned against Genome Reference Consortium Human Build 37 (hg19), followed by coverage analysis and variant calling using the coverage analysis and variantCaller plugins from the Torrent Suite, respectively. Secondary data analyses of the variant caller files were annotated, filtered and mined following an in-house pipeline (15).

Variant Selection, Genotyping, and Quality Assessment

In the subset of 32 sequenced samples, variants that passed the quality control assessment were considered for validation in the entire sample set using the iPLEX® MassARRAY system from Agena Bioscience™. IPLEX assays were designed and analyses were performed by the service provider Inqaba Biotech (Inqaba Biotechnology Pretoria South Africa). Assays were designed using the Assay Design Suite (ADS) software and dbSNP for metadata. gDNA was amplified in 96 microtiter plates using iPLEX reagent kits and a nano dispenser RS1000 was used to transfer samples from microtiter plates to a SpectroCHIP® array. Data were obtained from the SpectroCHIP® array using the MassARRAY® analyser. Reports were automatically generated by Typer (Company). Genotype calls were made in real-time during MALDI-TOF analysis and data was automatically saved to the MassARRAY database. Variants were assessed for quality, and tested for adherence to Hardy-Weinberg equilibrium (HWE) (16) by using Haploview and modified Pearson chi-square (χ^2) test. Adherence to HWE was set at $P < 0.001$.

Urine Sample Collection and Iodine Analysis

From the 98 women participating in the 6-month follow-up, we collected a midstream spot urine sample (10–40 ml) into clean polystyrene cups between 07:00 and 12:00 noon, and approximately 5 ml was decanted into iodine-free screw-capped cups. The research team ensured that the urine samples were not used for any routine assessments using dipsticks (potential contamination with iodine). Samples were aliquoted and stored on-site at −20°C for a maximum of 7 days. Thereafter, samples were transported on dry ice to the CEN laboratories, for storage at −80°C until analysis.

Urinary iodine concentration (UIC) in spot urine samples was measured in duplicate using the Pino modification of the Sandell-Kolthoff reaction with spectrophotometric detection at CEN laboratories (17, 18). All analyses were done using nanopure grade water and all laboratory glassware and plasticware were acid washed before use. Internal and external controls were used to ensure the quality of the analysis. Iodine concentrations in spot urine samples are expressed as median concentrations (μg/L). A UIC cut-off of <100 μg/L was used to indicate insufficient iodine intake in lactating women (19).

Breast Milk Sample Collection and Iodine Analysis

From 58 lactating women who participated in the 6-month follow-up, a breast milk (foremilk) sample (≈5 ml) was collected by manual expression into an iodine-free screw-capped cup before feeding the infant. Iodine concentrations in breast milk (in μg/L) were measured using a multi-collector inductively coupled plasma mass spectrometer [MC-ICP-MS (Finnigan NEPTUNE, Thermo Scientific™ Waltham, MA, USA)] as described by Dold

et al. (10). A BMIC cut-off of <100 μg/L was used to indicate inadequate maternal iodine intake (20).

Iodine Excretion Calculations

Individual UIC and BMIC measures were used to calculate the estimated daily iodine excretion through the urine and breast milk by assuming a total daily urine volume of 1.5 L (12, 21, 22) and breast milk volume of 0.78 L (12, 22). Estimated total daily iodine excretion was calculated as the iodine excretion in urine added to the iodine excretion in breast milk (12). Furthermore, fractional iodine excretion in urine and breast milk as percentages of estimated total daily iodine excretion were also calculated (12, 22).

Statistical Analyses

Raw data were captured in Microsoft Access and 20% of all data were randomly checked for correctness. All genetic, UIC and BMIC, data were captured in Excel Windows XP (Microsoft, Seattle, WA, USA). Data processing and statistical analysis of data were performed using SPSS software (SPSS Inc, Chicago, IL, USA).

Data were tested for normality using the Kolmogorov-Smirnov test. UIC and BMIC data were log-transformed for further analysis. For UIC, values above the cut-off indicative of excessive iodine intake (UIC >500 μg/L) were considered outliers. These UIC outliers (n = 2) were excluded from the analysis because high intakes of iodine have previously been reported to lead to improved BMIC in an individual that harbored a *SLC5A5* variant associated with the lower transfer of iodine into breast milk (23). Normally and non-normally distributed data are expressed as means ± standard deviation (SD) and medians (25^{th} percentile, 75^{th} percentile), respectively. Categorical data are expressed as frequencies and percentages.

TABLE 1 | Genetic variants identified with next generation sequencing.

	Reference ID	Location bp[a]	Reference allele[a]	Alternative allele[a]	Exon[a]	Variant type	Minor allele frequency (MAF)
Annotated variants							
1	rs371875541	17983314	G	T	1/15[b]	synonymous	0.031
2	rs775249401	17985454	G	A		intron	0.125
3	rs55910163	17994635	C	T		intron	0.188
4	rs866765676	17982992	C	T	1/15	5′ UTR	0.031
5	rs73518702	17983105	C	A	1/15	5′ UTR	0.188
6	rs575147988	17983038	C	T	1/15	5′ UTR	0.063
7	rs34850953	17983503	G	T		intron	0.125
8	rs150814476	17988717	C	G		intron	0.125
9	rs116154266	17983093	C	T	1/15	5 prime UTR	0.063
10	rs8103545	17986763	C	T	5/15	Splice site	0.188
11	rs35209536	17988794	C	T	7/15	synonymous	0.188
12	rs73520743	17994573	A	C	11/15	synonymous	0.313
13	rs112076606	17994594	A	G		intron	0.281
14	rs73520745	17994836	G	A	12/15	missense	0.188
15	rs4808708	18001686	G	A		intron	0.281
16	rs4808709	18001839	A	G		intron	0.438
17	rs112077649	17983075	C	T	1/15	5′ UTR	0.094
18	rs77947605	17994644	C	T		intron	0.188
19	rs118133504	17983059	C	T	1/15	5′ UTR	0.031
20	rs113740966	17985371	AT	A		intron	0.031
21	rs369522814	17994641	C	G		intron	0.063
22	rs121909177	17999206	C	T	13/15	synonymous	0.031
23	rs7255301	17984834	G	A		intron	0.313
24	rs200868271	17994619	A	C		intron	0.031
25	rs8108188	17988825	T	G	7/15	missense	0.031
26	rs190567881	17988721	C	G		intron	0.031
Novel variants							
1		17983034	C	T[c]	1/15	5′ UTR	0.188
2		17999293	G	C[c]		intron	0.031
3		17999295	T	A[c]		intron	0.031

[a]*Source: Ensembl release 96—April 2019, retrieved 03 April 2019 (24),* [b]*Exon number in which the single nucleotide is found out of the 15 exons of the SLC5A5 gene.* [c]*Alternative allele as observed in our population.*

Participants were stratified according to UIC categories (UIC <100 μg/L and UIC ≥100 μg/L) or BMIC categories (BMIC <100 μg/L and BMIC ≥100 μg/L). The between-group analyses were performed using the Mann Whitney U test. Overlaid scatterplots were used to depict the relationship between total daily iodine excretion, fractional iodine excretion in breast milk and fractional iodine excretion in urine. Unadjusted general linear models were performed to compare UIC and BMIC between genetic variants with the recessive genetic model (GG vs. GA + AA) as categorical variables. For the significant models, effect sizes were calculated using Cohens' *d* and partial eta squared. Significance was set at $p < 0.05$.

RESULTS

Characterization of the Genetic Variants in the SLC5A5 Gene Locus

Genomic DNA was isolated from 246 samples and the *SLC5A5* gene of 32 randomly selected samples were sequenced following a targeted gene sequencing approach. Variants were quality controlled and 27 genetic variations passed the quality control assessment. Of the 27 variants, 26 had known annotation and one was novel located in the 5' untranslated region (UTR). Of the annotated variants, six were coding and 13 were in intronic regions, whereas the remaining seven variants were located in the 5' UTR (**Table 1**). Variants were inspected for possible functionality based on genomic position as well as association with regulatory sites and or available literature. Ten variants ($rs121909177_{(C/T)}$, $rs4808708_{(G/A)}$, $rs7255301_{(G/A)}$, $rs73520743_{(A/C)}$, $rs112076606_{(A/G)}$, $rs73520745_{(G/A)}$, $rs775249401_{(G/A)}$, $rs34850953_{(G/T)}$, $rs8103545_{(C/T)}$ and the novel variant) were validated in all 246 samples (see **Table 2**). All variants adhered to Hardy-Weinberg Equilibrium (HWE). The minor allele frequencies (MAF) of the variants studied in the lactating women ($n = 55$) compared well with the MAF of the total group. In the total group of pregnant women ($n = 246$), none of the women were homozygous for the alternative alleles for $rs121909177_{(C/T)}$, $rs73520745_{(G/A)}$, $rs73520743_{(A/C)}$, and $rs8103545_{(C/T)}$. In addition, the lactating women had no homozygosity for the alternative alleles of $rs7255301_{(G/A)}$ and $rs34850953_{(G/T)}$ variants too. The total study sample ($n = 246$) was monomorphic for the CC genotype of the novel variant (19: 17983034) and the lactating women all harbored the CC genotype for $rs121909177_{(C/T)}$ (**Table 2**).

Participant Characteristics

A total of 246 women from the NuPED study consented to participate in the genetic study of which 98 mothers and their infants were assessed at 6-months postpartum. Of these, 58 women indicated to breastfeed their infants and provided a breastmilk sample. Characteristics of the women who were lactating ($n = 58$) and non-lactating ($n = 40$) at 6 months postpartum are given in **Table 3**. The two groups were similar in age, height, weight, BMI, MUAC and UIC (**Table 3**). The median UIC of all women ($n = 98$) was 104.7 (66.2–154.0) μg/L, whereas the lactating and non-lactating women had a median UIC of 95 (64–146) μg/L and 122 (73–207) μg/L, respectively ($P = 0.259$). The lactating women had a median BMIC of 99.4 (60.1–167.9) μg/L. Of the lactating women, 53% had a UIC < 100 μg/L and 51% had a median BMIC < 100 μg/L. Lactating women had a total estimated iodine excretion of 227.2 (167.4–346.7) μg/d, with 36% iodine excreted in breast milk and 64% in urine.

Associations of SLC5A5 Gene Variants With BMIC in Lactating Women

An unadjusted general linear model was applied to study the associations of BMIC and UIC in the subset of lactating women ($n = 55$) with the recessive genetic model for $rs4808708_{(G/A)}$, $rs7255301_{(G/A)}$, $rs73520743_{(A/C)}$, $rs112076606_{(A/G)}$, $rs73520745_{(G/A)}$, $rs775249401_{(G/A)}$; $rs34850953_{(G/T)}$ and $rs8103545_{(C/T)}$ (**Table 4**). There was a significant difference in UIC between the genotypes of the $rs4808708_{(G/A)}$ variant ($P =$

TABLE 2 | Molecular characteristics of SLC5A5 genetic variants among pregnant women ($n = 264$) and the subset of lactating women ($n = 55$).

				All women ($n = 246$)					Sample of lactating women ($n = 55$)				
	Location	Gene structure[a]	Allele[a]	HWE	MAF	Genotype (*n*)			HWE	MAF	Genotype (n)		
	Chr:bp[a]		(M):(m)	*P*-value		MM	Mm	mm	*P*-value		MM	Mm	mm
rs121909177	19:17888397	Exon	C:T	1.0	0.007	225	3	-	1.0	0	52	-	-
rs4808708	19:17890877	Intron	G:A	1.0	0.137	176	56	4	1.0	0.087	41	11	1
rs7255301	19:17874025	Intron	G:A	0.0023	0.194	126	76	1	0.2802	0.188	30	14	-
rs73520743	19:17883764	Exon	A:C	1.0	0.02	-	9	223	1.0	0.03	-	44	6
rs112076606	19:17883785	Intron	A:G	0.448	0.214	2	55	81	1.0	0.214	1	16	17
rs73520745	19:17884027	Exon	G:A	1.0	0.046	217	22	-	1.0	0.038	46	5	-
rs775249401	19:17874645	Intron	G:A	1.0	0.056	217	25	1	1.0	0.028	43	9	1
rs34850953	19:17872694	Intron	G:T	0.7938	0.077	202	33	2	1.0	0.038	45	7	-
rs8103545	19:17875954	Exon	C:T	1.0	0.05	217	24	-	1.0	0.087	46	7	-
Novel	19:17983034	5′ prime UTR	C:C	1.0	0.0	218	-	-	1.0	0	47	-	-

Chr, chromosome; bp, base pair; M, reference allele; m, alternative; HWE, Hardy-Weinberg equilibrium; MAF, minor allele frequency. [a]Ensembl release 96—April 2019, retrieved 03 April 2019 (24).

TABLE 3 | Participant characteristics.

Characteristics	All ($n = 98$)	Lactating women ($n = 58$)	Non-lactating women ($n = 40$)	*P-value
Age (years)	27.2 ± 4.8[α]	28.1 ± 4.7	27.9 ± 5.0	0.750
Weight (kg)	69.4 (60.1–81.6)[β]	70.4 (59.4–81.5)	68.6 (61.2–79.7)	0.702
Height (m)	159.1 ± 6.3	159.4 ± 6.3	158.4 ± 5.7	0.634
MUAC (cm)	29.8 (27.0–33.5)	30.0 (27.0–33.6)	29.0 (27.0–33.5)	0.757
BMI (kg/m^2)	27.5 (24.3–32.4)	28.1 (24.1–32.4)	26.2 (24.8–32.3)	0.831
Country of birth ($n = 95$) [n(%)]				
South Africa	68 (69.4)	36 (64.3)	29 (82.9)	0.044
Zimbabwe	26 (26.5)	20 (35.7)	5 (14.3)	
Lesotho	1 (1.0)	–	1 (2.9)	
Years in South Africa ($n = 95$) [n(%)]				
1–2 years	3 (3.1)	3 (5.4)	–	0.183
>2–5 years	6 (6.1)	5 (8.9)	1 (2.9)	
>5 years	86 (87.8)	48 (85.7)	34 (97.1)	
Ethnicity ($n = 97$) [n(%)]				
Black	86 (88.6)	52 (89.7)	31 (88.6)	0.676
Colored	10 (10.2)	5 (8.6)	4 (11.4)	
Indian	1 (1.0)	1 (1.7)		
Education ($n = 97$) [n(%)]				
None/primary	4 (4.1)	2 (3.4)	2 (5.7)	0.324
Grade 8-10	14 (14.3)	7 (12.1)	6 (17.1)	
Grade 11-12	55 (56.1)	31 (53.4)	22 (62.9)	
Tertiary	24 (24.5)	18 (31.0)	5 (14.3)	
Employment ($n = 97$) [n(%)]				
Unemployed	44 (44.9)	28 (48.3)	15 (42.9)	0.640
Self-employed	5 (5.1)	3 (5.2)	2 (5.7)	
Wage earner	46 (46.9)	25 (43.1)	18 (51.4)	
Other	2 (2.0)	2 (3.4)	–	
Marital status ($n = 97$) [n(%)]				
With partner	64 (66.0)	40 (69.0)	21 (60.0)	0.378
Without partner	33 (34.0)	18 (31.0)	14 (40.0)	
Grants ($n = 92$) [n(%)]				
None	65 (66.3)	44 (81.5)	19 (55.9)	0.061
Child support	24 (24.5)	10 (18.5)	12 (35.3)	
Social relief	1 (1.0)	–	1 (1.0)	
Disability	1(1.0)	–	1 (1.0)	
Old age	1 (1.0)	–	1(1.0)	
LSM score [n(%)]				
1–4 (Low)	3 (3.0)	2 (3.4)	1 (2.8)	0.250
5–7 (Medium)	62 (63.3)	32 (55.2)	26 (72.2)	
8–10 (High)	33 (33.7)	24 (41.4)	9 (25.0)	
HIV status				
HIV-infected [n (%)]	28 (28.6)	16 (44.4)	11 (19.0)	0.008
Median UIC (μg/L)	105 (66–154)	95 (64–145.5)	122 (73–207)	0.259
Median BMIC (μg/L)	–	104 (60–169)	–	
Estimated daily iodine excretion (μg/d)				
Urine[γ]	157.0 (99.3–231.0)	142.4 (96.5–218.3)	178.4 (168.8–309.5)	0.259
Breast milk[δ]	–	80.9 (46.8–132.1)	–	
Total daily estimated daily iodine excretion[ε]	–	227.2 (167.4–346.7)	–	

*MUAC, mid-upper arm circumference; BMI, body mass index; LSM, living standards measure; UIC, urinary iodine concentration; BMIC, breast milk iodine concentration, [α]Mean ± SD (all such values), [β]Median; 25th and 75th percentiles in parenthesis (all such values), [γ]Individual estimated iodine daily excretion in urine was calculated by multiplying UIC by an assumed total daily urine volume of 1.5 L (12, 21, 22), [δ]Individual estimated iodine daily excretion in breast milk was calculated by multiplying individual BMIC by an assumed total daily breast milk volume of 0.78 L (12, 22). [ε]Estimated total daily iodine excretion is the sum of iodine excretion in urine and iodine excretion in breast milk (12). *p-value for the Mann Whitney U test significance set at $p \leq 0.05$.*

TABLE 4 | Association of SLC5A5 variants with breast milk iodine concentrations and urinary iodine concentrations ($n = 55$).

Genetic variant	(M):(m)	MM	Mm + mm	*P*-value*
Breast milk iodine concentration (μg/L)				
rs4808708	G:A	108.1 (67.3–175.5)	107.9 (46.8–149.7)	0.612
rs7255301	G:A	112. 9 (72.5–155.0)	103.7 (44.1–150.1)	0.802
rs73520743	C:A	103.7 (57.7–168.9)	109.2 (83.4–191.1)	0.589
rs112076606	A:G	142.1 (69.4–201.0)	83.3 (39.4–143.4)	0.051
rs73520745	G:A	109.2 (63.0–172.2)	89.1 (68.0–148.4)	0.807
rs775249401	G:A	86.8 (54.9–167.9)	143.9 (122.4–169.3)	0.042
rs34850953	G:T	97.6 (60.5–147.1)	142.1 (41.5–219.4)	0.792
rs8103545	C:T	108.4 (89.1–51.1)	127.1 (89.1–51.1)	0.561
Urinary Iodine concentration (μg/L)				
rs4808708	G:A	111.4 (71.2–154.8)	70.1 (45.6–82.8)	0.050
rs7255301	G:A	108.0 (66.4–137.5)	82. 4 (60.8–165.1)	0.967
rs73520743	C:A	103.2 (66.2–137.6)	91.1 (65.0–155.1)	0.267
rs112076606	A:G	133.0 (72.2–153.7)	81.4 (58.4–119.1)	0.081
rs73520745	G:A	91.1 (62.2–133.9)	137.7 (69.6–178.3)	0.409
rs775249401	G:A	102.0 (66.4–137.4)	81.1 (48.6–145.2)	0.912
rs34850953	G:T	102.0 (67.2–137.6)	66.1 (47.2–154.0)	0.739
rs8103545	C:T	91.1 (54.7–133.9)	137.7 (66.4–183.1)	0.345

*M, reference allele in this study population; m, alternative allele in this study population, *p-value for the unadjusted general linear model, significance set at $p \leq 0.05$.*

0.05). Homozygote rs4808708$_{(GG)}$ had higher UIC compared to (A)-allele carriers rs4808708$_{(AG+AA)}$ ($P = 0.05$), whereas BMIC were comparable between the two genotypes ($P = 0.612$) for the same variant. BMIC were different between the rs775249401$_{(G/A)}$ genotypes, whereby the homozygous GG genotype had lower BMIC compared to the (A)-allele carriers rs775249401$_{(AG+AA)}$ ($P = 0.042$). No difference in UIC were apparent for rs775249401$_{(G/A)}$ genotypes. The variant rs112076606$_{(A/G)}$ showed a trend toward a significant association with both BMIC and UIC ($P = 0.051$ and $P = 0.081$). The homozygotes AA genotype had higher median concentrations compared to the G-allele carriers rs112076606$_{(GA+GG)}$.

Furthermore, the estimated breast milk iodine excretion for rs775249401$_{(GG)}$ was 67.7 (42.9–131.1) μg/d, while estimated breast milk iodine excretion of the (A)-allele carriers of rs775249401$_{(AG+AA)}$ was 112.2 (95.5–132.1) μg/d. All the (A)-allele carriers (100%) of rs775249401$_{(AG+AA)}$ had a BMIC above 100 μg/L, whereas 39.5% the homozygous GG [rs775249401$_{(GG)}$] had a BMIC ≥100 μg/L. **Figure 1** shows the associations between estimated total daily iodine excretion and fractional excretion in urine and breast milk for rs775249401$_{(G/A)}$. The homozygotes GG genotype had a higher fraction of iodine excreted in urine (63.2%) than in breast milk (36.8%) (**Figure 1A**). The fractional excretion of iodine in urine and breast milk remained constant across the range of estimated total daily iodine excretion. In the (A)-allele carriers rs775249401$_{(AG+AA)}$, fractional iodine excretion in urine and breast milk plotted against total daily iodine excretion (**Figure 1B**) shows that a higher fraction of iodine was excreted in breast milk than urine when estimated total daily iodine excretion was lower than 350 μg/day, while the fraction of iodine excreted in breast milk was lower than urine above this threshold.

Figure 2 shows the association between estimated total daily iodine excretion and fractional excretion in urine and breast milk in homozygotes GG (rs775249401$_{(GG)}$) and in the (A)-allele carriers of rs775249401$_{(AG+AA)}$, stratified by UIC (UIC<100 μg/L vs. UIC≥100 μg/L). The fractional excretion of iodine in breast milk for rs775249401$_{(GG)}$ with UIC <100 μg/L was lower than in urine when estimated total daily iodine excretion was low, but higher with higher estimated total daily iodine excretion and exceeded the proportion of iodine excreted in urine (**Figure 2A**). In contrast, fractional excretion of iodine in breast milk of the(A)-allele carriers with UIC <100 μg/L, was higher than in urine when the estimated total daily iodine excretion was low, but lower with a higher estimated total daily iodine excretion (**Figure 2B**). In both the rs775249401$_{(GG)}$ and rs775249401$_{(AG+AA)}$ genotype groups with UIC ≥100 μg/L, fractional excretion of iodine in breast milk was lower than fractional excretion of iodine into the urine (**Figures 2C,D**, respectively) and remained constant across estimated total daily iodine excretion. The association between rs775249401$_{(G/A)}$ and BMIC had a Cohens' d of 0.88 and a partial eta squared $\eta^2 = 0.078$.

DISCUSSION

To our knowledge, this is the first study to assess the role of genetics in the transfer of iodine from plasma to breast milk in healthy lactating women of African descent using a targeted NGS approach. Genetic variants in the *SLC5A5* gene in women of African descent living in urban South Africa were characterized and studied in the context of iodine transfer from plasma to breast milk during lactation. Our results suggest rs775249401$_{(G/A)}$ to be a candidate variant

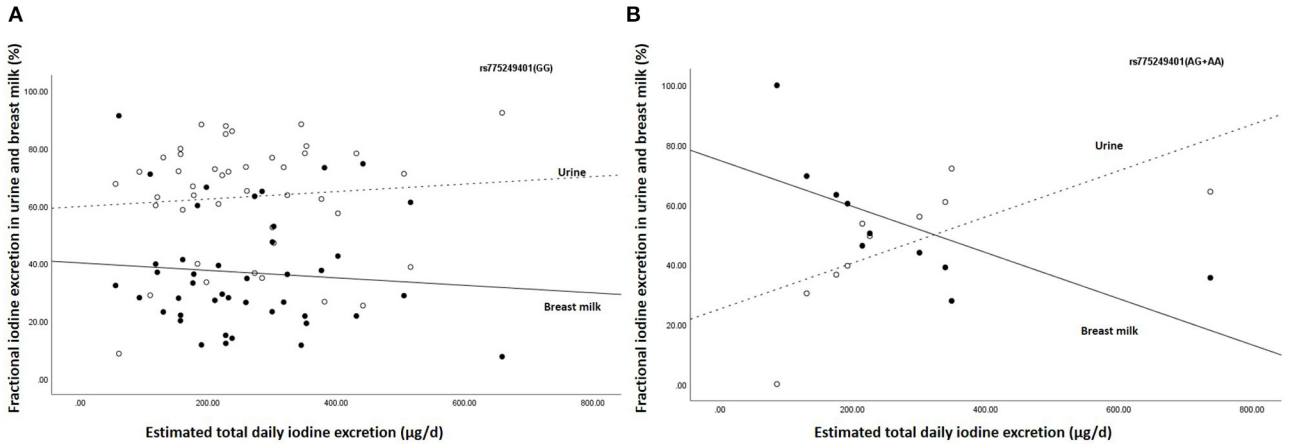

FIGURE 1 | Association between estimated daily iodine excretion and fractional iodine excretion in breast milk and urine for **(A)** the homozygous G group ($n = 43$) and **(B)** the (A) allele carriers (AA + AG) ($n = 10$). The open data points represent fractional excretion of iodine in urine and the filled data points, the fractional excretion in breast milk. The dotted line is the regression line for fractional excretion of iodine in urine. The solid line is the regression line for fractional excretion of iodine in breast milk.

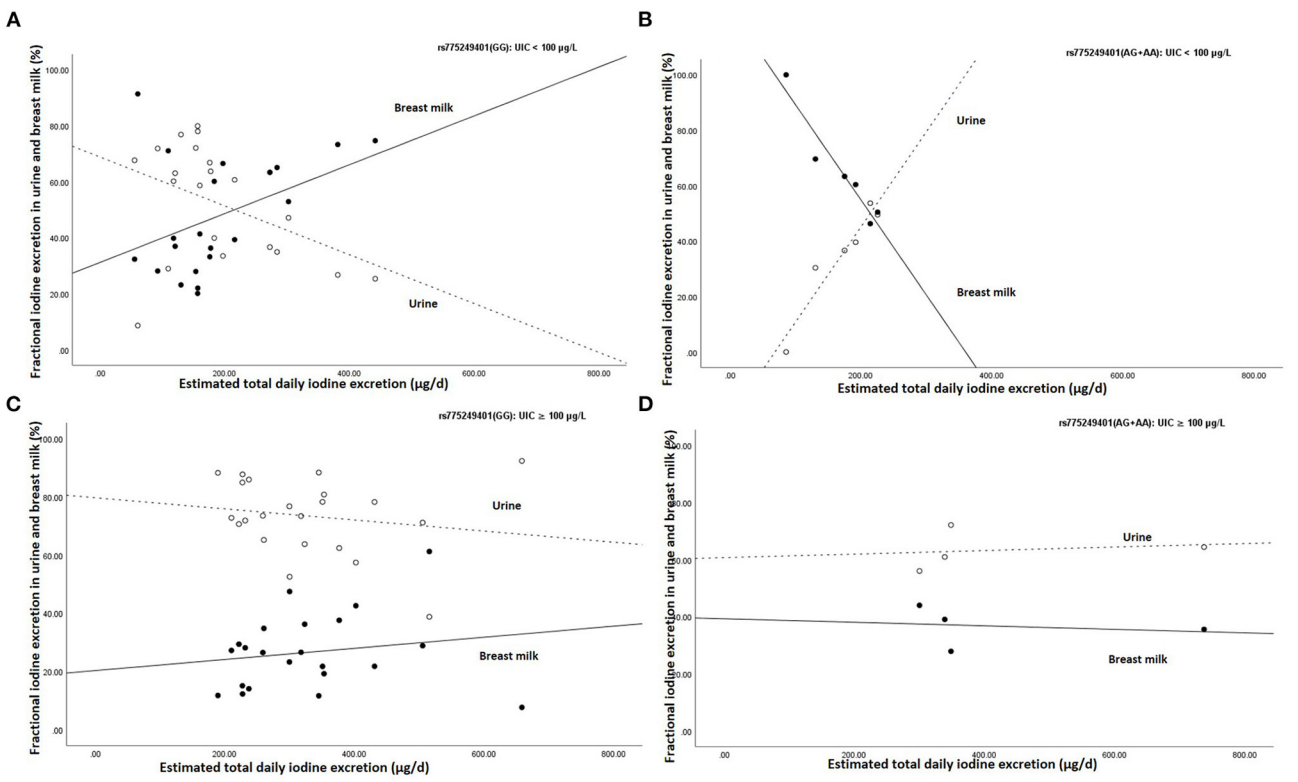

FIGURE 2 | Association between estimated daily iodine and fractional iodine excretion in breast milk and urine **(A)** the homozygous G group ($rs775249401_{(GG)}$) with UIC <100 µg/L ($n = 21$), **(B)** the (A) allele carriers for the $rs775249401_{(AG+AA)}$ with UIC <100 µg/L ($n = 6$), **(C)** the homozygous G group ($rs775249401_{(GG)}$) with UIC ≥100 µg/L ($n = 22$) and **(D)** the (A) allele carriers for the $rs775249401_{(AG+AA)}$ with UIC ≥100 µg/L ($n = 4$). The open data points represent fractional excretion of iodine in urine and the filled data points, the fractional excretion in breast milk. The dotted line is the regression line for fractional excretion of iodine in urine. The solid line is the regression line for fractional excretion of iodine in breast milk.

for mediating the transfer of iodine from plasma into breast milk, specifically among lactating women with poor iodine status. The function of the variant may not be observed when the iodine status is adequate, suggesting that adequate

iodine intake may counter the low iodine transfer associated with rs775249401$_{(GG)}$.

We observed that in individuals harboring the rs775249401$_{(GG)}$ genotype fractional excretion of iodine into the breast milk was lower than fractional excretion of iodine into urine with low iodine status, while in the rs775249401$_{(AG+AA)}$ group fractional excretion of iodine into the breast milk was higher even when iodine status was low. Thus, lactating women carrying an (A) allele for rs775249401$_{(AG+AA)}$ (19%; 10/53) had an adaptive advantage to maintain optimal levels of BMIC despite suboptimal iodine status. Our findings suggest a positive genetic drift from the ancestral rs775249401$_{(GG)}$ to the alternative allele to rs775249401$_{(AG+AA)}$, which leads to preferential excretion of iodine into the breast milk when iodine status is low. This preferential excretion of iodine in breast milk rather than in urine is likely a result of an increased expression of the NIS in the lactating breast and further supports the plausibility of a regulatory potential by the rs775249401$_{(G/A)}$.

The rs775249401$_{(G/A)}$ variant is located in a promoter region between exon 4 and 5 (24), which is responsible for transcriptional control. It is speculated that the variant interferes with the affinity of transcription factors (TFs) to bind to their idyllic binding sites (25), thus altering affinity to transcription factors (25). The adaptation observed in our study suggests a physiological benefit for the infant, in that it ensures sufficient iodine in breast milk, especially during periods of low iodine intakes. Furthermore, the rs775249401$_{(G/A)}$ variant is mainly present in African and not in Caucasian populations according to the Genome Aggregation Database (26). The variant rs775249401$_{(G/A)}$ has a minor allele frequency (MAF) of 0.028 in this study population, which is higher than reported in the genome-wide association study for Africa populations (0.015) (24). A total of 14 missense and non-sense mutations in the *SLC5A5* gene have been previously described in association with iodide transport defects in the thyroid (27). However, a better understanding of the impact that genetic variations in the *SLC5A5* gene have on BMIC is as important.

Out of the 10 variants explored in our study, only rs775249401 was significantly associated with BMIC. One other variant, rs4808708$_{(G/A),}$ was associated with UIC but not BMIC. The variant rs112076606$_{(A/G)}$ showed a trend toward significance with both BMIC and UIC, whereby participants with the genotype rs112076606$_{(AA)}$ had a higher BMIC and UIC compared to rs112076606 $_{(AG+GG)}$ carriers, suggesting AA homozygotes to have a higher overall iodine excretion compared to their G allele carrier counterparts.

To our knowledge, this is the first study to assess the role of genetics in the transfer of iodine from plasma to breast milk in healthy lactating women. Furthermore, our participants were healthy participants with no known history of thyroid disease. However, a major limitation of this study is the small sample size; based on an *a posteriori* sample size calculation, our study was only powered to determine associations of medium to large effect sizes. Thus, exploring the relationship of variants in the SLC5A5 gene locus in other studies with larger sample sizes is highly recommended.

CONCLUSION

Our results indicate that genetics may play an important role in the transfer of iodine into breast milk. The *SLC5A5* gene variant rs775249401$_{(G/A)}$ seems to be a candidate variant for further investigation. The A-allele carriers of rs775249401$_{(AG+AA)}$ are likely to have higher iodine transfer into breast milk when in an iodine-deficient state compared to the homozygous GG group. Our results suggest that genetic variations in the SLC5A5 gene may play an important role in the transfer of iodine from plasma into breast milk and may partially explain variability in BMIC independent of maternal iodine intake. Therefore, these findings could contribute toward the body of evidence to improve precision nutrition strategies.

DATA AVAILABILITY STATEMENT

The data generated for the study are deposited in the sequence read archive (SRA), bioproject number (PRJNA735618), https://www.ncbi.nlm.nih.gov/bioproject/PRJNA735618.

ETHICS STATEMENT

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving research study participants were approved by the Human Research Ethics Committee of the North-West University and the University of the Witwatersrand, Johannesburg. Permission to perform the NuPED study was given by the CEO of RMMCH, the RMMCH research review committee, the Gauteng Department of Health and the Johannesburg Health District's District Research Committee. Written informed consent was obtained from all participants before enrolment. Consent for genetic testing were obtained.

AUTHOR CONTRIBUTIONS

LZ, JB, JN, and SS conceptualized and designed the study and wrote the first draft of the manuscript. SS, JB, LZ, EAS, LM, and CMS executed the study and collected data. LZ, MS, JB, and SS performed biochemical and statistical analyses. All authors critically evaluate the manuscript.

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Plant Extracts Rich in Polyphenols as Potent Modulators in the Growth of Probiotic and Pathogenic Intestinal Microorganisms

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Medicinal plants and their extracts contain substantial quantities of polyphenols. As metabolically active plant metabolites, polyphenols are food components with a wide range of biological activities. Given their poor absorbability in the digestive tract their activity toward the human host is typically mediated through interaction with intestinal microbes. As a result, polyphenols comprise a novel group of prebiotics. In this study, we tested the effect of five polyphenol-rich extracts from four medicinal herbs on the growth of probiotic and pathogenic microbes. The studied medicinal herbs were *Gentiana asclepiadea* L. (willow gentian), *Hypericum perforatum* L. (St. John's wort), *Satureja montana* L. (winter savory), and *Achillea millefolium* L. (yarrow). All these plants are traditionally used for the treatment of digestive problems. Extracts were prepared using safe solvent combinations. We tested the impact of addition of plant extracts on the growth of three probiotic lactobacilli and probiotic yeast *Saccharomyces boulardii*. The effect of addition of plant extracts to liquid media (concentration range 0.25–10 mg/mL) on the growth of probiotics, was tested *in vitro*. The antimicrobial activity of the extracts was tested against several opportunistic bacteria and yeast. St. John's wort, winter savory, and willow gentian extracts showed a stimulative effect on probiotic yeast growth, while the highest growth-stimulating effect was achieved when microwave-assisted yarrow extract was used in the concentration of 0.5 mg/mL. Under these conditions growth of *S. boulardii* was increased 130-fold. In addition, the yarrow extract stimulated the growth of *Lactiplantibacillus plantarum* 299v. The growth of two *Lacticasibacillus rhamnosus* strains was not stimulated by the addition of any extracts. Our results show that plant polyphenol-rich extracts can influence the growth of microorganisms that are typical members of the intestinal microbiota. For the first time we demonstrate that probiotic yeast growth can be stimulated by extracts of medicinal herbs, which when accompanied by suppression of *Candida* yeasts suggests a potential benefit of the treatment in diseases that are associated with fungal dysbiosis.

Keywords: prebiotics, medicinal herbs, polyphenols, antimicrobial activity, *Gentiana asclepiadea* L., *Hypericum perforatum* L., *Satureja montana* L., *Achillea millefolium* L.

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INTRODUCTION

An individualized approach toward nutrition and medication is becoming increasingly important. While genetic and epigenetic host-factors contribute significantly to the individualized response to any compound that is taken up from the environment, another important factor of variation is human microbiota. The human microbiota is a complex ecosystem of microbes that inhabit various niches of the human body, with highest densities being on the skin, the oral cavity, and gastrointestinal tract. Of particular importance is colonic microbiota. It has been suggested that this anatomic part of the human body could be considered as a bioreactor in which an extremely dense microbial consortium is performing various functions that are important for systemic health (1). Gut microbiota is comprised of members of all three domains of life—Bacteria, Archaea and Eukarya; however, large individual differences hamper the complete description of the ecosystem (2). Despite its complexity and variability, recent research has clearly shown that interaction between gut microbiota and undigested food components, as well as medicaments, can yield various metabolites that could promote or jeopardize human health (3). Different types of studies contribute to resolving the puzzle between gut microbiota/food/health interactions, including *in vitro* tests, animal studies, and clinical trials. This is particularly important since many diseases, including obesity, diabetes, inflammatory bowel diseases, and colorectal cancer, have been associated with an imbalance in gut microbial composition, i.e., dysbiosis. Dysbiosis can occur due to decrease in the number of beneficial bacteria, overgrowth in detrimental species, and loss in microbial diversity (4, 5). One of the strategies promoting gut health is diet modification. Given that it has been shown that polyphenols and their metabolites have a positive impact on gut health (6), in this study, we aim to assess the impact of selected polyphenol-rich medicinal plant extracts on the growth of pathogenic and probiotic microorganisms, which in turn contributes to the modification of gut microbiota.

Polyphenols are plant metabolites, present in vegetables, fruits, spices, and medicinal plants with a wide range of biological activities, such as antioxidant, antimicrobial, anticancer, and anti-inflammatory (7, 8). Their dietary intake has been related to the prevention of some chronic and degenerative diseases, such as some types of cancers, inflammatory, cardiovascular diseases, diabetes, or neurodegenerative disorders like Alzheimer's and Parkinson's diseases. However, the exact mechanisms of polyphenol beneficial activity still remain to be defined (7–10). It has been estimated that <5–10% of the ingested polyphenols are resorbed in the small intestine, while the remaining compounds reach the large intestine where they are metabolized by the gut microbiota (8). Given their poor absorption in the digestive tract, their activity toward the human host is typically mediated through interaction with intestinal microbes, such that polyphenols comprise a novel group of prebiotics (11). *In vitro* studies suggest that polyphenols may exert a doubly positive effect, simultaneous inhibition of pathogens, and stimulation of beneficial bacteria (12). Beneficial species belonging to genera *Lactobacillus* and *Bifidobacterium* are often reported to thrive in the presence of polyphenols, whereas the growth of detrimental species is inhibited (13). Species of *Bifidobacterium* and *Lactobacillus* are usually used as probiotics, but some *Escherichia coli* and *Bacillus* strains, and the yeast *Saccharomyces boulardii* are used as well (14). *S. boulardii* is an extremely potent probiotic that is effective in preventing antibiotic-induced diarrhea, as well as in protecting the intestine from *Clostridium difficile* and cholera toxins (15). Additionally, the World Gastroenterology Organization recommends *S. boulardii* for the treatment of nine different intestinal diseases (14). The impact of polyphenol-rich medicinal plant extracts on the growth of probiotic or pathogenic microbes has not been assessed yet, while no previous study has assessed the effect of polyphenols on *S. boulardii* growth.

Many medicinal herbs have been used in folk medicine for treatment of gastrointestinal disorders (16). In this study, we evaluated the effect of different extracts of four medicinal plants that are traditionally used to treat digestive problems. *Achillea millefolium* L., commonly known as yarrow, has been used in traditional medicine for inflammatory and spasmodic gastrointestinal disorders, as an appetite-enhancing drug, for wound healing and diabetes (17, 18). *Hypericum perforatum* L., commonly known as St. John's Wort, has been used for poor appetite, difficulty sleeping, nervousness, the treatment of burns, skin ulcers, and cuts (19). *Satureja montana* L., commonly known as winter savory, is used for digestive complaints, such as diarrhea, and colic (20). Lastly, *Gentiana asclepiadea* L., commonly known as willow gentian, is traditionally used for improving appetite, digestive problems, and hepatitis infections (21). All listed medicinal plants have proven antimicrobial, antioxidant, anticancer, and anti-inflammatory properties (18, 19, 22–25). The major active compounds in the St. John's wort are flavonoids (quercetin, biapigenin, hyperoside, rutin, quercitrin, and isoquercitrin), phenolic acids (chlorogenic, caffeic, *p*-coumaric, and ferulic acid), naphthodianthrones (hypericin and pseudohypericin), and phloroglucinols (hyperforin and adhyperforin) (19, 22, 26). In the yarrow, predominant bioactive compounds are phenolic acids (chlorogenic, and 1,5-, 3,4-, 3,5- and 4,5-dicaffeoylquinic acids) and flavonoids (apigenin, luteolin, luteolin-7-O-glucoside, hyperoside, and rutin), tannins and sesquiterpene lactones (achillin and achillicin) (17, 18, 27). Winter savory has a diverse composition of secondary metabolites, such as phenolic monoterpene (carvacrol and thymol), phenolic acids (protocatechuic, syringic, vanillic acids caffeic, sagerinic, salvianolic, *p*-coumaric, ferulic, and rosmarinic acid), and flavonoids (luteolin, quercetin, rutin, epicatechin, catechin, and epicatechin) (24, 28–30). In the willow gentian, the major active compounds are secoiridoids (gentiopicrine, sweroside, and swertiamarine), flavonoids (isovitexin and homoorientin), phenolic acids, xanthone-C-glycosides (mangiferin), and alkaloids (25). Currently, forty-four medicinal plants are recommended in the European Union for treatment of gastrointestinal disorders, including the yarrow that can be used in mild, spasmodic gastro-intestinal complaints, and bloating (16). In this study, we assessed the effect of five

extracts obtained from four medicinal plants on the growth of four selected probiotics, two strains of *Lacticaseibacillus rhamnosus* [previously *Lactobacillus rhamnosus* (31)], one *Lactiplantibacillus plantarum* [previously *Lactobacillus plantarum* (31)], and yeasts *S. boulardii*. The effect of plant extracts on the growth of opportunistic pathogens, *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Candida albicans* was also tested.

MATERIALS AND METHODS

Plant Material and Chemicals

The St. John's wort, winter savory, and yarrow were obtained from the Institute of Medicinal Plant Research "Dr Josif Pancic," Pančevo, Serbia, while the willow gentian was obtained from "Bilje Borča," Belgrade, Serbia.

A growth medium Tryptic Soy Broth (TSB), MRS broth, agar, and yeast extract were obtained from the Institute of Immunology and Virology, Torlak, Belgrade, Serbia. Resazurin was purchased from Acros Organics, Geel, Belgium, and ethanol was obtained from Zorka Pharma-Hemija d.o.o., Šabac, Serbia.

Bacterial Strains and Culture Conditions

The following strains of bacteria and yeast were used: *Enterococcus faecalis* ATCC 29812, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27833, *Listeria monocytogenes* IM2002 (Institute of Meat Hygiene and Technology, Belgrade), *Lacticasibacillus rhamnosus* ATCC 7469, *Lacticasibacillus rhamnosus* GG (ATCC 53103), *Lactiplantibacillus plantarum* 299v, *Candida albicans* ATCC 10259, and *Saccharomyces boulardii* (*Saccharomyces cerevisiae* var. *boulardii* HANSEN CBS 5926), from the collection of the Department of Biochemical Engineering and Biotechnology of the Faculty of Technology and Metallurgy, Belgrade. Lactobacilli were grown overnight at 37°C in MRS broth in CO_2 enriched conditions, while all other microorganisms were grown in TSB, supplemented with 0.6% w/v yeast extract at 37°C.

Extraction of Polyphenols From Medicinal Plants

A total of five extracts were prepared from four medicinal plants, including yarrow, winter savory, St. John's wort, and willow gentian. The extracts were prepared either using standard procedures of the European Medicines Agency (EMA) or under optimized conditions for microwave-assisted extraction (MAE) that yields extracts with the highest polyphenol content. The optimization of the MAE extraction parameters was previously published for yarrow (32), while for the other used medicinal plants the manuscripts describing the optimization process are submitted/prepared for publication elsewhere.

Two extracts were prepared from St. John's wort. The first one was prepared using the optimized MAE procedure, in which three grams of St. John's wort was extracted for 40 s, with 150 mL of 30% ethanol at 170 W of microwave power. The second extract was prepared following the EMA procedure according to which ten grams of St. John's wort's waste powder was extracted with 100 mL of 50% ethanol in the dark at room temperature for 72 h (33). The yarrow extract was prepared using the optimized MAE under the following conditions 33 s of extraction time, 70% of ethanol concentration, 40 mL/g of liquid/solid ratio, and 170 W of microwave power (32).

The winter savory extract was prepared using the optimized MAE procedure for obtaining the extract with the highest polyphenol content in which three grams of plant powder were mixed with 60 mL of 55% ethanol and extracted for 80 s at 170 W of microwave power.

The willow gentian extract was prepared following the optimized procedure for obtaining an extract with the highest polyphenol content in which two grams of plant powder were extracted with 60 mL of 25% ethanol, in the dark, for 72 h.

After extractions, solids were separated from liquids using vacuum filtration and the ethanol was evaporated from all the extracts on a rotary evaporator (Büchi, Switzerland) at 150 mm Hg pressure and 60°C. The percentage of dry matter in the extract was measured on a moisture analyzer (Kern MLS-A, Balingen, Germany), and the extracts were stored at 4°C.

Determination of Total Polyphenol Content

Total polyphenol content in the extracts was determined following the previously described method (32). The results were expressed as mg gallic acid equivalents (GAE)/g extract dry matter, using the gallic acid calibration curve. All experiments were performed in triplicate.

Antimicrobial Activity

Antimicrobial activity of the extracts was determined using a broth microdilution method and resazurin as an indicator of microbial growth (34). For all pathogenic microorganism [three Gram-positive bacteria (*E. faecalis*, *L. monocytogenes*, and *S. aureus*), two Gram-negative bacteria (*E. coli*, and *P. aeruginosa*), and yeast *C. albicans*], and for all probiotic microorganisms (two strains of *L. rhamnosus*, *L plantarum*, and *S. boulardii*) the minimal inhibitory concentration (MIC) was determined.

In brief, 100 μL of TSB or MRS broth for lactobacilli was pipetted in all the wells of 96 well-microtiter plate. Afterward, 100 μL of plant extract stock solution (40 mg/mL) was pipetted in the first row and 2-fold serial dilution throughout the column was achieved by transferring 100 μL of the extract and nutrient broth mixture to the next row, so that the final volume in each well was 100 μL. The concentration of the plant extracts varied in the range from 0.016 to 20 mg/mL. Subsequently, 10 μL of microbial suspension was added to each well to achieve a final concentration of 5×10^6 CFU/mL. Lastly, 10 μL of the resazurin solution (32.7 mM) was pipetted into all wells. Every plate contained a negative and a positive control. The negative control contained 10 μL of nutrient broth instead of the microbial suspension, while the positive control was prepared by mixing all solutions except the plant extract. The plates were incubated at 37°C for 24 h, and MIC values were determined visually based on color change. In

the presence of growing cells purple resazurin is reduced to pink resorufin. The lowest concentration at which no color change occurred was considered as MIC. All tests were run in biological triplicate.

Prebiotic Activity

The prebiotic activity of five plant extracts was tested in terms of growth stimulation potential. Liquid cultures using the plate count method were applied (35) and two *L. rhamnosus* strains, *L plantarum* 299v, and *S. boulardii* were tested. In short, fresh TSB or MRS (2 mL) were inoculated with an overnight growth culture of *S. boulardii* or lactobacilli (0.1 mL) to achieve a final concentration of 5×10^6 CFU/mL. Plant extracts were added to the culture media to obtain a final concentration of dry matter in the range from 0.25 to 10 mg extract dry matter/mL, which are concentrations of extracts expected to be found under physiological conditions (33, 36). One control sample remained without any plant extract. After incubation (37°C for 24h), the samples were diluted with sterile normal saline and plated on TSA or MRS agar medium and incubated for 24 h at 37°C. The effect of plant extracts on lactobacilli species and *S. boulardii* was determined by comparing the number of colony-forming units (CFU) in the samples containing plant extracts and appropriate controls. All tests were run in biological triplicate.

Statistical Analysis

Statistical analysis of the total polyphenol content in the extracts was performed in the program Origin Pro 8. All data were reported as mean ± standard deviation of three independent measurements. Significant differences between data were analyzed using analysis of variance (one-way ANOVA) and the Tukey test, with the criterion of $p < 0.05$.

Significant differences between the number of CFU on the plates with the plant extracts and the control were analyzed using a paired *t*-test with cut off value of $p < 0.05$ for significant difference. Previously, the Kolmogorov-Smirnov test was used to determine whether the data follows normal distribution, for which a paired *t*-test is a suitable tool for analysis.

RESULTS

Total Polyphenol Content

Total polyphenol content in the tested extracts is presented in **Table 1**. Among all tested plants, the highest polyphenol content was identified in winter savory and the lowest content was determined in willow gentian. Comparing two St. John's wort extracts obtained using the MAE and EMA procedures, the total polyphenol content was higher in the MAE extract by 11.58%.

Antimicrobial Activity

Antimicrobial activity of all extracts was determined for three Gram-positive opportunistic pathogens (*E. faecalis*, *S. aureus*, and *L. monocytogenes*), two Gram-negative strains (*P. aeruginosa*, and *E. coli*) and yeast *C. albicans*. Antimicrobial activity was determined for probiotics as well, two *L. rhamnosus* strains, *L plantarum*, and *S. boulardii* (**Table 2**). Generally, Gram-positive bacteria were more sensitive to the plant extracts compared to Gram-negative. The most sensitive pathogen to the tested plant extracts was *L. monocytogenes,* as indicated by the lowest MIC value of 0.16 mg/mL. Only *S. aureus* and *L. monocytogenes* were inhibited by all the extracts. *E. coli* and *C. albicans* were sensitive only to winter savory and willow gentian, with MIC values of 5 and 20 mg/mL for *E. coli*, and 20 and 5 mg/mL for *C. albicans*, respectively.

Interestingly, the plant extracts showed higher inhibition to pathogens than to probiotics. Two St. John's wort extracts showed

TABLE 2 | Antimicrobial activity of plant extracts.

	Yarrow	St. John's wort[a]	St. John's wort[b]	Winter savory	Willow gentian
Microorganism	**MIC, mg/mL**				
S. aureus	5	10	10	5	10
L. monocytogenes	1.25	2.5	2.5	0.16	20
E. faecalis	5	5	5	1.25	-
E. coli	-	-	-	5	20
P. aeruginosa	2.5	5	5	0.63	-
C. albicans	-	-	-	20	5
L. rhamnosus ATCC 7469	-	-	20	10	-
L. rhamnosus GG	-	-	10	10	-
L. plantarum	-	-	-	-	-
S. boulardii	-	-	-	20	20

[a] *St. John's wort extract obtained using the MAE procedure.*
[b] *St. John's wort extract obtained using the EMA procedure.*
All tests were run in biological triplicate.

TABLE 1 | Total polyphenol content in tested medicinal plant extracts.

	Extract				
	Yarrow	**St. John's wort[a]**	**St. John's wort[b]**	**Winter savory**	**Willow gentian**
TPC, mg GAE/g	204.61 ± 5.49*	282.01 ± 6.20*	252.75 ± 3.92*	386.75 ± 2.58*	19.21 ± 0.13*

TPC, Total polyphenol content; GAE, gallic acid equivalent.
[a] *St. John's wort extract obtained using the MAE procedure.*
[b] *St. John's wort extract obtained using the EMA procedure.*
**Mean comparison was used to evaluate the significant difference among total polyphenol content in different plant extracts with the criterion of $p < 0.05$.*

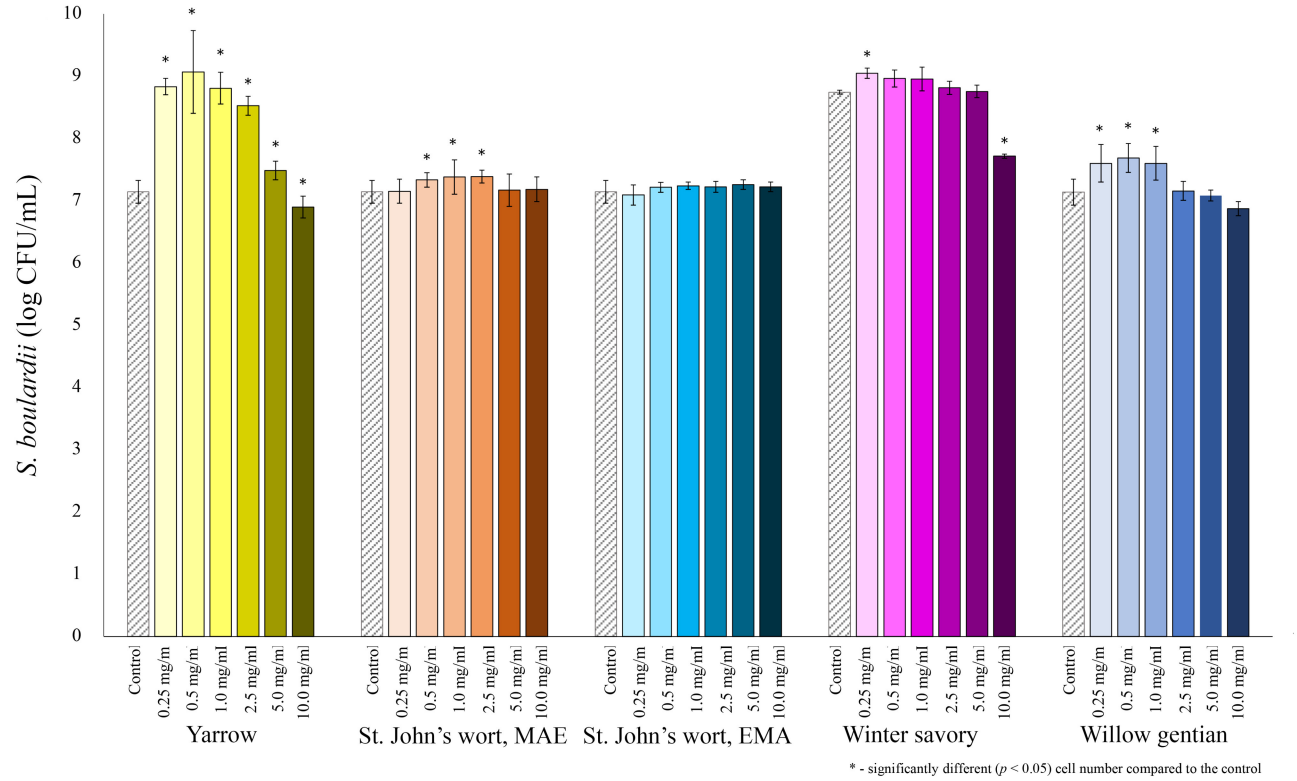

FIGURE 1 | Growth of *S. boulardii* (log CFU/mL) after 24 h of growth at 37°C in the growth medium supplemented with plant extracts. Tests were run in biological triplicate.

the same inhibition against pathogenic microorganisms, while the inhibition of probiotics growth was different. The MAE extract did not inhibit the growth of the tested probiotics, while the EMA extract inhibited the growth of two *L. rhamnosus* strains, with different MIC values (**Table 2**). This result suggested different chemical compositions of the two St. John's wort extracts.

Willow gentian suppressed the growth of *S. aureus, L. monocytogenes, E. coli*, and *C. albicans*, with MIC values ranging from 5 to 20 mg/mL, while it did not affect the growth of Gram-positive probiotics. It suppressed the growth of both yeasts, however, the MIC value for the *C. albicans* was 4-fold lower than the MIC value for *S. boulardii*.

The winter savory inhibited the growth of *L. rhamnosus* ATCC 7469 and *L. rhamnosus* GG, with equal MIC values. Antimicrobial activity toward the tested pathogens was stronger compared to the probiotic bacteria. The MIC values for pathogens varied in the range from 0.16 to 5 mg/mL, while it was 10 mg/mL for the two probiotics.

Prebiotic Activity

The prebiotic potential of five plant extracts obtained from four medicinal plants was tested in terms of their ability to stimulate the growth of selected probiotics. The concentration of extracts in the growth medium was varied, and optimal concentration for growth stimulation of each extract–probiotic pair was identified. Among all tested probiotics, the yeast *S. boulardii* was the most stimulated by the addition of the yarrow MAE extract (**Figure 1**). The highest growth-stimulating effect was achieved when the MAE yarrow extract was added in the final concentration of 0.5 mg extract dry matter/mL of the growth medium. Under these conditions, the growth of *S. boulardii* was increased 130-fold. A significant 2-fold stimulation was also observed when the willow gentian or winter savory extracts were added to the growth medium in the final concentration of 0.5 or 0.25 mg/mL. However, both extracts inhibited the growth of *S. boulardii* when added in a concentration higher than 20 mg/mL. At this point, it should be noted that the growth performance of the control differed considerably when testing the winter savory and all other extracts. The tests were not performed in the same period, and for the winter savory a new lot of the same *S. boulardii*, strain was used. The strain from the new lot grew better in the control nutrient broth.

Among lactobacilli, the growth of *L. plantarum* was stimulated with the addition of the yarrow extract, and the St. John's wort MAE extract (**Figure 2**). Growth stimulation was achieved when the concentration of yarrow MAE and St. John's wort MAE extracts was in the range 0.25–2.5 and 0.25–0.5 mg/mL, respectively. When the concentrations of the extracts further increased, the cell count significantly decreased compared to the control. The growth of two *L. rhamnosus* strains was not

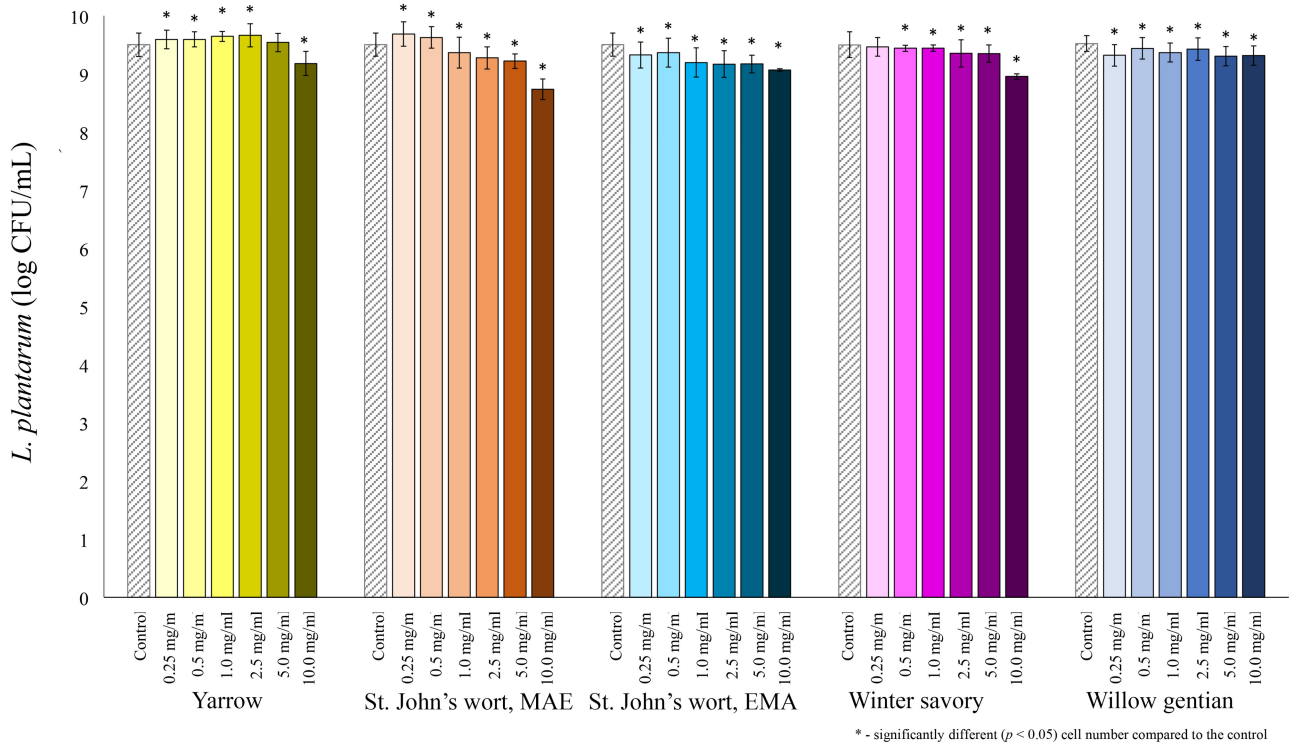

FIGURE 2 | Growth of *L. plantarum* (log CFU/mL) after 24 h of growth at 37°C in the growth medium supplemented with plant extracts. Tests were run in biological triplicate.

stimulated by the addition of any of the five plant extracts in the tested concentrations (**Figures 3**, **4**).

DISCUSSION

The present study provides new findings on the impact of yarrow, St. John's wort, winter savory, and willow gentian extracts on the growth of selected pathogens and probiotics. We demonstrate that these medicinal plants, which are traditionally used for the treatment of digestive problems, could suppress the growth of pathogens, while the presence of the same concentration could stimulate the growth of probiotics. Hence, one potential mechanism of the beneficial effect of medicinal plants could be modulation of the gut microbiota composition and/or activity.

It is important to emphasize that in this study the growth stimulation of probiotics and antimicrobial activity against pathogens was achieved with a concentration of medicinal plant extracts that can be expected to be found under physiological conditions. We could evaluate the intake only for St. John's extract prepared according to EMA procedure, as only for this extract, official recommendations exist from among all tested extracts (33). Daily recommended intake of St. John's extract is 10 ml. Based on extract concentration, this would require intake of ∼180 mg of extract dry matter. Given that the exact dilution factor under physiological conditions can be only estimated, it is important to perform *in vivo* human intervention studies. Nevertheless, we would like to note that an estimated dilution of the maximal dose of St. John's EMA extract in 200 mL would facilitate the maximal concentration tested in our growth stimulation experiments. Furthermore, one intervention on healthy subjects, showed that intake of cocoa-derived flavanols in a dosage similar to that achieved following EMA recommended daily intake of St. John's extract, was sufficient to induce changes in gut microbiota (36).

The selected medicinal plants have proven antioxidant, antimicrobial, and anti-inflammatory properties, among others, due to the presence of bioactive compounds, such as polyphenols. It is known that the concentration of polyphenols in plants depends on the species of plant, environmental conditions, rainfall and ultraviolet radiation, geographical origin, and storage conditions (37). Further, the total polyphenol content in the extract depends on the extraction technique and solvent used in the process. Ethanol and water mixtures were used in the preparation of all five extracts. Ethanol has GRAS (generally regarded as safe) status and can be used for preparation of extracts that are safe for human consumption (38). Of the four tested plants, winter savory is the richest in polyphenolic compounds, followed by St. John's wort, yarrow, and willow gentian. These findings are consistent with literature data (21, 29, 39, 40). Comparing the extracts obtained from the same plant material, but with different extraction techniques, it can be concluded that MAE was more efficient than the maceration. Using modern extraction

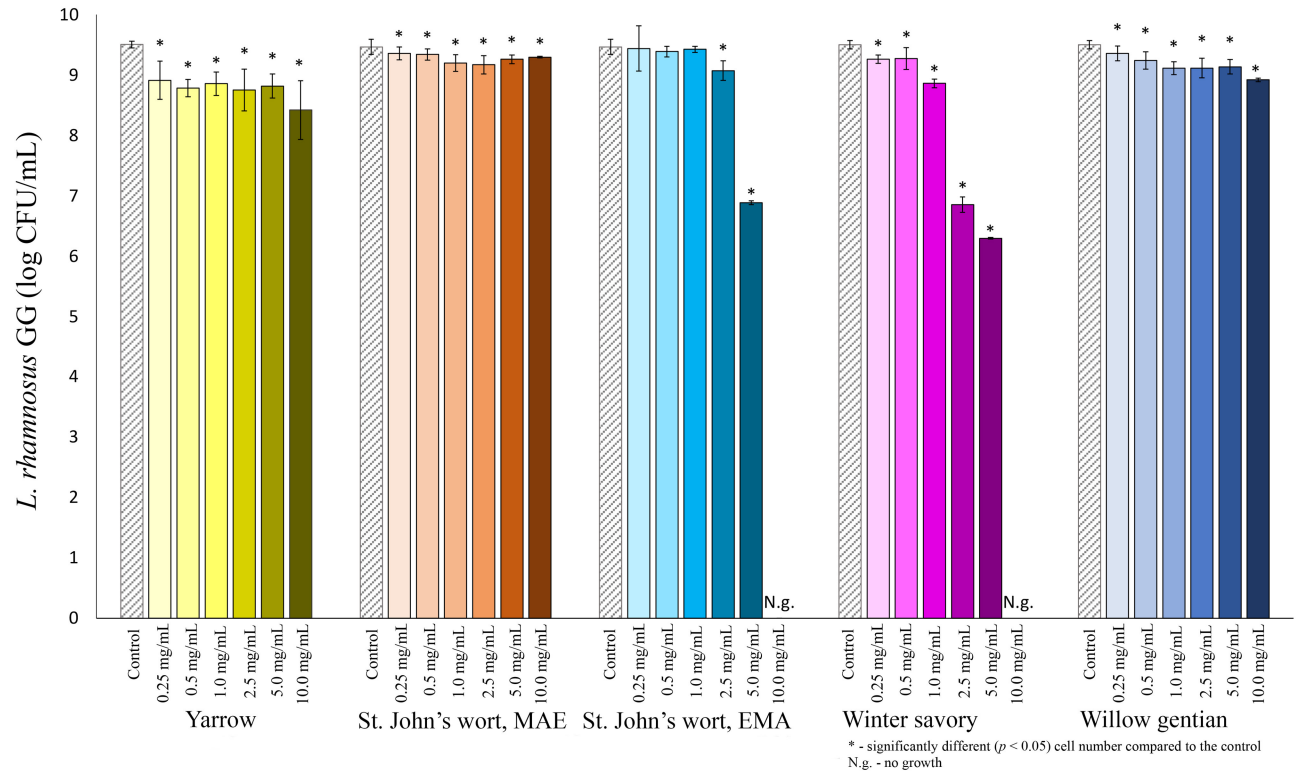

FIGURE 3 | Growth of *L. rhamnosus* GG (log CFU/mL) after 24 h of growth at 37°C in the growth medium supplemented with plant extracts. Tests were run in biological triplicate.

techniques, such as MAE, the yield of polyphenols can be increased, while extraction time and solvent usage are reduced (41). Additionally, polyphenol composition may differ when two extraction techniques are used (41). This was most likely the case for the two St. John's wort extracts, as indicated by their different biological activity.

Of the four tested plants, winter savory contained the highest concentration of polyphenols and this extract had the highest antimicrobial effect on all six tested pathogens. The highest antimicrobial activity was against *L. monocytogenes*, while the lowest was against *C. albicans* (**Table 2**). The two St. John's wort extracts inhibited the growth of four pathogens with equal MIC values, while the effect on probiotics was different. While the EMA extract inhibited the growth of two *L. rhamnosus* strains, the MAE extract stimulated the growth of *S. boulardii* and *L. plantarum*, indicating that there is a difference in the composition of polyphenols in the two extracts. All five extracts showed stronger antibacterial activity against Gram-positive bacteria than against Gram-negative ones (**Table 2**). Generally, the antibacterial effect of plant extracts was usually more effective against Gram-positive than Gram-negative bacteria (13). The exact mechanism of action of polyphenols as antimicrobial agents is not yet fully understood. Polyphenols could react with the cell membrane, inactivate essential cellular enzymes, while lipophilic flavonoids could disrupt microbial membranes and cause the loss of cell's macromolecules and subsequent cell death (42–44). Also, a synergistic effect of polyphenolic compounds present in the plant extract contributes to higher antimicrobial activity compared to a single compound's reaction, which may be due to the different mechanisms of action of each of the active components (45). Among all tested extracts, the willow gentian showed the highest antimicrobial activity toward *C. albicans* (**Table 2**), while the concentration of polyphenols in this plant extract is lower than in all other plant extracts. Besides polyphenols, the major compounds in willow gentian are secoiridoids (gentiopicrin), and it has been reported that the gentiopicrin inhibited the growth of Gram-negative, Gram-positive pathogen bacteria, as well as yeast *C. albicans* (46). Additionally, the predominant constituents of St. John's wort besides polyphenols are naphthodianthrones (hypericin and pseudohypericin) and phloroglucinols (hyperforin and adhyperforin) (26). It has been reported that hypericin and hyperforin are components responsible for the antimicrobial activity of the St. John's wort extract (26). Antimicrobial activities of all four medicinal plants have been previously published (47–54). However, in most of the studies, different and often toxic solvents were used for the preparation of extracts. As stated above all five extracts analyzed in this study were prepared using GRAS solvents (ethanol and water mixtures), which makes the extracts safe for human consumption (38). Compared to our results, only Mekinić and colleagues (47) reported higher antimicrobial activity of ethanol yarrow extract,

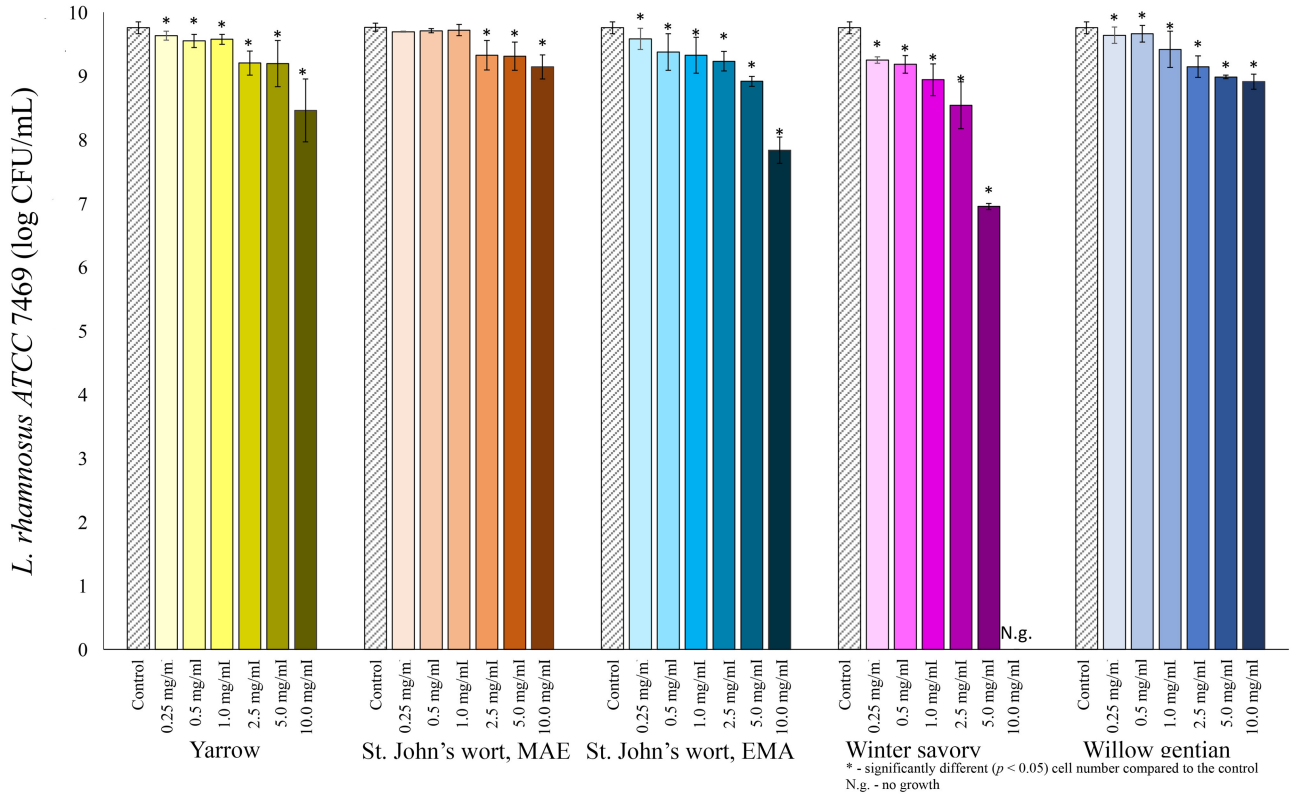

FIGURE 4 | Growth of *L. rhamnosus* ATCC 7469 (log CFU/mL) after 24 h of growth at 37°C in the growth medium supplemented with plant extracts. Tests were run in biological triplicate.

with the MIC values for *L. monocytogenes* and *S. aureus*. Moreira et al. (29) reported lower antimicrobial activity of winter savory extract compared to our results. Their extract inhibited the growth of *S. aureus* and *L. monocytogenes* with MIC values of 20 mg/mL, which are 4 and 125 times higher than the MIC values obtained in this study, respectively. Since the total polyphenol content obtained in this study is only slightly higher than in Moreira et al. (24.62 ± 0.16 vs. 23.43 ± 4.33 mg GAE/g plant dry weight), it can be expected that the MAE enhanced the extraction of more potent antimicrobial compounds. In addition, the MAE winter savory extract inhibited the growth of *E. coli*, which is in contrast to previous reports (20, 29). Moreover, the antimicrobial activity of medicinal plants depends on the date of plant collection. Borchardt et al. showed that a St. John's wort plant sample collected in August inhibited the growth of some pathogens, while the sample collected in July had no antimicrobial activity (55). This indicates that polyphenol content of medicinal plant extracts and consequent biological activity is affected by numerous factors.

The prebiotic effect of polyphenols from medicinal plants has not been assessed previously, but the influence of some plant extracts rich in polyphenols on the growth of pathogen and probiotic bacteria has been studied (35, 56–58). In our study, among all tested extracts, the highest growth-stimulating effect showed yarrow MAE extract on the yeast *S. boulardii*. With the dosage of 0.5 mg/mL in the growth medium, the maximal 130-fold stimulation of *S. boulardii* was achieved. The addition of St. John's wort MAE, willow gentian, and winter savory extracts caused an increase of *S. boulardii* growth, but not as strong as yarrow MAE extract. The exact mechanism by which polyphenols increase the growth of probiotics is not known. Most of the published papers discuss the effect of the plant extract on the pathogens and probiotic species of *Lactobacillus* and *Bifidobacterium*. To the best of our knowledge, the influence of plant extracts, or polyphenol compounds, on the growth of *S. boulardii* was not previously studied. Both winter savory and willow gentian stimulated the growth of *S. boulardii* ∼2-fold; however, the concentration of polyphenols between these two extracts was significantly different, indicating that other bioactive compounds from willow gentian could stimulate the growth of the probiotics. As reported before, the major compounds in willow gentian extracts are secoiridoids (gentiopicrin), besides polyphenols; however, there is no data in the literature on their influence on the growth of probiotic bacteria or yeast (21). It has been reported that some species of lactobacillus are able to metabolize gentiopicrin by the action of their β-glucosidase, producing aglycon and sugar moiety (59). Given the impact that the tested plant extracts showed on the pathogenic yeast *C. albicans*, it can

be concluded that consumption of the selected plants could have a beneficial effect on the *Candida*/*Saccharomyces* ratio, which could be relevant for treatment of fungal dysbiosis that was reported for inflammatory bowel disease and the diarrhea predominant irritable bowel syndrome (60, 61). Therefore, our data indicates that tested medicinal plant extracts could be used in precise nutrition/medical treatment of conditions affected by fungal dysbiosis. However, it should be noted that particular impact on the gut microbiota can be determined only in clinical trials.

Of all tested probiotic bacteria, only *L. plantarum* was stimulated by the addition of two plant extracts. The highest stimulation, 1.5-fold was achieved by the addition of St. John's wort extract in the final concentration of 0.25 mg/mL, followed by the yarrow MAE extract. Additionally, the yarrow MAE extract induced much higher, 6-fold growth stimulation of one more strain of *L. rhamnosus*, *L. rhamnosus* A71, with a concentration of 2.5 mg/mL in the growth medium (62), while it suppresses the growth of three Gram-positive and one Gram-negative bacteria, with the MIC values from 1.25 to 5 mg/mL. This suggested a potential use of the yarrow MAE extract as a dietary supplement that could initiate the change of the gut microbiota ecosystem in the direction of dysbiosis repair, contributing to increase in the relative abundance of probiotics and decrease in the relative abundance of opportunistic pathogens. Similar results could be expected for other extracts, such as winter savory, which showed the lowest growth-stimulation on tested probiotics among all four plants. It also inhibited the growth of *L. rhamnosus* ATCC 7469 and *L. rhamnosus* GG with equal MIC values, which was higher than the MIC values for pathogenic microorganisms. While our data is promising, it should be emphasized that the exact impact on the gut microbiota ecosystem can be assessed properly only in clinical trials. Based on published data on the effect of polyphenols on the growth of probiotic bacteria, one possible explanation for the stimulation of growth is that probiotics could metabolize polyphenols during growth (63, 64). Some microorganisms can hydrolyze O-glycosylated polyphenols to aglycone and glucose, which they can use for their growth, as a sole source of energy and carbon (65–67). Also, *L. plantarum* can degrade *p*-coumaric, caffeic, ferulic, coumaric, gallic and protocatechuic acid to obtain energy (13, 68, 69). For example, Duda-Chodac reported that the aglycons naringenin and quercetin inhibited the growth of *Lactobacillus* sp., while rutin showed slight stimulation on the growth (70). Rutin, *p*-coumaric, and chlorogenic acids are found in the St. John's wort and yarrow extracts which could explain their stimulatory role on the *L. plantarum* growth. Even though the bioactivity of the plant is related to its major components, the combined effect of all components in the plant can be more important for the effect of the herbal products than the activity of specific compounds (24). Therefore, the prebiotic activity could be due to the combined effect of all compounds present in the extract (24). Moreover, polyphenols in extracts could reduce oxidative stress in the medium caused by metabolic activities, thus providing better conditions for the growth of probiotic microorganisms (35).

CONCLUSIONS

The present work provides new findings on the influence of extracts from four medicinal plants—yarrow, winter savory, St. John's wort, and willow gentian—on the growth of probiotic and pathogenic microorganisms. For the first time, it was shown that polyphenol-rich medicinal plants could stimulate the growth of *S. boulardii* while suppressing the growth of pathogenic *Candida* yeast. This opens the possibility for the application of these plants in the treatment of fungal dysbiosis which could be particularly relevant for patients suffering from conditions in which the *Candida*/*Saccharomyces* disturbed ratio has been reported. Among all tested plants, the strongest antimicrobial activity was shown for the winter savory extract, which inhibited the growth of all tested pathogens. Interestingly, all tested plant extracts showed the ability to promote the growth of some of the tested probiotics and suppress the growth of some of the tested pathogens. Based on the provided data it can be speculated that medicinal plants, and particularly their polyphenol-rich extracts, could have the ability to modulate gut microbiota *in vivo*. Given that gut microbiota shows tremendous interindividual variation, and that polyphenol effects on microbial species might even be strain-dependent (65), it can be expected that the effect of medicinal herbs on microbiota will be highly individualized. This could potentially explain the high variability in response to the plant bioactive compounds, which is well-documented (71). Our results provide the first hint of the important interaction between medicinal herbs polyphenols and fungal and bacterial constituents of gut microbiota. Further studies, primarily ones based on clinical trials, will elucidate their role in microbiota-dependent personalized nutrition and medical treatment.

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.

AUTHOR CONTRIBUTIONS

MR-S created the main conceptual ideas. MM performed the experimental work and statistical analysis. MM, SD-B, and MR-S contributed to the organization and writing of the manuscript. All authors contributed to the article and approved the manuscript for publication.

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Zinc as a Biomarker of Cardiovascular Health

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The importance of zinc (Zn) for cardiovascular health continuously gains recognition. As shown earlier, compromised Zn homeostasis and prolonged inflammation are common features in various cardiovascular diseases (CVDs). Similarly, Zn biochemistry alters several vascular processes, and Zn status is an important feature of cardiovascular health. Zn deficiency contributes to the development of CVDs; thus, Zn manipulations, including Zn supplementation, are beneficial for preventing and treating numerous cardiovascular (CV) disorders. Finally, additional long-term, well-designed studies, performed in various population groups, should be pursued to further clarify significant relationships between Zn and CVDs.

Keywords: zinc, cardiovascular diseases, zinc deficiency, zinc supplementation, obesity, hypertension, cardiovascular health

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INTRODUCTION

Zinc (Zn) is one of the most essential micronutrients involved in numerous crucial biological functions, i.e., cell differentiation and proliferation, cellular transport, DNA synthesis, endocrine, immune, and central nervous system functioning, reproduction, gene expression, and homeostasis (1). With the capacity to bind more than 300 enzymes and over 2,000 transcriptional factors, it is often regarded as a multipurpose trace element (2). Zn is a major antioxidant mineral responsible for inhibiting expansion and negative effects of free radicals and regulating the oxidant-antioxidant balance of cells (3). Zn deficiency significantly affects the functioning of biological systems, creates dysfunctions in humoral and cell-mediated immunity, consequently, increases the vulnerability to infections—predisposing people to disturbances in gut microbiota activity, increases the incidence of bacterial, viral, and fungal infections, and leads to the progression of chronic and degenerative diseases, i.e., type 2 diabetes mellitus (T2DM), cardiovascular diseases (CVDs), and cancers (3). CVDs are the leading cause of morbidity and mortality worldwide, and 17.9 million people died from CVDs in 2016, representing 31% of global deaths (4). CVD-related deaths are projected to reach 23.6 million annually by 2030 (1). Three-quarters of these deaths occur in low-income and middle-income countries (4). The deficiency of Zn affects 17% of the global population, up to 35% in low-income populations, i.e., South Asia and Africa (1). An association between Zn intake and Zn status with the pathogenesis of CVDs is demonstrated by several experimental and clinical studies (5, 6). Imbalances in Zn homeostasis contribute significantly to the development of CVDs, such as coronary heart disease (CHD), congestive heart failure (HF), ischemic cardiomyopathy (CM), myocardial infarction (MI), sudden cardiac death (SCD), and CVD mortality, in general (5). Antioxidant and prooxidant functions of Zn may have various positive effects on CV health and could prevent the development of CVDs (6).

This study provides a concise and thorough overview of the relationship between Zn homeostasis and CVDs. The importance and potential suitability of Zn status as a biomarker of CV health are discussed, highlighting present controversies and research gaps that entail further research studies.

ZINC DEFICIENCY—A CONTRIBUTING FACTOR FOR DEVELOPING CVDs

Zinc is a major component of numerous enzymes within the human body. It controls the functioning of metalloenzymes, transcription factors, angiotensin-converting enzymes, desaturases, superoxide dismutases, and many others (1). Consequently, deficiency of Zn leads to apoptosis, inflammation, and oxidative stress, all well-acknowledged risk factors for the development of CVDs (7). Perturbations in Zn homeostasis affect the vascular endothelium (8). Zn deficiency weakens vascular health, impairs appropriate fatty acid and carbohydrate metabolism, and negatively impacts the cell structure of the aorta (9). Impaired Zn homeostasis is associated with common genomic and proteomic modifications that relate to CVDs (10). Zn controls the arteriosclerotic process, and inadequate Zn intake leads to increased oxidative stress, disrupted nitric oxide (NO), and nuclear factor kappa-light-chain-enhancer of activated B-cell (NF-kB) signaling and contributes considerably to endothelial damage and development of arteriosclerosis (ARS) (6). The rate of ARS, ischemic injuries, ischemic CM, and ischemic HF amplifies in line with decreasing plasma Zn levels (11, 12). Likewise, dietary Zn intake and Zn deficiency are adversely linked to subclinical ARS as demonstrated through carotid intima-media thickness (13, 14). Heart development is sensitive to Zn deficiency, and maternal Zn deficiency is linked to a high incidence of fetal heart abnormalities (15). Furthermore, Zn inadequacy prevents adequate development of cardiac tissues and increases blood pressure in fetuses and infants (16). Excessive embryonic cell death occurs after episodes of Zn deficiency (15). Proatherogenic factors, released during Zn deficiency, increase the incidence of arrhythmias, strokes, CM, and many other CV system pathologies (5, 17). There is an inverse relation between the serum Zn concentrations and the risk of CVDs in high-risk populations (18). Besides, lower serum Zn levels are associated with a higher risk of CVDs, with the greatest relations reported in most vulnerable populations, i.e., patients with diabetes and coronary angiography (18). Stimulated expression of inflammatory cytokines, i.e., interleukin 6 (IL-6), interleukin 2 (IL-2), interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and increased oxidative stress are aggravated under Zn deficiency conditions (19). Similarly, cytokines can upregulate or downregulate the expression of particular cellular Zn transporters (20). Twenty-four Zn transporters are found within the human heart muscle tissue, so disturbances in Zn homeostasis may lead to CVDs (21). Turbulences in Zn homeostasis contribute to the development of hypertension (HT) (22). Through the renin-angiotensin-aldosterone system, Zn regulates arterial pressure and plays an important role in the etiopathogenesis of arterial HT (23).

Adequate Zn levels are a critical component in peroxisome proliferator-activated receptor signaling during atherosclerosis (ATS) (23). Furthermore, patients with coronary heart disease have poor Zn status (17). Zn deficiency contributes to the thickening of the vascular wall due to enhanced proliferation and hypertrophy (24). Low serum Zn levels are measured in people with HF (25, 26). Zn also has a role in redox signaling pathways, and it improves antiapoptotic, anti-inflammatory, and antioxidant activities (27). Deficiency of Zn can degenerate essential proteins like protein creatine kinase (C kinase), stimulate the production of inflammatory cytokines and C-reactive proteins, and may trap constituents in monocytes and macrophages (19). Serum Zn levels are considerably diminished in patients with left ventricular hypertrophy (LVH), and a significant inverse relation is seen between Zn status and LVH (5). Patients with ischemic stroke have lower serum Zn levels than healthy subjects (13). Similarly, lower serum Zn levels are seen in patients with HF and patients with left ventricular diastolic function (28). Besides, serum Zn levels are inversely associated with diminished glucose homeostasis and insulin resistance (29). Low serum Zn concentration predicts mortality in patients that need coronary angiography (30). What is more, serum Zn levels could be a valid diagnostic indicator for acute MI (31). According to the meta-analysis data, an increased prevalence of coronary artery disease (CAD) is linked to a lower dietary Zn intake, with a direct association between Zn status and MI (31).

ZINC INTERVENTIONS ALLEVIATE RISK FACTORS FOR CVDs

Cohort studies, randomized trials, and meta-analyses of these studies propose that higher consumption of dietary Zn is linked to reduced risk of CVDs. Administration of Zn stimulates myocardial healing and improves arrhythmias (32). Besides, Zn is a wound-healing agent that supports cardiac steam cell survival, a critical element of cardiac healing (12). Zn supplementation has an atheroprotective effect (20) and contributes to a higher concentration of high-density lipoprotein cholesterol (HDL-C) and apoproteins, and lower total cholesterol (TC) levels (33). Higher serum Zn concentrations are associated with a decline in relative risk of death of CVDs (12). Reduced prevalence of CAD and T2DM is correlated with higher dietary Zn intakes (34, 35). Additionally, higher plasma Zn concentration is associated with a diminished risk of mortality of vascular disease (VD) (30, 36). Zn supplementation could potentially increase the effectiveness of currently used therapeutic drugs for managing CVDs (37). Finally, recently presented data of a systematic review and meta-analysis point out that low-dose and long-duration Zn interventions are of identical or in some instances of even larger magnitude and with even more beneficial effects compared to high-dose and short-duration interventions. Long-duration Zn studies, for 12 weeks or longer, alleviated risk factors for T2DM and CVDs, such as blood glucose, total fats, triglycerides (TGs), and low-density lipoprotein cholesterol (LDL-C), while the longer duration of low Zn doses affected a larger number of risk factors (38).

LIMITED KNOWLEDGE ON VASCULAR ROLES OF ZINC TRANSPORTER PROTEINS

Twenty-four Zip transporters are present within human heart muscle tissues, so disturbances in Zn homeostasis are strongly related to CVDs (21). Zrt, Irt-like protein2 (Zip2), Zip12, Zip14, and Zn transporter1 (ZnT1) and ZnT2 are linked to the vascular biology of CVDs (37).

For example, Zip2 has a beneficial role in the post-conditioning cardioprotective process (32). Zip2 polymorphism is associated with human carotid artery disease in the elderly (39). In addition, Zip12 is involved in the uptake of Zn into the vascular wall (22). Yet, limited information is available on vascular roles of Zip14, ZnT1, and ZnT4 (37).

Furthermore, ZnT1 is involved in cardiac electrophysiological effects of Zn and increased ZnT1 expression is seen in patients with atrial fibrillation (40, 41). Zn has a central role in the generation of NO and actions that have multiple implications for vascular endothelial and smooth muscle functions, i.e., vascular smooth muscle relaxation, antiplatelet properties, and protection of vascular endothelium against oxidative damage (42). The availability and function of NO are disturbed in Zn deficiency (37). The action of NO is controlled by both Zn and metallothionein (MT), so an insufficient supply of endothelial Zn will make NO ineffective as a CVD therapeutic agent (37).

Investigation of the genetic polymorphism of Zn transporters gains more and more attention. The polymorphism of Zn transporters confers a predisposition to various chronic and age-related diseases, such as chronic CVDs (43, 44). A common polymorphism in the ZnT8 gene, on the C allele, is associated with a higher risk of developing T2DM and metabolic syndrome (45, 46). Several single-nucleotide polymorphisms modulate Zn intake and status (47). There is an interaction between certain dietary components (i.e., omega three fatty acid intake) with Zn transporters in relation to the risk factors for CVD development (48). MT polymorphisms, MT1A, MT1B, MT2, and MT4, are often associated with dietetic neuropathy, blood pressure, inflammatory cytokine levels, DM, and CVDs (49–51). Similarly, there is an indirect involvement of uncoupling proteins in the MT-dependent reduction in the free radical-induced cardiac toxicity (52). Finally, ZnT1, ZnT4, ZnT5, ZnT6, ZnT7, and ZnT9 polymorphisms are linked to T2DM, dyslipidemia, and insulin resistance, all well-known CVD risk factors (53, 54).

ZINC AND INDEPENDENT RISK FACTORS OF CVDs

Several risk factors (i.e., T2DM, obesity, and HT) that predispose to VD are linked to irregularities in Zn homeostasis in individual organs or the whole body (55). A direct association between serum Zn and metabolic risk factors for the development of CVD, i.e., serum lipids, T2DM, and obesity, is shown (35, 55, 56). Zn plays an important role in insulin synthesis, crystallization, storage, and secretion in the pancreatic β-cells (57). Oxidative stress, a key risk factor in the pathogenesis of diabetes mellitus (DM), is aggravated under Zn deficiency states (58). Zn has insulin-mimicking properties, stimulates glucose uptake in insulin-dependent tissues, and regulates gluconeogenic enzymes (59).

ZnT8, located on dense core vesicles in β-cells, has a central role in the transportation of Zn into insulin secretory granules of β-cells and is identified as a novel therapeutic target in patients with diabetes (18, 60). Diabetes is often accompanied by hypozincemia and hyperzincuria (33, 61). Furthermore, Zn stimulates insulin binding to hepatocyte membranes and low Zn status considerably decreases the reaction of tissues to insulin (62). There is an inverse correlation between femur Zn and serum glucose concentrations (63).

Interestingly, a moderately high Zn intake could reduce the risk of diabetes by 13%, up to 40% in people living in rural areas (64). Zn supplementation improves glycemic control and reduces hemoglobin A1c (HbA1c) levels in patients with T2DM (63, 65). Besides, Zn improves glucose metabolism and contributes to glucose uptake into the relevant tissues (66). By inhibiting the activation of cytokines, Zn deficiency contributes to apoptosis and insulin resistance of β-pancreatic cells (57). The highest amount of Zn within the human body is stored in the pancreatic β-cells, so Zn ameliorates the consequences of immune-mediated free radicals in pancreatic islet cells (67). In addition, Zn stimulates phosphorylation of insulin receptor substrates and improves insulin sensitivity (68). Insulin resistance of adipocytes increases the release of fatty acids into the circulation and consequently improves fatty acid flux to the liver leading to hypertriglyceridemia (63). Similarly, Zn affects lipid metabolism directly. Zn maintains adipose tissue functioning *via* the activity of Zn finger proteins involved in the regulation of lipid metabolism (69). Zn-α2-glycoprotein inactivates hormone-sensitive lipase and accordingly reduces lipogenesis and increases lipolysis in adipose tissues (66). Zn modulates postprandial lipemia, and Zn deficiency markedly reduces the absorption rate of TGs, brings compositional alterations of chylomicrons, and reduces their production rates and uptake by the liver (70). Thus, Zn deficiency is often linked to obesity, due to chronic inflammation and oxidative stress. Zn levels in obese subjects are lower than in controls (71–73), and supplementation of Zn reduces plasma insulin resistance, leptin, and inflammatory biomarkers in obese individuals (74, 75).

ZINC HOMEOSTASIS AND CVDs—THE EXISTING CONTROVERSIES

The link between HT and Zn status is not decisive, and contradictory findings are reported over the years. Some studies demonstrate an inverse association (76, 77), while others found

a direct positive link between serum Zn levels and blood pressure (57, 78). There are also data signifying no association between the two variables (74–81). Similarly, discrepancies in findings are reported for the risk of developing ARS in relation to serum Zn levels, and certain data reveal a direct link (6, 82), while others show no association between the serum Zn concentrations and ARS (83). The first randomized controlled trial (RCT) in humans shows adverse effects of Zn supplementation on HDL-C in healthy subjects (20). However, opposite findings exist, and a positive relation between serum Zn and HDL-C and LDL-C concentrations is observed (79). Lower consumption of dietary Zn is related to low HDL-C levels (31).

In addition, Zn supplementation has a beneficial effect on plasma lipid parameters, and it noticeably reduces TC, LDL-C, and TG levels in healthy individuals (10, 33). The benefits of Zn supplementation are more evident in non-healthy population groups. The meta-analysis data show that Zn supplementation leads to a significant reduction in LDL-C, TC, and TG levels in non-healthy patients, while in healthy people a noteworthy decline in TC levels is seen (33). HDL-C levels increase under Zn supplementation (20, 33, 77). Large longitudinal prospective cohort studies provide inconsistent findings on the association between supplementary Zn intake and risk of T2DM, showing both a direct, beneficial (34, 49, 83, 84), an inverse (85), and no relation (86, 87).

Likewise, there are no definitive conclusions on the relationship between Zn status and T2DM: no association (88) and an inverse link are reported (89) but, lower serum Zn levels are generally associated with an increased risk of T2DM (43, 90).

Different health status of participants, dissimilarities in the design, assessed outcomes across studies and influence of confounding factors and their appropriate adjustments, (i.e., medication, duration of the disease, dietary habits, and physical activity), differences in Zn assessment methods, lack of distinction in dietary Zn sources, variations in dietary data collection, and the inconsistency in utilized statistical models may all be potential reasons for observed discrepancies in findings among studies.

RESEARCH GAPS AND RECOMMENDATIONS FOR FURTHER RESEARCH STUDIES

The precise role of Zn deficiency mechanisms in the pathogenesis of CVDs is still not known. The biological properties of Zn, playing a role in the physiology and pathology of CVDs, should be examined further. Additional community-based observational cohort studies may be useful for obtaining more precise and evidence-based conclusions on the relation between Zn and CVDs. It is essential to clarify the instances when inadequate dietary Zn intake and low Zn status are a result rather than a cause of CVDs. Particular attention should be paid to exclude the negative effects of medications of CVDs, i.e., diuretic furosemide, angiotensin receptor blockers, and angiotensin-converting enzyme inhibitors, on Zn status. Larger, well-designed randomized clinical trials are necessary to thoroughly examine the effect of Zn intake on CV health. Potential interactions with other dietary factors and micronutrients that could modulate Zn intake should be considered. Benefits, clinical applications, risks, and contraindications of dietary and supplemental Zn intake on main CV events should be examined further. The impact of the baseline Zn status on the efficacy of Zn interventions on CVD risk factors is of great importance and should be appropriately assessed and reported. Risk factors related to CVDs should be examined as primary outcomes of these interventions, and they should aim to examine the development and progression of these conditions. Further research studies should investigate the interaction between Zn intake and Zn status data with present preventative schemes and currently employed treatment methods that could help in the prevention and management of many ensuing CVDs.

As Zn status is affected by various factors, a careful selection of confounders should be made. Zn deficiency may not only be caused by an inappropriate dietary intake and/or bioavailability but also by factors such as age, physical activity, and alcohol or drug addictions. Further research studies should explore molecular mechanisms that support the sensing and distribution of Zn in various tissues. The interaction between Zn and inflammation deserves further research studies. The limitations of biomarkers of Zn status should be taken into consideration. Circulating plasma/serum Zn concentrations are affected by inflammation, time of the last meal, infections, and some other factors. All these elements have to be suitably deliberated. Newly proposed biomarkers of Zn status should be taken into consideration and investigated to CVD-related factors.

The mechanisms of action of Zn transporter proteins require additional research studies. Detailed and careful analysis of the activities of these transporters is required to improve our knowledge on the pathogenesis of CVDs. The transfer of information from Zn intake/status to cellular functions needs further extrapolation. New studies are needed to provide a more thorough understanding of MT and ZnT roles and the effects of their common genetic variations. Additional studies are required to explain the interactions between specific genetic profiles and zinc status. Further research studies should clarify gene-nutrient interactions and their relationship with Zn status and CVDs. It would be beneficial to develop suitable methods for measuring endothelial Zn as a biomarker of vascular Zn deficiency. The interplay between Zn and NO levels should be further investigated. The expression and functions/dysfunctions of Zn transporters in vascular tissues and genetic risk factors associated with Zn transporters should be additionally tested. Zn homeostasis is altered early in CVDs, so an intervention with Zn-related therapy could provide significant benefits. Preventative CVD actions should include programmed Zn nutrition approaches. The possibility of therapeutic manipulations of CVDs by Zn-based treatments exists; however, further low-dose short- and/or long-duration

well-designed studies, across a variety of populations, are needed. The role of Zn supplementation in the process of recovery from CVDs should be more intensively investigated to find safe and desirable levels of Zn supplementation and, additionally, to determine the dose and duration that would be most beneficial primarily for the prevention of and, if need be, for the treatment of various ensuing CVD-related pathologies. Appropriate dietary recommendations, food fortification, and agronomic biofortification strategies should all be investigated and employed so that majority of people, both in developing and developed countries, can attain sufficient levels of dietary Zn in daily diets and potentially diminish the risk of developing CVDs.

AUTHOR CONTRIBUTIONS

MK conceptualized and wrote the manuscript and prepared the manuscript for submission. MG revised the final version of the manuscript. Both authors contributed to the article and approved the submitted version.

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Memorable Food: Fighting Age-Related Neurodegeneration by Precision Nutrition

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Healthcare systems worldwide are seriously challenged by a rising prevalence of neurodegenerative diseases (NDDs), which mostly, but not exclusively, affect the ever-growing population of the elderly. The most known neurodegenerative diseases are Alzheimer's (AD) and Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis, but some viral infections of the brain and traumatic brain injury may also cause NDD. Typical for NDD are the malfunctioning of neurons and their irreversible loss, which often progress irreversibly to dementia and ultimately to death. Numerous factors are involved in the pathogenesis of NDD: genetic variability, epigenetic changes, extent of oxidative/nitrosative stress, mitochondrial dysfunction, and DNA damage. The complex interplay of all the above-mentioned factors may be a fingerprint of neurodegeneration, with different diseases being affected to different extents by particular factors. There is a voluminous body of evidence showing the benefits of regular exercise to brain health and cognitive functions. Moreover, the importance of a healthy diet, balanced in macro- and micro-nutrients, in preventing neurodegeneration and slowing down a progression to full-blown disease is evident. Individuals affected by NDD almost inevitably have low-grade inflammation and anomalies in lipid metabolism. Metabolic and lipid profiles in NDD can be improved by the Mediterranean diet. Many studies have associated the Mediterranean diet with a decreased risk of dementia and AD, but a cause-and-effect relationship has not been deduced. Studies with caloric restriction showed neuroprotective effects in animal models, but the results in humans are inconsistent. The pathologies of NDD are complex and there is a great inter-individual (epi)genetic variance within any population. Furthermore, the gut microbiome, being deeply involved in nutrient uptake and lipid metabolism, also represents a pillar of the gut microbiome–brain axis and is linked with the pathogenesis of NDD. Numerous studies on the role of different micronutrients (omega-3 fatty acids, bioactive polyphenols from fruit and medicinal plants) in the prevention, prediction, and treatment of NDD have been conducted, but we are still far away from a personalized diet plan for individual NDD patients. For this to be realized, large-scale cohorts that would include the precise monitoring of food intake, mapping of genetic variants, epigenetic data, microbiome studies, and metabolome, lipidome, and transcriptome data are needed.

Keywords: neurodegenerative diseases (MeSH), epigenetics (DNA methylation, histone modifications), gut microbiota, aging, precise nutrition

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INTRODUCTION

Because of increasing life expectancy and decreasing birth rates, the world's population aged 60 years and older is expected to total 2 billion by the year 2050, and 80% of the elderly will be living in low- and middle-income countries, according to a World Health Organization report (1). Healthcare systems all over the world are seriously challenged by a rising prevalence of disabling chronic diseases, including cancer, cardiovascular disease and neurodegenerative disease (NDD), which affect mostly, but not exclusively, the ever-growing population of the elderly. Gradual and progressive severe damage in neuronal cells lead to severe memory and behavioral impairment (dementia) and loss of movement control (ataxia and paralysis) making NDD the major cause of disability and morbidity among older people worldwide. The most common NDDs are Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS), but some viral infections of the brain and traumatic brain injury may also cause NDD. Moreover, a significant proportion of the older population is affected by "age-related cognitive decline," which is independent of dementia and has an incidence 70% higher than dementia alone (2). These patients experience increasing deficits in daily living activities, productivity losses, and subsequently need constant and long-term care, which is connected with overwhelming economic and societal cost (3, 4). Thus, healthy aging and the prevention of neurodegeneration is emerging as a global ultimate goal.

Several cellular and molecular determinants of aging have been identified so far, including loss of protein homeostasis (proteostasis), stem-cell exhaustion, mitochondrial dysfunction, genomic instability, epigenetic alterations, telomere attrition, cellular senescence (i.e., permanent proliferation arrest), deregulated nutrient sensing, altered intracellular signaling, and synaptic dysfunction (5–7). The complex interplay of all the above-mentioned factors is a fingerprint of neurodegeneration, with different diseases being affected to different extents by specific factors.

The phenotypes of aging can be modified to increase longevity and to prevent or to delay the onset and/or to ameliorate the clinical course of neurodegeneration. Besides drugs approved by the Food and Drug Administration (FDA), such as acetylcholine esterase and levodopa for PD, that ameliorate the symptoms and slow down the progression of NDD, many other medications, such as rapamycin, senolytics, metformin, acarbose, spermidine, and NAD^+ enhancers, may improve the quality of life by preserving functional capacity and decreasing disease burden in the elderly and are currently being intensively investigated (8). There is increasing evidence that regular exercise and healthier dietary patterns, balanced in macro- and micro-nutrients, can also have beneficial effects on brain health and cognitive functions by modifying the above-mentioned age-related molecular determinants.

ROLE OF DIET IN NEURODEGENERATION

The important role of deleterious dietary behavior (overfeeding, a high caloric/low dietary fiber diet, or the low consumption of antioxidant nutrients), environmental factors (smoking, alcohol, stress, drugs, and exposure to pesticides), and a sedentary lifestyle throughout the entire life span, including early life, in the development of neurodegeneration is now well-recognized (9). The unbalanced diet during pre-conception, pregnancy, and the first 2 years of life is associated with the inheritance of epigenetic alterations that promote neurodegeneration and are transmissible to offspring and to subsequent generations (10). In addition, an unhealthy diet may alter the gut microbiota, including the neonate's microbiota *via* breastfeeding as a result of the mother's diet, and promote the development of NDD (11). Generally, the consumption of diets rich in antioxidants and anti-inflammatory components and reduced caloric intake may lower age-related cognitive decline and the risk of NDD (12). Fruits, vegetables, beverages, green tea, coffee, spices, nuts, and cereal products are major sources of plant-derived antioxidants—polyphenols (phenolic acids, flavonoids, anthocyanins, lignans, and stilbenes), carotenoids (xanthophylls and carotenes), and vitamins (vitamins E and C)—and their beneficial effect in NDD has been previously reviewed (13). *In vitro* and *in vivo* studies have proposed the neuroprotective properties of vitamin D (14), B vitamins (B12, B6 and riboflavin) (15), vitamin K (16), and trace elements such as selenium, copper, magnesium, iron, lithium, and zinc (17) on neurocognitive disorders, mitochondrial dysfunction, immune dysfunction, inflammatory conditions, cognition, and memory. The neuroprotective role of polyunsaturated fatty acids (PUFAs) and their positive effect in prevention and treatment in NDD is documented in nutritional epidemiology studies, prospective population-based surveys and clinical trials (18, 19). Metabolic and lipid profiles in NDD can be improved by healthy dietary patterns, such as the Mediterranean diet.

The traditional diet consumed in Mediterranean countries is characterized by a high intake of vegetables, legumes, fruits, nuts, and wholegrains, a moderate intake of fish, poultry, and red wine (with meals), and a low intake of red and processed meats, with olive oil used as the main fat source; as a whole the diet has a positive effect on diabetes, cardiovascular disease, and many other chronic conditions (20), as well as aging (5). The nutritional value of this diet implies the consumption of antioxidants, vitamins, trace elements, and PUFAs, in particular ω-3 PUFA. It has been reported that an increased adherence to the Mediterranean diet over a longer period (above 4–6 years) contributes to neuronal integrity (increases cortical thickness and brain volume, slows down the rate of hippocampal atrophy and amyloid accumulation, and improves structural connectivity), as well as cognition, memory, and executive function (21).

As such findings are fragmented and sometimes inconsistent, the optimal daily doses of particular macro- and micro-nutrients in preventing, slowing, and reversing neurodegeneration are still to be established in different population subgroups. Precision nutrition and precision medicine, based on the phenotype of

aging, food preferences, clinical history, and lifestyle patterns, are becoming important issues with regard to neurodegeneration. The mechanisms by which neurodegeneration can be fought through "memorable" food, with a focus on epigenetic mediation, intervening in the gut microbiota's composition, the reversal of low-grade inflammation and anomalies in lipid metabolism, and caloric restriction, are further discussed.

EPIGENETICS IN NEURODEGENERATION

An increasing body of evidence suggests the role of epigenetic modifications in the development of NDD. Epigenetics encompasses a wide range of stable inheritable and reversible modifications that result in changes in gene expression and function, without affecting the DNA sequence (22). The epigenome constantly changes during the lifespan of an individual. Some of the epigenetic modifications are intrinsically programmed and essential for normal development, growth, and differentiation. The others result in inappropriate epigenetic reprogramming. Although epigenetic modifications are quite stable, they can be modulated by physiological and pathological conditions as well as by the environment (23).

These modifications typically arise owing to DNA methylation or hydroxymethylation, histone post-translational modifications, synthesis of microRNA (miRNAs) and long non-coding RNAs (lncRNAs), and changes in nucleosome positioning, thereby regulating patterns of gene expression. In normal cells these changes are well-balanced and affected by genetic factors (24), environmental factors (25), and stochastic (undetermined) factors. The influence of hereditary factors in epigenetic changes over time has been shown in studies of monozygotic twins (26), dizygotic twins (27), as well as by the familial clustering of DNA methylation found in longitudinal studies (28). In addition, the epigenetic process can also be affected by nutritional and environmental factors and thereby be dynamically changed during the lifespan of an organism (29, 30). Although methylation was originally thought to serve as a stable mark of gene silencing, nowadays it is known that these changes in DNA methylation can be both rapid and reversible (31). Several studies have shown that nutrition- and environment-induced epigenetic modifications can occur at any stage of life, from the *in utero* period throughout adult life and aging, and they can be maintained through multiple offspring generations (32). Epigenetic changes may lead to mutations, and, conversely, mutations are frequently observed in genes that modify the epigenome (33).

DNA METHYLATION

Among all epigenetics processes, the most common and investigated is the methylation of DNA.

As presented in **Figure 1**, the mechanism of DNA methylation implies the presence of S-adenosyl methionine (SAM) as the universal methyl donor for DNA and histone proteins. SAM donates the methyl group to the C5 atom of the cytosine moieties followed by guanines, the so-called CpG dinucleotide. This conversion involves the action of DNA methyltransferases (DNMTs). In turn, SAM becomes S-adenosylhomocysteine (SAH), which acts as a competitive inhibitor of methyltransferases, including DNMTs (34) (**Figure 1**). The inhibition is limited as SAH is rapidly hydrolyzed to adenosine and homocysteine. The main role of DNA methylation is to reduce or impair the binding of transcription factors to the regulatory regions, i.e., promoters of genes. Moreover, DNA methylation results in the recruitment of methyl-binding proteins (MBPs) and histone deacetylases (HDACs) at the methylated site of promoter regions, thereby repressing the expression of genes. In line with this, actively transcribed genes have hypomethylated promoters, whereas hypermethylated promoters are normally associated with silenced, non-expressed genes (35).

There is growing evidence that altered DNA methylation contributes to the occurrence of several diseases (36). Different types of cancers and allergic, immunological, and inflammatory diseases are closely associated with the epigenetic changes of DNA (37–39). Besides tumors, the main class of diseases associated with epigenetic modifications is neurodegenerative disease (40). The association between DNA methylation and NDD is confirmed in Parkinson's disease (41), Alzheimer's disease (42), amyotrophic lateral sclerosis (43), and multiple sclerosis (44). In these diseases, some of the genes are hypermethylated while others are hypomethylated. Thus, in AD, several genes are hypermethylated (*APOE, MTHFR, MAPT, SORB3*), while others included in Aβ peptide production (*PSEN1, APP, PP2A, CREB5, S100A2, BACE*) are hypomethylated (45). Studies have indicated a positive effect of SAM donors on cognitive function and AD in animals and humans through downregulation of the *PSEN1* gene (46, 47). Thus, in a few animal studies conducted in the mice model for AD, supplementation with SAM as the methyl donor modulates the methylation in *PSEN1*, which leads to not only restoring the methylation potential but also losing the symptoms linked with AD (48, 49).

Besides DNA methylation, histone acetylation is a reversible epigenetic modification controlled by histone acetyltransferases and deacetylases. The acetylation of lysine residues on histones decreases the electrostatic attraction between the histones and the DNA backbone and consequently increases transcription. In NDD, histone acetylation homeostasis is markedly impaired, shifting toward hypoacetylation. Enhanced histone acetylation, promoted by HDAC inhibitors, improves learning and memory and has a neuroprotective effect (50).

Many different environmental stressors, such as diet, pollutants, pesticides, chemical species, drugs, physical exercise, and stress, are known to be causative of epigenetic changes (35). They can switch on/off corresponding genes either by direct interaction with DNA, RNA, or chromatin receptors or indirectly using various enzymes or other epigenomic-associated pathways (51–53).

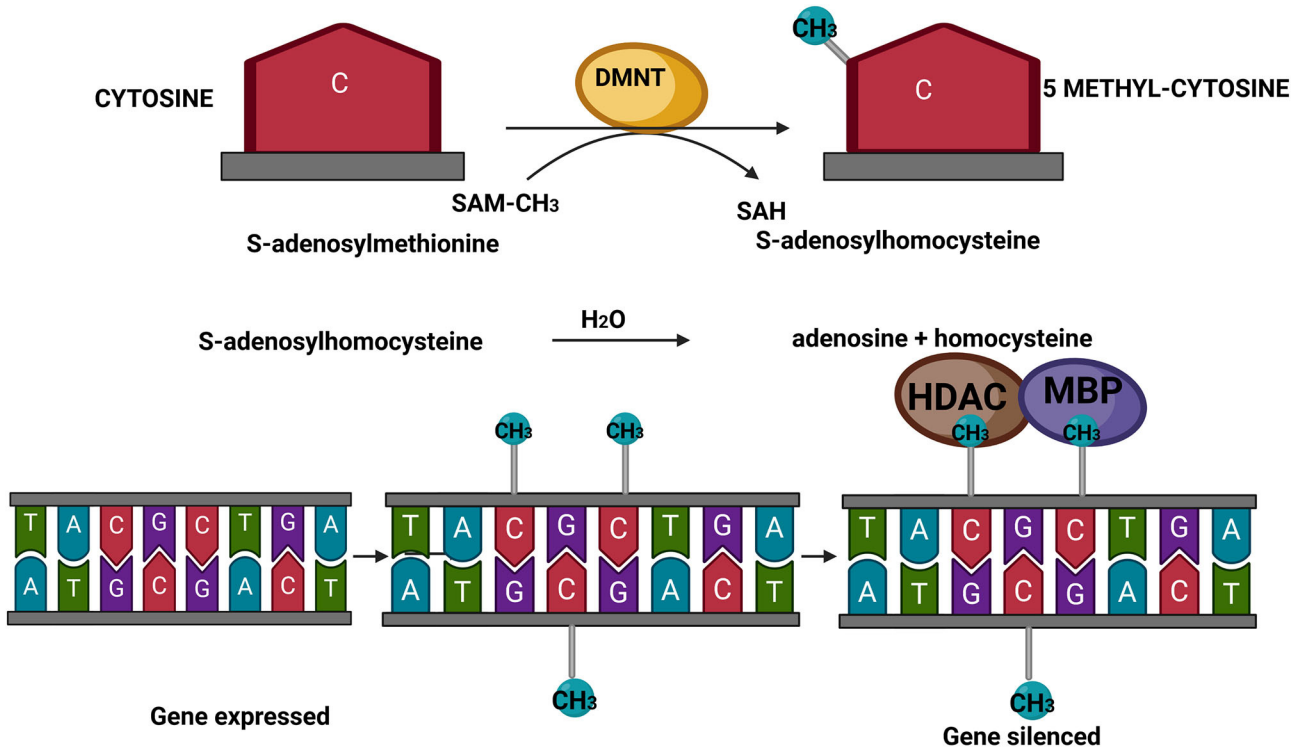

FIGURE 1 | Schematic presentation of DNA methylation. SAM, S-adenosyl methionine; SAH, S-adenosylhomocysteine; DNMTs, DNA methyltransferases; MBPs, methyl-binding proteins; HDACs, histone deacetylases. Created with BioRender.com.

NUTRIEPIGENOMICS

Many nutrients from food interact with the DNA, and these interactions are studied by nutriepigenomics. Nutrients affect human health *via* epigenetics without alterations in the DNA sequence in two ways—by promoting epigenetic modifications and by reversing the previous or inherited changes. With regard to food, there are differences between synthetic xenobiotics (bisphenol and glyphosate), which are consumed with food and have an epigenomic effect, and diet and its many micro- and macro-nutrients that have also demonstrated epigenetic effects in *in vitro* and *in vivo* studies, as well as in clinical trials. Toxic xenobiotics may induce DNA methylation in different ways: directly *via* the inhibition of DNA methyltransferases, which leads to hypomethylation, or by subtracting methyl groups from the physiological reactions in which they are included (53).

On the other hand, some nutrients can not only prevent the hypermethylation of DNA but also promote demethylation and the reversal of genes silenced by previous DNA methylation. Thus, molecules such as B vitamins act as methyl donors and might avert the loss of DNA methylation induced by air pollution or some other cause (54). In addition, some bioactive compounds can reverse the epigenome dysregulation induced by bisphenol A (55), while dietary folic acid supplementation can prevent the adverse effects caused by heavy metals (56).

In general, vegetables and fruits and their active molecules have epigenetics potential, and they can modulate DNA methylation. To date, many bioactive components, such as lycopene, hesperidin, phloretin, genistein, coumaric acid, caffeic acid, isothiocyanates, and epigallocatechin gallate, have been identified as those with strong epigenetic potential (**Table 1**). These molecules exert different effects on the levels of DNA methylation. While some of them show hypermethylating activity, there are those with hypomethylating effects. Thus, tea flavonoids, such as catechin, epicatechin, epicatechin 3-gallate, epigallocatechin, and quercetin, or parsley's apigenin inhibit DNA methylation, leading to demethylation and the reactivation of genes previously silenced by methylation (57). Many of them inhibit DNA methylation in a direct manner by forming hydrogen bonds between different residues in the active site of DNMT (34) or indirectly by decreasing the level of SAM and increasing the levels of both SAH and homocysteine, which subsequently leads to the inhibition of DNA methylation (58). Similarly, resveratrol, found in grapes and red wine and also in peanuts, mulberries, and cranberries, modulates DNA methylation and histone modification *via* the inhibition of DNMTs and histone deacetylase activities (59). In addition, several studies indicate that caffeic acid, present in coffee and barley grain (60), and polyphenols from olive oil can induce the inhibition of DNA methylation (61). In addition, some bioactive molecules, such as curcumin, have both hyper- and hypo-methylating effects on different genes in different cancers, with the same outcome on the tumor (62–64). They can activate some tumor suppressor genes and inactivate oncogenes. Although the

protective effects of Ginkgo biloba extract and its flavonoid kaempferol ellagitannin have been widely investigated in AD, its role in the epigenetic alterations related to AD pathogenesis has not been fully finalized. Namely, the inhibition of HDAC activity by kaempferol is confirmed in human-derived hepatoma and colon cancer cells but not in NDD (65).

EPIGENETIC EFFECTS OF FATTY ACIDS

Apart from polyphenols, fatty acids may also be involved in epigenetics transformation. Dietary PUFAs play a significant role in regulating the epigenome, especially in modifying DNA methylation (66). On other hand, there is an opposite correlation, i.e., epigenetic processes can be involved in PUFA biosynthesis processes. However, like polyphenols, the epigenetics roles of PUFAs have been mostly investigated in tumor cells, but not in NDD. Thus, Huang et al. demonstrated that treatment with ω-3 PUFA induced decreased tumor incidence and tumor size in a colorectal cancer rat model (67). They showed that there was a close correlation between the anticancer effects of ω-3 PUFA and increased genomic DNA hydroxymethylation, leading to the silencing of some genes. On the other hand, Sarrabi et al. indicated that PUFA treatment caused the decreased methylation of different oncogenes and suggested that PUFAs can alter both DNA methylation and the expression of DNMTs in colorectal cancer cells (68). Similarly, Ceccarelli et al. showed that ω-3 PUFA directly regulates and demethylates DNA in hepatocarcinoma cell lines (69). Thus, dietary supplementation with bioactive compounds and PUFA may lead to better prognoses for diseases that are associated with epigenetics modulation, including NDD.

Epigenetics development and the identification of highly sensitive, specific, and easily accessible epigenetic biomarkers and applying them, along with genetic biomarkers, is a key step toward successful personalized treatment. Besides personal genetic and epigenetic information, other data, including gender, age, gut microbiota, and presence of diseases, should be taken into account for personalizing prevention and treatment (70). These differences among individuals result in different responses to similar treatments and suggest the need for a personalized approach.

MICROBIOTA AND GUT–BRAIN AXIS

Apart from (nutri)epigenetics, the latest research has shown that the gut microbiota affects the brain's physiological, behavioral, and cognitive functions, although the exact mechanisms have not been fully clarified. The intestinal microbiota represents about 10^{14} microbial cells including bacteria, archaea, viruses, fungi, and protozoa populating the gastrointestinal tract and maintaining a symbiotic relationship with the host. Most of the microbial species in the human gut belong to five phyla: *Firmicutes* and *Bacteroidetes* are dominant, whereas *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* represent minor constituents. Dysbiosis, the imbalance in the composition and function of the gut microbiota, has been implicated in the development of chronic diseases, including gastrointestinal, autoimmune, metabolic, and neurodegenerative diseases (71).

Numerous factors may have harmful effects on the microbiome such as diet, food additives, pesticides, antibiotics, and stress. The balance of and symbioses with the gut microbiome that have been established during human evolution and the rapid change of diet in the last 100 years may outpace the time necessary for the adaptation of the digestive system, resulting in increased occurrence of chronic diseases. Ultra-processed food and excessive energy intake are dominating hallmarks of the Western diet. This diet, abundant in saturated fat and refined carbohydrates, negatively impacts on gut microbiome composition and consequently on the immune system and brain health (72). On the other hand, epidemiological data suggest that caloric restriction and dietary intervention using certain macronutrients (fish), micronutrients (B vitamins, vitamins C, E, and D, flavonoids, and omega-3 PUFA), probiotics, and prebiotics may prevent cognitive decline and/or delay age-related neurodegeneration (73). Such a type of diet is the Mediterranean diet.

The gut microbiota communicates with distant organs and the brain through a complex neuro-humoral connection called the gut–brain axis, which includes: the central nervous system (CNS), the autonomic nervous system, the enteric nervous system (ENS), the hypothalamic–pituitary–adrenal axis, and the immune system. The ENS is the largest part of the peripheral nervous system, consisting of ∼200 million neurons and enteric glial cells (the digestive equivalent of brain astrocytes) located along the gastrointestinal tract and often referred to as the "second brain" due to its ability to control gut behavior with and without input from the CNS. Important players in the gut–brain axis are the enteroendocrine cells (EECs). They are specialized cells localized within the intestinal epithelium and represent the sensors of the gut microbiota and its metabolites. In response to luminal content, the EECs secrete hormones and cytokines that can act in a paracrine manner on the ENS or send information *via* the vagus nerve to the CNS (**Figure 2**). In addition, they are involved in the motility of the gastrointestinal tract, the gut barrier, and mucosal immunity. The vagus nerve, as the main component of the parasympathetic nervous system, establishes one of the connections between the gut, the brain, and inflammation and represents an important link between nutrition and diseases. The CNS affects digestion by regulating gut motility, secretion, and immunity *via* the sympathetic and parasympathetic nervous systems. Neurotransmitters, hormones, and peptides released by the ENS and transported through the bloodstream cross the blood–brain barrier and can act synergistically with the signals sent "down" from the brain through the efferent vagus nerve to regulate food intake and appetite (74). The gut microbiota reacts to these changes by producing neurotransmitters and microbial metabolites, such as short-chain fatty acids (SCFAs), secondary bile acids, and tryptophan- and polyphenol-derived products, which all affect the host's CNS. These processes and connections are schematically presented in **Figure 2**.

The major metabolites secreted by the colon microbiota, after anaerobic degradation of non-digestible carbohydrates

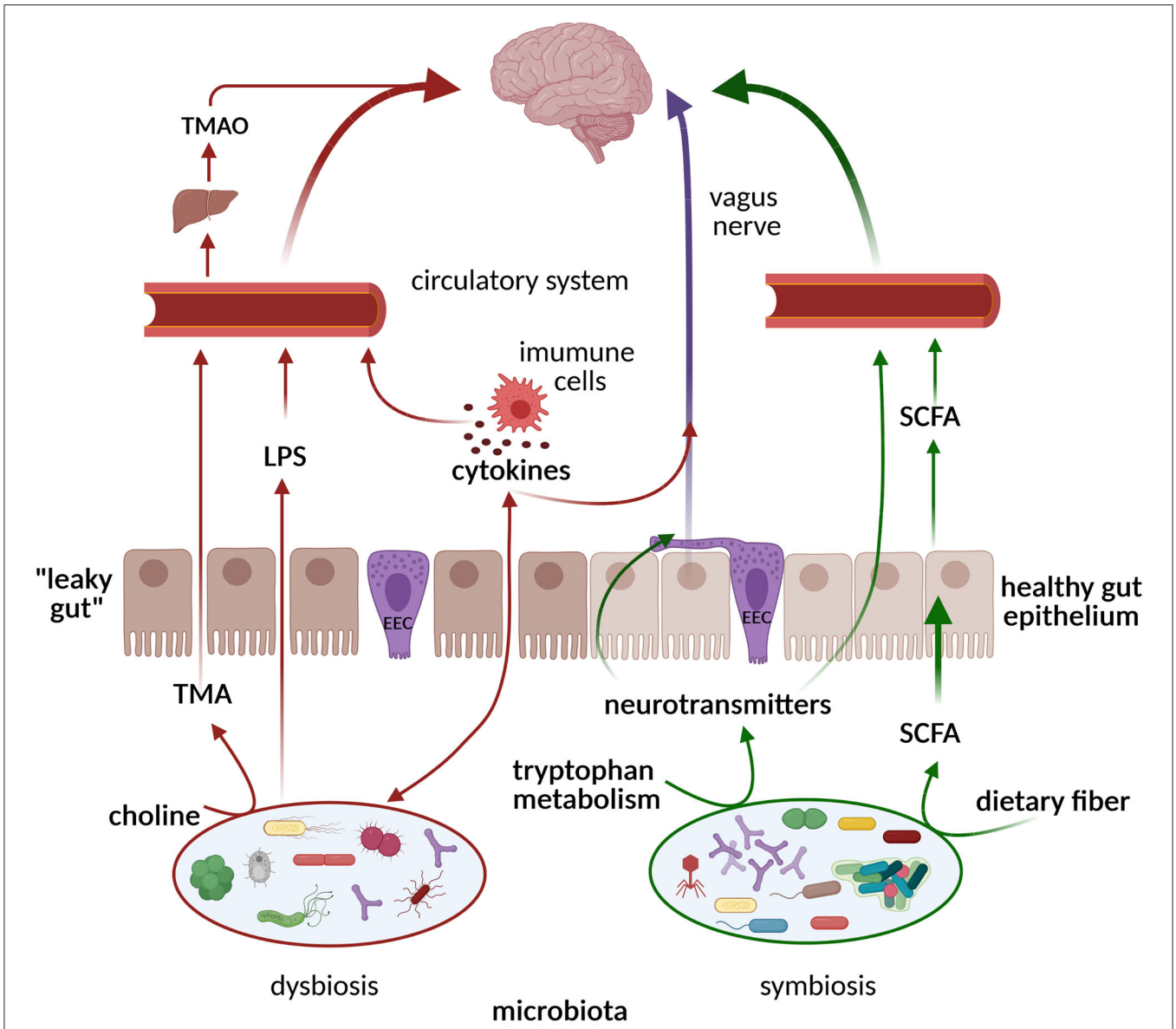

FIGURE 2 | Pathways of communication along the gut microbiome–brain axis. In the state of symbiosis, the gut microbiota produces SCFAs, tryptophan metabolites, and neurotransmitters that exert neuroprotective effects on the brain *via* circulation and the vagus nerve (green arrows). In the state of dysbiosis, TMA/TMAO, pro-inflammatory cytokines, and LPSs are formed, inducing cognitive impairment (red arrows). ECCs, enteroendocrine cells; SCFAs, short-chain fatty acids; LPSs, lipopolysaccharides; TMA, trimethylamine; TMAO, trimethylamine N-oxide. Created with BioRender.com.

(dietary fibers), are SCFAs: mainly butyric, propionic, and acetic acids (75). Acetate and propionate are produced by the *Bacteroidetes phylum*, while species of the *Firmicutes phylum* preferentially produce butyrate. Different sources of fibers, such as resistant starches (from whole grains and legumes) or fructo-oligosaccharides from bananas, onions, and asparagus, yield different levels of butyrate and other SCFAs. Some prebiotic fibers, such as inulin and fructo-oligosaccharides, promote the growth of commensal bacteria that produce high amounts of butyrate in the gut (76). SCFAs contribute to gut health by regulating the integrity of the intestinal barrier, mucus production, and controlling inflammation by inducing Treg differentiation. Butyrate is used as an energy source by the colonocytes, while the liver clears the majority of propionate and butyrate from the portal circulation (77). However, a minor fraction of colon-derived SCFAs reaches the bloodstream and can be transported to the brain by passing through the blood–brain barrier to exhibit a neuroprotective effect (78). SCFAs are involved in maintaining blood–brain barrier permeability and the CNS immune system by regulating microglial function. The epigenetic effects of butyrate have also been documented, as it is a well-known inhibitor of HDAC, affecting gene expression in

the gut and associated immune tissue, as well as in the nervous system. Treatment with sodium butyrate in animal models of Parkinson's disease has been shown to prevent neuronal cell death, while in Alzheimer's disease and traumatic brain injury models memory and learning improved (79). However, chronic, slightly elevated blood propionate and concomitant increased ammonia levels in the circulation may play a role in cognitive impairment and dementia (80).

Gut microbiota species also produce other bioactive compounds, such as folate (vitamin B9), and neurotransmitters, such as serotonin (5-hydroxytryptamine; 5-HT), dopamine, and γ-aminobutyric acid (GABA), as summarized in a recent review (81). The tryptophan microbial metabolite indole also represents an important link between the microbiota and the host, playing a role in the modulation of intestinal epithelial integrity and intestinal inflammation, and it positively correlates with longevity (82). A reduced level of the neurotransmitter serotonin, found in dysbiosis, is related to cognitive impairment (**Figure 2**).

DYSBIOSIS—A LINK BETWEEN DIET, AGING, AND NDD

Dysbiosis is a state of imbalanced abundance and composition in the gut microbiota with changes in microbial-derived products. It often leads to the overgrowth of otherwise low-abundance and/or harmful bacteria. A family of Gram-negative bacteria, *Enterobacteriaceae*, are the most commonly overgrown gut microbes in a wide range of pathologic conditions, including inflammation. Gut inflammation, on the other hand, causes damage to and the death of mucosal epithelium cells. This results in an increase in phospholipids from the membrane lipids of dead cells, which can be used as a source of carbon and/or nitrogen by a variety of species in the *Firmicutes*, *Actinobacteria*, and *Proteobacteria phyla*, as well as pathogenic species such as *Salmonella* and *Pseudomona*s. An inflamed gut favors the growth of anaerobic bacteria (such as *E. coli*) and mucin-degrading bacteria (*Akkermansia musiniphila* and *B. acidifaciens*), leading to a depletion of commensal bacteria (*Bacteroidetes* and *Clostridia phyla*) and favoring a growth of *Enterobacteriaceae* and pathogens such as *S. Typhimurium* and *Clostridium difficile* (83). Inflammation results in increased intestinal permeability, referred to as "leaky gut," allowing the translocation of microorganisms and/or their components and metabolites from the gut to the bloodstream. Endotoxins (lipopolysaccharides; LPSs), a major component of the outer membrane of Gram-negative bacteria, after entering the bloodstream and binding with LPS-binding protein (LBP) and CD14 receptor, launch the secretion of pro-inflammatory cytokines. In this way LPSs induce neuroinflammation, which triggers and perpetuates the neurodegenerative process (**Figure 2**). Although small concentrations of LPSs are detectable in healthy individuals (endotoxemia), elevated postprandial LPS levels after fat-rich meals, referred to as "metabolic endotoxemia," have been proposed as a major cause of inflammation, including chronic low-grade inflammation (84). Several studies have demonstrated that chronic gastrointestinal syndromes, such as inflammatory bowel syndrome (85), small intestinal bacterial overgrowth (86), and celiac disease, are associated with neurological disease development (87). Dysbiosis has been implicated in worsened outcomes after traumatic brain injury, which has been considered as a non-genetic risk factor for several NDDs, including ALS, AD, and PD (88).

Aging is concomitant with changes in gut physiology, including lower levels of stomach acid and changes in gastric motility and in the ENS, that consequently affect the composition and function of the gut microbiota. The most prominent feature in the microbiota of elderly individuals is a reduced *Firmicutes*-to-*Bacteroidetes* ratio compared with young adults (89) and decreased beneficial *Lactobacillus* and *Bifidobacterium*. Decreased microbial diversity in elderly individuals is associated with increased frailty, blood inflammatory markers, and decreased nutritional diversity. Perturbations in the gut microbiota, such as decreased abundance of bacteria involved in SCFA production and an enrichment of low-abundance pathobionts, further promote and sustain pro-inflammatory conditions (90). Low-grade chronic systemic inflammation accompanied with physiological aging is defined as "inflammaging." Altered, aged gut microbiota compositions have been proposed to contribute to this heightened pro-inflammatory status characterized with increases in pro-inflammatory cytokines (IL-6 and TNF-α), acute-phase reactants (C-reactive protein), and decreases in IL-10 (91). Inflammaging contributes to the development of age-related diseases: metabolic, cardiovascular, and neurodegenerative (92).

Some recent metabolomic investigations have shown that individual gut microbiomes become increasingly more unique with age, and uniqueness is positively associated with health and longevity. Although, healthy elderly individuals showed a decline in core taxa (dominant genera) and replacement by less common taxa, their gut microbiome continued to show a distinct composition (82). Metabolomic studies correlated three markers of longevity (phenylacetylglutamine—PAG; p-cresol sulfate—PCS; 2-hydroxybenzoate-—2-HB) from the urine of elderly individuals and centenarians with the gut microbiome. PAG and PCS are formed by the microbial catabolism of proteins (phenylalanine and tyrosine metabolites), while 2-HB originates from fruits and vegetables. PAG was positively correlated with *Proteobacteria* species, both PCS and PAG correlated to *Vibrio et. rel.*, and 2-HB was found to be positively correlated with *Proteus et. rel.* (93).

Emerging evidence suggests that changes in the function and composition of the gut microbiota contribute to the pathogenesis of NDD by the induction of epigenetic modifications. Epigenetic changes associated with NDD mostly include DNA methylation and histone modifications, which are controlled by several enzymes, such as acetylases and methylases (94). These enzymes are regulated by the metabolites generated by the host's gut microbiota. Such metabolites are short-chain fatty acids, folates, biotin, and trimethylamine-N-oxide (11). The bidirectional interaction between the gut microbiota and epigenetics has been documented, although the nature and significance of this relationship has not been fully elucidated. They may act in

TABLE 1 | Bioactive compounds from food with effects on NDD.

Food	Compound	Action	Role in NDD	References
Nuts	Ellagic acids	Inhibit HATs; Activate HDACs	Reverse brain atrophy in AD	(123, 124)
Fish oil	ω-3 PUFA	Activates or inhibits DNMTs in different cells	Prevents age-associated cognitive decline	(18, 68, 69)
Olive oil	Gallic acid	Inhibits HATs	Potential prevention of AD	(123, 125)
Ginkgo biloba extract	Kaempferol	Inhibits HDACs	Improves cognitive function in patients with mild dementia during long-term administration	(65, 126)
Red wine	Resveratrol	Activates HATs; Inhibits DNMTs and HDACs	Reduces the risk of AD	(59, 115, 127)
Berries	Gallic and ellagic acids	Inhibit HATs; Activate HDACs and DNMTs	Delay the development of age-related cognitive decline	(123, 128)
Tea	Epigallocatechin-3-gallate	Inhibits DNMTs and HATs	Low prevalence of AD	(57, 129, 130)
Turmeric	Curcumin	Activates HDACs; Inhibits HATs, DNMTs, and miRNA	Corrects the dysregulation of several pathways in NDD	(62–64, 131, 132)
		Alters the relative abundances of bacterial species	Gut microbiota produces neuroprotective metabolites from curcumin	(117)
Cocoa	Epicatechin	Inhibits HATs; Activates HDACs; Increases the presence of "healthy" bacteria	Decreases cerebral inflammation	(119, 133)
Pomegranate	Ellagitannins	Inhibits HATs	Protective effects against AD	(122, 123)
Morinda officinalis	Oligosaccharides	Not determined	Regulates the synthesis and secretion of neurotransmitters in rats	(120)
Algae	Oligomannate	Not determined	Inhibits AD progression in AD mouse models	(121)

AD, Alzheimer's disease; NDD, neurodegenerative disease; HATs, histone acetyltransferases; HDACs, histone deacetylases; HMTs, histone methyltransferases; miRNA, microRNA.

synchrony to modulate the pathogenesis and progression of NDD (94, 95). On the other hand, metabolites produced by the gut microbiota may also reverse some of the previously induced epigenetic modifications (96) and thereby prevent the development or progression of NDD.

MICROBIOTA IN DIFFERENT NDDS

Recent studies have shown decreased intestinal microbial diversity in AD patients, with increased abundance of the *Bacteroides phylum* and decreased abundance in *Firmicutes* (97). Such conditions favored the growth of pro-inflammatory Gram-negative bacteria, such as *Escherichia coli, Shigella, Helicobacter,* and *Odoribacter*, while reducing beneficial commensals such as *Bifidobacterium* and SCFA-producing bacteria (98). This state promotes the formation of amyloid plaques, typically found in AD patients. Another possible mechanism involves bacteria-derived amyloids, produced by *E. coli, Salmonella spp., Pseudomonas fluorescens, Klebsiella pneumonia, Staphylococcus aureus, Bacillus subtilis, Streptomyces coelicolorcan,* that can function as initiators to cross-seed and through molecular mimicry aggregate host amyloids (99). In addition, a chronic *H. pylori* infection could trigger the release of both inflammatory mediators and amyloids in AD patients (100), while the eradication of *H. pylori* has been shown to be associated with a decreased progression of AD symptoms (101). As Aβ plaques have been detected in the intestinal mucosa of both AD animal models and human patients, it is hypothesized that endogenous Aβ production starts in the gut and subsequently spreads to the CNS. Another possible mechanism that causes cognitive deterioration is a release of certain microbial-derived metabolites, such as trimethylamine N-oxide—a product of the metabolic transformation of dietary choline by the gut microbiota to trimethylamine, which is then oxidized by the host's liver (**Figure 2**) (102). Both trimethylamine and its oxide are involved in aging and neurodegeneration.

Parkinson's disease (PD) is characterized by a loss of movement control due to a decrease in brain dopamine production as a result of the degeneration of substantia nigra neurons. Non-motor symptoms are also present, such as constipation, which may precede the onset of motor deficits years or decades before. A hallmark of PD pathology is an aggregation of misfolded α-synuclein (αSYN), so-called Lewy bodies, in the brain; although it has been found that αSYN aggregates can be present in the ENS of clinically healthy individuals years before they develop PD. Misfolded α-synuclein has also been shown to spread from cell-to-cell and, in a prion-like fashion, from the periphery to the

CNS *via* the vagus nerve (103, 104). A large number of studies have shown diversity and abundance in the microbial species present in the gut of PD patients (105). Moreover, a positive correlation between the increased abundance of *Lactobacillaceae* and *Enterobacteraceae* and disease severity has been shown. In PD, observed decreases in the fecal amounts of *Prevotella spp.* and *Clostridium spp.*, major producers of SCFAs, folate (vitamin B9), and thiamine (vitamin B1), may have an impact on intestinal epithelial barrier permeability. Furthermore, studies indicate alterations of the bacteriophage community in people with PD (106). Epidemiological data indicate that PD is associated with a variety of enteral dysfunctions: inflammatory bowel disease, *H. pylori* infection, and constipation (105). Recent studies have revealed changes in the gut microbiome as a result of usual PD treatments (107). On the other hand, *Enterococcous faecalis* has been found to metabolize levodopa, a commonly used drug for PD, suggesting that the gut microbiome may reduce the peripheral availability of levodopa and thereby affect the efficacy of the PD treatment (108, 109).

Amyotrophic lateral sclerosis affects the brain and spinal cord neurons, leading to paralysis, respiratory failure, and death. In ALS patients, reduced relative microbial abundances have been found in butyrate-producing *Anaerostipes, Oscillibacter*, and *Lachnospira*, while that of glucose-metabolizing *Dorea* has been found to be significantly increased. ALS patients also have elevated LPS levels in plasma (110), which support neuroinflammation through microglia activation. Multiple sclerosis is an autoimmune NDD, which also appears to be associated with an altered gut microbiota. An increased relative abundance of *Akkermansia* has been shown to be correlated with symptom expression. In addition, MS patients have been shown to exhibit decreased levels of *Parabacteroides distasonis*, a species associated with anti-inflammatory activity (111).

PRECISION NUTRITION IN NDD

Studies with caloric restriction have shown neuroprotective effects in animal models, but the results in humans are inconsistent. However, the Mediterranean diet (112) and the Mediterranean–ketogenic diet (113) improved cognitive function in older people by altering intestinal microbiota and their metabolites.

In addition to the effects on the gut microbiota, diets rich in olives, nuts, or ω-3 PUFA affected genes associated with infection and inflammation. The Mediterranean diet downregulates transcriptional repressor *NFIL3*, which is involved in the regulation of cytokine expression, the development of immune cells, and the circadian clock. Olive oil and nuts have been found to downregulate IL-8, a key mediator of inflammation, and a coagulation factor (SERPINB2) involved in adipose tissue development. Moreover, they downregulate the expression of *RGS1*, a regulator of the G-protein superfamily, which is linked to chronic inflammatory diseases such as celiac disease, MS, and AD. However, the supplementation of a diet with olives, nuts, or ω-3 PUFA dysregulated different genes in AD patients (114).

Numerous nutraceuticals such as curcumin, epigallocatechin-3-gallate, resveratrol, Ginkgo biloba extract, genistein, flavonoids from berries, and polyphenols from extra virgin olive oil and red wine are able to delay neurodegeneration (115). Besides their role in epigenetics, as discussed above, the health benefits of these nutraceuticals in the prevention and alleviation of NDD are due to their ability to change the gut microbiota. Moreover, the bioavailability of polyphenols, PUFAs, and antioxidants with neuroprotective effects depends on the colon microbiota. The differences in gut microbiota composition may explain the inter-individual variability in the outcome of supplementation in clinical trials, emphasizing the need for precision nutrition.

The relationship between microbiota and nutraceuticals is bidirectional. This has been shown for curcumin (116). Curcumin was found to significantly alter the relative abundances of bacterial species, several of which have been associated with the development of AD, while the biotransformation of curcumin by gut bacteria produces neuroprotective metabolites (117). Further, turmeric and curcumin have been shown to exert potential to alter the gut microbiota but with significant variation over time and an individualized response to treatment (118). Flavanols from red wine and cocoa have a positive effect on the gut microbiota by increasing the presence of "healthy" bacteria, such as *Firmicutes* and *Bacteroidetes*, which have been found decreased in cerebral inflammation (119). Probiotic and prebiotic supplementation have shown moderate beneficial effects in AD patients, although oligosaccharides from *Morinda officinalis* (120) and oligomannate from algae (121) exhibit promising effects on animal models of AD. On the other hand, gut-microbiota-derived metabolites from plants may have protective effects against AD, e.g., urolithins, which are metabolites of ellagitannin in, for example, pomegranate fruit (122).

The reversible nature of epigenetic changes has pointed out the specific nutritional interventions aimed at reversing epigenetic modifications to prevent or treat NDD. Although diets rich in bioactive compounds have demonstrated beneficial effects in preventing and modifying NDD, the application of precision nutrition appears to be markedly more effective than the traditional approach. The pathologies of NDD can exert variable clinical characteristics in the patients with the same disease. A combination of genetic and epigenetic factors, lifestyle, and microbiome data could provide a full overview of an individual patient and enable the stratification of patients into specific nutritional groups in order to avoid non-responders to a specific diet and to get a maximum response from the precision nutrition for each patient.

CONCLUSIONS

In summary, NDDs remains an important public health challenge because of the lack of effective prevention or treatment. The reasons for the slow progress in both prevention and therapy may lie in the fact that current recommendations are designed for the general population, without taking into account

individual genetic, epigenetic, and lifestyle factors. However, NDDs have complex pathologies with great inter-individual (epi)genetic variance within the population. In addition, the gut microbiome is linked with the development and progression or alleviation of NDDs, through the gut microbiome–brain axis. The relationship between the gut microbiota and epigenetic modifications in NDD is bidirectional and markedly dependent on nutrition. These individual differences in both microbiota and epigenetic signatures suggest the need for personalized dietary plans for NDD patients. A possible target is the creation of personalized dietary interventions containing specific bioactive nutrients that can modify epigenetic changes and/or the gut microbiota. Although numerous studies on the role of different nutraceuticals in the prevention, prediction, and treatment of NDDs have been conducted, we are still far away from a personalized diet plan for individual NDD patients, which is undoubtedly the future of NDD therapy. To achieve this goal as soon as possible, large-scale cohort studies that would include the precise monitoring of food intake, mapping of genetic variants, epigenetic data, microbiome studies, and metabolome, lipidome, and transcriptome data are urgently needed.

AUTHOR CONTRIBUTIONS

VV and MM designed the review. MM, AA, and ZC performed the literature analysis and wrote the manuscript. VV critically revised the text and gave a substantial scientific contribution. All authors have approved the final version of the manuscript for publication.

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Certain Associations Between Iron Biomarkers and Total and γ' Fibrinogen and Plasma Clot Properties Are Mediated by Fibrinogen Genotypes

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Introduction: Evidence for the relationship between body iron and cardiovascular disease (CVD) is inconsistent and mechanisms involved remain poorly understood. Therefore, we first investigated whether there are linear or non-linear relationships between iron status and total and γ' fibrinogen as well as plasma fibrin clot properties and, second, determined whether there are interactions with iron biomarkers and fibrinogen and *FXIII* single nucleotide polymorphisms (SNPs) in relation to fibrinogen concentration and functionality.

Methods: In this cross-sectional analysis of 2,010 apparently healthy Black South Africans we quantified total and γ' fibrinogen, serum iron, ferritin and transferrin using standardized methods and calculated transferrin saturation (TS). Clot architecture and lysis were explored with a global analytical turbidity assay. The SNPs were determined through an Illumina BeadXpress® platform.

Results: Total, but not %γ', fibrinogen negatively correlated with serum iron concentrations, although both decreased over iron tertiles. %γ' fibrinogen correlated negatively with transferrin and decreased over the transferrin tertiles. A weak negative association between total fibrinogen and TS was detected with fibrinogen decreasing over the TS tertiles and categories based on TS. Lag time correlated positively with transferrin and increased over transferrin tertiles, when adjusting for fibrinogen. Before adjusting for fibrinogen, lag time was shorter in those with adequate iron status based on TS than other iron subcategories. Clot lysis time (CLT) negatively correlated with ferritin and was longer in the first than in the third ferritin tertile. Among iron status categories based on ferritin, only CLT differed and was longer in those with adequate iron than with iron-overload. CLT negatively correlated with TS, albeit weakly, shortened over the TS tertiles and was shorter in those with adequate iron based on TS categories. Interactions were observed between *FGB* SNPs and some of the markers of iron status investigated, in relation to the clot properties with the most prominent associations detected in homozygous carriers of the variant alleles for whom increased iron status

was more beneficial than for those harboring the wild-type alleles. Iron modulated the influence of the SNPs so that for the majority iron was beneficial in respect of clot properties, but even more so for a minority group harboring specific variant alleles.

Conclusion: This is the first large-scale epidemiological study to relate fibrinogen concentration and functionality to markers of iron status and to take genetic factors into consideration. We have detected a relationship between iron biomarkers and fibrinogen as well as clot characteristics that are influenced by the genetic make-up of an individual.

Keywords: coagulation, ferritin, fiber density, fibrinolysis, lag time, lateral aggregation, transferrin, turbidity

INTRODUCTION

Cardiovascular disease (CVD) continues to soar on a trajectory that will cripple many health services (1), despite being preventable by appropriate lifestyle modification (2), including diet (3). Micronutrients (4) such as the dietary trace mineral, iron, may play a role in CVD (5, 6). In those with optimal iron status, iron has been shown to have a neutral effect, but CVD risk is modestly increased in those with a deficiency or in individuals with iron-overload (5, 7). The mechanisms for this association remain to be determined. However, plausible pathways include effects on fibrinogen concentration, blood clot structure and properties (8, 9), which have themselves been implicated in CVD (10–12). The circulating glycoprotein fibrinogen is, upon activation, converted to fibrin, which provides the matrices for blood clots. Furthermore, fibrinogen has a common splice variant, γ' fibrinogen, with a physiological range of 8–15% (13), known to influence fibrin clot structure and mechanics (14). Factor XIII (FXIII) is another constituent of blood, which crosslinks fibrin fibers to protect the clot against premature lysis to allow wound healing (15). Single nucleotide polymorphisms (SNPs) within the fibrinogen α, β and γ chain genes (*FGA*, *FGB,* and *FGG*) influence total and γ' fibrinogen concentrations (16, 17) and have also been demonstrated together with those of *FXIII* to influence clot properties (16, 17). Undesirable iron stores (due to either iron overload or iron deficiency) are associated with oxidative stress (18–21), which can lead to post-translational modifications, but have also been shown to direct binding of iron to the fibrinogen molecule, leading to conformational changes and thereby hypercoagulability (22–24).

There are various iron biomarkers, representing different components of iron status. The gold standard for measuring iron stores is to evaluate iron deposits in a bone marrow aspiration sample (25). However, this procedure, being invasive and expensive, is not feasible in epidemiology (5). Ferritin was asserted to be the most powerful indicator for simple iron deficiency in populations (26), being a storage protein which is proportional to total-body iron stores and decreases when the body needs more of this essential trace mineral. Transferrin is a transport protein, which increases when the body needs more iron to increase absorption. Concentrations of iron in serum represent the circulating iron bound to transferrin and ferritin. From the latter markers, transferrin saturation (TS) can be calculated to increase the accuracy of iron deficiency detection (27). TS signifies the total iron availability in the body (indicating deficiency, adequacy or overload) and the equilibrium between the release of iron from storage areas and its use by bone marrow for erythropoiesis (28). TS is deemed a good indicator of iron reserves in the bone marrow (29). We included both serum ferritin and TS in our investigation to provide the best possible assessment of iron status (27). Hemoglobin and hematocrit are late indicators of iron status (30) and dietary intake is inadequate as a sole indicator of depletion (31), therefore these criteria should be interpreted with caution.

The aim of this study was 2-fold: to increase our understanding of the manner in which iron stores associate with fibrinogen concentration and functionality (fibrin clot properties), and how these associations are influenced by specific variations within the fibrinogen and *FXIII* genes. To this end, we first determined linear and non-linear relationships between total fibrinogen and—to our knowledge for the first time—the γ' splice variant of fibrinogen, and clot properties and iron markers, in a large population-based study. Second, because fibrinogen and *FXIII* SNPs might interact with iron status markers to modulate total or γ' fibrinogen concentrations as well as the kinetics of clot formation, structural clot properties and fibrinolysis, we examined whether there were such interactions. This work could potentially contribute to personalized nutrition in which dietary recommendations are tailored to genetic makeup, to enhance the therapeutic success of diet-based lifestyle interventions on disorders contingent on hemostasis.

MATERIALS AND METHODS

Study Design, Population Selection, and Demographic Characteristics

This cross-sectional research is nested within the South African arm of the *P*rospective *U*rban and *R*ural *E*pidemiology (PURE) study that forms part of a multinational investigation tracking risk factors for chronic non-communicable diseases (32). A three-stage sampling process was followed with sample size based on power calculations using a previous investigation (33). After obtaining gatekeeper permission from local authorities, 6,000 houses were randomly selected from urban and rural strata. From the households, 4,000 eligible individuals with no reported use of chronic medication (excluding that for blood pressure) or any self-reported acute illness were identified. During the baseline

period (data reported in this paper), biological samples were collected from 1,006 rural and 1,004 urban, ostensibly healthy Black South African men and women, who provided written informed consent. The study protocol was approved by the Health Research Ethics Committee of the authors' University (NWU-00016-10-A1 and NWU-00034-17-A1-02), honoring the guidelines laid down in the Declaration of Helsinki of 1975 and revised in 2000.

Lifestyle Factor Assessment

Questionnaires were interviewer-based and conducted by trained fieldworkers in the participant's home language. Details regarding demographic and lifestyle factors, including tobacco and alcohol use, were obtained. These results were published previously (34).

Anthropometrical and Blood Pressure Assessment

Participants were weighed and their heights measured to calculate body mass index (BMI, kg/m^2). Waist circumference was obtained at the narrowest point between the 10th rib and iliac crest via a measuring tape by level 1 anthropometrists. Blood pressure—systolic and diastolic—was measured with the OMRON HEM-757 apparatus (Omron Healthcare, Kyoto, Japan) with the cuff on the participant's left arm.

Blood Collection, Sampling, and Storage

Fasting blood samples were collected by registered nurses between 07:00 and 11:00 from participants' antebrachial vein branches and centrifuged at 2,000 × g for 15 min. Sodium citrate-treated tubes were used to collect samples for the determination of fibrinogen concentration and clot properties. For high-sensitivity C-reactive protein (CRP), gamma glutamyl transferase (GGT) and lipids, tubes not containing anti-coagulants were used for blood collection. Ethylenediaminetetraacetic acid (EDTA) tubes were used for the quantification of serum ferritin, transferrin, iron and glycated hemoglobin A1c (HbA1c). Following removal of serum and plasma from the blood collection tubes, the remaining buffy coat was removed for the purpose of DNA isolation. Samples were transferred to collection tubes (500 μL), snap-frozen on dry ice and stored at −20°C for 2–4 days prior to transfer to the cryofreeze facilities at the NWU, where they remained at −80°C until analyzed.

Biochemical Analyses

Total fibrinogen concentrations were quantified using the modified Clauss method on the Dade Behring BCS coagulation analyzer (Multifibrin U-test Dade Behring, Deerfield, IL, USA). An enzyme-linked immunosorbent assay (ELISA) using a Santa Cruz Biotechnology (Santa Cruz, Dallas, TX, USA) 2.G2.H9 mouse monoclonal coating antibody against human γ', and an Abcam (Cambridge, MA, USA) goat polyclonal horseradish peroxidase (HRP) conjugated antibody against human fibrinogen, were used to determine fibrinogen γ' concentrations. Fibrinogen γ' is expressed as % of total fibrinogen for the purpose of this paper.

To obtain plasma fibrinolytic potential, turbidimetric analysis (A405 nm) (35) with modified tissue factor and tissue-plasminogen activator (tPA) concentrations was used to obtain clot lysis times (CLT) of between 60 and 100 min. Final concentrations were: tissue factor (125x diluted—-an estimated final concentration of 59 pM; Dade Innovin, Siemens Healthcare Diagnostics Inc., Marburg, Germany), $CaCl_2$ (17 mmol/L), tPA (100 ng/mL; Actilyse, Boehringer Ingelheim, Ingelheim, Germany) and phospholipid vesicles (10 μmol/L; Rossix, Mölndal, Sweden). Kinetics of clot formation (lag time and slope) and structural plasma fibrin clot properties (maximum absorbance) and CLT were calculated from the turbidity curves as described previously (36). Lag time represents the time required for fibrin fibers to attain a sufficient length to allow lateral aggregation and activation of the coagulation cascade. Slope reflects the rate of lateral aggregation and maximum absorbance indicates clot density.

Serum ferritin and transferrin concentrations were determined using an enzyme immunoassay procedure (Ramco Laboratories, Inc, Stafford, TX, USA) and serum iron, CRP and GGT were measured with a particle-enhanced immunoturbidimetric assay using a Sequential Multiple Analyzer Computer (SMAC) using the Konelab20iTM auto analyzer (Thermo Fisher Scientific Oy, Vantaa, Finland). Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula. HbA1c concentrations were quantified using the D-10 Hemoglobin testing system (Bio-Rad Laboratories, Hercules, CA, USA). Whole blood was used for the determination of HIV status, making use of the First Response rapid HIV card test (PMC Medical, India). If positive, the investigation was repeated with the Pareeshak card test (BHAT Bio-tech, India).

DNA Extraction, Sequencing, and Genotyping

Genomic DNA was extracted using FlexiGene™ kits (QIAGEN, catalog number 51206). If insufficient yield was established, the Maxwell® 16 blood DNA purification kit was used for the second isolation attempt. *FGA* 2224G/A (rs2070011), *FGA* 6534A/G (rs6050), *FGB* 1038G/A (rs1800791), *FGB* Arg448Lys, G/A (rs4220), *FGB* −148C/T (rs1800787), *FGG* 10034C/T (rs2066865), and *FGG* 9340T/C (rs1049636), the *FXIII* His95Arg A/G (rs6003) and *FXIII* Val34Leu, C/A (rs5985) were genotyped. Details of the genotyping are described elsewhere (16).

Quality control included a check for individuals successfully genotyped (missingness), minor allele frequencies (MAF), conformance to Hardy–Weinberg equilibrium (HWE) and a tally of Mendelian errors. Haploview version 4.2 software (37) was used to calculate the level of pairwise linkage-disequilibrium (LD) between the SNPs, as reported before (38). For this study population, three SNPs (rs7439150, rs1800787, and rs1800789) were in high, albeit not complete, LD. Therefore, we report the SNPs separately.

Statistical Analysis

The computer software package *Statistica*® version 13.3 (TIBCO Software Inc., Tulsa, OK, USA) and R version

3.5.0 (39) were used for computation. TS was calculated as follows (Fe/transferrin × 70.9 (40). A sensitivity analysis was included in which all statistical analyses were conducted before and after those with a CRP concentration ≥15 mg/L were excluded (this cut-off was used because the tympanic temperature of all participants included was <37.0°C, to rule out acute infection and the population is known to have a high basal CRP due to genetics (41) and socio-economic factors (42). However, because exclusion did not result in major differences, we opted to report the results for the whole population with adjustment for, rather than excluding, CRP.

The variables data were tested for normality using the Shapiro–Wilk W-test and the Kolmogorov–Smirnov test and descriptive statistics were calculated. Because variables were non-normally distributed, data are reported as medians (lower and upper quartiles).

Spearman correlations were performed to test for statistical dependence between variables. LDL-C, age, HbA1c, GGT and BMI were identified as possible confounders. We adjusted for these as well as CRP in subsequent analyses including in the Spearman partial correlations.

Kruskal–Wallis ANOVA and Mann–Whitney *U*-tests were used to detect differences in hemostatic markers among the tobacco use and HIV status subgroups as well as between men and women. Differences in total and %γ' fibrinogen and fibrin plasma clot properties (lag time, slope, maximum absorbance and CLT), among tertiles created for the iron biomarkers, ferritin categories (iron deficient <15 μg/L; adequate =15–≤200 μg/L; overload >200 μg/L) and TS categories (<20% iron deficient; ≥20% adequate) were analyzed using generalized linear models with adjustments and *post hoc* tests.

To investigate whether the biomarkers of iron status had interactive effects with the fibrinogen and *FXIII* SNPs in predicting total and γ' fibrinogen concentrations and clot properties, general linear models with adjustments and interactions were performed using the biomarkers as continuous variables. The low prevalence of homozygous carriers of the variant allele among the study participants did not allow further subdivision of the population into tertiles based on iron biomarkers within the interaction analyses. Interactions that remained after adjusting for multiple testing are reported in **Table 4**. The method of Benjamini-Hochberg (43) was used to account for multiple testing and false discovery rates in terms of the interactions. This procedure indicated that the statistical threshold for significance was $p < 0.0017$ [1/16*3*3(0.25)] when the false discovery rate was set at 25%. The number of SNPs investigated was 16, the iron biomarkers—based on associations with one another—were regarded as three, whereas total and γ' fibrinogen concentrations and fibrin clot properties were also regarded as three. To describe the interactions, rho values were determined through Spearman partial correlations adjusting for confounders including CRP and additionally for fibrinogen when the interaction was related to one of the clot properties.

TABLE 1 | Specific characteristics of participants.

			Median (interquartile range) or (n: n)
			Whole group#
Sex (men: women)*			747: 1,263
Age (yr)			48.0 (41.0–56.0)
Blood pressure (mmHg)			
Systolic			130 (116–147)
Diastolic			87.0 (78.0–97.0)
Tobacco use (%)@			
Never (1)			52.7
Former (2)			4.14
Current (3)			42.7
BMI (kg/m²)			22.9 (19.3–28.6)
Waist circumference (cm)			77.5 (70.2–87.7)
HIV status❁			
(Unaffected: affected)			1,668: 326
Biochemical markers	**Cardiovascular and clot properties**		
		TC (mmol/L)	4.82 (4.01–5.87)
		LDL-C (mmol/L)	2.79 (2.08–3.65)
		HDL-C (mmol/L)	1.42 (1.06–1.87)
		Triglycerides (mmol/L)	1.08 (0.82–1.55)
		HbA1c (%)	5.50 (5.30–5.80)
		CRP (mg/L)	3.29 (0.96–9.34)
		Total fibrinogen (g/l)	2.90 (2.30–5.00)
		Fibrinogen γ' (%)	10.2 (7.14–14.6)
		Lag time (min)	6.52 (5.10–7.78)
		Slope (x10^{-3} AU/s)	8.90 (6.49–11.9)
		Maximum absorbance (nm)	0.42 (0.33–0.52)
		CLT (min)	57.2 (50.9–63.8)
	Iron status biomarkers		
	Iron (μmol/L)	Whole	15.9 (10.8–23.9)
		Tertile 1	9.30 (6.80–10.8)
		Tertile 2	15.9 (14.3–17.7)
		Tertile 3	28.5 (23.9–38.8)
	Transferrin (g/L)	Whole	2.72 (2.33–3.27)
		Tertile 1	2.18 (1.94–2.33)
		Tertile 2	2.71 (2.58–2.83)
		Tertile 3	3.56 (3.23–4.02)
	Ferritin (μg/L)	Whole	93.9 (36.4–208)
		Tertile 1	23.0 (11.5–36.4)
		Tertile 2	93.9 (72.7–121)
		Tertile 3	268 (208–445)
	TS (%)	Whole	23.08 (15.6–34.9)
		Tertile 1	13.0 (9.22–15.6)
		Tertile 2	23.1 (20.6–26.2)
		Tertile 3	34.9 (34.9–57.4)

#Total sample was 2,010, but numbers are slightly different for several variables due to random missing data.

**Total fibrinogen, maximum absorbance and CLT differed between the sexes.*

@CLT differed among the tobacco use subgroups.

❁Total fibrinogen, %γ' fibrinogen, slope and maximum absorbance differed between the HIV status groups.

BMI, body mass index; CLT, clot lysis time; CRP, high-sensitivity C-reactive protein; HbA1c, glycosylated hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; HIV, human immunodeficiency virus; LDL-C, low-density lipoprotein cholesterol; n, number of patients; TC, total cholesterol; TS, transferrin saturation.

TABLE 2 | Correlations (r) of biomarkers of iron status with total fibrinogen and γ' fibrinogen concentrations and clot properties.

Participant characteristics	Total fibrinogen	%γ'fibrinogen	Lag time	Slope	Maximum absorbance	CLT
Iron (μmol/L)	−0.22***	−0.04	0.03	−0.13***	−0.08**	−0.01***
	−0.12**	−0.03	−0.02	−0.08*	0.05	0.02
	–	–	−0.009	−0.05	0.08*	0.02
Transferrin (g/L)	0.06**	−0.09***	0.016	0.03	0.08***	0.12***
	0.03	−0.06	0.09*	−0.02	0.06	0.01
	–	–	0.09*	−0.02	0.05	0.01
Ferritin (μg/L)	0.06	−0.05	−0.02	0.008	−0.03	−0.1**
	0.09*	−0.07	−0.05	0.04	−0.03	−0.1**
	–	–	−0.06	0.02	−0.06	−0.1**
TS (%)	0.24***	−0.002	0.03	−0.13***	−0.11***	−0.15***
	−0.1**	0.02	−0.04	−0.06	0.03	0.01
	–	–	−0.02	−0.03	0.06	0.02

*$p < 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.
The first r-value is for Spearman correlation; second r-value for Spearman partial correlations of iron biomarkers with clot properties, adjusting for CRP, LDL-C, age, HbA1c and GGT; third r-value is for Spearman correlations of iron biomarkers with clot properties, adjusting for CRP, LDL-C, age, HbA1c and GGT as well as fibrinogen.
CLT, clot lysis time; TS, transferrin saturation.

RESULTS

The demographic and clinical characteristics of participants are presented in **Table 1**. We observed ferritin concentrations of <15 μg/L, indicative of iron deficiency (44) in 11.7% of the study population, and >200 μg/L, indicative of a risk of iron overload (44) in 26.7% of the volunteers; 39.4% presented with a TS of <20%, also indicative of iron deficiency (25, 44, 45).

Spearman and partial Spearman correlations are presented in **Table 2**. Noteworthy correlations observed were a negative association of serum iron with total fibrinogen, and a positive association between TS and total fibrinogen. TS also correlated negatively with CLT. Most of the correlations observed between clot properties and markers of iron status disappeared after adjustment for fibrinogen. In **Table 3** we divided the iron biomarkers into tertiles to explore their relationship with fibrinogen and clot structure.

Total fibrinogen but not γ' fibrinogen negatively correlated with serum iron concentrations, although both decreased over iron tertiles. %γ' fibrinogen correlated negatively with transferrin and decreased over the transferrin tertiles. A weak negative association between fibrinogen and TS was detected, with total fibrinogen decreasing over the TS tertiles. Lag time correlated positively, albeit weakly, with transferrin and increased over transferrin tertiles after adjustment for fibrinogen. Despite lag time not being correlated with ferritin, we detected a difference therein among ferritin tertiles. The negative correlation between circulating iron and CLT was weak and the decrease in CLT over the serum iron tertiles disappeared after *post hoc* tests. CLT negatively correlated with ferritin and was longer in the first than in the third ferritin tertile. CLT negatively correlated with TS, albeit weakly, and shortened over the TS tertiles. However, because clinical guidelines regarding iron status based on ferritin and TS cut-offs exist, we also report the significant differences observed among and between these categories. Among iron status categories based on ferritin, fibrinogen concentrations tended to be higher in the iron-adequate and overload groups than in the deficient group ($p = 0.057$) and CLT was shorter in the iron overload group than in those with an adequate status ($p = 0.017$). Between iron status categories based on TS, total fibrinogen ($p = 0.00003$), maximum absorbance ($p = 0.02$; after additional adjustment for fibrinogen $p = 0.09$) and CLT ($p = 0.00008$; after additional adjustment for fibrinogen $p = 0.002$) were lower in the iron-deficient than the iron-adequate group.

In **Table 4** only the significant interactions ($p < 0.0017$) between the SNPs and the biochemical markers of iron status are presented in relation to fibrinogen as well as plasma clot structure and function. Serum iron interacted with the *FGB* SNP at rs4463047 in relation to slope. Participants harboring the wild type at this locus displayed an inverse, albeit weak, correlation between the rate of lateral aggregation and concentrations of serum iron, whereas slope and iron did not correlate in carriers of the variant allele. Ferritin interacted with the *FGB* SNP at rs2227388 in relation to lag time. Among the different genotypes at this locus, only heterozygous individuals presented with a negative relationship between ferritin and lag time, with the homozygous individuals of the minor allele being under-represented. A negative association, albeit weak, was seen with carriers of the wild-type genotype of the *FGB* SNP at rs4463047 between TS and slope, whereas there was none in individuals with the minor allele. The *FGB* SNP at rs1800787 was modulated by TS in relation to CLT. Negative associations between TS and CLT were seen in the major C allele carriers.

DISCUSSION

To date, limited information is available on the associations between iron and fibrinogen concentration, clot structure and properties (46). Furthermore, reports available on severe iron status abnormalities and their association with fibrinogen and blood clot data are scarce. However, based on the limited

TABLE 3 | Iron biomarker tertiles in relationship to total and γ' fibrinogen as well as clot properties (i.e., lag time, slope, maximum absorbance, and CLT).

		Adjusted means with 95% CI determined through GLM		
Serum iron Model 1	**<12.4 (μmol/L)**	**12.4–20.45 (μmol/L)**	**>20.45 (μmol/L)**	***p*-value**
Fibrinogen (g/L)	3.94 (3.79–4.10)*#$	3.70 (3.54–3.85)*#$	3.41 (3.25–3.57)*#$	0.00004
Fibrinogen γ' (%)	13.1 (12.4–3.83)*#	11.7 (11.1–2.43)*	11.5 (10.8–2.23)#	0.004
Lag time (min)	6.52 (6.36–6.68)	6.55 (6.40–6.70)	6.36 (6.21–6.52)	0.23
Slope (x 10^{-3}AU/s)	9.74 (9.39–10.1)	9.56 (9.22–9.90)	9.64 (9.29–10.0)	0.76
Maximum absorbance (nm)	0.44 (0.42–0.45)	0.43 (0.42–0.45)	0.42 (0.41–0.44)	0.35
CLT (min)	58.6 (57.7–9.52)	57.0 (56.1–57.9)	56.1 (55.2 –57.0)	0.001
Model 2				
Lag time (min)	6.45 (6.29–6.61)	6.46 (6.30–6.62)	6.36 (6.20–6.52)	0.66
Slope (x 10^{-3} AU/s)	9.57 (9.22–9.91)	9.50 (9.17–9.84)	9.81 (9.46–10.15)	0.45
Maximum absorbance (nm)	0.43 (0.41–0.44)	0.43 (0.41–0.44)	0.43 (0.42–0.44)	0.97
CLT (min)	58.6 (57.6–59.5)	57.3 (56.4–58.2)	56.3 (55.3–57.2)	0.004
Transferrin Model 1	**<2.46 (g/L)**	**2.46–3.04 (g/L)**	**>3.04 (g/L)**	
Fibrinogen (g/L)	3.58 (3.39– 3.71)	3.67 (3.51–3.84)	3.74 (3.59–3.90)	0.20
Fibrinogen γ' (%)	13.3 (12.6–14.0)*#	11.8 (11.1–12.6)*	11.4 (10.8–12.1)#	0.0006
Lag time (min)	6.43 (6.30–6.60)	6.41 (6.25–6.58)	6.60 (6.44–6.75)	0.23
Slope (x 10^{-03} au/s)	9.35 (9.00–9.70)	9.96 (9.60–10.3)	9.51 (9.17–9.85)	0.05
Maximum absorbance (nm)	0.42 (0.41–0.43)	0.43 (0.42–0.45)	0.43 (0.42–0.44)	0.35
CLT (min)	57.2 (56.2–8.09)	57.1 (56.1–8.02)	57.5 (56.6–8.35)	0.83
Model 2				
Lag time (min)	6.35 (6.19–6.52)*	6.31 (6.14–6.48)#	6.58 (6.43–6.74)*#	0.04
Slope (x 10^{-3} AU/s)	9.36 (9.01–9.72)	9.92 (9.57–10.3)	9.47 (9.15–9.80)	0.07
Maximum absorbance (nm)	0.42 (0.40–0.43)	0.42 (0.41–0.44)	0.43 (0.42–0.44)	0.43
CLT (min)	57.4 (56.4–58.4)	57.2 (56.3–58.2)	57.5 (56.6–58.4)	0.92
Ferritin Model 1	**<54.9 (μg/L)**	**54.9–152.6 (μg/L)**	**>152.6 (μg/L)**	
Fibrinogen (g/L)	3.95 (3.73–4.16	3.91 (3.70–4.13)	3.78 (3.56–4.00)	0.54
Fibrinogen γ' (%)	13.1 (12.1–4.11)	12.1 (11.1–3.12)	12.5 (11.5–13.5)	0.37
Lag time (min)	6.53 (6.33–6.73)	6,20 (6.00–6.40)	6.44 (6.24–6.64)	0.03
Slope (x 10^{-3} AU/s)	9.71 (9.25–10.2)	9.71 (9.26–10.2)	9.82 (9.36–10.3)	0.93
Maximum absorbance (nm)	0.46 (0.44–0.47)	0.44 (0.43–0.46)	0.42 (0.40–0.44)	0.06
CLT (min)	60.1 (58.9–61.2)*	58.6 (57.4–59.7)	57.0 (55.9–58.1)*	0.002
Model 2				
Lag time (min)	6.54 (6.33–6.75)	6.16 (5.95–6.36)	6.32 (6.11–6.53)	0.04
Slope (x 10^{-03} au/s)	9.68 (9.21–10.1)	9.64 (9.19–10.1)	9.84 (10.1–9.38)	0.81
Maximum absorbance (nm)	0.45 (0.43–0.46)	0.44 (0.43–0.46)	0.42 (0.40–0.44)	0.09
CLT (min)	60.2 (59.0–61.4)*	58.4 (57.3–59.6)	57.4 (56.2–58.6)*	0.005
TS Model 1	**<18.02 (%)**	**18.02–30.01 (%)**	**>30.01 (%)**	
Fibrinogen (g/L)	3.97 (3.82–4.13)*#$	3.64 (3.48–3.91)*#$	3.42 (3.26–3.58)*#$	0.00001
Fibrinogen γ' (%)	3.97 (3.82–4.13)	3.64 (3.48–3.79)	3.42 (3.26–3.58)	0.4
Lag time (min)	12.7 (12.0–13.4)	11.6 (10.9–12.3)	12.1 (11.4–12.8)	0.54
Slope (x 10^{-3} AU/s)	9.78 (9.44–10.1)	9.57 (9.23–9.91)	9.58 (9.23–9.93)	0.64
Maximum absorbance (nm)	0.43 (0.42–0.45)	0.44 (0.42–0.45)	0.42 (0.41–0.43)	0.07
CLT (min)	58.7 (57.8–59.6)*#$	57.2 (56.3–58.0)*#$	55.8 (54.9–56.7)*#$	0.0009
Model 2				
Lag time (min)	6.41 (6.25–6.57)	6.46 (6.30–6.62)	6.40 (6.24–6.56)	0.86
Slope (x10^{-3} AU/s)	9.59 (9.26–9.93)	9.57 (9.24–9.90)	9.69 (9.34–10.0)	0.9
Maximum absorbance (nm)	0.43 (0.42–0.44)	0.43 (0.42–0.44)	0.42 (0.41–0.43)	0.41
CLT (min)	58.6 (57.6–59.5)*#$	57.4 (56.5–58.3)*#$	56.1 (55.2–57.1)*#$	0.002

The first model for each iron biomarker is adjusted for age, LDL-C, BMI, HbA1c, GGT, and CRP, and the second additionally for fibrinogen. All tertiles contain ~670 individuals. Bonferroni post hoc test revealed significant differences between the categories indicated with symbols. CI, confidence interval; CLT, clot lysis time; GLM, general linear models; TS, transferrin saturation.

TABLE 4 | *FGB*, gene polymorphisms' interactions with biomarkers of iron status in relation to clot properties.

Interaction	Interaction *p*-value	Genotype	*r*	*p*-value	*r*	*p*-value
Serum iron in relation to slope						
***FGB*:** rs4463047	0.001 (0.003)	TT	−0.08	0.01	−0.06	0.06
		TC	−0.07	0.23	0.08	0.21
		CC	0.12	*	0.40	*
Ferritin in relation to lag time						
***FGB*:** rs2227388	0.0009	GG	−0.03	0.50	0.03	0.50
	(0.001)	AG	−0.30	>0.05	−0.30	>0.05
		AA	−0.07	0.78	−0.20	0.50
TS in relation to slope						
***FGB*:** rs4463047	0.0008	TT	−0.08	0.01	−0.06	0.06
	(0.0004)	TC	0.08	0.21	0.09	0.16
		CC	0.47	*	0.72	*
TS in relation to CLT						
***FGB*:** rs1800787	0.001 (0.002)	CC	0.02	0.60	0.02	0.63
		CT	−0.08	0.55	−0.06	0.63
		TT	−0.25	*	−0.40	*

First, r and p-value are for a GLM interaction adjusted for age, LDL-C, HbA1c, GGT, and BMI; and the second with additional adjustment for fibrinogen. Key: A, adenine; C, cytosine; FGB, fibrinogen beta chain; G, guanine; rs, reference sequence; T, thymine; TS, transferrin saturation.

**Stratification resulted in too few individuals to perform analysis.*

available literature, it seemed plausible that fibrinogen and clot properties are affected negatively in both iron deficiency and overload. Therefore, to complement the linear relationship detected by correlation analysis, we created tertiles of the iron status markers in addition to clinical cutoffs used for ferritin and compared the subgroups. In this study, we confirmed that inverse linear relationships exist between iron biomarkers and fibrinogen as well as clot characteristics and detected new linear relationships that have not been reported previously. Only fibrinogen concentrations tended to be higher in the iron-adequate and overload groups than in the deficient group (based on the clinical cut-offs for ferritin values). We did not observe any other U- or J-shaped relationships. This work also contributes to the field in a novel way by considering genetics.

Total fibrinogen but not γ' fibrinogen negatively correlated with serum iron concentrations, with total and γ' fibrinogen decreasing over the serum iron tertiles. The negative relationship between serum iron and total fibrinogen observed here is in agreement with the findings of others on Black South Africans (47, 48). Here, despite not detecting a correlation between ferritin and fibrinogen, fibrinogen tended to be higher (borderline significant) in the iron-adequate and overload groups than in the deficient group. Studies are conflicting regarding the relationship between ferritin and fibrinogen, with one finding no association (49) and others observing a positive association in healthy men and patients with stable angina, myocardial ischemia and unstable angina (50, 51). Reasons for this inconsistent relationship observed between ferritin and fibrinogen are unclear, but could be due to the different target populations that were investigated (apparently healthy vs. having CVD). A weak negative correlation between fibrinogen and TS was detected after *post hoc* correction for covariates. The inverse association was further supported with fibrinogen decreasing over the TS tertiles and being lower in the TS category corresponding to adequate iron status. The negative relationship of TS and total fibrinogen we report contrasts with data from a study involving patients with chronic kidney disease (52). Here we noted novel negative relationships between γ' fibrinogen and serum iron/transferrin. %γ' fibrinogen negatively correlated with transferrin, albeit weakly, and decreased over the transferrin tertiles.

Regarding the clot properties, lag time demonstrated a weak positive association with transferrin (after adjustment for fibrinogen). Additionally, lag time differed between TS iron status categories (before adjustment for fibrinogen), being lower in the iron-deficient group than those with adequate status. CLT correlated negatively with ferritin and was longer in the first than in the third ferritin tertile and shorter in the iron overload group than those with an adequate status based ferritin. CLT was shorter in those with adequate status based on TS cut-offs. In kidney disease patients, D-dimer, a fibrin degradation product indicating coagulation activation and fibrinolysis, seems to be augmented in those with anemia (53). In 2017, a case study reported elevated D-dimer in a patient presenting with anemia after iron sucrose infusion (54). Experimental studies have shown iron-mediated increased coagulation (8, 22, 24, 55). Hydroxyl free radicals are produced by poorly chelated iron ions accumulating in the circulation and converting soluble human fibrinogen into an insoluble and plasmin-resistant polymer. Fibrin clots of this polymer are resistant to enzymatic degradation (56). The proposed biological mechanism for iron-mediated hypercoagulability is not just via oxidative stress, but also involves direct binding of iron to fibrinogen, causing conformational changes in this glycoprotein (57). Lipinski et al. added iron ions

(ferrous chloride) to whole blood and observed enhanced fibrin fiber formation with thrombin, delayed fibrinolysis in a concentration-dependent manner and more densely matted fibrin deposits (23). The ferrous ions caused the appearance of dense deposits of matted fibrin, similar to those observed in the plasma of stroke patients (55). In iron overload, a decrease in the time of onset of coagulation was observed (22). These *in vitro* findings do not seem to apply to the epidemiological setting. Here, fibrinogen concentrations seemed to be higher and CLT shorter in those with iron-overload (as defined by ferritin and TS concentrations) than those with adequate status or in the first ferritin tertile than the third, whereas no other marker of clot properties differed after Bonferroni correction. Because we observed a negative association between serum iron and total and γ' fibrinogen and no relationship between ferritin and fibrinogen and the CLT relationship remained after adjusting for fibrinogen, we attribute the increased lysis rate to other factors that also influence fibrinolysis.

To our knowledge, we are the first to relate a unique set of variables reflecting fibrinogen functionality—that is, total fibrinogen or γ' fibrinogen concentrations as well as the kinetics of clot formation, structural properties and fibrinolysis—with iron status biomarkers and to take the genetic make-up of individuals into account. Here we report that certain *FGB* genetic variants modified the association of iron biomarkers with fibrin clot characteristics. Iron biomarkers modulated the influence of the SNPs so that, for the majority of the study population, having adequate iron status was beneficial in respect of clot structure/properties. Optimal iron was evidently even more beneficial for a minority group harboring specific variant alleles. The interactions observed require further replication and functional validation for a mechanistic understanding. However, we know that iron status has the ability to perturb normal patterns of DNA methylation (58) and influence epigenetic regulatory mechanisms (59). We speculate that these methylation changes can translate into altering fibrinogen expression due to conformational changes at the *FGB*: rs4463047 (downstream), *FGB*: rs2227388 (5'upstream) and *FGB*: rs1800787 (5' upstream) loci, leading to the interaction we observed. However, after additional adjustment for fibrinogen the interactions remained. Therefore, future research should investigate the mechanisms behind the interactions observed per locus; here we simply demonstrate the nuanced relationships of iron biomarkers and clot structure/properties due to genetic factors and that they are probably not due to changes in fibrinogen concentration.

Overall, we observed increasing iron biomarkers to be beneficial in terms of fibrinogen and clot properties and did not detect iron-hypercoagulability as previous researchers did (8). However, we acknowledge certain limitations. It is possible that we did not have enough participants presenting with extreme iron deficiency or overload, because we included ostensibly healthy individuals who did not have pronounced iron status abnormalities. To interpret iron status, we accounted for the degree of inflammation by conducting a sensitivity analysis and by adjusting for inflammation; however, maintaining statistical power became problematic when dealing with the study population in terms of particular genotypes. Whereas, two *FXIII* SNPs were included in this study, we could not quantify FXIII levels to provide additional evidence for the association of FXIII levels with fibrin clot structure. Serum transferrin receptor (TfR) concentration and the ratio of serum TfR to serum ferritin may be another indicator of iron deficiency to consider in future (5). Even though hemoglobin is a late indicator of iron deficiency, because it is widely used in clinical settings, its inclusion may aid healthcare workers in giving medical advice. Additionally, viscoelastic testing complimentary to the already collected clot properties could be investigated in future research. We believe that, even with these limitations and the observational nature of the study design, these results still contribute usefully to current knowledge on this topic. Moreover, we have highlighted the idiosyncrasies that result from the genetic differences in how iron status modulates fibrinogen concentration and functionality by investigating blood clot structure and properties.

CONCLUSION

We show relationships between fibrinogen, fibrin formation and fibrinogen lysis and biomarkers of iron status that are modulated by certain fibrinogen SNPs. CVD and iron status have both been associated with increased mortality and morbidity. Fibrinogen and fibrinogen functionality may form part of the mechanisms linking these conditions. Therefore, in terms of overall health both iron insufficiency and overload should be detected and treated. Whether manipulation of dietary iron status to influence fibrinogen and fibrin blood clot formation in the presence of certain genetic characteristics can reduce CVD, still needs to be determined. Healthcare workers could potentially use these findings, when enough evidence such as we provide here has accumulated, to modify iron status of individuals to ultimately reduce CVD in the emerging field of personalized medicine.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The data that support the findings of this study are available upon reasonable request and with the permission of the Health Research Ethics Committee of North-West University and the principal investigator of the PURE-SA-NW study, at North-West University's Africa Unit for Transdisciplinary Health Research. Requests to access these datasets should be directed to lanthe.kruger@nwu.ac.za.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by North-West University Health Research Ethics Committee (NWU-00016-10-A1 and NWU-00034-17-A1-02). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PR: statistical analysis and interpretation of data, drafting and finalizing the manuscript, and final approval. CN-R: study concept, acquisition of data and analysis, drafting, finalizing and critical revision of the manuscript, final approval, and study supervision. ZL-L: data generation and interpretation, critical revision of the manuscript, and final approval. MP: obtained funding, acquisition of data, interpretation of data, critical revision of the manuscript, final approval, and study supervision. All authors contributed to the article and approved the submitted version.

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Vitamin D Status and Its Correlation With Anthropometric and Biochemical Indicators of Cardiometabolic Risk in Serbian Underground Coal Miners in 2016

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The status of vitamin D in underground working coal miners and its association with their cardiometabolic health is rarely studied. This study aimed to examine vitamin D (VitD) status in Serbian underground coal miners and to correlate it with anthropometric and laboratory indicators of cardiometabolic risk. Nutritional data (food frequency questionnaire, FFQ, and two times repeated 24 h recall), anthropometric data (including segmental analysis by bio-impedance analyzer TANITA BC-545N), arterial tension, and biochemical and hematological data of 103 coal miners (aged 22–63 years) were correlated with their late summer (early September) serum 25 (OH)D levels (measured by HPLC). 68.9% of the studied coal miners were overweight/obese, and 48.5% had metabolic syndrome. Their mean VitD nutritional intakes were low: 5.3 ± 3.8 μg/day (FFQ) and 4.9 ± 8 μg/day (24 h recalls), but their mean serum 25 (OH)D levels were surprisingly high (143.7 ± 41.4 nmol/L). Only 2.9% of the coal miners had 25(OH)D levels lower than 75 nmol/L (indicating an insufficient/deficient status), while 63.2% had values above 125 nmol/L (upper optimal limit), and even 10.7% had values above 200 nmol/L. There were no statistical differences in 25 (OH)D levels in the coal miners with or without metabolic syndrome (or overweight/obesity). Interestingly, 25(OH)D levels had significant positive correlations with body mass index (BMI), fat mass (FM), fat mass percentage (FM%), limbs FM%, serum triglycerides, GGT, AST, ALT, and ALT/AST ratio, and had significant negative correlations with serum HDL-cholesterol and age. All these correlations were lost after corrections for age, FM, FM%, and legs FM%. In Serbian coal miners, high levels of early September VitD levels were observed, indicating sufficient non-working-hour sun exposure during the summer period. Furthermore, the unexpected positive correlations of VitD levels with anthropometric and biochemical parameters indicative of obesity, metabolic syndrome, and fatty liver disease were found. More research is needed on the VitD status of coal miners (particularly in the winter period) and its relationship with their cardiometabolic status.

Keywords: coal miners, Vitamin D, physical activity, cardiometabolic health, metabolic syndrome, obesity, lipids

INTRODUCTION

The relationship between vitamin D (VitD) status and obesity, metabolic syndrome, diabetes, and cardiovascular diseases has gained scientific attention over the last decades. Low serum vitamin D levels have been associated with obesity, metabolic syndrome, and increased risk for diabetes and cardiovascular diseases (1–4). However, inconsistent findings exist, and cause/effect relationships still need to be confirmed (1–5).

On the other hand, in parallel with the increasing worldwide prevalence of obesity, metabolic syndrome, diabetes, and cardiovascular diseases (6–8), with the latter two being among the first four leading causes of death across the world, there is an increasing prevalence of VitD insufficiency/deficiency (9, 10). Some populations are at increased risk for low VitD levels (10–12). The modern lifestyle in big cities, with high buildings, air pollution, and low insolation of streets; everyday commuting by closed vehicles and predominantly indoor work and leisure activities (without enough outdoor activities and direct exposure to the sun), leads to an increased prevalence of VitD insufficiency/deficiency among populations (10, 13) where it was not expected to appear (e.g., in regions with low latitude and high insolation) (14, 15). Some professions and working conditions are more associated with low VitD levels. For example, shift work, night work, and working indoors, without direct sun exposure, are associated with lower VitD serum levels (16, 17). All of those are characteristics of underground mining work. Nevertheless, insufficient data exist on the serum levels of VitD in this population of workers (17–21), and the data do not show the lower levels compared with the general population or surface workers from the same geographic region (17–21). Therefore, more data are needed on this topic. Furthermore, there are insufficient data on the cardiometabolic status of coal miners, with only few recently published studies (22–26).

Given that, this study aimed to examine the following: (1) the levels of serum VitD among a sample of underground working coal miners in Serbia; (2) the anthropometric and biochemical indicators of cardiometabolic risk among them; (3) the association of VitD levels with indicators of cardiometabolic risk.

MATERIALS AND METHODS

Participants and Study Design

This cross-sectional study included 103 male coal miners, aged 22–63 years, from the underground Brown Coal Mine "Rembas" (shaft "Vodna," Strmosten), in the Resavica coal mining basin in Serbia, who during the period May 9, 2016, to September 9, 2016, (late summer) underwent their periodic (annual) preventive systematic medical examinations, obliged by the law and performed by the Institute of Occupational Medicine Niš. The study was a joint project of the Institute of Medical Research of the University of Belgrade and the Institute of Occupational Medicine Nis and supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia. The study was in accordance with the ethical principles of the Declaration of Helsinki and approved by the Institute of Occupational Health Niš Ethics Board.

According to official data (27), in 2016, in Serbia, there were 2,750 underground coal miners, working in shafts, and the vast majority of them were employed in the Resavica basin (76.4%). The required study sample size was 45 subjects, which was calculated according to the previously published data on summer Serbian general male population VitD levels (28) and the decided precision (margin of error) of ± 5 nmol/L at 95% confidence level, and the formula: needed sample size = $(1.96)^2$ * $(SD)^2$/(decided precision)^2 (29). Considering possible unfitness, dropout, and planned further correlation analyses, we decided to initially include (i.e., invite to participate in the study) all 140 employed miners in this coal mine who attended their preventive systematic medical examinations, which would represent about 5% of Serbian coal miners.

On the day of the examination, the coal miners attended the center for preventive medical checkups at 07:00, after fasting overnight for 12 h. Detailed examinations to obtain information on their health were performed by medical specialists (including specialists of occupational health and internal medicine), and incorporated, *inter alia*, blood pressure measurements and fasting blood, and urine sampling for laboratory biochemical analyses. Supplementary analyses for this study involved the following: (1) anthropometric measures (including, among others segmental body composition analysis by bioelectrical impedance, BIA); (2) dietary surveys [such as the two times repeated 24 h recall and FFQ, which was validated for VitD intake (30)], (3) and further analyses of the obtained biochemical serum samples to determine VitD status by high-performance liquid chromatography (HPLC).

From the 140 coal miners who attended the medical preventive checkups and were invited to participate in the study, a total of 103 agreed to participate in the study, signed written consent, and performed all the tests and analyses required for this study (i.e., the participants who did not manage to perform all of the required examinations, presented here, or fulfilled the exclusion criteria, were excluded from the study). The exclusion criteria were the following: the presence of serious medical conditions; established diagnosis of diabetes mellitus with regular use of glucose-lowering therapy; established diagnosis of hyperlipidemia with regular use of lipid lower drugs; a known liver disease with non-metabolic etiology, excluding non-alcoholic fatty liver disease (NAFLD); taking any antihypertensive and glucose/lipid-lowering agents in the previous 24 h).

All the underground miners worked in three rotating shifts (2 days in first shift: 07:00–15:00; 2 days in second shift: 15:00–23:00; 2 days in third shift: 23:00–07:00; then, 2 days off) and always stayed underground, in the shaft during their shifts. They all performed heavy physical work (31), such as hewing coal, loading coal, and erecting roof supports.

Anthropometric Measurements and Measurement of Arterial Tension

Measurements of body height, body weight, fat mass (FM), fat mass percentage (FM%), fat-free mass (FFM), muscle mass, bone

mass, waist circumference, and hip circumference were taken by a trained medical doctor, following standard techniques (32, 33). The subjects were measured fasted at 8 am, after emptying their bladder and bowels, wearing only underwear. Body height was measured to the nearest 0.01 m with a wall-mounted stadiometer. Bodyweight, FM, FM%, FFM, muscle mass, and bone mass were measured on a portable scale Tanita Inner Scan V BC-545N Segmental Body Composition Monitor (Tanita, Aerolit d.o.o., Belgrade, Serbia), by BIA. The body weight was measured to the nearest 0.1 kg. The measurements were performed according to the recommendations provided by the manufacturer. Additional segmental analyses included muscle mass of trunk, arms, and legs; fat percentage of the trunk, arms, and legs; and visceral fat level (VFL), calculated according to the Tanita algorithm by the BIA segmental body analysis (34, 35). Body mass index (BMI: kg/m^2) was calculated by dividing the weight (in kilograms) by the square of height (in meters), and according to their BMI, the coal miners were classified into three groups: normal weight (BMI: 18.5–24.9 kg/m^2), overweight (BMI: 25–29.9 kg/m^2), and obese (BMI 30 kg/m^2) (36). Increased adiposity was defined according to %FM $\geq$ 21–22 (depending on age) (37). Waist and hip circumferences were measured with a flexible, stretch-resistant tape, to the nearest centimeter. Waist circumference was measured at the approximate midpoint between the lower margin of the last palpable rib and the top of the iliac crest, at minimum respiration. Hip circumference was measured at the widest points over the hip at the level of the greater trochanter, with the tape parallel to the floor (33). Abdominal obesity was defined by waist circumference $\geq$ 102 cm (33). Supplementary calculations included waist to hip ratio (WHR), and WHR $\geq$0.91 was considered as indicative of centripetal obesity, i.e., truncal fat distribution (33).

Arterial blood pressures were measured in the morning, after sitting for at least 10 min, on the left upper arm, with a calibrated mechanic sphygmomanometer and stethoscope (Becton Dickinson, Franklin Lakes, NJ, United States), and the average of two measurements was presented as the result. Hypertension was defined by systolic blood pressure $\geq$ 140 mmHg and/or diastolic blood pressure $\geq$ 90 mmHg, while pre-hypertension was defined by systolic blood pressure between 130 and 140 mmHg and/or diastolic blood pressure between 85 and 90 mmHg (38).

Laboratory Biochemical Analyses of Blood Samples

Fasting blood samples were collected between 07:00 and 08:00 in specific Vacutest Kima® blood collection tubes, available from Vacutest Kima® S.R.L., Italy (Yunycom d.o.o., Belgrade, Serbia), including the following: Vacutest® Kimased sodium citrate 3.8% 1.6 ml tubes (for erythrocyte sedimentation rate, tubes, erythrocyte sedimentation rate (ESR), analysis), Vacutest® K3EDTA 3 ml tubes (for blood cell count, hematocrit, and hemoglobin analysis), Vacutest® Serum Separator Clot Activator 6 ml tubes for analysis of serum glucose, total cholesterol, high-density lipoproteins (HDL)-cholesterol and low-density lipoproteins (LDL)-cholesterol, triglycerides, urea, creatinine, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and VitD.

Following the instructions of the manufacturer for processing and handling of the blood collection tubes, ESR analyses were performed with the Westergren's method at 18°C, using the Kimased ESR rack with scaling. One-hour Westergren values were determined by reading the distance (in mm) from the plasma meniscus to the top of the column of sedimented erythrocytes 30 min, immediately (maximum 1 h) after blood collection. Analyses of blood cell counts, hematocrit, and hemoglobin were performed on Phoenix NCCNCC-2310 (NeoMedica d.o.o., Nis, Serbia), and the serum coagulation tubes (after allowing them to clot for at least 30 min in an upright position at room temperature) were centrifuged at 3,000 × g for 10 min at room temperature. Following centrifugation, standard photometric assays were performed using an ARCHITECT c8000 Abbott clinical chemistry analyzer (Abbott Laboratories S.A., Belgrade, Serbia), and commercially available kits (Abbott Laboratories S.A., Belgrade, Serbia). Intra- and inter-assay coefficients of variation (CVs) for all measurements were <5%.

The criteria for the existence of diabetes and impaired fasting glucose were set according to the American Diabetes Association (ADA) 2016 guidelines (39). The existence of dyslipidemia was established based on the National Cholesterol Education Program—Adult Treatment Panel III (NCEP-ATP III) criteria from 2001 (40). The atherogenic risk was also calculated as the ratio of total cholesterol/HDL-cholesterol, the ratio of LDL-cholesterol/HDL-cholesterol, and the ratio of triglycerides/HDL-cholesterol, as all of these ratios have clinical implications (41, 42). Metabolic syndrome was defined according to the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) 2005 criteria (43).

Serum VitD Analysis by HPLC Method

Serum total 25 (OH) vitamin D, calculated as a sum of 25 (OH) vitamin D2 and 25 (OH) vitamin D3, was determined using an Agilent 1100 series HPLC system equipped with a diode array detector (DAD) and the RECIPE's (RECIPE Chemicals + Instruments GmbH, Munich, Germany) ClinRep® HPLC Complete Kit for 25 (OH) VitD2/D3 in plasma and serum (containing a ClinTest® Test Solution, ClinCal® Serum Calibrators, ClinChek® Serum Controls, sample preparation vials, the precipitation agent, internal standard, mobile phase, and analytical column), according to the instructions of the manufacturer.

Immediately after the analyses with the ARCHITECT c8000 clinical chemistry analyzer were performed, 1.5 ml of serum was separated from the serum coagulation tubes in 2 ml Eppendorf microtubes and kept frozen at −80°C in a freezer until further VitD assays were performed.

After 24 months of storage at −80°C (which should not influence the results, since vitamin D is quite stable during prolonged storage at −80°C, up to 6–30 years) (44, 45), serum samples were defrosted and vortex-mixed and 0.4 ml of the samples were transferred to the sample preparation vials, filled with 0.5 ml of the precipitation agent. Afterward, 0.4 ml of

internal standard was added (cooled at temperature −20°C), and the vials were vortex-mixed for 30 s and centrifuged for 5 min at 10,000 × g. After centrifugation, 50 μl of the upper liquid phase of the supernatant was injected into the Aligent 1100 HPLC system, with a flow rate of 1 ml/min, and the column heater at 40°C. The sample components were separated on the analytical column, and the analytes, 25 (OH) VitD2 and 25 (OH) VitD3, were detected with the DAD detector at a wavelength of 264 nm, with retention times of 7.3 and 6.4 min, respectively. Quantification was performed comparing the peak areas of 25 (OH) VitD2 and 25 (OH) VitD3 with the peak area of the internal standard (its retention time was 8.3 min). In addition, the ClinChek® Serum Controls were used after running every 10 examined samples to perform quality control. The precision of measurement (expressed as CVs) for 25 (OH) VitD2 and 25 (OH) VitD3 was 6.5 and 6%, respectively.

Taking into consideration different cutoff values for defining VitD deficiency/insufficiency (9, 10, 46–48), the serum levels of 25 (OH) VitD below 50 nmol/L were defined as a deficient state, while those between 50 and 75 nmol/L were defined as an insufficient state (9). Deficiency below 50 nmol/L was defined as mild to moderate and below 30 nmol/L was defined as severe (10). The values above 175 nmol/L were defined as mildly increased, above 200 nmol/L was defined as moderately increased (the lowest literature-reported level associated with toxicity in subjects with normal renal function and without primary hyperparathyroidism), and above 250 nmol/L was defined as excessive (hypervitaminosis) (46). The values between 75 and 175 nmol/L were defined as VitD sufficiency ("normal range"), while the values between 75 and 125 nmol/L were defined as "optimal." (46, 49).

Assessment of Dietary Intakes

Dietary intake data were collected by a trained medical doctor using a validated food frequency questionnaire (FFQ) (30) and the two times repeated 24 h recall method, in accordance with the European Food Safety Authority (EFSA) EU Menu methodology (32, 50). The FFQ and the first 24 h recall were performed on the day of physical examination and blood sampling, while the second 24 h recall was performed with a minimum of 7 days apart, by a telephone call. The average intakes of energy, macronutrients, and VitD were calculated with the DIET ASSESS & PLAN (DAP) software (51, 52), which was evaluated by the EFSA (32, 50) using the national validated Food Atlas for Portion Size Estimation (53) and Serbian Food Composition Database, developed in compliance with EuroFIR standards (54). Calculated data for VitD intakes were evaluated by comparison with the adequate intake (AI) defined by the EFSA in 2016 (i.e., minimum 15 μg/day for adults) (48).

Statistical Analysis

Statistical analyses were performed using the SPSS 22.0 (SPSS Inc., Chicago, IL, United States) statistical software. Data are presented as the mean ± SD, along with minimum and maximum values. The normality of data distribution was tested by the Kolmogorov–Smirnov test. Differences in VitD levels between subjects with and without overweight/obesity or metabolic syndrome were analyzed by the Mann–Whitney *U*-test. Correlation between VitD levels and biochemical data, anthropometric measurements, daily energy, and macronutrient/micronutrient nutrient intake was assessed by Pearson's correlation coefficient (for parametric data) and Spearman's correlation coefficient (for non-parametric data). Partial correlations were used when one or more controlling variables were included in the analyses. Linear stepwise regression models were made to explore the strength and independence of associations between VitD levels and characteristics of analyzed subjects. *P*-value was expressed as a number rounded up to three decimal places; statistical significance was assumed at a two-tailed $P < 0.05$.

RESULTS

Descriptive Statistics: Characteristics of the Studied Population of Coal Miners

Table 1 presents general, anthropometric, and blood pressure data, **Table 2** presents the data on biochemical analyses, and **Table 3** gives the data on dietary intakes of energy, macronutrients, and VitD.

The mean age was 40.6 ± 9.6 years, but 10-year age categories were not represented equally, and only 11.7% of the participants were above 52-year-old (**Supplementary Figure 1**). More than half (53.5%) of the participants were smokers, and the huge majority (79.6%) lived in rural areas (the rest lived in semi-urban areas).

The average BMI belonged to the category of overweight (27.2 ± 4.5 kg/m^2), and almost half of the participants (45.6%) were overweight, while about one-quarter (23.3%) were obese, with these two BMI categories making together above two-third of the studied population (68.9%). Less than one-third (28.2%) had normal weight, and only 2.9% were underweight. Almost half of the participants (45.6%) had increased adiposity, according to % FM ≥ 21–22 (depending on age). According to waist circumference, almost half of the participants (45.6%) had abdominal obesity, even though the average waist circumference value was under the cutoff value for abdominal obesity (i.e., < 102 cm). Centripetal obesity/fat distribution according to WHR was present in 75.7% of the participants.

The average measured values of fasting serum glucose (**Table 2**) were above the ADA cutoff values for the diagnosis of impaired fasting glucose ("pre-diabetes"), and 63.1% of the participants had serum glucose values above this value (i.e., ≥5.6 mmol/L), while at the same time 11.7% of the participants also met the criteria for diabetes (i.e., fasting serum glucose ≥ 7 mmol/L). Also, the average blood lipid levels were above the ATP III cutoff values for triglycerides (i.e., ≥1.69 mmol/L), total cholesterol (i.e., ≥5.17 mmol/L), and LDL-cholesterol (i.e., ≥3.34 mmol/L), while the average HDL-cholesterol levels were below the recommended values for men (i.e., <1.03 mmol/L) (**Table 2**). In total, 53.4, 62.1, and 45.6% of the participants had elevated triglycerides, total cholesterol, and LDL-cholesterol, respectively, while only 26.2% of the participants had reduced HDL-cholesterol levels. In total, 84 participants (81.6%) had

TABLE 1 | Demographic, anthropometric, and arterial BP data of the studied coal miners ($N = 103$, men), and correlation with serum 25 (OH) vitamin D levels.

	Descriptives			Correlation with vitamin D levels (non-adjusted)	Correlation with vitamin D levels (adjusted for age, FM, and FM%)	Correlation with vitamin D levels (adjusted for age, FM, FM%, and legs FM%)
	Mean	*SD*	*(Min–Max)*	*r*	*r*	*r*
Age (years)	40.6	9.6	(22.0–63.0)	**−0.283****		
Body height (cm)§	177.4	6.5	(162.0–196.0)	0.014	−0.116	−0.124
Body weight (kg)	86.0	16.6	(49.4–126.3)	**0.201***	0.068	0.034
BMI (kg/m^2)	27.2	4.5	(16.5–38.1)	**0.243***	**0.205***	0.185
FM (kg)	18.0	8.9	(2.7–39.2)	**0.210***		
FM% (%)	19.9	7.2	(5.0–36.2)	**0.200***		
FFM (kg)	68.1	9.8	(40.6–88.8)	0.150	0.068	0.034
Muscle mass (kg)	64.7	9.4	(38.4–84.6)	0.151	0.070	0.036
Skeletal mass (kg)	3.4	0.5	2.1–4.3)	0.159	0.081	0.048
Segmental analysis						
Arms FM% (%)	18.1	5.9	(4.6–39.4)	0.162	−0.076	−0.137
Trunk FM% (%)	21.2	8.6	(5.0–35.9)	0.171	−0.163	0.148
Legs FM% (%)	18.4	6.4	(3.8–42.7)	**0.250***	**0.208***	
Arms muscle mass (kg)	7.8	1.4	(3.7–12.1)	**0.194***	0.149	0.090
Trunk muscle mass (kg)	35.2	5.1	(21.6–48.8)	0.120	0.078	0.022
Legs muscle mass (kg)	21.7	3.2	(13.1–28.9)	0.182	0.007	0.046
VFL	8.0	4.2	(1.0–18.5)	0.077	0.046	0.167
Waist circumference (cm)	98.9	12.9	(69.0–129.0)	0.143	−0.032	−0.050
Hip circumference (cm)	104.4	8.3	(86.0–124.0)	**0.211***	0.091	0.074
WHR	0.95	0.07	(0.78–1.11)	0.021	−0.098	−0.104
Systolic BP (mmHg)§	127.1	14.2	(100.0–160.0)	0.059	0.091	0.105
Diastolic BP (mmHg)§	83.1	8.0	(60.0–120.0)	−0.005	0.034	0.025

§*Spearman's rank correlation coefficients—r_s (otherwise—data represent Pearson's correlation coefficients—r).*

Bolded coefficients are statistically significant.

**p < 0.05; correlation is significant at the 0.05 level (two-tailed).*

***p < 0.01; correlation is significant at the 0.01 level (two-tailed).*

BMI, body mass index; FM, fat mass; FM%, percentage of fat mass; FFM, fat-free mass; VFL, visceral fat level; WHR, waist to hip ratio; BP, blood pressure; SD, standard deviation; r, Pearson's correlation coefficients; r_s, Spearman's rank correlation coefficients; p, statistical significance of correlations.

dyslipidemia according to at least one of the ATP III criteria, while 11 participants (10.7%) fulfilled all the ATP III criteria for dyslipidemia (at the same time having increased triglycerides and LDL-cholesterol levels, and decreased HDL-cholesterol levels). The ratios of total cholesterol, LDL-cholesterol, and triglycerides to HDL-cholesterol were increased (i.e., respectively, ≥5, ≥3.3, and ≥0.87) in 43.7, 34, and 73.8% of the participants, respectively.

The average systolic and diastolic pressures were in the normal range, but 51.5% of the participants met the criteria for pre-hypertension or hypertension.

Only three participants used lipid-lowering agents (but they did not use them regularly), while 11 participants used antihypertensive drugs. No other medicaments used were reported, and no medications were used on the morning of the examination.

According to the obtained data on glucose, triglycerides, HDL-cholesterol, blood pressure, and waist circumference (or regular use of relevant medicaments), 48.5% of the participants fulfilled the 2005 AHA criteria for the diagnosis of metabolic syndrome. The serum levels of ALT above 30 U/L, GGT above 30 U/L, and ALT/AST ratio higher than 1.25 [indicative for NAFLD (55–57)] had 36.9, 48.5, and 40.8% of the participants, respectively.

The average energy intake, according to the repeated 24 h recalls, was 3955.2 ± 1095.4 kcal/day, which is in accordance with existing recommendations (58), but there was a huge inter-individual difference (individual intakes ranged from 1,600 to 7,200 kcal) (**Table 3**). On average, proteins, fats, carbohydrates, and alcohol provided 12.9 ± 2.1, 46.8 ± 6.7, 38.1 ± 7.1, and 2.2 ± 3.5% of total daily energy intake, respectively. Daily alcohol intake (average for 2 days, according to 24 h recalls) varied from 0 g (in 54.4% of coal miners) to 126.7 g. Similarly, according to FFQs, only 58.3% of the coal miners reported alcohol intake.

The average intake of VitD was quite low and far below the recommended values by EFSA: 5.3 ± 3.8 μg/day (according to FFQs) and 4.9 ± 8 μg/day (according to 24 h recalls) (**Table 3**). Only two of the participants had an adequate nutritional intake of VitD, above 15 μg/day. There was a statistically significant

TABLE 2 | Hematological and biochemical data of the studied coal miners (N =103, men), and correlation with serum 25 (OH) vitamin D levels.

	Descriptives			Correlation with vitamin D levels (non–adjusted)	Correlation with vitamin D levels (adjusted for age, FM, and FM%)	Correlation with vitamin D levels (adjusted for age, FM, FM%, and legs FM%)
	Mean	*SD*	*(Min–Max)*	r_s	r_s	r_s
ESR (mm/h)	6.8	3.5	(3.0–22.0)	−0.112	−0.037	−0.026
WBC ($\times 10^9$/L)	7.8	2.1	(3.8–14.4)	0.038	0.018	0.003
RBC ($\times 10^{12}$/L)	5.0	0.5	(3.2–6.3)	**0.208***	0.129	0.096
HGB (g/L) §	155.1	10.1	(135.0–183.0)	0.142	0.073	0.047
HCT (L/L)§	0.4	0.0	(0.3–0.5)	0.144	0.112	0.096
MCV (fL)§	87.8	5.1	(69.3–102.4)	−0.169	−0.036	−0.013
MCH (pg)	30.9	2.4	(23.0–41.2)	−0.108	−0.059	−0.058
MCHC (g/L)	352.7	17.7	(324.0–430.0)	0.022	−0.065	−0.077
PLT ($\times 10^9$/L)	240.2	56.7	(131.0–427.0)	−0.028	0.001	−0.012
Glucose (mmol/L)	6.0	1.2	(3.5–11.5)	−0.015	−0.030	−0.037
Triglycerides (mmol/L)	2.0	1.0	(0.5–4.9)	**0.249***	**0.237***	0.190
Total cholesterol (mmol/L)	5.8	1.3	(3.5–9.8)	0.169	**0.198***	0.135
LDL-cholesterol (mmol/L)	3.4	1.1	(1.1–7.5)	0.118	0.134	0.071
HDL-cholesterol (mmol/L)	1.3	0.4	(0.7–2.7)	**-0.250***	**−0.209***	−0.145
TC/HDL	5.0	2.0	(1.5–11.5)	**0.300****	**0.293****	**0.220***
LDL/HDL	2.9	1.4	(0.4–7.4)	**0.245***	**0.235***	0.159
TG/HDL	1.9	1.4	(0.2–7.1)	**0.273****	**0.251***	0.192
AST (U/L)	24.5	7.5	(12.0–64.0)	**0.235***	0.194	0.191
ALT (U/L)	30.3	14.8	(11.0–93.0)	**0.351****	**0.254***	0.187
ALT/AST	1.23	0.43	(0.58–2.44)	**0.276***	0.170	0.089
GGT (U/L)	37.3	31.0	(10.0–245.0)	**0.203***	0.165	0.107
ALP (U/L)	56.3	16.4	(14.0–99.0)	0.084	−0.002	0.007
Total bilirubin (μmol/L)	11.7	6.6	(3.7–35.4)	−0.169	−0.191	−0.198
Urea (mmol/L)	4.9	1.0	(2.8–8.3)	0.036	0.097	0.089
Creatinine (μmol/L)	72.8	10.1	(54.5–120.3)	**0.244***	**0.207***	0.157

§*Pearson's correlation coefficients—r (otherwise—data represent Spearman's rank correlation coefficients—r_s).*

Bolded coefficients are statistically significant.

**$p < 0.05$; correlation is significant at the 0.05 level (two-tailed).*

***$p < 0.01$; correlation is significant at the 0.01 level (two-tailed).*

FM, fat mass; FM%, percentage of fat mass; ESR, erythrocyte sedimentation rate; WBC, white blood cell count; RBC, red blood cell count; HGB, hemoglobin; HCT, hematocrit; MCV, mean volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet count; LDL, low-density lipoprotein; HDL, high - density lipoprotein; TC/HDL, total cholesterol/HDL-cholesterol ratio; LDL/HDL, LDL-cholesterol/HDL-cholesterol ratio; TG/HDL, triglycerides/HDL-cholesterol ratio; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; SD, standard deviation; r_s, Spearman's rank correlation coefficients; r, Pearson's correlation coefficients; p, statistical significance of correlations.

correlation between VitD intakes by FFQs and 24 h recalls (Spearman's rho correlation coefficient 0.268, $p = 0.04$). Only four of the coal miners consumed supplements of VitD.

Vitamin D Status

The average value of serum 25(OH) vitamin D was way above the lower cutoff value for the sufficient state: 143.7 ± 41.4 nmol/L, ranging from 43.5 to even 247.5 nmol/L. Only three (2.9%) of the coal miners had levels of VitD lower than 75 nmol/L (indicating an insufficient/deficient status, with two subjects having mild deficiency), while 63.2% had values above 125 nmol/L (upper optimal limit). In total, 22.3% of the coal miners had 25(OH) VitD above 175 nmol/L (the upper limit for normal range), and even 10.7% had values above 200 nmol/L (the lowest literature-reported level associated with toxicity). None of the coal miners had VitD values above 250 nmol/l (hypervitaminosis) (For graphical presentation, refer to **Supplementary Figure 2**.)

Most of the total serum 25 (OH) VitD was 25(OH) vitamin D3 (143.5 ± 41.4 nmol/L, ranging from 43.5 to 247.5 nmol/L), while 25(OH) VitD2 made only a small part (0.2 ± 1.6 nmol/L, ranging from 0 to 15 nmol/L).

The results on 25 (OH) VitD levels were not much different if the subjects who consumed supplements of VitD were excluded from the analysis (mean total serum 25 (OH) VitD was 144.1 ± 42.1 nmol/L, again ranging from 43.5 to 247.5 nmol/L). Considering the four coal miners who consumed the

TABLE 3 | Nutritional intakes data of the studied coal miners (N =103, men), and correlation with serum 25 (OH) vitamin D levels.

	Descriptives			Correlation with vitamin D levels (non-adjusted)	Correlation with vitamin D levels (adjusted for age, FM, and FM%)	Correlation with vitamin D levels (adjusted for age, FM, FM%, and legs FM%)
	Mean	*SD*	*(Min–Max)*	r_s	r_s	r_s
Total caloric intake (kcal/d)	3955.2	1095.4	(1609.0–7170.2)	0.119	0.117	0.078
Protein intake (kcal/d)	509.8	171.2	(186.2–1199.0)	0.056	0.050	0.022
Fat intake (kcal/d)	1851.6	565.6	(864.0–3547.5)	0.018	0.045	0.019
Carbohydrates intake (kcal/d)	1497.4	504.4	(492.4–2963.6)	0.167	0.144	0.106
Alcohol intake (kcal/d)	96.4	157.6	(0.0–886.9)	0.158	0.123	0.088
Total caloric intake (kcal/d)–day 1[§]	3957.8	1190.3	(2025.0–7284.4)	0.182	0.181	0.158
Protein intake (kcal/d)–day 1[§]	510.4	181.3	(228.8–1036.3)	0.116	0.121	0.101
Fat intake (kcal/d)—day 1[§]	1830.1	639.7	(514.8–3634.4)	0.080	0.081	0.072
Carbohydrates intake (kcal/d) – day 1[§]	1533.1	568.0	(419.0–3288.0)	**0.211***	0.186	0.162
Alcohol intake (kcal/d) – day 1[§]	84.3	188.0	(0.0–1108.8)	**0.225***	0.192	0.135
Vitamin D intake (μg/d)	4.9	8.0	(1.1–65.2)	−0.089	−0.063	−0.079
Vitamin D intake (μg/d) – day 1[§]	5.5	11.4	(0.9–98.0)	−0.040	−0.015	−0.033
Vitamin D intake FFQ (μg/d)[¥]	5.3	3.8	(1.2–18.5)	−0.007	−0.139	−0.170

[§]*Data referring only to the first 24 h recall, representing the day before sampling of blood (otherwise—data represent the mean of two recalls).*

[¥] *Data derive from FFQ (otherwise—data derive from 24-h recalls, representing the mean of two recalls, or only referring to day 1, the day before sampling of blood).*

Bolded coefficients are statistically significant.

$p < 0.05$; correlation is significant at the 0.05 level (two-tailed).

FM, fat mass; FM%, percentage of fat mass; FFQ, food frequency questionnaire; SD, standard deviation; r_s, Spearman's rank correlation coefficients; p, statistical significance of correlations.

supplements, their VitD levels ranged from 105.5 to 149.5 nmol/L (in average 123.2 ± 18.7 nmol/L), only nine subjects were sunbathing by the seaside during the summer, and the levels of VitD were statistically marginally higher in them: 172.3 ± 46.5 vs. 141 ± 40.1 nmol/L (Mann–Whitney test $p = 0.05$).

There was no statistically significant difference in VitD levels between those who lived in rural and semi-urban areas (141.6 ± 42.2 vs. 152.1 ± 37.6 nmol/L, respectively), or smokers and non-smokers 140.8 ± 43.2 vs. 146.4 ± 40.3 nmol/L, respectively).

There was no correlation between serum 25 (OH) VitD levels and VitD nutritional intake, according to both FFQs and 24 h recalls (**Table 3**).

When only nutritional data from the day before blood sampling were analyzed, VitD levels correlated with 24 h recall carbohydrates and alcohol intake the previous day (**Table 3**).

Association of VitD Levels With Cardiometabolic (Anthropometric and Biochemical) Parameters

There were no statistically significant difference in 25 (OH) VitD levels in the coal miners with or without metabolic syndrome, or with or without overweight/obesity (148.9 ± 42.0 vs. 138.8 ± 40.5 nmol/L, and 147 ± 37.1 vs. 136.4 ± 49.5 nmol/L, respectively, Mann–Whitney test $p > 0.05$), even though in subjects with those conditions VitD levels seemed somehow higher.

Tables 1, **2** show the correlation of VitD levels with cardiometabolic (anthropometric and biochemical) parameters.

Serum 25 (OH) vitamin D levels had significant negative correlations with age and HDL-cholesterol levels and had significant positive correlations with body weight, BMI, FM, FM%, limbs FM%, arms muscle mass, hip circumference, serum triglycerides, GGT, AST, ALT, total cholesterol/HDL-cholesterol, LDL-cholesterol/HDL-cholesterol, triglycerides/HDL-cholesterol ratios, and ALT/AST ratio (**Tables 1**, **2**). Leg FM% was the anthropometric parameter that correlated most with VitD levels. At the same time, the above-mentioned biochemical variables had significant positive inter-correlations with anthropometric parameters indicative of increased adiposity (FM and FM%) and, surprisingly, some of them more with indicators of peripheral (gluteo-femoral) rather than with indicators of centripetal (truncal) adiposity (e.g., lipid levels correlated more with hip circumference and leg FM%, than with waist circumference and trunk FM%, **Supplementary Table 1**). Serum 25 (OH) VitD levels also positively correlated with erythrocytes count and creatinine (**Table 3**), while creatinine also had a positive correlation with muscular mass (**Supplementary Table 1**).

To eliminate the possible confounding, we performed Pearson's and Spearman's rank partial correlations. The correlations with lipids, creatinine, and ALT remained significant even after correcting for age, FM, and FM%, but were lost after additional corrections for leg FM% (with exception of total cholesterol/HDL-cholesterol ratio, which only remained significant correlation with VitD levels after this additional correction) (**Table 3**).

The stepwise linear regression analysis (which included age, and biochemical and anthropometric data as predictor variables), identified that the most significant predictors of VitD levels were age, leg FM%, and triglyceride levels (the models 1 and 2 have accounted for a statistically significant amount of VitD variance, and explained 23.8 and 23% of VitD variance, respectively) (**Table 4**).

DISCUSSION

This study is among the very rare ones that examined the levels of VitD in a population of underground coal miners. We found surprisingly high levels of late-summer serum 25 (OH) VitD, which were much higher compared with the same season VitD data on an urban-living, general male Serbian population from similar geographic latitude (28), and previously published values for coal miners from other geographic regions (18–21).

Apart from this, to the best of our knowledge, this is the first published study that examined the association of VitD levels in coal miners with their cardiometabolic status. Again, we have found unexpected positive correlations with anthropometric data and biochemical parameters indicative of obesity, metabolic syndrome, and fatty liver disease, which is in contrast with previously published data (28, 59).

Such unexpected findings require explanation and more exploration.

The high levels of serum 25 (OH) vitamin D can be explained by the late summer season (beginning of September) when the serum levels of VitD are expected to be highest, and by living in rural/semi-urban areas, with high isolation, where additional agricultural work is quite common in that period (leading to additional sun exposure during non-coal mining working hours). Therefore, it would be necessary to examine the winter/early spring levels of VitD in the same group, when there would be much less sun exposure. The nutritional intake of VitD in this study was quite low, and far below the current EFSA recommendations, and only four subjects consumed VitD supplements. Therefore, it could be expected to find much lower values during winter/early spring.

However, the higher vitamin D levels can be also a consequence of their heavy physical work, leading to increased lipolysis and release of VitD from fat and muscular depots (60–62). Studies indicate that serum VitD levels increase after intensive physical activity (60, 63–65) and remain increased even after 24 h (60, 63, 64). People who exercise regularly, even indoors, have higher VitD levels compared with sedentary people (60, 65–67). The data on increased VitD levels after weight loss (accompanied by increased lipolysis) also support this hypothesis (68). This can additionally explain the difference in VitD status with the urban-living Serbian general male population, not performing hard physical work, in which levels were almost three times lower (49.8 ± 17.2 nmol/L), despite quite similar BMI (28 ± 6.3 kg/m^2), waist circumference (98.5 ± 18 cm), and slightly higher FM% (24.8 ± 10.3%) (28). However, this cannot explain the difference in VitD status with other published data on summer VitD status in coal miners around the world (18–21). The main explanations for the difference with the Newcastle (UK) coal miners [where average values of 73.8 ± 73.4 nmol/L were found during the summer period (18), which were two times lower compared with these data] are geographic latitude and the lower level of insolation, apart from the earlier time of sampling (from May to July). In the study from India, samples were taken over a whole year, not specified if summer or winter (21), and in two other studies from UK and Turkey, the samples were from the winter/early spring period (19, 20). Nevertheless, the values in all those studies were much lower, particularly in Turkish workers (20).

However, there can be some additional explanations for the high vitamin D levels in the coal miners of this study.

In this study, vitD levels had a significant positive association with alcohol intake the previous day. Nevertheless, it is quite possible that the coal miners consciously withheld their alcohol consumption both in the 24 h recalls and FFQs (since it was an official medical checkup), and that their alcohol intake was much higher, since some data place this profession among the professions with highest alcohol consumption (21, 69, 70). Several cross-sectional studies showed positive associations of alcohol consumption with serum 25 (OH) VitD levels (66, 71–74). Even though these could be confounding findings, there can be a direct association between alcohol intake and VitD metabolism. It was shown in rats that chronic alcohol intake increases serum 25 (OH) VitD3, while it decreases serum 1, 25 $(OH)_2$ VitD3 (75). The effect is most probably conveyed by a direct inhibition of parathyroid hormone (PTH) secretion by alcohol (76, 77). PTH stimulates the 1-alpha hydroxylation of 25 (OH) VitD3 to 1,25 $(OH)_2$ VitD3. Nevertheless, only a small fraction of 25 (OH) VitD3 is converted to 1,25 $(OH)_2$ VitD3 (78), and it is less likely that the inhibited 1-alpha hydroxylation significantly contributed to the considerably increased 25 (OH) VitD3 levels in this study. In accordance, in one study, only about a 10 nmol/L increase in serum 25 (OH) VitD was noted 5 years after parathyroidectomy connected with significant decreases in PTH (79). However, we cannot exclude the effect of habitual alcohol consumption on VitD levels in this study, even though regression models did not identify alcohol consumption as a significant predictor of VitD levels.

Probably the most difficult is to explain the positive association of vitamin D with the level of adiposity, triglycerides, and liver enzymes indicative of fatty liver disease, and the negative association with HDL-cholesterol. Most literature data contrast with these findings and show the negative association with the level of adiposity (3), blood lipids (80, 81), and liver enzymes (82), even though there are also some conflicting findings (1–4, 83).

In line with this study findings of increased prevalence of obesity and metabolic disturbances among coal miners despite their heavy physical activity, in a recent NHANES study, only increased recreational physical activity was found to be associated with an improved metabolic profile, while occupational physical activity was not (84). In contrast, in a recent study of Chinese coal miners, the higher level of occupational physical activity was related to a decreased prevalence of metabolic syndrome among them (23).

TABLE 4 | Linear (stepwise) regression models for assessing the impact of specific characteristics of coal miners (general, anthropometric, biochemical, and nutritional) on serum 25 (OH) vitamin D levels.

Model	R^2	Adjusted R^2	Coefficients	β	B	95% *CI* for *B*	
						Lower bound	Upper bound
1	0.268	0.238	(Constant)		58.609	[43.829, 73.388]**	
			Age	−0.364	−0.630	[−0.947, −0.312]**	
			Legs FM%	0.279	0.719	[0.216, 1.221]**	
			Triglycerides	0.204	3.263	[0.433, 6.093]*	
			ALT	0.136	0.152	[−0.056, 0.359]	
2	0.252	0.230	(Constant)		62.273	[48.292, 76.254]**	
			Age	−0.390	−0.674	[−0.987, −0.361]**	
			Legs FM%	0.325	0.839	[0.361, 1.316]**	
			Triglycerides	0.220	3.529	[0.707, 6.351]*	

*$p < 0.05$; regression coefficients are significant at the.05 level (two–tailed).

**$p < 0.01$; regression coefficients are significant at the 0.01 level (two–tailed).

FM%, percentage of fat mass; ALT, alanine aminotransferase; R^2, coefficient of determination; β (beta), standardized linear regression coefficients; B, unstandardized linear regression coefficients; CI, confidence intervals; p, statistical significance.

Even though most (almost 70%) of the subjects were overweight and obese (according to BMI), only less than half of them had increased adiposity, centripetal obesity, or metabolic syndrome, which can be explained by the increased muscular mass related to their heavy physical work and the effect of physical activity on waist circumference and metabolic parameters (85–87). However, in this study, hyperglycemia was detected in more than 60%, while dyslipidemia (according to at least one lipid parameter) was detected in even more than 80% of the study subjects. In accordance with this study, in a study of Indian coal miners, serum triglycerides, total cholesterol, very-low-density lipoproteins (VLDL)-cholesterol, and LDL- cholesterol levels were found to be increased, while HDL- cholesterol levels were found to be decreased, in the coal miners compared with other workers, irrespective of the level of adiposity, which suggests that some other factors (inappropriate diet or working conditions, e.g., shift work) additionally contribute to metabolic disturbances (22).

In this study, the most prevalent type of dyslipidemia was hypertriglyceridemia. However, the levels of HDL-cholesterol were not decreased in the same magnitude (only in one-quarter of the subjects were they decreased), which can be explained by the positive effect of heavy physical activity on HDL-cholesterol, which is more sensitive to physical activity compared with LDL-cholesterol and triglycerides (87).

The positive association of serum 25 (OH) vitamin D levels with the levels of adiposity in this study can be explained by increased lipolysis and VitD release from adipose tissue depots during and after heavy physical work (60). Consistent with this, in the study of Sun et al. (64), increased 25 (OH) VitD levels after physical activity persisted for 24 h, and the strongest positive predictor of the incremental area under the curve was %FM, which is in agreement with the here presented findings. In this study, there were also positive correlations of VitD levels with limb muscle mass (although on the borderline statistical significance), which also confirms that muscular depots contribute to VitD status, although to a lesser extent (60–62).

In adipose tissue, most of the stored vitamin D is in its non-hydroxylated form (60). After the non-hydroxylated form of VitD is released from fat and muscular depots, it binds to a VitD binding protein, (DBP) and enters the liver where it is activated (25–hydroxylated) and, without delay, is released again into circulation, to be stored again in fat and muscular depots (and other target organs), with a half-life of ∼2–3 weeks, which ensures that the circulating 25 (OH) VitD levels are quite stable and not significantly influenced by transient dietary intake and sun exposure changes (60). This is in accordance with the findings of this study that the previous day VitD intake did not correlate with the measured 25 (OH) VitD levels.

The negative correlation of vitamin D status with age in this study is in agreement with previously published data since older people produce less cutaneous VitD3 (46), and we have used age as a controlling factor to examine the association of VitD with other parameters. Since in this study both VitD status and different types of dyslipidemia had a positive association with the level of adiposity (but not centripetal obesity), we used adiposity parameters (FM and FM%) as additional controlling factors. In the adjusted analyses, the associations with TG, HDL, lipid ratios, ALT, and creatinine remained significant, as well with leg FM%. Only after additionally correcting for leg FM% was the significance of these associations with biochemical parameters lost, indicating that leg FM% was an additional factor affecting the association of VitD and biochemical parameters. Similarly, in the regression analysis, the only significant predictors of VitD status were age, leg FM%, and triglyceride levels.

The association with leg FM% can be explained by the fact that leg (femoral) fat depots represent an important resource of lipolysis during coal mining, even though the upper body subcutaneous fat generally contributes more to lipolysis in men during rest and exercise (88, 89). However, there are some conflicting data, indicating that in younger overweight men (in

contrast to older overweight men) femoral fat depots respond more to exercise by lipolysis, compared with abdominal fat depots, which was related to the initial size of the depot (89, 90). In addition, even though leg fat is considered to have a more protective metabolic function and to be associated with a more beneficial metabolic profile (88), the findings from this study indicate the opposite. In this study, different types of dyslipidemia had a positive association with the level of adiposity, but, surprisingly, more with indicators of peripheral (gluteo-femoral) rather than centripetal (truncal) adiposity (e.g., lipid levels more correlated with hip circumference and leg FM% than with waist circumference and trunk FM%).

Unfortunately, there are not enough data on the difference between distinct subcutaneous and visceral fat depots in the storage and release capacity for VitD, particularly on the difference between distinct subcutaneous depots (60, 91). In one study (91), the concentrations of the non-hydroxylated form of VitD in visceral fat depots were moderately higher compared with abdominal subcutaneous fat depots, in both obese and non-obese women (with no difference between them). Nevertheless, considering a significantly larger amount of subcutaneous fat depots (4–6-fold), these depots contribute the most to circulating VitD levels (60).

On the other hand, the association of vitD with adipose tissue is bidirectional, and vitD can influence fat accumulation and re-distribution: in one study (92), supplementation with VitD decreased truncal subcutaneous fat without changing total fat mass, and this effect can be attributed to the direct effects of VitD on adipose tissue (probably through adipose tissue VitD receptor actions, or through its effects on calcium metabolism) and lower PTH levels (93–96).

Since high 25 (OH) vitD3 levels have a reverse relationship with PTH levels (78), we suppose that in the population of coal miners (with high VitD levels), the levels of PTH were lower than in the general Serbian male population. PTH is known to simulate truncal fat distribution, thus centripetal obesity and related metabolic disturbances (i.e., metabolic syndrome) are often observed in primary hyperparathyroidism (79, 97–99). Therefore, lowering the levels of PTH can lead to a more favorable peripheral fat distribution and metabolic profile (79, 98, 99). Increased/decreased PTH secretion can be a possible explanation for commonly observed negative relationships of serum VitD with centripetal obesity and metabolic syndrome (28) and the in this study found positive association with peripheral fat accumulation (hip circumference and leg FM%). Unfortunately, we did not measure the levels of PTH in this study, as it was not originally planned in the study design to measure PTH, and even though (after measuring VitD) we had some leftovers of the serum samples of coal miners, with an adequate quantity for PTH measurements, the low stability of PTH in the serum samples during long-term storage (100) did not allow us to perform reliable measurements. Nevertheless, in this study, three-quarters of the coal miners had a centripetal body fat distribution (according to WHR), despite the very high levels of VitD (and probably low levels of PTH), and there was no association of VitD with WHR or waist circumference, which indicates that the role of VitD and PTH on body fat distribution was insignificant in the coal miners. This is in accordance with data on the imperceptible effects of VitD on obesity treatment (94, 101) and with data that show that in subjects with high levels of VitD, there is no effect of additional supplementation with VitD on body fat re-distribution (96, 101).

Particularly interesting is the finding of a positive association of serum 25 (OH) VitD levels with serum triglycerides levels. In general, most of the published data (but not all) indicate a negative association of VitD with triglyceride levels, and a positive association with HDL-cholesterol levels (80, 81). Triglycerides and HDL-cholesterol generally have an inverse association (102, 103), which we confirmed in this study (Spearman's coefficient of correlation $= -0.615$, $p < 0.001$), and the linear regression analysis identified only triglycerides as significant predictors of VitD levels. Interestingly, and in line with this study findings, some studies show that VitD supplementation increased the levels of triglycerides (104–106), even though both unchanged (107–109) and decreased (110) levels were found in other studies. Therefore, we hypothesize that the observed positive relationship of VitD with serum triglycerides reflects the effect of positive association with lipolysis after an enduring physical work (89). The increased lipolysis during prolonged physical work not only increases the release of VitD from adipose and muscular depots but also increases the supply of free fatty acids (FFAs), released from fat tissue, to the liver. In the liver, accumulated FFAs stimulate the production and secretion of VLDL, the main transporters of serum triglycerides, which are consequently cleared, mostly by muscle and adipose tissue (89, 111).

In general, under fasting and resting conditions, circulating triglycerides are in direct proportion with the rates of release and oxidation of FFAs (111). After physical activity, lipolysis is still increased, and this effect can last up to 24 to 42 h (89, 112, 113). The increased FFAs' release after physical activity increase the FFAs' supply to the liver. The increased supply of FFAs to the liver increases triglycerides secretion from the liver (in the form of VLDL) (111), but one could expect that triglycerides will be rapidly cleared from circulation by lipoprotein lipase (LPL), whose activity in a muscle is concomitantly increased during recovery after a physical activity (87), which would lead to a drop in triglycerides levels. However, quite the opposite, increases in triglyceride levels have also been described several hours after physical activity, indicating that their increased production can prevail over their increased clearance (87, 114). In accordance, the increased FFAs supply to muscle and fat tissue after physical activity can interfere with triglyceride clearance by LPL (111). Alcohol consumption additionally decreases LPL activity (115). Finally, studies show that total energy replacement after physical activity (what coal miners do after their work) diminishes/completely abolishes the beneficial effects of the physical activity on the next-day fasting and postprandial triglyceride and HDL-cholesterol levels (87, 114, 116, 117).

Nevertheless, these hypotheses need to be confirmed by further studies, and there can also be other mechanisms of the found associations of VitD levels with cardiometabolic parameters.

In accordance with the present study findings of high vitD values associated with worse metabolic profile among coal miners, some studies show a U-shaped association of VitD levels

with cardiovascular mortality and risk, which requires further investigation (46).

Although not a primary aim of this study, the positive correlation of VitD with creatinine can be explained by the confounding effect of increased physical activity and release from muscular depots, since after a heavy physical activity the levels of creatinine also increase (118, 119), apart from the levels of VitD. Similarly, after heavy physical activity, the levels of ALT, AST, and GGT increase and can stay increased even after 24 h (118). The positive correlation with erythrocyte number could be explained by the direct effects of VitD on erythropoiesis and/or erythrocytes survival (120, 121), but can also be another confounding effect of physical activity (connected with increased erythrocyte release, due to splenic contraction, and hemoconcentration) (122).

This study has several limitations. The main limitation of this study is that only the late summer VitD levels are measured, without additional measurements of the winter/early spring VitD levels [which are expected to be much lower (19, 20)]. An additional limitation is the non-existence of a direct control group of non-coal miners from the same area, matched by sex, age, and anthropometric variables. However, we compared the data with recent literature findings for the Serbian general male population (urban-living and not working in physically demanding jobs but with similar age and anthropometric features), even though the method for VitD measurement (chemiluminescent immunoassay, CLIA) and the month and year of study sampling (July 2014) were not the same (28). Nevertheless, we had some serum samples from a small group of urban-living general population from the same latitude, from the beginning of September 2016 (the same as in the present study) and analyzed by HPLC at the same time with the samples from the coal miners, but that group was very small and mostly made of women (22 women and two men), and the data only served for HPLC method verification. The mean values in this group were 55.1 ± 30.9 nmol/L, again almost three times lower than in the coal miners and similar to data in reference (28). Another limitation is that the sample of the miners was not randomly taken (as it was a part of systematic medical examinations of all employed coal miners from one coal mine/shaft in the Resavica basin) and was only from one region for coal mining in Serbia, but we think that also in other regions situation will be quite similar (since they are very near geographically), and the most of Serbian coal miners work in the sampled region (76.4%) (27). Furthermore, we performed HPLC, but liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the gold standard for VitD measurement (46, 78). We did not measure PTH levels, nor the free (i.e., bioavailable, not bound with DBP) VitD form, the active form of VitD (1,25 $(OH)_2$ VitD), other VitD metabolites or epimers (e.g., C-3 epimers), which could modify or more explain these results (63, 78). In addition, some data show that VACUETTE® tubes with a serum clot activator can interfere with immunoassays for 25 (OH) VitD measurements (123, 124), but there are no such data on the HPLC method with Vacutest Kima® tubes with a serum clot activator (125), and even though a bit higher values were observed in immunoassays with VACUETTE® tubes with a serum clot activator, they correlated well with the ones measured by LC-MS/MS (123, 124). Moreover, TANITA BIA segmental analysis is not that accurate and precise in assessing regional body composition as other methods (e.g., dual-energy X-ray absorptiometry, DXA; computerized tomography, CT; magnetic resonance imaging, MRI), particularly in some populations (126). However, the mentioned techniques are costly and cannot be easily applied in field settings, and there is still a fairly good estimation with an eight-contact electrode BIA system, as confirmed by some validity studies (34). Finally, we did not directly measure or precisely estimate the level of individual physical activity, since it was not initially planned. Nevertheless, we controlled for physical activity in the questionnaire. All the coal miners were asked about their self-reported physical activity levels and the specific jobs they perform during coal mining. All of them were involved in hewing coal, loading coal, or (to a much lesser extent) erecting roof supports, and were placed in the heavy occupational work physical activity level (PAL) category (31). There are some publications which describe physical activities and directly measure physical activity and energy expenditures during coal mining (25, 127), and there was no huge difference between hewing coal and loading coal in energy expenditure. Nevertheless, it would be of interest to directly measure or at least precisely estimate the individual level of physical activity, and to relate it to individual VitD status in coal miners, to support the presented hypotheses.

Coal miners are a population of workers with specific nutritional needs due to their heavy physical work and unfavorable working conditions: working in shifts, working underground, and working with chronic occupational exposure to coal mine dust, which has pro-oxidative, pro-inflammatory, and pro-cancerogenic properties (128–130). Therefore, in the view of the science of precision nutrition, measuring the levels of VitD (which has the important metabolic, antioxidant, and anti-inflammatory activities) is very important in this population of workers to achieve their optimal nutrition, to adjust their nutrition (and possible supplementation) and meet their specific nutritional needs (131).

Considering the unexpected findings of this study, which are not easy to explain, and the above-mentioned study limitations, more studies are warranted to confirm the results and further explore the topic of VitD status and cardiometabolic health among coal miners.

CONCLUSION

In this study, we have found surprisingly high levels of late summer serum 25 (OH) VitD in a population of Serbian coal miners, much higher compared with the same season VitD data from an urban-living Serbian general male population with similar anthropometric characteristics, and previously published data for coal miners from other countries. Furthermore, we have found unexpected positive correlations of serum 25 (OH) VitD levels with anthropometric and biochemical parameters indicative of obesity, metabolic syndrome, and fatty liver disease. The linear regression analysis identified that the most significant positive predictors of VitD levels were age, leg FM%, and triglyceride levels. Such unexpected findings require more exploration. Data on VitD levels in the population of coal miners are scarce, as well as data on their cardiometabolic health.

More research is needed on the VitD status of coal miners (particularly in the winter period) and its relationship with their cardiometabolic status.

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 Institute of Occupational Health Niš Ethics Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IS wrote the manuscript and performed anthropometric measurements, nutritional interviews, software exploration of nutritional data, biochemical analyses, and all the statistical analyses. JJ organized and performed preventive medical examinations, clinical assessments of coal miners, and biochemical analyses. MZec and ZP performed VitD measurements by HPLC. JD-M performed biochemical analyses. MZek and JM were involved in the dietary assessment. MGu and MGl led the project under which the study was conducted. All authors contributed to the writing 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.2021.689214/full#supplementary-material

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Genetic Link Determining the Maternal-Fetal Circulation of Vitamin D

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Vitamin D is an essential micronutrient whose demand is heightened during pregnancy to support the growth of the fetus. Furthermore, the fetus does not produce vitamin D and hence relies exclusively on the supply of maternal vitamin D through the placenta. Vitamin D inadequacy is linked with pregnancy complications and adverse infant outcomes. Hence, early predictive markers of vitamin D inadequacy such as genetic vulnerability are important to both mother and offspring. In this multi-ethnic Asian birth cohort study, we report the first genome-wide association analysis (GWAS) of maternal and fetal vitamin D in circulation. For this, 25-hydroxyvitamin D (25OHD) was measured in the antenatal blood of mothers during mid gestation ($n = 942$), and the cord blood of their offspring at birth ($n = 812$). Around ~7 million single nucleotide polymorphisms (SNPs) were regressed against 25OHD concentrations to identify genetic risk variants. About 41% of mothers had inadequate 25OHD (≤75 nmol/L) during pregnancy. Antenatal 25OHD was associated with ethnicity [Malay ($B = -22.32$ nmol/L, $p = 2.3 \times 10^{-26}$); Indian ($B = -21.85$, $p = 3.1 \times 10^{-21}$); reference Chinese], age ($B = 0.47$/year, $p = 0.0058$), and supplement intake ($B = 16.47$, $p = 2.4 \times 10^{-13}$). Cord blood 25OHD highly correlated with antenatal vitamin D ($r = 0.75$) and was associated with ethnicity [Malay ($B = -4.44$, $p = 2.2 \times 10^{-7}$); Indian ($B = -1.99$, $p = 0.038$); reference Chinese]. GWAS analysis identified rs4588, a missense variant in the group-specific component (*GC*) gene encoding vitamin D binding protein (VDBP), and its defining haplotype, as a risk factor for low antenatal ($B = -8.56$/T-allele, $p = 1.0 \times 10^{-9}$) and cord blood vitamin D ($B = -3.22$/T-allele, $p = 1.0 \times 10^{-8}$) in all three ethnicities. We also discovered a novel association in a SNP downstream of *CYP2J2* (rs10789082), a gene involved in 25-hydroxylation of vitamin D, with vitamin D in pregnant women ($B = -7.68$/G-allele, $p = 1.5 \times 10^{-8}$), but not their offspring. As the prevention and early detection of suboptimal vitamin D levels are of profound importance to both mother and offspring's

health, the genetic risk variants identified in this study allow risk assessment and precision in early intervention of vitamin D deficiency.

Keywords: vitamin D, ethnicity, genome-wide association study, pregnancy, offspring, GUSTO

INTRODUCTION

Vitamin D is a steroid hormone that plays an important role in calcium homeostasis and metabolic pathways, and is hence linked with multiple human health outcomes (Theodoratou et al., 2014). The most abundant form of vitamin D is vitamin D3 (cholecalciferol), which is synthesized in the skin by exposure of 7-dehydrocholesterol to UV B radiation from sun. Alternatively, vitamin D, either as vitamin D2 (ergocalciferol) or vitamin D3, can also be consumed through diet. Vitamin D obtained from solar radiation is often influenced by spatiotemporal factors (latitude, altitude, seasonality, time of day, and air pollution) and individual-specific factors (skin pigmentation and preference for outdoor activity, clothing, and sunscreen use; Wacker and Holick, 2013).

After synthesis in the skin or consumption through diet, vitamin D circulates in the bloodstream and is rapidly converted to 25-hydroxycholecalciferol (25OHD) by cytochrome P450 (CYP) enzyme in the liver. Subsequent 1-hydroxylation in the kidney converts 25-hydroxycholecalciferol to the active metabolite 1,25-dihydroxycholecalciferol [1,25(OH)2D]. Vitamin D binding protein (VDBP) is the principal transporter of vitamin D and its metabolites in the blood stream and helps mobilize them to their target tissues. Many tissues express the vitamin D receptor (VDR), which binds to 1,25(OH)2D and heterodimerises with the retinoic X receptor (RXR) to form a transcription factor. Since RXR is involved in cell proliferation, differentiation, and organogenesis, it plays a critical role in pregnancy and fetal development (Szanto et al., 2004).

Measurement of circulating 25OHD, the major form of vitamin D in the bloodstream, is recommended to evaluate the vitamin D status (Holick et al., 2011). However, there is no consensus on cut-offs for deficient and insufficient vitamin D concentrations for pregnant women and newborns and even for the non-pregnant adult population. For example, the Institute of Medicine (IOM) guideline (Ross et al., 2011) defines vitamin D status as severely deficient (<30 nmol/L), insufficient (30–49 nmol/L), and sufficient (>50 nmol/L), while the Endocrine Society Clinical Practice Guideline (Holick et al., 2011) defines vitamin D status as deficient (<50 nmol/L), insufficient (50–75 nmol/L), and sufficient (>75 nmol/L). Various other thresholds are also used in practice (Nassar et al., 2011).

During pregnancy the maternal demand for calcium increases with calcification of the fetal skeleton, and this is corroborated with the increased circulation of maternal vitamin D and its metabolites. Furthermore, the fetus does not produce vitamin D and hence relies exclusively on the supply of maternal vitamin D through placenta. There is a strong correlation reported between infant cord blood and maternal 25(OH)D concentrations, especially toward late gestation and delivery (Sachan et al., 2005; Bodnar et al., 2007; Lee et al., 2007; Parlak et al., 2015; Rodda et al., 2015). The antenatal blood levels of VDBP have also been reported to increase by 40–50% during pregnancy, suggesting that VDBP may play a role in vitamin D homeostasis during gestation (Zhang et al., 2014; Karras et al., 2018). Thus, the heightened demand for vitamin D in pregnant women elevates their risk of developing vitamin D deficiency and many studies have shown high prevalence of vitamin D deficiency and insufficiency during pregnancy worldwide and in Asia (Sachan et al., 2005; Bodnar et al., 2007; Lee et al., 2007). The Endocrine Society Clinical Practice Guideline recommends that pregnant women require at least 600 IU/day of vitamin D and may need 1,500–2,000 IU/day to maintain their circulating 25OHD concentration of >75 nmol/L (Holick et al., 2011).

Insufficient vitamin D concentrations during pregnancy have been associated with gestational diabetes, pre-eclampsia, bacterial vaginosis, antenatal and postnatal depression, and small for gestational age/low birth weight infants (Christesen et al., 2012; Aghajafari et al., 2013, 2018; Theodoratou et al., 2014). In this study involving the Growing Up in Singapore Towards healthy Outcomes (GUSTO) mother-offspring cohort (Soh et al., 2014), maternal vitamin D inadequacy was found to be associated with higher fasting glucose in Malays and increased risk of emergency cesarean section in Chinese and Indian women (Loy et al., 2015), as well as poor sleep quality and night-time eating during pregnancy (Cheng et al., 2017) and higher abdominal subcutaneous adipose tissue volume in the infants (Tint et al., 2018). However, although maternal vitamin D supplementation was shown to prevent neonatal vitamin D deficiency (Rodda et al., 2015), no evidence of benefit for pregnancy or birth outcomes have been demonstrated from vitamin D supplementation (Roth et al., 2015, 2017).

Genetic variation has been shown to contribute to vitamin D metabolism and risk of vitamin D insufficiency. Previous genome-wide association studies (GWAS) to identify risk factors for vitamin D have focused primarily on vitamin D measurements obtained from adults (Benjamin et al., 2007; Ahn et al., 2010; Wang et al., 2010; Moy et al., 2014; Manousaki et al., 2017; Jiang et al., 2018) and children (≥4 years; Lasky-Su et al., 2012; Anderson et al., 2014) of European descent. Recently, two GWAS extended the investigations to include the African and Hispanic Americans populations (Hong et al., 2018; O'Brien et al., 2018). Only one GWAS has been conducted exclusively for vitamin D in an Asian population (Sapkota et al., 2016).

In this study, we measured vitamin D concentrations in the blood plasma of mothers of Chinese, Indian, and Malay descent in mid-pregnancy and in the cord blood of their offspring at birth. We investigate the epidemiological and genetic risk factors associated with antenatal and cord blood vitamin D concentrations. To our knowledge, this is the first GWAS investigating vitamin D concentrations of women during pregnancy, and their offspring at birth, and also in a multi-ethnic Asian cohort.

MATERIALS AND METHODS

Study Population

Data were obtained from GUSTO, a mother-offspring prospective cohort study in Singapore (Soh et al., 2014). Briefly, 1,247 pregnant women with singleton pregnancies were recruited at 11–14 weeks of gestation from two hospitals in Singapore, KK Women's and Children's Hospital (KKH) and National University Hospital (NUH), from June 2009 to September 2010. The inclusion criteria included age range between 18 and 50 years, intention to reside in Singapore for the next 5 years, intention to deliver in KKH and NUH, and willingness to donate antenatal and cord blood. We included only Chinese, Malay, and Indian women whose parents and whose partner's parents were of the same ethnicity in the study. Informed written consent was obtained from all women. The study was conducted according to the guidelines laid down in the Declaration of Helsinki. Ethical approval was obtained from the Domain Specific Review Board of Singapore National Healthcare Group (reference D/09/021) and the Centralized Institutional Review Board of SingHealth (reference 2009/280/D).

Maternal Characteristics

Demographic data on ethnicity, maternal age, educational levels, and pre-pregnancy weight was self-reported by participants at recruitment visit. Height, weight, and gestational diabetes status was measured at the GUSTO visit at 26–28 weeks gestation. Briefly, women underwent a 75 g oral glucose tolerance test (OGTT) and gestational diabetes was defined using the WHO 1999 definition (Alberti and Zimmet, 1998; fasting glucose ≥7.0 mmol/L or 2-h post-OGTT glucose ≥7.8 mmol/L). Mothers reported dietary supplements that they were consuming during pregnancy and trained research nutritionists contemporaneously coded the nutrient information from supplements to determine presence of vitamin D in supplements. The ethnicity of the study population was verified using the genotype data with the 1000 Genomes as reference population and samples not matching the self-reported ethnicity were removed.

Infant Characteristics

Gestational age was assessed by ultrasonography in the first trimester in a standardized manner at both hospitals by trained ultrasonographers. Offspring sex and birthweight were obtained by trained research coordinators from birth records at the time of delivery.

Antenatal Vitamin D Measurement

As described previously (Ong et al., 2016), maternal blood was collected during the clinical visit at 26–28 weeks gestation (same time as OGTT) in EDTA tubes, centrifuged at 1,600 *g* for 10 min at 4°C within 4 h of collection and the plasma was frozen at −80°C until analysis. Plasma 25OHD and its metabolite concentrations was analyzed by isotope-dilution liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS). The intra- and inter-assay CVs for 25OHD were ≤10.3%, and the detection limit was <4 nmol/L (Maunsell et al., 2005). The contribution of 25(OH)D2 was negligible (detected in <1% of antenatal samples). Thus, we used only 25(OH)D3 for analysis throughout this paper.

Cord Blood Vitamin D Measurement

Cord blood was collected from infant umbilical cords either by directly dripping into EDTA tubes for normal deliveries, or extracted through a syringe for cords delivered through Cesarean section deliveries, then processed in the same way as maternal samples until analyses. Plasma 25OHD concentrations was analyzed in the laboratories of Bevital AS[1] using LC-MS/MS (Midttun and Ueland, 2011). The intra- and inter-assay CVs for 25OHD were 4–5 and 7–8%, respectively, and the detection limit was 3.3 nmol/L (Midttun and Ueland, 2011). The contribution of 25(OH)D2 was again negligible (detected in 1.3% of cord blood samples) and hence the subsequent analysis was restricted to 25(OH)D3 only.

DNA Extraction, Genotyping, and Imputation

Mother's DNA was extracted from blood collected at mid-gestation and infant DNA from cord tissue or blood and father's DNA from buccal swabs. DNA extraction for mother and infants were as described previously (Lin et al., 2017). Isohelix DNA buccal swabs stored at −80°C were equilibrated to room temperature for 1 h and 1 ml of ATL lysis buffer (Qiagen) was added and incubated at room temperature for 30 min. About 100 µl of proteinase K (Qiagen) was then added and incubated at 60°C with shaking for 30 min to 1 h. DNA extraction from the lysates was performed using QIAsymphony DNA kits as per the manufacturer's instructions.

The extracted DNA was genotyped using Illumina OmniExpress plus Exome array. DNA hybridization arrays and scanning were performed by Expression Analysis, Inc. (Morrisville, NC). Data were processed using GenomeStudio Genotyping Module version 1.0 (Illumina, Inc.). Briefly, genotyping calls were made by the GenCall software and genotypes with a GenCall score less than 0.15 are not assigned genotypes. Samples with genotyping call rate <97%, not matching self-reported ethnicity or discrepant in sex or with incongruent offspring-parent relationship (expected PI_HAT = 0.5) were removed.

Genotype imputation was done for each ethnicity separately (for details and scripts[2]). Briefly, SNPs with minor allele frequency (MAF) <5%, call rate <95% or fail Hardy-Weinberg Equilibrium at value of $p < 10^{-6}$ (all parameters was estimated using parents only) were excluded in each ethnicity using PLINK version 1.90 (Chang et al., 2015). The data were aligned to GRCh37 build and further processed using a published pipeline[3] before haplotype phasing using SHAPEIT2 with duoHMM method (O'Connell et al., 2014), which incorporates the family structure for better accuracy. We imputed the phased haplotypes with

[1]www.bevital.no
[2]https://bitbucket.org/sics-sb/gusto-imputation.git
[3]https://github.com/natacha-beck/imputePrepSanger

TABLE 1 | Characteristics for antenatal vitamin D concentration.

	N (%)/mean (SD)	Univariate model, *p*	Multivariate model[b]	
			B	*p*
N	942			
Antenatal 25OHD concentration in nmol/L, mean (SD)	81.08 (27.16)			
Ethnicity, *N* (%)				
Chinese	520 (55.2)	*ref*	*ref*	*ref*
Indian	175 (18.6)	1.1×10^{-21}	−21.85	3.1×10^{-21}
Malay	247 (26.2)	3.3×10^{-28}	−22.32	2.3×10^{-26}
Age in years at recruitment, mean (SD)	30.48 (5.05)	1.2×10^{-7}	0.47	0.0058
Highest level of education attained, *N* (%)		0.076[c]		
Primary and secondary	285 (30.2)			
Post-secondary	324 (34.4)			
University	320 (34.0)			
Not answered	13 (1.4)			
Monthly income of household SGD, *N* (%)		0.0017[c]		*ns*
<$1,000	17 (1.8)			
$1,000–1,999	121 (12.8)			
$2,000–3,999	273 (29.0)			
$4,000–5,999	220 (23.4)			
≥$6,000	250 (26.5)			
Unknown or refused to answer	61 (6.5)			
Pre-pregnancy BMI in kg/m^2, mean (SD)[a]	22.67 (4.29)	2.0×10^{-5}		*ns*
Smoked or exposed to tobacco smoke during pregnancy, *N* (%)[d]	51 (5.4)	0.40		
Gestational weight gain in kg, mean (SD)[a]	8.62 (4.44)	0.59		
Gestational diabetes mellitus using WHO 1999, *N* (%)[a]	161 (17.7)	0.22		
Consumption of vitamin D containing supplements, *N* (%)				
Yes	700 (74.3)	*ref*	*ref*	*ref*
No	147 (15.6)	1.5×10^{-7}	−16.47	2.4×10^{-13}
Unknown	95 (10.1)			
Male offspring, *N* (%)	490 (52.1)	0.23		
With genotype data, *N* (%)	919 (97.6)			
With genotype data and information on supplements, *N* (%; i.e., sample size for **Figure 1A** and antenatal GWAS)	827 (87.8)			

[a]*Contains missing values: pre-pregnancy BMI (n = 78), gestational weight gain (n = 80), AND gestational diabetes mellitus (n = 34).*
[b]*For the multivariate model, we included all variables that was significant in an univariate test ($p < 0.05$) followed backward elimination. Pre-pregnancy BMI and household income was not significant in the multivariate model and thus dropped in favor of a more parsimonious model (ns, not significant at $p < 0.05$).*
[c]*Values of p shown is for the F-test from the ANOVA model.*
[d]*We considered a mother to be smoking during pregnancy if she self-reported to be smoking during pregnancy (n = 29) or if the antenatal blood cotinine levels were >56.5 nmol/L (n = 20). We considered a mother to be exposed to tobacco smoke if her cotinine levels were between 11 and 56.5 nmol/L (n = 11).*

PBWT (Durbin, 2014) using the Sanger Imputation Service (McCarthy et al., 2016) using the 1000 Genomes Phase 3 (1000 Genomes Project Consortium et al., 2015) as reference panel. We analyzed 6,978,879 SNPs that passed stringent quality control (MAF > 5% and imputation INFO > 0.50) in at least one ethnicity.

Statistical Analysis

25-hydroxyvitamin D was measured in 942 antenatal samples and 812 cord blood samples. While, we briefly report the proportion of women with sufficient concentrations (>75 nmol/L) based on recommendations by Endocrine Society Clinical Practice Guideline (Holick et al., 2011), we analyze 25OHD concentrations as a continuous measure due to the lack of a well-established clinical cut-offs for insufficiency especially in pregnancy and for cord blood concentrations. Summary values are reported as mean ± SD.

To identify significant covariates for the GWAS analysis, we first regressed the variable of interest with 25OHD using univariate linear regression models. Variables that were statistically significant (value of $p < 0.05$) were included in a multivariate model and the most parsimonious model was selected using backward elimination procedure, i.e., start with the saturated model and drop a term if the χ^2 value of $p > 0.05$ for the reduction in Akaike's information criteria.

Genome-wide association analysis for antenatal 25OHD was adjusted for ethnicity, consumption of vitamin D containing supplements and maternal age at recruitment. Similarly, GWAS for cord blood 25OHD was adjusting for antenatal 25OHD concentrations and ethnicity. SNPs were coded using the additive model of alleles. All analyses and plots were conducted in R version 3.3.2 unless stated otherwise. Associations reaching the genome-wide significance threshold (value of $p \leq 5 \times 10^{-8}$) were considered statistically significant and regional association plots using LocusZoom (Pruim et al., 2010) and ethnicity-stratified boxplots were generated. We also used Haploview (Barrett et al., 2005) to investigate the genetic linkage for selected loci. We attempted to elucidate the

TABLE 2 | Characteristics for cord blood vitamin D levels.

	N (%)/ mean (SD)	Univariate model, *p*	Multivariate model[b]	
			B	*p*
N	812			
Cord blood 25OHD levels in nmol/L, mean (SD)	34.05 (13.76)			
Antenatal 25OHD levels in nmol/L, mean (SD)[a]	79.00 (27.29)	2.0×10^{-120}	0.35	9.6×10^{-99}
Ethnicity, *N* (%)				
Chinese	399 (49.1)	*ref*	*ref*	*ref*
Indian	166 (20.4)	4.0×10^{-15}	−1.99	0.038
Malay	247 (30.4)	8.3×10^{-29}	−4.44	2.2×10^{-7}
Gestational age in weeks, mean (SD)	38.75 (1.36)	0.69		
Birth Weight in kg, mean (SD)	3.10 (0.44)	0.073		
Male offspring, *N* (%)	432 (53.3)	0.88		
With genotype data, *N* (%)	777 (95.6)			
With genotype data and mother's antenatal 25OHD measurement, *N* (%; i.e., sample size for **Figure 1B** and cord blood GWAS)	656 (80.8)			

[a]*Contains missing values: Antenatal vitamin D (n = 144).*
[b]*For the multivariate model, we included all variables that was significant in an univariate test (p < 0.05) followed backward elimination.*

presence of second independent signals by including the selected SNP as a covariate in the model.

Data Statement

Clinical data are not publicly available due to ethical restrictions but can be obtained from the authors upon reasonable request and subject to appropriate approvals from the GUSTO cohort's Executive Committee.

RESULTS

Vitamin D Inadequacy Is Very Common in Pregnant Mothers Especially in Malays and Indians

Vitamin D was measured at mid-gestation in 942 women (**Supplementary Figure 1**) from three major Asian ethnicities (520 Chinese, 175 Indian, and 247 Malay). 25(OH)D3 was the predominant vitamin D metabolite detected, with mean concentration of 81.08 ± 27.16 nmol/L (**Supplementary Figure 2**). Overall, 41% of the mothers had inadequate vitamin D (≤75 nmol/L). In a multivariate regression model (**Table 1**), low concentrations of antenatal vitamin D strongly associated with being Malay ($B = -22.32$ nmol/L, $p = 2.3\times10^{-26}$) or Indian ($B = -21.85$, $p = 3.1\times10^{-21}$) compared to Chinese mothers; not consuming supplements containing vitamin D ($B = -16.47$, $p = 2.4\times10^{-13}$) and being younger at recruitment ($B = 0.47$ per year, $p = 0.0058$). These covariates collectively explained 21.1% of variability in antenatal vitamin D.

Majority of the mothers (84.6%) reported consuming supplements containing vitamin D and despite this 36.8% of these mothers (59.6% in Malay, 53.5% in Indian, and 19.2% in Chinese mothers; **Figure 1A**) had inadequate antenatal vitamin D. As expected, the proportion of pregnant women with vitamin D inadequacy is much higher at 53.8% among those not consuming vitamin D containing supplements (81.8% in Malay, 73.1% in Indian, and 42.3% in Chinese mothers).

Cord Blood Vitamin D Is Highly Correlated With Maternal Antenatal Concentrations

Vitamin D was measured in cord blood from 812 infants (**Supplementary Figure 1**), and again the predominant vitamin D metabolite detected was 25(OH)D3, with a mean concentration of 34.05 ± 13.76 nmol/L (**Supplementary Figure 2**). In a multivariate regression model (**Table 2**), low concentration of cord blood vitamin D was strongly associated with being Malay ($B = -4.44$, $p = 2.2\times10^{-7}$) or Indian ($B = -1.99$, $p = 0.038$) compared to Chinese offspring. These covariates collectively explain 57.4% of variability in cord blood vitamin D. There was a high correlation between cord blood and maternal antenatal vitamin D with a Pearson correlation of 0.75 (**Figure 1B**) and this correlation persisted even when stratified by ethnicity (0.70 in Chinese, 0.69 in Indian, and 0.70 in Malay).

A Missense Variant (rs4588) in *GC* Is Associated With Decreased Concentrations of Vitamin D

We conducted GWAS for vitamin D concentrations measured in 827 pregnant women and 656 cord blood samples (**Supplementary Figures 1–4** for sample selection flow diagram, phenotype distribution, QQ, and Manhattan plots). The strongest signal for maternal antenatal and cord blood vitamin D (**Table 3**) was in the *GC* gene, which encodes the VDBP (**Figure 2**).

The antenatal vitamin D GWAS identified an intronic variant rs1352846 in *GC* as the most statistically significant association ($p = 6.5\times10^{-10}$). However, this variant is in a very high linkage disequilibrium ($r^2 = 0.957$ in Chinese, $r^2 = 0.983$ in Indian, and $r^2 = 0.985$ in Malay; **Supplementary Figure 5**) with rs4588 ($p = 1.0\times10^{-9}$), which is a missense variant that has been previously reported to be associated for vitamin D in European, African American, and Hispanic descent populations (Lasky-Su et al., 2012; Anderson et al., 2014; Hong et al., 2018; Jiang et al., 2018; O'Brien et al., 2018). rs4588 was also the most significant risk variant associated with cord blood vitamin D concentrations ($p = 1.0\times10^{-8}$). Therefore, we postulate rs4588

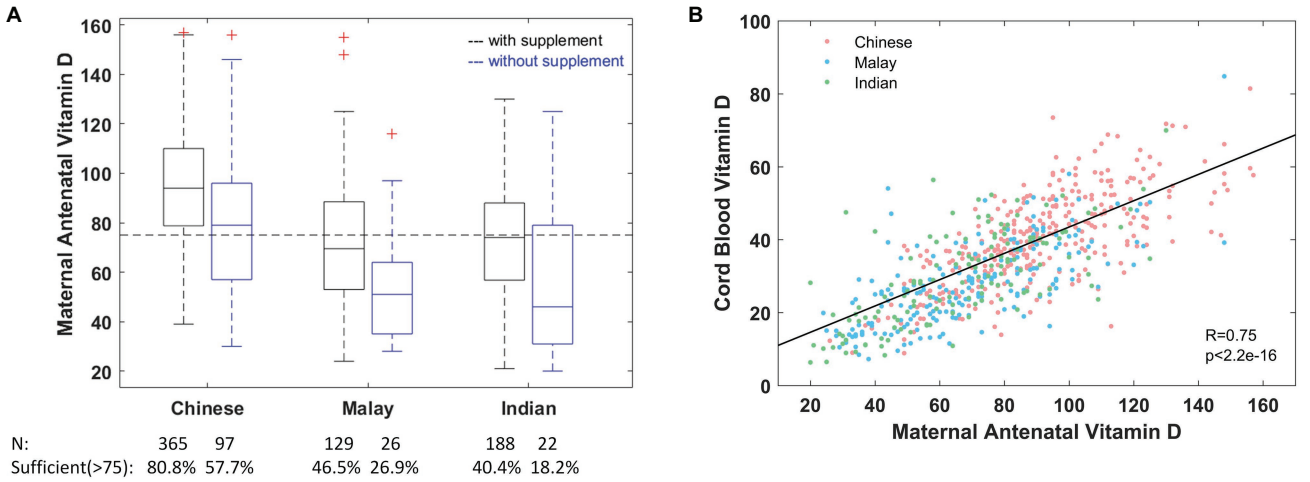

FIGURE 1 | (A) Maternal antenatal vitamin D concentration stratified by ethnicity and consumption of vitamin D containing supplements. **(B)** Cord blood vitamin D concentration is strongly correlated with maternal antenatal vitamin D across all ethnicities. The dashed horizontal line marks the level of sufficiency (>75 nmol/L) as recommended by the Endocrine Society Clinical Practice Guidelines (ESCPG; Holick et al., 2011). For visualization purposes, the *y* axis in **(A)** is truncated at 160 nmol/L, which excludes two high values (187, 195) from Chinese mothers who consumed vitamin D containing supplements.

TABLE 3 | Variants reaching genome-wide significance.

Gene	dbSNP	Chr	Position	Reference allele	Risk allele	Risk allele frequency	*B*	*p*	Source
Variants reaching genome-wide significant with antenatal vitamin D concentration:									
CYP2J2	rs10789082	1	60,357,969	A	G	29.4%	−7.68	1.5×10^{-8}	Typed
GC	rs17467825	4	72,605,517	A	G	24.9%	−8.53	3.3×10^{-9}	Imputed
GC	rs2282680	4	72,608,364	C	T	24.7%	−8.41	3.4×10^{-9}	Imputed
GC	rs2282679	4	72,608,383	T	G	24.7%	−8.36	3.2×10^{-9}	Typed
GC	rs3755967	4	72,609,398	C	T	24.7%	−8.37	3.1×10^{-9}	Imputed
GC	rs2298850	4	72,614,267	G	C	25.1%	−8.58	9.8×10^{-10}	Imputed
GC	rs11723621	4	72,615,362	A	G	25.1%	−8.58	9.9×10^{-10}	Imputed
GC	rs1352846	4	72,617,775	A	G	24.1%	−8.88	6.5×10^{-10}	Imputed
GC	rs4588	4	72,618,323	G	T	25.1%	−8.56	1.0×10^{-9}	Typed
Variants reaching genome-wide significant with cord blood vitamin D concentration:									
GC	rs2298850	4	72,614,267	G	C	25.0%	−3.18	1.5×10^{-8}	Imputed
GC	rs11723621	4	72,615,362	A	G	25.0%	−3.18	1.5×10^{-8}	Imputed
GC	rs1352846	4	72,617,775	A	G	24.6%	−3.22	1.5×10^{-8}	Imputed
GC	rs4588	4	72,618,323	G	T	25.0%	−3.22	1.0×10^{-8}	Typed

is most likely the causal variant in GC. There was no secondary signal in *GC* gene after conditioning on rs4588. The T-allele is the risk allele for rs4588 and is associated with a reduction of 8.6 nmol/L per allele in antenatal vitamin D ($p=1.0\times10^{-9}$) and a reduction of 3.2 nmol/L per allele in cord blood vitamin D ($p=1.0\times10^{-8}$). This variant explains 3.3 and 2.0% of the variability in antenatal and cord blood vitamin D, respectively, after accounting for the factors included in multivariate models in **Tables 1** and **2**. The directionality of this association is consistent across ethnicities (**Figure 2**), while the risk allele frequency (RAF) varies from 29.9% in Chinese, 23.5% in Indian, and 15.7% in Malay mothers.

The missense variant rs4588 (p.Thr436Lys) and missense variant rs7041 (p.Glu432Asp) are frequently studied together as haplotype-tagging SNPs in *GC*. While rs7041 only had a modest statistical association in our single-SNP GWAS ($p=2.8\times10^{-4}$ for antenatal and $p=0.01$ for cord blood), the results for haplotype association (**Table 4**) were highly significant (ANOVA $p=2.0\times10^{-7}$ for antenatal and $p=3.0\times10^{-6}$ for cord blood). We find that the vitamin D concentrations are highest in individuals with haplotypes 1s/1s or 1s/1f and lowest in those with haplotype 2/2, in agreement with the findings of a large study with 11,704 Norwegian adults (Jorde and Grimnes, 2015).

A Variant (rs10789082) Downstream of *CYP2J2* Is Associated With Decreased Antenatal Vitamin D

The second strongest genetic association was for the SNP rs10789082 (**Table 3**; **Figure 3**), which is located 1,011 bases downstream from the 3' end of the last exon (exon 9,

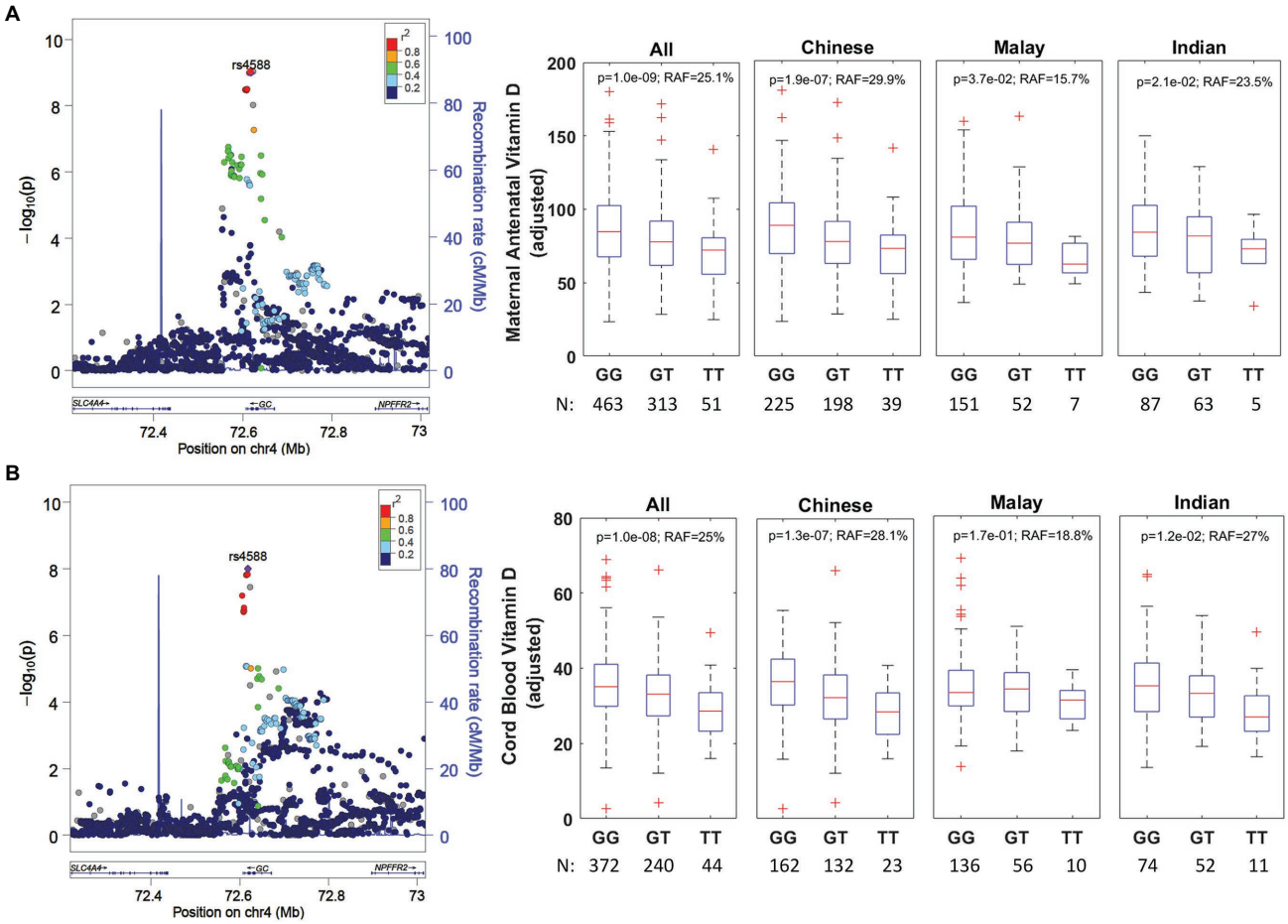

FIGURE 2 | (A) Regional association plot for rs4588 group-specific component (*GC*) and ethnicity-stratified levels for antenatal vitamin D. The regional association plot (left) is based on all individuals with complete data for analysis. The data presented as boxplots (right) has been adjusted for ethnicity, mother's age at recruitment and consumption of vitamin D containing supplements. RAF, risk allele frequency. **(B)** Regional association plot for rs4588 (*GC*) and ethnicity-stratified levels for cord blood vitamin D. The regional association plot (left) is based on all individuals with complete data for analysis. The data presented as boxplots (right) has been adjusted for ethnicity and antenatal vitamin D levels.

Supplementary Figure 6) of the *CYP2J2* (*Cytochrome P450 Family 2 Subfamily J Member 2*) gene. The G-allele is the risk allele for rs10789082 and is associated with a reduction of 7.7 nmol/L per allele in antenatal vitamin D ($p = 1.5 \times 10^{-8}$) but this SNP was not associated with cord blood vitamin D ($p = 0.65$). This variant explains an additional 2.0% of the variability in antenatal vitamin D after accounting for the factors included in multivariate models in **Table 1**. We note that this SNP is not in high linkage disequilibrium with any of the SNPs in the region of the *CYP2J2* gene but this variant is a genotyped SNP with good separation of the allele signals for genotype calling (**Supplementary Figure 7**). Furthermore, the directionality of this association is consistent across ethnicities (**Figure 3**).

DISCUSSION

In this mother-offspring study of three major Asian ethnicities (Chinese, Indian, and Malay), which represents 40% of the global population, we measured the vitamin D concentrations in blood collected mid-pregnancy and from the umbilical cord of their offspring. First, we found that many pregnant women, particularly those of Malay and Indian descent, do not achieve the recommended concentrations of 75 nmol/L and above of vitamin D in pregnancy. We also observed a substantial level of vitamin D insufficiency despite majority of mothers reporting consumption of vitamin D containing supplements. This discrepancy may be due to the low amounts of vitamin D in the supplements consumed as there is a heightened demand for vitamin D during pregnancy and up to 1,500–2,000 IU/day may be needed to maintain their circulating 25OHD concentration of >75 nmol/L (Holick et al., 2011). An additional reason could be the limitations of the self-reporting questionnaire to collect details of duration of supplement consumption and compliance to the recommended dosage. Next, we found that the vitamin D concentrations in cord blood are strongly correlated with the mothers' mid-pregnancy vitamin D concentrations across all three ethnicities. Mothers with

TABLE 4 | Analysis of *GC* haplotypes for the antenatal and cord blood vitamin D genome-wide association analysis (GWAS).

Haplotype and definition			Antenatal vitamin D ($N = 827$)[a]				Cord blood vitamin D ($N = 656$)[b]				Published values for Jorde and Grimnes (2015)	
Haplotype	**rs4588[c]**	**rs7041[c]**	***N***	**mean ± SD**	***B***	***p***	***N***	**mean ± SD**	***B***	***p***	***N***	**mean ± SD**
1s/1s	GG	CC	114	86.7 ± 24.8	*ref*	*ref*	99	35.5 ± 9.2	*ref*	*ref*	3,621	55.4 ± 16.8
1s/1f	GG	AC	210	86.5 ± 24.8	−1	0.74	147	35.9 ± 8.4	0.43	0.72	2,456	53.3 ± 17.2
1f/1f	GG	AA	139	85.6 ± 24.9	−1.78	0.59	126	35.7 ± 9.1	0.23	0.85	510	52.2 ± 16.8
1s/2	GT	AC	150	80.1 ± 24.8	−7.45	0.015	113	33.2 ± 8.7	−2.36	0.052	3,315	50.3 ± 15.6
1f/2	GT	AA	163	76.5 ± 20.4	−11.3	3.4×10^{-4}	127	32.6 ± 8.6	−2.97	0.016	1,104	50.5 ± 16.3
2/2	TT	AA	51	69.7 ± 20.9	−18.3	1.6×10^{-5}	44	28.9 ± 7.1	−6.67	3.7×10^{-5}	698	46.9 ± 15.1

[a]*Mutually adjusted for ethnicity, consumption of vitamin D containing supplements and age at recruitment. The mean ± SD column is after adjusting for these covariates. The B and value of p are from the estimates from the linear regression.*

[b]*Mutually adjusted for antenatal vitamin D levels and ethnicity. The mean ± SD column is after adjusting for these covariates. The B and value of p are from the estimates from the linear regression.*

[c]*All of the alleles are reported on the positive strand.*

inadequate vitamin D concentrations and their offspring are at risk of pregnancy complications and early life adverse outcomes (Nassar et al., 2011; Christesen et al., 2012; Aghajafari et al., 2013, 2018; Theodoratou et al., 2014). To develop more insights into vitamin D insufficiency, we examined the genetic association with antenatal and cord blood vitamin D concentrations, which revealed two interesting loci.

Our GWAS identified rs4588, a missense variant in the gene *GC* of both mother and the offspring, as a significant risk factor for decreased vitamin D concentrations in antenatal and cord blood. The key haplotype-tagging SNP rs4588 has been previously reported as a risk factor for vitamin D in adult and non-pregnant populations of European, African American, and Hispanic descent (Manousaki et al., 2017; O'Brien et al., 2018). Several other variants in *GC* (rs2282679, rs3755967, and rs17467825) in high linkage disequilibrium with rs4588 have also been associated with vitamin D concentrations previously (Ahn et al., 2010; Wang et al., 2010; Lasky-Su et al., 2012; Anderson et al., 2014; Hong et al., 2018; Jiang et al., 2018). The *GC* gene encodes the VDBP, a protein that stores and transports both 25OHD and the active form of vitamin D, 1,25(OH)2D (Speeckaert et al., 2006), thus variants affecting VDBP activity would likely influence measured circulating 25OHD concentrations. Our findings augment the results of previous GWAS on vitamin D. The rs4588 and rs7041 haplotype GC 1F has been shown to affect VDBP binding to vitamin D (Arnaud and Constans, 1993), thus may affect the half-life of 25OHD and/or the bioavailability of free 25OHD. The majority of circulating 25OHD is bound to VDBP (Karras et al., 2018). Pregnant women have increased VDBP due to estrogen-dependent production and have increased 25OHD concentrations likely due to higher concentrations of VDBP and/or vitamin D supplementation (Karras et al., 2018). The effects of dysregulation of VDBP may play a role in the associations of vitamin D insufficiency and adverse pregnancy-related outcomes (Karras et al., 2018).

We also identified rs10789082 downstream of *CYP2J2* to be associated with antenatal vitamin D. This is a novel association in humans and is consistent across all three ethnicities. It is interesting to find this association with antenatal vitamin D concentrations but not cord blood vitamin D concentrations. This may be because the fetus depends on the mother for 25-hydroxylation of vitamin D. 25OHD crosses the placenta and fetal vitamin D concentrations are dependent on maternal vitamin D concentrations (Salle et al., 2000; Hollis and Wagner, 2017). The fetus appears to obtain active 1,25OHD largely from fetal kidney activity but 1,25OHD may also cross the placenta (Salle et al., 2000). SNPs in *CYP2J2* have been reported to be associated with serum vitamin D status in beef cattle (Casas et al., 2013) but not in human. The gene *CYP2J2* encodes the enzyme CYP, family 2, subfamily J, and polypeptide 2. The CYP superfamily of enzymes catalyze the oxidation of small organic compounds and are involved in drug metabolism and activation of steroid hormones such as vitamin D. The human CYP2J2 enzyme has been demonstrated to hydroxylate vitamin D2, vitamin D3, and 1α-hydroxyvitamin D3 (Aiba et al., 2006). SNPs in related genes *CYP2R1* and *CYP24A1* have been previously associated with vitamin D concentrations (Ahn et al., 2010; Wang et al., 2010; Hong et al., 2018; Jiang

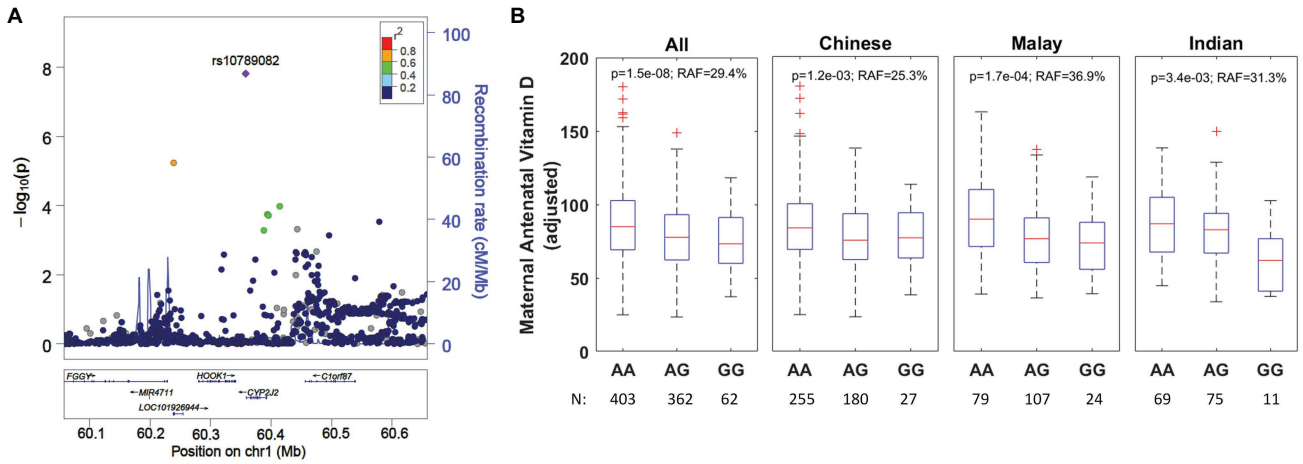

FIGURE 3 | (A) Regional association plot for rs10789082 (*CYP2J2*) and ethnicity-stratified levels for antenatal vitamin D. The regional association plot is based on all individuals with complete data for analysis. **(B)** Boxplots adjusted for ethnicity, mother's age at recruitment, and consumption of vitamin D containing supplements. RAF, risk allele frequency.

et al., 2018; O'Brien et al., 2018) but these reported associations were not significant in our cohort ($p > 0.01$). 25-hydroxylation of vitamin D is thought to primarily be due to CYP2R1 with CYP27A1, CYP2J2, and CYP3A4 also contributing and subsequent 1-alpha-hydroxylation by CYP27B140. Perhaps CYP2J2 plays a larger role in 25-hydroxylation of vitamin D during pregnancy or in Asian populations.

The present study has several strengths. To our knowledge, this is the first GWAS to investigate vitamin D in the context of antenatal blood, paired offspring cord blood and also in a multi-ethnic Asian cohort. Our cohort offers the unique opportunity to compare three major Asian ethnicities in a standardized manner as all participants live in a geographically small region with constant sunshine, little seasonal variation, and, where data was collected in a uniform manner. We used the LC-MS/MS method to quantify vitamin D concentrations, which is the proposed reference method for 25OHD measurement (Vogeser et al., 2004), as the competitive binding assays commonly used for measuring 25OHD may underestimate vitamin D concentrations due to differences in antibody affinity (Ong et al., 2012).

There are also some limitations to this study. One limitation is the single measurement of vitamin D in pregnancy, as it is known that pregnancy is marked by dynamic changes in physiology and vitamin D increases during the course of pregnancy. However, the time point chosen for this study is mid-gestation, which corroborates with the rise in antenatal vitamin D levels and fetal bone development (Moon et al., 2015). We have not measured the physiologically active form of vitamin D, 1,25(OH)2D, which has been shown to increase by 100% or more during pregnancy (Christesen et al., 2012); however, 25(OH)D reflects vitamin D stores and is the form of vitamin D recommended to evaluate vitamin D status (Holick et al., 2011). Second, we have not accounted for diet in our model, although, we anticipate sun exposure to have a greater role in determining vitamin D concentrations as the major vitamin D metabolite detected in plasma was 25(OH)D3. Third, we have also not accounted for individual-specific factors (skin pigmentation, preference for outdoor activity, and clothing preferences) in our models. We observe that the ethnicity specific effects are still present after accounting for the genetic associations identified here, suggesting that these factors may explain some of the difference in vitamin D concentrations between ethnic groups. Fourth, although, we adjusted for reported consumption of vitamin D containing supplements, we are unable to adjust for exact amounts consumed as we cannot ascertain the compliance and frequency of consuming the supplements with high degree of confidence. Fifth, we did not have weather data on amount of sunshine, although Singapore is a small island lying 1.5° north of the equator with sunshine throughout the year with no true distinct seasons. Therefore, we should anticipate very little impact due to the usual spatiotemporal factors that might affect the participants. Finally, the genotype association with rs10789082 downstream of *CYP2J2* needs to be replicated in other studies, preferably in Asian ethnicities first.

In conclusion, we found high prevalence of vitamin D inadequacy in pregnant women in a country with predominantly Asian population receiving sunlight all year round. Both Malay and Indian ethnic groups had more vitamin D inadequacy compared to Chinese. Our genetic finding on VDBP, the primary transporter of vitamin D in circulation, augments the understanding of vitamin D deficiency in pregnancy and its downstream impact on the availability of vitamin D to the growing fetus when the demands for it are critical during gestation. We have also identified a new SNP downstream of *CYP2J2*, which may influence vitamin D concentrations in pregnancy. The association of these genetic risk factors are similar across all three Asian ethnic groups studied here. These results may help to identify individuals who are genetically predisposed to vitamin D insufficiency in Asian population and advise further studies of the impact of this genetic predisposition on the association between vitamin D and different pregnancy and child health outcomes.

DATA AVAILABILITY STATEMENT

Clinical data are not publicly available due to ethical restrictions but can be obtained from the authors upon reasonable request and subject to appropriate approvals from the GUSTO cohort's Executive Committee.

ETHICS STATEMENT

The study was conducted according to the guidelines laid down in the Declaration of Helsinki. Ethical approval was obtained from the Domain Specific Review Board of Singapore National Healthcare Group (reference D/09/021) and the Centralized Institutional Review Board of SingHealth (reference 2009/280/D). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AS, AR, and LC performed the statistical analysis and genome-wide associations. AR, KT, and NK interpreted the data and wrote the manuscript. YC, PG, FY, KG, MC, and NK were responsible for data generation (clinical and genotyping data) in GUSTO cohort. NK conceptualized and supervised the study. 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/fgene.2021.721488/full#supplementary-material

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Zinc Deficiency, Plasma Fatty Acid Profile and Desaturase Activities in Hemodialysis Patients: Is Supplementation Necessary?

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Background: Desaturation and elongation are critical processes in endogenous metabolic fatty acid pathways. Zinc (Zn) is a cofactor for desaturases and elongases enzymes. There is limited evidence regarding the relationships between biomarkers of Zn status, nutritional intake, plasma phospholipid fatty acid profile and clinical outcomes among patients undergoing hemodialysis (HD).

Objective: To examine the relationships between dietary and serum levels of Zn and Cu/Zn ratio and to explore associations of these micronutrients with PUFA profile and estimated desaturase and elongase enzyme activities in serum phospholipids among HD patients.

Methods: This study included 40 adult patients undergoing hemodialysis treatment. Repeated 24-h recalls were applied for dietary intake assessment. Serum concentration of Zn and Cu were determined using inductively coupled plasma mass spectrometry and fatty acid composition by gas-liquid chromatography. Desaturase and elongase activities were calculated from product-precursor fatty acid ratios.

Results: Inadequate dietary Zn intake was found in 55% of HD patients. They all had serum Zn concentration below the reference value of 60 μg/dL (mean 38.8 ± 7.72 μg/dL). Adequate zinc intake was accompanied with significantly higher intake of energy, total fats, SFA, MUFA and proteins. There was no correlation between Zn serum status and Zn intake estimates. Serum Cu/Zn ratio was high, (2.76 ± 0.68), directly and significantly associated with HD period, CRP, BMI, VFA, and inversely with Kt/V, albumin, iron, and iPTH. The n-6/n-3 ratio in plasma phospholipids was elevated (12.25 ± 3.45) and patients with inadequate Zn intake had lower n-3 PUFA intake and status compared to those with adequate intake. Serum Zn concentrations were inversely correlated with linoleic/dihomo-γ-linolenic acid ratio (LA/DGLA) ($p = 0.037$), related to D6-desaturase activity ($p = 0.033$) and directly with DGLA relative abundances ($p = 0.024$). Cu status

was inversely associated with EPA level ($p = 0.03$) and estimates of elongase activity ($p = 0.001$). Furthermore, positive relationship was found between the Cu/Zn ratio and determined elongase value ($p = 0.01$).

Conclusion: Findings of this study underpin the high prevalence of Zn deficiency and inadequate n-3 PUFA intake and status among subjects undergoing HD. The results obtained indicate that the assessment of Zn status should be a standard parameter of nutritional status screening in HD patients while emphasizing the importance of Cu/Zn determination. Although further research is warranted, Zn and-n-3 PUFA supplementation in HD patients might be beneficial for the prevention and attenuation of adverse health outcomes

Keywords: zinc, fatty acid, Cu/Zn ratio, LA/DGLA, hemodialysis

INTRODUCTION

Chronic kidney diseases (CKD) is recognized as a global public health concern with significant clinical, economic, and humanistic burdens. Patients on hemodialysis treatment have a high incidence of cardiovascular diseases (CVD) and an increased mortality rate (1). Risk factors such as dyslipidemia, insulin resistance, disturbances in fatty acid metabolism, increased oxidative stress, and inflammation are considered accountable for the endothelial impairment and cumulative alterations of vascular function in hemodialysis patients (2). Zinc is an essential micronutrient with numerous roles is fundamental biologic processes. Due to unique antioxidant and anti-inflammatory properties and implications in enzyme activity, membrane stabilization and apoptosis inhibition, zinc is essential for endothelial integrity (3). Consequently, zinc deficiency could lead to severe endothelial damage and amplification of the detrimental impact of certain fatty acids such as linoleic acid, and inflammatory cytokines on vascular function (4). There is accumulating evidence that dysregulation of zinc metabolism and suboptimal status could be associated with the development of CVD (5). The risk of atherosclerosis in zinc-deficient patients with renal failure plays an important role in the progression and complications of CKD (6, 7). Lower zinc levels are related to end-stage renal disease (8).

Zinc deficiency increases oxidative stress and a cytokine-mediated inflammatory process leading to atherosclerotic complications (3). Among patients with CKD, deterioration of kidney function causes impaired elimination of an array of uremic toxins. They have been shown to promote inflammatory state and aggravate the production of reactive oxygen species. The damaging effect of these processes is intensified by the hemodialysis procedure itself, and, in such circumstances, zinc exerts a significant role in antioxidant systems. Numerous studies underpinned Cu/Zn-superoxide dismutase gradient as an effective oxidative stress marker for the evaluation of progressive renal damage (8). It has been proposed that the serum Cu/Zn ratio reflects the reciprocal reaction of Cu and Zn better than serum Cu or Zn concentrations separately (9, 10). Furthermore, it was found that the Cu/Zn-ratio significantly increases with aging (11). This biomarker, used also for the evaluation of oxidative stress burden, is associated with a higher risk of incident infection (10) and elevated cardiovascular mortality rate in the elderly population (12–14). Zinc, as an antioxidant and anti-inflammatory mediator, regulates the function of lymphocytes, making the body's immune system less susceptible to various infections (15–17). Recently, the protective role of zinc in vulnerable groups for COVID-19 such as hemodialysis patients has been mentioned (18).

Several studies confirm a high prevalence of zinc deficiency in hemodialysis patients (5, 19–23). This could be caused by multiple factors such as: inadequate nutritional intake due to protein restriction, reduced gastrointestinal dietary absorption, increased zinc excretion or loss into the dialysate, altered cellular and tissue distribution, as well as phosphate-binder drugs interaction and oral iron supplementation (16, 24–26). The main inhibitor of intestinal zinc absorption is phytate, which is found in unrefined cereals, pulses, oilseeds, and nuts. The evidence indicates that zinc deficiency could lead to erythropoietin-resistant anemia and increased susceptibility to renal damage in patients with diabetes (27, 28).

Previous studies, including ours, have reported disturbances in the proportion of serum fatty acids in hemodialysis patients particularly with lower n-3 polyunsaturated fatty acids (PUFA) and higher monounsaturated fatty acids (MUFA) content compared to healthy subjects (29–32). Depending on stages of CKD severity, there is an increase of MUFA and a gradual progressive decrease in the content of n-3 and n-6 PUFA, which contributes to the occurrence of dyslipidemia in CKD patients (33). The mechanism by which disturbance in fatty acid profile such as decline of n-3 PUFA/arachidonic acid (AA) ratio in hemodialysis still remains unclear (34). Endogenous fatty acid

Abbreviations: AA, arachidonic acid; ALA, alfa-linolenic acid; Alb, albumin; BMI, body mass index; CKD, chronic kidney disease; CRP, C-reactive protein; Cu, copper; CVD, cardiovascular disease; D5D, Δ-5-desaturase; D6D, Δ-6-desaturase; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; ELO, elongase; EPA, eicosapentaenoic acid; HD, hemodialysis; Hg, hemoglobin; ICP-MS, inductively coupled plasma mass spectrometry; iPTH, intact parathyroid hormone; LA, linoleic acid; MAC, mid arm circumference; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; RDI, recommended daily intake; *sCr, serum creatinine;* SFA, saturated fatty acids; Tg, Triglyceride; TC, total cholesterol; VAI, visceral adiposity index; VFA, visceral fat area; WC, waist circumference; Zn, zinc.

metabolism is regulated by D5, D6, and D9 desaturase enzymes. Zinc is a cofactor for desaturases and elongases enzymes (35). Therefore, the alterations of zinc serum levels could affect the activities of these enzymes and consequently modulate the regulation of fatty acid metabolism. Recently, the linoleic (LA)/dihomo-γ-linoleic acid (DGLA) ratio has been proposed as a novel biomarker of zinc status (36, 37). There is a scarcity in available scientific data regarding the interaction between zinc intake and status and PUFA fatty acid in CKD.

Thus, the aim of the present study was to examine the relationship between dietary zinc intake, its serum concentrations and Cu/Zn ratio and to explore associations of these micronutrients with PUFA profile in serum phospholipids and estimated desaturase and elongase enzyme activities in hemodialysis patients.

MATERIALS AND METHODS

Study Design

This was a cross-sectional study conducted at the Department of Hemodialysis, Clinic of Nephrology, Military Medical Academy, Belgrade, Serbia.

Study Population

We studied 40 patients undergoing hemodialysis treatments between February 2017 and January 2019. All patients were dialyzed three times per week for 4 h with high-permeability membranes. The blood flow was in the range of 250–300 mL/min with a dialysis rate flow of 500 mL/min. Oral iron supplementation, based on ferrous citrate or an iron containing phosphate binder was administered to all the participants. The exclusion criteria were heart failure (NYHA III or IV), acute myocardial infarction, and acute infectious disorders within three months of recruitment. Patients were not obese and had no severe malnutrition (BMI 20–30 kg/m^2). After the initial eligibility screening, subjects consuming zinc-salts and omega 3 dietary supplements were excluded. Involved patients voluntarily provided the written informed consent to participate in this research. This study was conducted in accordance with standards and a principle stated in the Declaration of Helsinki and was approved by the Ethical Review Board of the Military Medical Academy, Belgrade, Serbia (Approval Project No 8/15-17).

Analysis of Biochemical Parameters

All blood samples were obtained immediately before the mid-week dialysis session after a 12-hour fast. The following serum parameters were considered: urea, creatinine, and potassium, C-reactive protein (CRP), hemoglobin (Hb), total cholesterol, HDL- and LDL-cholesterol, triglycerides, albumin, iron, vitamin D, and intact parathyroid hormone (iPTH). After the dialysis procedure serum urea, creatinine and potassium analyses were repeated. Concentrations of biochemical blood parameters were obtained spectrophotometrically using the Siemens Dimension Rxl Max analyzer. The value of CRP was calculated *via* enhanced turbidimetric-immunoassay (PETIA) using the Siemens Dimension RxlMax analyzer. The Kt/V value quantifying the hemodialysis efficiency was calculated using the formula proposed by Daugirdas and Blake (38). Aliquots of the remaining serum were made immediately, and these samples were stored at −80 °C for further analysis of trace metal profiles (zinc, copper) and fatty acids.

Anthropometric Measurements

Assessment of anthropometric parameters included height, weight, mid-arm circumference (MAC) and waist circumference (WC). Measurement was performed with individuals wearing light clothes, without shoes. Height was measured using wall-mounted stadiometer to the nearest 0.5 cm. Body weight and % of body fat was measured using a body composition analyzer InBody720 (Biospace Co., Ltd., Seoul, Korea). Visceral fat area (VFA) was measured at the level of the umbilicus with a dual bioelectrical impedance analyzer (DUALSCAN, Omron Healthcare Co, Kyoto, Japan). WC was measured from the midpoint between the lateral iliac crest and the lowest rib to the nearest 0.5 cm. The visceral adiposity index (VAI) was calculated using the formula by Amato et al. (39).

Assessment of Dietary PUFA, Zinc and Copper Intake

Dietary intake evaluation was based on participants' subjective retrospective report, using the repeated 24-h recalls as the assessment method. Complete consumption of food and beverages was recorded on three occasions (dialyses day, one non-dialyses weekday and one non-dialyses weekend day) for each individual. Data was collected within in-depth multiple-pass direct structured interviews administered by skilled survey staff. Quantities of food consumed were estimated in reference to common household measures, natural units, and standard measuring kitchen tools as well as container size or packaging information for commercial products. Regionally specific, previously validated Food Atlas containing 135-items (simple foods and composite dishes) was applied as an additional two-dimensional portion-size estimation aid (40). Questionnaires were processed and analyzed *via* Diet Assess & Plan, an advanced nutritional software tool (41). Conversion of food to nutrient intake was performed based on mean values for three recall replicates and all the listed food items were matched and coded using the national Serbian Food Composition Database (42) in the Institute for Medical Research. Estimates of zinc intake were compared against reference values proposed specifically for hemodialysis patients, i.e.10–15 mg for men and 8–12 mg for women (43, 44). Adequacy assessment for copper intake was based on European Food Safety Authority (EFSA) recommendations for general population (1.3 mg/day for women, 1.6 mg/day for men) (45).

Fatty Acid Determination and Estimation of Desaturase Activity

Total serum lipids were extracted according to the method of Folch (46). As previously described, plasma phospholipids were isolated by one-dimensional thin-layer chromatography (TLC) in neutral solvent system hexane-diethyl-ether acetic acid (87:12:1 v/v) using Silica Gel GF plates (C. Merck, Darmstadt, Germany)

and trans esterified to methyl fatty acid (47). Afterwards, phospholipids' fatty acid methyl ester samples were analyzed by gas–liquid chromatography on the Shimadzu chromatograph GC 2014 (Kyoto, Japan), equipped with a flame ionization detector on an Rt × 2330 column (60 m × 0.25 mm ID, film thickness of 0.2 μm; RESTEK, Bellefonte, PA, USA) with initial oven temperature 130 °C held for 10 min and then programmed to increase at 3 °C/min to a final oven temperature of 220 °C. Fatty acid methyl esters were identified by comparing peak retention times with retention times obtained for fatty acids components of standard mixtures PUFA−2 and/or 37 FAMEs mix (Supelco, Bellefonte, PA, USA). The relative abundances of individual fatty acids were calculated and expressed as a percentage of total identified FAs. Activities of fatty acid desaturases (D) were estimated as ratios of their relative abundances as follows: 18:1n-9/18:0 for stearoil-CoA D18(SCD-18), 16:1n-7/16:0 stearoil-CoA D16 (SCD-16), 20:3n-6/18:2n-6 for delta-6D (D6D), 20:4n-6/20:3n-6 delta- (D5D) and 22:5n-3/20:5n-3 for elongase (ELO) (48).

Serum Zinc and Copper Determination

Serum samples were prepared according to Stojsavljević et al. (49). All chemicals used were of high grade and were supplied by Merck (Darmstadt, Germany). Briefly, all samples for metal analysis were diluted ten times with an aqueous solution containing 0.05% nitric acid, 0.1% Triton X-100, and 3% 1-butanol. Copper and zinc were determined by inductively coupled plasma-mass spectrometry (ICP-MS) in the collision mode with internal standardization. Linearity of the calibration curve above 0.999 was obtained for both trace metals in the range from 1 to 250μg/L. The accuracy of the method used, expressed as recovery and determined by analysis of standard reference material (Seronorm™ Trace Elements Serum L-2), was from 95.2 to 103.3% for ^{65}Cu and from 96.2 to 102.7% for ^{66}Zn. Serum zinc concentration below 60 μg/dL was defined as zinc deficient (50).

Statistical Analysis

Complete Statistical analysis was performed with IBM SPSS 23 software package (Chicago, IL, USA) and principal component analysis (PCA) in PLS ToolBox, v.6.2.1, Matlab 7.12.0 (R2011a) statistical package. The distribution of data was evaluated using Shapiro-Wilk's test and Grubbs' test-a and principal PCA to test outliers. Performed test showed that there were no outliers. The results obtained were presented as the mean value ± standard deviation (SD) for normally distributed, and medians (interquartile range) for scattered variables. Mean values of normally distributed data were compared by unpaired Student's *t*-test and non-parametric Mann-Whitney test was performed on non-transformed scattered values data. A logarithmic transformation was performed for scattered variables prior to further analyses with Pearson's/Spearmen's test for correlation analysis. For all the analyses a p-value < 0.05 was considered indicative for statistical significance.

RESULTS

Dietary Zinc, Copper and PUFA Intake in Hemodialysis Patients

Forty participants with a mean age of 56 ± 15 years were enrolled in the present study. The sample comprised thirty-one (78%) male and nine female (22%) hemodialysis (HD) patients. As it is shown in **Table 1**, 22 (55%) participants had inadequate dietary zinc intake compared to recommended values for HD patients (10–15 mg/day for men, 8–12 mg/day for women). Distribution of food groups of zinc dietary sources among study participants, based on 24 h recalls, is presented in **Figure 1**. Biomarker analyses revealed that all HD patients had zinc deficiency (i.e. serum zinc concentration below the 60 μg/dL threshold) with a mean value of 38.8 ± 7.8 μg/dL. Accordingly, patients' subdivision into categories based on zinc status was not possible. With a median value of 629 mg/day, copper intake was significantly below the recommended level for the general population (45). Serum copper concentration and the Cu/Zn ratio were 97.3 μg/dL and 2.8, respectively (**Table 1**).

Dietary intake data of HD patients is presented in **Table 1**. Considering the estimated dietary intake of PUFA, the nutritional n-6/n-3 ratio was notably high among HD patients. The median contribution of PUFA to total energy intake was 6.84 ± 3.28%. Further data analyses indicated higher n-3 PUFA intake among HD patients with adequate zinc intake in comparison with those whose intake was below the recommendations: 0.69 (0.56–0.94) vs. 0.53 (0.39–0.69) g/day. This difference reach statistical significance ($p = 0.028$). There were no differences for n-6, n-6/n-3, total PUFA and copper intake comparing these groups (**Table 1**). Group comparison based on zinc intake adequacy revealed that HD patients with appropriate dietary zinc intake had higher caloric intake ($p = 0.03$), protein ($p = 0.003$), SFA ($p = 0.005$), and MUFA ($p = 0.012$).

To acknowledge the impact of diverse dietary sources on zinc bioavailability and subsequent status, the contribution of various food groups to total zinc intake is presented in **Figure 1A**. In this study, the main dietary source of zinc were food items belonging to meat and meat product group with mean contribution of 3.26 mg/day, representing 34.72% of the total estimated dietary intake of zinc, so we presented the distribution of zinc daily intake from foods from meat and meat products group in **Figure 1B**. In this food group, a dominant source was beef meat, followed by pork and chicken meat. Based on food consumption analysis, other major sources were grains and grain products, miscellaneous food products, as well as milk, milk products and substitutes with 14.03, 13.77 and 10.39% contribution, respectively. In the miscellaneous product group important zinc dietary sources were bakery yeast and seasonings such as powdered red paprika, dried parsley leaves, ground nutmeg, and powdered garlic. Only 2.66% of total zinc intake was attributed to seafood and related products.

TABLE 1 | Demographic characteristic, dietary intake data, zinc and copper status in hemodialysis patients.

Variable	All patients	Adequate Zn intake	Inadequate Zn intake
Age (years)	56 ± 15	56 ± 14	53 ± 15
Gender (M/F) n (%)	31/9 (78/22%)	15/3	16/6
BMI (kg/m^2)	24.49 ± 3.76	25.09 ± 4.12	24.62 ± 3.44
HD duration (years)	5 (2–11.5)	3.5 (2–5)	5 (2–11)
Smokers/non-smokers n (%)	17/23 (42/58%)	9/9	8/14
Dietary zinc intake (mg/day)	9.35 ± 3.38	12.13 ± 2.56	7.07 ± 1.95***
M (RDI in HD 10-15 mg/day)	12.63 ± 2.49		
F (RDI in HD 8-12 mg/day)	9.63 ± 2.10		
M < 10 mg/day n (%)	16/31 (52%)		
F< 8 mg/day n (%)	6/9 (67%)		
Dietary copper intake (μg/day)	629 (425–774)	664 (512–799)	525 (402–749)
Energy intake (kcal/day)	1,858 (1,687–2,177)	2,093 ± 420	1822 ± 232*
Protein (g/day)	72.0 (59.0–87.7)	87.7 (78.9– 99.8)	63.6 (55.8–72.8)**
Carbohydrate (g/day)	230 ± 45	233 ± 62	228 ± 26
Total fiber (g/day)	19.5± 5.9	19.18 ± 5.33	19.77 ± 6.44
Fats (g/day)	79.8 ± 24.1	89.1 ± 18.5	70.9 ± 22.9**
SFA (g/day)	30.77 ± 12.04	38.42 ± 10.71	27.72 ± 11.25**
MUFA (g/day)	24.84± 7.57	31.06 ± 6.67	23.82 ± 9.11*
Dietary PUFA intake (g/day)	15.53 (8.72–20.00)	16.60 (9.07–19.96)	15.20 (7.18–26.02)
n−3 PUFA (g/day)	0.581 (0.507–0.836)	0.687 (0.560–0.942)	0.528 (0.394–0.688)*
n-6 PUFA (g/day)	12.60 ± 6.88	12.85 ± 5.96	12.39 ± 7.68
n-6/n-3	17.86 (10.16–29.00)	19.12 (10.20–27.07)	16.70 (10.12–36.72)
Serum			
Zinc (μg/dL)	38.8 ± 7.72	35.2 ± 5.4	39.8 ± 9.3
Serum zinc deficiency			
(< 60 μg/dL) n (%)	40 (100 %)		
Correlation: Zn intake/status	$r = -0.200$		
	$p = 0.211$		
Copper (μg/dL)	973 (930–1,191)	1,037 (719–1,108)	980 (930–1,086)
Cu/Zn ratio	2.76 ± 0.68	2.85 ± 0.68	2.56 ± 0.51

*Continuous variables are shown as means ± SD for a normal distribution or medians (interquartile range) for a non-normal distribution. HD, hemodialysis; RDI, recommended daily intake; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; Cu, copper; Zn, zinc. *p < 0.05, **p < 0.01, ***p < 0.001 inadequate Zn intake vs. adequate Zn intake.*

Correlations of Biochemical and Anthropometric Parameters With Zinc Intake and Status

Group comparison based on zinc intake adequacy revealed that HD patients with appropriate dietary zinc intake had lower iPTH concentrations ($p = 0.047$), VAI values ($p = 0.020$) and higher MAC ($p = 0.031$) (**Figures 2A–C**). There were no other statistically significant differences in biochemical and anthropometric parameters between these two groups. Moreover, further analysis of obtained data showed that there are significant correlations between dietary zinc intake and iPTH, VAI and MAC values (**Table 2**). The only significant difference in the fatty acid profile in HD patients with inadequate zinc intake was a lower level of n-3 PUFA compared to HD patients with adequate zinc intake (**Table 3**). Nevertheless, low EPA+DHA values and high n-6/n-3 ratio were present in both groups.

Table 4 represents the associations of biochemical and anthropometrics parameters with dietary intake of zinc, serum zinc and copper status. Dietary zinc intake did not correlate with serum zinc concentration in HD patients (data not shown). The inverse associations were found between zinc intake estimates and LDL cholesterol ($r = -0.332$, $p = 0.036$) as well as iPTH levels ($r = -0.317$, $p = 0.046$) (**Table 4**). Dietary intake of zinc was directly associated with the anthropometric parameter MAC and inversely with VAI ($p < 0.05$, for both parameters). There were no significant correlations between the serum zinc concentrations and analyzed biochemical parameters. However, significant inverse correlations between the serum Cu/Zn ratio and serum albumin ($r = -0.404$, $p = 0.009$), iron ($r = -0.351$, $p = 0.026$), PTH ($r = -0.332$, $p = 0.036$) concentration and Kt/V ($r = -0.317$, $p = 0.046$) were noted. In addition, the serum Cu/Zn ratio was directly associated with HD period ($r = 0.355$, $p = 0.023$), CRP ($r = 0.315$, $p = 0.048$), BMI ($r = 0{,}384$, $p = 0.014$)

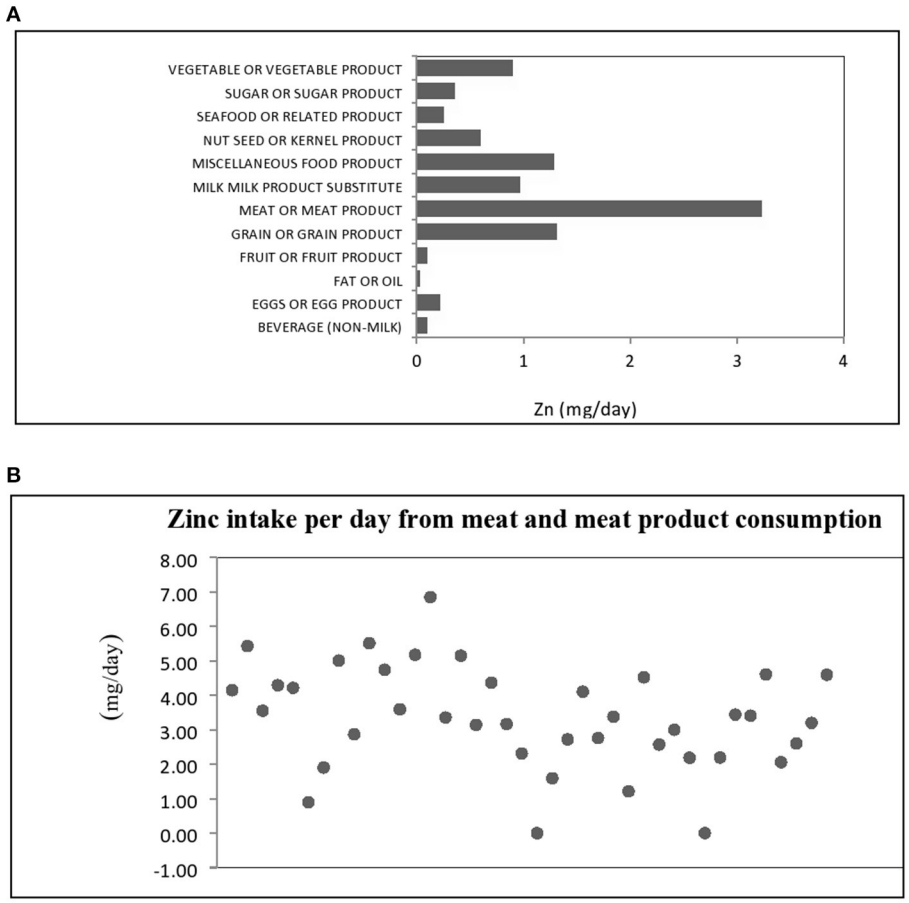

FIGURE 1 | (A) Dietary sources of zinc intake by food groups and **(B)** Distribution of zinc intake for meat and meat product in hemodialysis patients.

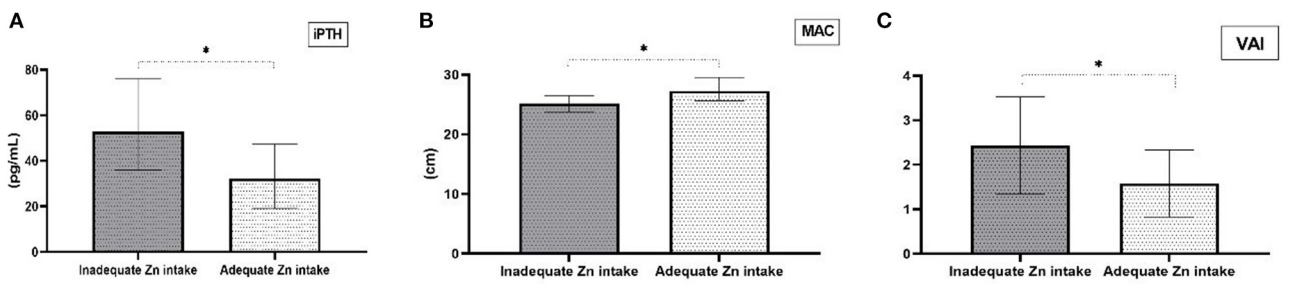

FIGURE 2 | Differences in clinical and anthropometric parameters comparing groups with adequate and inadequate dietary Zn intake. **(A)** Median values for intact parathyroid hormone (iPTH) levels (1st-3rd interquartile for inadequate Zn intake = 36–75.9; 1st-3rd interquartile for adequate Zn intake = 19–47.3), **(B)** Median values for mid-arm circumference (MAC) (1st-3rd interquartile for inadequate Zn intake =23.7–26.5; 1st-3rd interquartile for adequate Zn intake = 25.6–29.55), and **(C)** Mean ± standard deviation for visceral adiposity index (VAI). *$p < 0.05$ for inadequate Zn intake vs adequate Zn intake.

and VFA (r = 0.327, $p = 0.040$). Serum copper concentration directly correlated with BMI (r = 0.384, $p = 0.014$) and VFA ($r = 0.361$, $p = 0.023$) and inversely correlated with serum albumin concentration ($r = -0.449$ $p = 0.004$).

Correlations of PUFA and Estimates of Desaturase Activity With Zinc Status

As shown in **Table 5**, no statistically significant correlations were observed between dietary zinc intake and plasma

TABLE 2 | Characteristic of the hemodialysis patients.

Variable	All patients	Adequate Zn intake	Inadequate Zn intake
Hg (g/L)	101.0 ± 15.2	102.8 ± 11.8	102.6 ± 18.3
Urea (mmol/L)	23.9 ± 4.8	24.1 ± 5.8	23.3 ± 4.7
Creatinine (μmol/L)	913 ± 147	967 ± 135	876 ± 161
Kt/V	1.47 ± 0.32	1.34 ± 0.27	1.49 ± 0.37
Protein (g/l)	67.5 (63–70)	68 (66.5–69)	67.5 (63–70)
Albumin (g/l)	37.5 (36–40)	37 (35–39)	38 (37–40)
Total cholesterol (mmol/L)	4.52 ± 0.92	4.73 ± 0.92	4.40 ± 1.35
LDL cholesterol (mmol/L)	3.09 ± 0.78	2.95 ± 0.82	3.19 ± 0.93
HDL cholesterol (mmol/L)	F: 0.995 (0.91–1.08)	F: 0.955 (0.91–1)	F: 1.41 (1.09–1.46)
	M: 1.065 (0.90–1.29)	M: 1.185 (0.98–1.4)	M: 0.9 (0.64–1.14)
Triglycerides (mmol/L)	1.21 (1.025–1.575)	1.11 (1.02–1.51)	1.50 (1.00–1.93)
Tg/HDL	1.28 (0.91–1.69)	1.10 ± 0.48	1.52 ± 0.73
CRP ≤ 3 mg/L n (%)	12 (42%)	6/12	6/16
CRP > 3 mg/L n (%)	28 (58%)		
WC (cm)	F: 82.1 ± 10.8	F: 78.5 ± 11.3	F: 87.1 ±8.8
	M: 95.6 ± 11.4	M: 96.8 ±13.3	M: 94.5 ±9.5
% Body fat	F: 30.6 ± 10.8	F: 28.2 ± 2.3	F: 29.1 ±5.3
	M: 25.5 ± 8.4	M: 23.7 ±9.4	M: 26.1 ± 8.0
MAC (cm)	25.6 ± 3.8	27.25 (25.6–29.55)	25.1 (23.7–26.5)*
VFA (cm^2)	96.2 ± 44.6	93.2 ± 55.	102.7 ± 32.2
VAI	2.00 ± 1.01	1.58 ± 0.75	2.44 ± 1.09*
Iron (μmol/L)	14.8 ± 5.3	13.8 ± 4.4	15.2 ± 6.1
iPTH (pg/ml)	43.45 (19.4–64.7)	32.3 (19–47.3)	52.8 (36–75.6)*
Vitamin D (nmol/L)	35.87 ± 9.81	35.46 ± 11.46	34.94 ± 8.03

*Continuous variables are shown as means ± standard deviation for a normal distribution or medians (interquartile range) for a non-normal distribution. BMI, body mass index; F, female; M, male; CRP, C-reactive protein; Hg, hemoglobin; Tg, Triglyceride; WC, waist circumference; MAC, mid arm circumference; VFA, visceral fat area; VAI, visceral adiposity index; iPTH, intact parathyroid hormone. *$p < 0.05$ inadequate Zn intake vs adequate Zn intake, #distribution of data could not be tested as there are less than 5 data ($n = 3$).*

phospholipids' levels of individual n-6 and n-3 PUFAs neither with estimated desaturase activities among HD patients. Positive correlations were found for serum zinc concentration with DGLA ($r = 0.357$, $p = 0.024$) and D6D ($r = 0.311$, $p = 0.037$) and inverse associations with the DGLA/LA ratio ($r = -0.335$, $p = 0.033$). Serum copper concentration was inversely associated with EPA ($r = -0.465$, $p = 0.03$). The Cu/Zn ratio and serum copper concentration directly correlated with ELO ($r = 0.384$, $p = 0.01$ and $r = -0.575$, $p < 0.001$, respectively).

DISCUSSION

To the best of our knowledge, this is the first study evaluating zinc status in hemodialysis patients, considering dietary zinc intake, serum zinc levels and mutual relations with fatty acid profile, and estimated desaturase activities. Among studied patients, both zinc deficiency and altered Cu/Zn ratio were observed. Inadequate dietary zinc intake was present up to 55% of hemodialysis patients and there was no significant correlation between the estimated intake and serum zinc concentration. Based on our findings, zinc dietary intake did not correlate with serum PUFA status and estimated desaturase activity in hemodialysis patients. Furthermore, data revealed the absence of association between serum zinc concentration and analyzed biochemical parameters. It is noteworthy that all the studied hemodialysis patients had zinc deficiency, determined according to low serum zinc concentration as known as a sensitive biomarker of zinc status. This led to no statistically significant difference in the zinc levels and Cu/Zn ratio compared hemodialyzed patients with adequate and inadequate zinc intake. Results of this study indicate that the serum zinc and copper concentrations and their ratio were related to modified fatty acid PUFA profile in hemodialysis patients, with DGLA, EPA, LA/DGLA and estimated D6D activity.

Our results are in accordance with previously published data suggesting that hemodialysis patients have low serum zinc concentration (5, 19–23). As reported in the present study, dietary zinc intake is quite often not correlated with plasma/serum zinc status (37, 51). Fifty-five percent of recruited patients had zinc intake below the recommended level for patients with CKD and all had zinc deficiency (defined as serum concentration below 60 μg/dL). Severe zinc deficiency can manifest clinically when its serum values decrease to 40 μg/dL. Mean serum zinc concentration of 38.8 ± 7.72 μg/dL obtained in these samples could be considered as indicative for zinc supplementation. Although only 1% of total zinc in the body is present in circulation, serum zinc is still regarded as an important

TABLE 3 | Plasma fatty acid status in hemodialysis patients.

Fatty acid %	All patients	Adequate Zn intake *n* = 18	Inadequate Zn intake *n* = 22
16:0	28.60 ± 1.94	28.44 ± 2.03	28.31 ± 2.28
18:0	19.06 (17.68–19.81)	17.80 (17.04–19.70–24.82)	19.36 (18.36–20.29)
SFA	47.52 ± 2.32	46.85 ± 2.22	47.96 ± 2.30
16:1n−7	0.38 (0.26–0.46)	0.36 (0.22–0.45)	0.40 (0.27–0.48)
18.1n-9	9.61 (8.54–10.25)	9.73 (8.38–10.19)	9.87 (8.86–10.44)
18:1n−7	1.85 (1.70–2.09)	1.73 (1.66–2.02)	1.95 (1.74–2.08)
MUFA	11.84 (11.03–12.68)	11.81 (10.72–12.20)	12.31 (11.22–12.87)
18:2n-6 (LA)	21.89 ± 3.14	22.22 ± 2.93	21.64 ± 3.99
20:3n-6 (DGLA)	2.87 ± 0.66	3.02 ± 0.74	2.94 ± 0.63
20:4n-6 (AA)	11.78 ± 2.25	12.14 ± 2.06	11.61 ± 2.62
22:4n-6	0.66 (0.50–0.90)	0.67 (0.46–1.01)	0.60 (0.45–0.71)
n-6	37.27 ± 3.03	38.12 ± 2.69	36.79 ± 7.48
18:3n-3	0.11 (0.08–0.16)	0.12 (0.08–0.15)	0.09 (0.07–0.19)
20:5n−3 (EPA)	0.22 (0.16–0.31)	0.24 (0.14–0.32)	0.18 (0.16–0.32)
22:5n-3(DPAn-3)	0.56 (0.48–0.69)	0.62 (0.48–0.80)	0.54 (0.44–0.59)
22:6n-3 (DHA)	2.26 ± 0.57	2.36 ± 0.62	2.08 ± 0.41
n-3	3.25 ± 0.74	3.42 ± 0.83	2.97 ± 0.51*
n-6/n-3	11.99 ± 2.54	11.71 ± 2.66	12.68 ± 2.20
EPA+DHA	2.51 ± 0.60	2.61 ± 0.63	2.29 ± 0.42
PUFA	40.52 ± 3.31	41.54 ± 2.71	39.77 ± 3.61
D5D	4.28 ± 1.15	4.20 ± 1.09	4.06 ± 1.11
D6D	0.128 (0.106–0.153)	0.129 (0.109–0.149)	0.127 (0.102–0.185)
SCD16	0.013 (0.009–0.016)	0.012 (0.008–0.016)	0.014 (0.010–0.016)
SCD18	0.49 (0.43–0.57)	0.53 (0.45–0.57)	0.49 (0.44–0.57)
LA/DGLA	8.10 ± 2.53	7.80 ± 2.17	7.79 ± 2.72
ELO	2.47 (0.96–11.0)	3.29 (3.10–11.00)	2.51 (1.00–5.17)

*Continuous variables are shown as means ± standard deviation for a normal distribution or medians (interquartile range) for a non-normal distribution; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; LA, linoleic acid; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acids; D5D, Δ-5-desaturase; AA/DGLA (20:4 n-6/20:3 n-6), D6D, Δ-6-desaturase: DGLA/LA (20:3 n-6/18:2 n-6); SCD16 and 18, stearoil-CoA desaturase 16 and 18, ELO, elongase: (DPAn-3/EPA), *$p < 0.05$ inadequate Zn intake vs. adequate Zn intake.*

biomarker and useful indicator of dietary zinc restriction, zinc supplementation, and clinical deficiency. The recently determined interval for serum zinc concentration in the adult Serbian population (from 40 to 82,5 μg/ dL) strongly implies that even our healthy population could be at risk to develop zinc deficiency (52). Therefore, adequate oral zinc supplementation and/or foodstuff fortification should be considered to prevent the deleterious effects caused by zinc deficiency (53). It is important to notice that meta-analysis by Wang et al. (54) showed that zinc supplementation with median dose of 45 mg/day and duration of median 60 days results in higher serum zinc levels and lower CRP and malondialdehyde levels in hemodialyzed patients. Thus, evidence supported that zinc supplementation in doses that are much higher than recommended values for dietary intake (8–12 mg/day for women, 10–15mg/day for men) may improve serum zinc levels in hemodialysis patients. Increasing number of CKD patients is considered a major public health problem and there is the new evidence that low dietary zinc intake may increase the risk of CKD development in individuals with normal renal function (55). Low serum zinc concentrations among hemodialysis patients are supposed to result from zinc removal during the treatment itself, decreased albumin levels, diminished gastrointestinal zinc absorption, and antagonistic effects of copper to their common carrier – metalothionine, due to its higher affinity to copper compared to zinc (16, 56). In addition, a high intake of copper, iron or phytic acid could cause malabsorption of dietary zinc (57). The molar ratio of phytate and zinc has been shown to be a predictor of zinc absorption; however, data for dietary amounts of phytic acids are still scarcely available in food composition databases. A nutrition plan targeting plant-based food was not recommended to studied hemodialysis patients due to the high content of potassium and phosphates. Given that the major contributor to zinc intake were meat and meat products, participants of the present study dominantly obtained zinc from high bioavailability sources. Group comparison based on zinc intake adequacy revealed that our hemodialysis patients with appropriate dietary zinc intake had daily higher energy, protein, total fats and SFA, MUFA and n-3 PUFA consumption.

TABLE 4 | Correlation between zinc intake and status values with the different biochemical parameters in hemodialysis patients.

	Hg	Iron	CRP	HD per	Kt/V	Urea	sCr	Alb	TC	LDL	Tg	Tg / HDL	iPTH	Vit D	BMI	MAC	VFA	VAI
Dietary Zn																		
r	*0.021*	*−0.078*	*−0.140*	*−0.168*	*−0.132*	*−0.120*	*0.222*	*0.202*	*0.051*	*−0.332*	*−0.039*	*−0.168*	*−0.317*	*0.087*	*0.005*	*0.449*	*0.088*	*−0.317*
p	*0.899*	*0.629*	*0.388*	*0.300*	*0.415*	*0.460*	*0.170*	*0.211*	*0.750*	*0.036*	*0.809*	*0.300*	*0.046*	*0.589*	*0.977*	*0.004*	*0.589*	*0.046*
Serum Zn																		
r	*0.113*	*0.021*	*0.009*	*−0.089*	*0.132*	*−0.118*	*0.032*	*0.118*	*−0.039*	*0.051*	*0.083*	*0.001*	*0.113*	*0.021*	*0.083*	*0.010*	*0.113*	*0.069*
p	*0.486*	*0.900*	*0.955*	*0.582*	*0.415*	*0.469*	*0.842*	*0.470*	*0.809*	*0.753*	*0.611*	*0.998*	*0.485*	*0.896*	*0.611*	*0.925*	*0.485*	*0.669*
Serum Cu																		
r	*0.118*	*−0.214*	*0.231*	*0.010*	*−0.256*	*−0.032*	*0.118*	*−0.449*	*0.056*	*−0.064*	*0.010*	*−0.021*	*−0.177*	*−0.038*	*0.383*	*0.185*	*0.361*	*0.094*
p	*0.470*	*0.183*	*0.151*	*0.950*	*0.111*	*0.842*	*0.470*	*0.004*	*0.729*	*0.696*	*0.950*	*0.900*	*0.273*	*0.815*	*0.015*	*0.252*	*0.023*	*0.560*
Cu/Zn																		
r	*0.118*	*−0.214*	*0.231*	*0.010*	*−0.256*	*−0.032*	*0.118*	*−0.449*	*0.056*	*−0.064*	*0.010*	*−0.021*	*−0.177*	*−0.038*	*0.383*	*0.185*	*0.361*	*0.094*
p	*0.415*	*0.026*	*0.048*	*0.023*	*0.046*	*0.454*	*0.879*	*0.009*	*0.683*	*0.522*	*0.539*	*0.751*	*0.029*	*0.720*	*0.016*	*0.155*	*0.040*	*0.770*

Alb, albumin; sCr, serum creatinine; HD per, hemodialysis period; TC, total cholesterol.

TABLE 5 | Correlation between Zinc intake and status values with plasma PUFA profile and estimated desaturase activity in hemodialysis patients.

	LA	ALA	DGLA	AA	EPA	DHA	n-6/n-3	EPA + DHA	D5D	D6D	LA / DGLA	SCD16	SCD18	ELO
Dietary Zn														
r	*0.173*	*0.213*	*0.133*	*0.023*	*0.026*	*−0.048*	*0.007*	*0.058*	*−0.092*	*0.011*	*−0.025*	*−0.036*	*0.054*	*0.073*
p	*0.284*	*0.182*	*0.406*	*0.892*	*0.874*	*0.742*	*0.955*	*0.729*	*0.564*	*0.947*	*0.883*	*0.827*	*0.761*	*0.668*
Serum Zn														
r	*−0.125*	*−0.218*	*0.357*	*0.250*	*−0.292*	*−0.052*	*0.165*	*−0.112*	*−0.168*	*0.331*	*−0.335*	*−0.229*	*−0.183*	*0.220*
p	*0.442*	*0.174*	*0.024*	*0.118*	*0.064*	*0.748*	*0.307*	*0.491*	*0.296*	*0.037*	*0.033*	*0.155*	*0.277*	*0.168*
Serum Cu														
r	*−0.032*	*−0.147*	*0.256*	*0.089*	*−0.465*	*0.079*	*0.060*	*−0.027*	*−0.215*	*0.181*	*−0.202*	*0.165*	*−0.202*	*0.575*
p	*0.842*	*0.365*	*0.111*	*0.583*	*0.003*	*0.627*	*0.704*	*0.868*	*0.184*	*0.263*	*0.211*	*0.310*	*0.211*	*<0.001*
Cu/Zn														
r	*0.118*	*0.021*	*−0.023*	*−0.118*	*−0.364*	*0.093*	*−0.060*	*0.036*	*−0.087*	*−0.070*	*0.063*	*0.035*	*−0.027*	*0.384*
p	*0.470*	*0.899*	*0.907*	*0.470*	*0.090*	*0.566*	*0.715*	*0.827*	*0.589*	*0.666*	*0.697*	*0.829*	*0.867*	*0.014*

LA, linoleic acid; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; ALA, alfa-linolenic acid; EPA, eicosapentaenoic acid, DHA, docosahexaenoic acid; D5D, Δ-5-desaturase; AA/DGLA (20:4 n-6/20:3 n-6); D6D, Δ-6-desaturase; DGLA/LA (20:3 n-6/18:2 n-6); SCD16 and 18, stearoil-CoA desaturase 16 and 18; ELO, (DPAn-3/EPA).

It is assumed that disruption of zinc and copper levels could be the cause of clinical deterioration as well as negative outcomes in hemodialysis patients. Previous findings indicated that the increased plasma Cu/Zn ratio reflects the disturbance in zinc and copper homeostasis and could be applied as a clinical predictor for patients' inflammatory status (10). It is suggested that the optimum serum Cu/Zn-ratio lies between 0.7 and 1.0 (58). Participants in the present study exhibited abnormally increased Cu/Zn-ratio with a mean value of 2.76 ± 0.68. Trendafilov et al. (56) found a negative correlation between zinc status and markers of inflammation (CRP and IL-6), and a direct association of these parameters with copper status. Given the association found with the CRP as an indicator of the inflammatory response, clinical significance of Cu/Zn ratio for treatment outcome and further progression of renal failure is evident in our study population.

According to the European Society for Clinical Nutrition and Metabolism guideline for hospitalized patients with acute or CKD, zinc and copper levels should be measured for nutrition screening and supplemented accordingly (59). There is evidence implying that serum zinc concentration is reduced by hypoalbuminemia in CKD patients, as zinc is bound to albumin in the circulation (60). Our study showed that the Cu/Zn ratio is inversely correlated with albumin levels. This study did not identify such associations between serum zinc concentration and explored biochemical parameters. This might be due to the limited sample size, which resulted in low statistical power for detecting outcomes of the serum zinc variable. The Cu/Zn ratio has been recognized as a better indicator of the significance of zinc deficiency than the concentration of zinc and copper in serum separately. In the present study inverse association between the serum Cu/Zn ratio and serum iron and iPTH concentration was observed among hemodialysis patients.

Zinc deficiency is suspected to cause an increase in PTH due to its contribution in maintaining calcium homeostasis (61). Previously published studies showed a significant negative correlation between serum levels of iPTH and zinc in hemodialyzed children (62), but this correlation was not highlighted for the adult patient population (63). Based on our findings, estimated dietary zinc intake and Cu/Zn ratio were inversely associated with serum concentrations of iPTH, but there was no significant association between serum zinc levels and iPTH levels. Taken all together, our results indicate that zinc intake and its status together with Cu/Zn balance in serum are associated with iPTH levels and consequently calcium homeostasis. The mechanism of PTH effects on plasma zinc concentration in CKD was investigated by Chen et al. (64) who found that PTH enhanced extra renal zinc disposal and increased zinc uptake by liver, suggesting that the over-secretion of PTH could be present in zinc deficiency. Furthermore, low vitamin D status leads to reduced efficiency of intestinal calcium absorption, and the body reacts by increasing the secretion PTH. Prescription of activated vitamin D to a CKD patient with high PTH levels is, therefore, a justified clinical solution. It is noteworthy that all the studied patients in our sample had vitamin D deficiency. Nevertheless, serum vitamin D levels did not correlate with zinc status.

Previously published data revealed that zinc supplementation could significantly reduce total cholesterol, LDL-cholesterol, and triglycerides in the general population (63). However, a recent meta-analysis showed no effect on the lipid profile of zinc supplementation in patients subjected to maintenance hemodialysis (54). A statistically significant inverse association was found between LDL-cholesterol levels and estimated dietary zinc intake, but no significant relationships were found with serum zinc concentration and Cu/Zn ratio. We obtained significant differences for iPTH, VAI, and MAC between the groups with adequate and inadequate dietary zinc intake, and its association with these parameters. However, we did not obtain correlations of iPTH, VAI, and MAC with serum zinc concentration. This could indicate the influence of deviations of zinc homeostasis and redistribution in tissue and cell (e.g. zinc concentration in erythrocytes). Some authors declare that it is not clear yet whether low serum/plasma zinc levels in CKD patients represent genuine deficiency or its low levels are due to redistribution in different biological compartments (65). Recently, serum zinc levels were found to positively correlate with the nutritional status assessed by measuring abdominal fat area using computed tomography in patients with advanced CKD undergoing hemodialysis (66). In our study, no statistically significant correlations were determined between the serum zinc level concentration and anthropometric measurements. Nevertheless, based on our observations, the Cu/Zn ratio positively correlated with BMI and VFA. Furthermore, the results of our study indicated that dietary zinc intake directly correlated with the MAC and inversely with VAI, a reliable indicator of visceral fat function associated with cardiometabolic risk. There is substantial evidence that higher BMIs are associated with an increased risk of CVD and mortality in the general population, but a reverse relationship is observed in HD patients and this phenomenon is known as the "risk factor paradox" (67). It is, however, unclear whether the survival advantage associated with higher BMIs among hemodialysis patients is caused by increased muscle mass, fat mass, or both. Previous findings indicated that zinc supplementation increase body weight and BMI in hemodialysis patients (68, 69).

Blood fatty acid composition seems to be an independent risk factor for CVD in patients subjected to HD (34, 70–72). It reflects both nutritional intake and endogenous synthesis of fatty acids, but there is still not much available information on dietary fatty acid intake in patients undergoing hemodialysis. In a recent study, Khor et al. (73) reported suboptimal dietary intake of PUFAs (<5% of total calories). The American Heart Association recommends replacing saturated fatty acids with PUFAs (>10% of total calories) for the general population to reduce the risk for CVD (74). Inadequate intake of total PUFAs was found in 50% of hemodialysis patients in this study. The mean dietary intake of fish and seafood was 10.5 g/day i.e. 73.5 g/week. These findings suggest suboptimal intake of n-3 PUFA as well, since two servings of fish (180g) a week is recommended (75). Based on Wijendran et al. (76), if the consummation of LA, ALA, EPA+DHA were in accordance with the recommendations, the dietary n-6/n-3 ratio should be approximately 6. In the present study 33 of 40 participants had a higher dietary n-6/n-3 PUFA ratio. Therefore,

dietary habits of studied hemodialysis patients indicate the need for better nutritional strategies to achieve adequate fatty acid status. However, adequate zinc intake in our study population was accompanied with significantly higher intake of energy, total fats, SFA, MUFA and protein. According to results of our study red meat represented the main dietary source of zinc. Red meat represents a rich dietary source of zinc and proteins but also other nutrients such as SFA and cholesterol, limiting of its consumption is proposed strategy to reduce CVD risk in CKD patients, making even more difficult to reach adequate zinc intake by diet (77). Currently, there is no consensus in nutrition guidelines for the optimal dietary fatty acid intake targeting specifically CKD patients, but high dietary intake of zinc by consumption of foods abundant in zinc may lead to overconsumption of SFA. According to results of this study the zinc supplementation in CKD patients on hemodialysis, may be necessary to improve serum zinc status as the patients with adequate dietary zinc intake are shown to be zinc deficient, but could be a good strategy to ensure adequate zinc intake without high intake of some other nutrients such as SFA. Due to the recommended restriction of animal protein intake in patients with CKD supplementation is indicative for resolving zinc deficit in patients on hemodialysis.

A recent systematic review regarding the blood fatty acid status in dialysis patients from different countries revealed that the reported diversity of n-3 PUFA levels could be attributed to the variety of dietary habits (78). Among participants in this study, very low levels of EPA+DHA were observed in serum phospholipids. These findings are not unexpected since the dietary intake of fish and seafood was significantly below the recommendations. Our previous studies have already demonstrated markedly low n-3 PUFA status in dialyses population as well as its association with other clinical parameters, especially inflammation markers (29, 47, 79). This current study indicates that besides low n-3 PUFA status their dietary intake is also inadequate and accompanied by high n-6/n-3 ratio.

Essential fatty acids LA and ALA cannot be synthesized in humans and must be provided by the diet. However, they can be metabolized to long-chain PUFAs. DGLA, AA, and 22:4n-6 are the major products of LA desaturation/elongation metabolic pathway, while the ALA can be converted to EPA, DPAn-3, and DHA. Although these conversions seem to be relatively inefficient in humans, our previous research suggested beneficial effects of administration of ALA-rich seed mixture for improving EPA and DHA levels in hemodialysis patients (47). In fact, in the recent study authors hypothesized that uremic milieu found in hemodialysis patients could affect the n-3 PUFA metabolic pathway (73). Previous investigations have shown that serum/plasma zinc and copper levels could be associated with LA/DGLA values in healthy, dyslipidemic obese subjects, and children (35, 37, 80). To our best knowledge, there is no literature data regarding the relationship between zinc and copper status with estimates of desaturase activities and relative abundance of individual PUFA in HD population.

In the present study, zinc status was low in all patients undergoing hemodialysis, but the finding that it is directly associated with D6D is not unexpected. The inverse association between serum zinc status and LA/DGLA ratio has been determined in healthy subjects and dyslipidemic obese adult subjects, as well as children (35, 37, 80). Therefore, LA/DGLA was proposed as an emerging candidate biomarker for zinc status assessment (37). The obtained statistically significant inverse relationship between serum zinc concentrations and LA/DGLA confirms sensitivity of this biomarker in zinc deficient population. In line with other studies, we also found that DGLA levels were positively correlated with zinc status (35, 37). This fatty acid can be metabolized to one series of prostaglandins which possess anti-inflammatory properties, unlike two series of prostaglandins produced from AA generally viewed as pro-inflammatory (69). AA/DGLA ratio which is generally elevated in chronic renal patients undergoing HD is regarded as an important predictor of poor clinical outcomes (72). In this study, copper levels correlated inversely with EPA levels and directly with estimates of ELO activity, while the Cu/Zn ratio directly correlated with ELO activity and was not associated with EPA levels. These findings could be important as there is no literature data for copper status association with EPA levels and estimated ELO activity and scientific evidence suggests that EPA supplementation could decrease the all-cause mortality in patients undergoing HD treatment (71).

Based on a contrite meta-analysis of early and preliminarily available data, CKD seems to be associated with enhanced risk of severe COVID-19 infection. Multiple aspects of the immune system are affected by zinc. The prevalence of CKD in COVID-19 patients has been reported to be about 0.09–47.1% (81). Novel data suggest that oral zinc supplementation (80 mg/day of elemental zinc) administered at the first signs and symptoms of COVID-19 could be a promising therapeutic strategy (82). In general, a large body of evidence shows that up to one year of zinc supplementation in doses of 220 mg/d to 660 mg/d in chelated form (corresponding to approximately 50 mg to 150 mg of elemental zinc) seems to be a safe regimen for patients with CKD (54). Recently published studies demonstrated that the administration of oral zinc acetate could increase a risk for copper deficiency in hypoalbuminemic hemodialysis patients (83). A personal nutrition therapeutic approach is necessary for patients with CKD and hemodialysis sessions represent a valuable opportunity to monitor nutritional status and prescribe the appropriate interventions (84–86).

LIMITATIONS

Several limitations of this study should be acknowledged. First, the sample size was relatively small, and our findings should be confirmed and clarified by further larger-scale studies. All involved hemodialyzed patients were zinc deficient, so it was not possible to divide them into groups according to serum zinc levels. Second, dietary intake of zinc, copper and fatty acids were estimated using self-reported, retrospective 24 h nutritional surveys. All methods for the assessment of dietary exposure have their inherent limitations and 24 h surveys may not be the best nutritional tool to estimate the consumption of foods that are not consumed on a daily basis, such as fish, seafood, and

seafood products. Finally, statistical analysis was not performed with sex-specific considerations since female participants were underrepresented with only 20% in the studied patient group. Zinc deficiency could cause changes in a plethora of biochemical and clinical parameters that reflect renal function, the efficiency of hemodialysis treatment, dyslipidemia, calcium homeostasis (iPTH and vitamin D), body composition, inflammatory status, and anemia. In addition to factors explored in this study, there are other important parameters such as glucose homeostasis, redox status, and immune function that are relevant for patients undergoing HD, and further research is needed to provide a better understanding of their relationships with zinc status.

CONCLUSION

In conclusion, due to the observed zinc deficiency in hemodialysis patients, it is necessary to determine serum zinc levels together with the Cu/Zn ratio as a standard in screening nutritional status. These indices could play an important role in desaturation/elongation of PUFA in this patient population group. Our results underline the need for zinc supplementation in patients undergoing hemodialysis as all patients had suboptimal serum status of zinc including those with adequate dietary intake. Zinc supplementation with high doses that cannot be provided by diet in hemodialysis patients in combination with n-3 PUFA supplements potentially could induce even more favorable change in fatty acids profiles than n-3 PUFAs treatments alone. Importantly, zinc deficiency with an altered n-3 fatty acid profile can affect disease progression and associated with various risk for CVD. More interventional studies are warranted to confirm the benefits of zinc and PUFA supplementation in hemodialysis patients for the prevention and attenuation of adverse health outcomes.

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 Ethical Review Board of the Military Medical Academy, Belgrade, Serbia. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MT contributed reagents/materials/analysis tools and performed the statistical analysis and review. MZ accountable for dietary data analysis, presentation of data, review, and editing. BT and MM data collected and review. AS completed mineral analysis, reviewing, and editing. SR responsible for carrying out the human study, for its design and coordination, and editing. DRM conceptualization/study design, methodology, manuscript preparation, review, and editing. All authors contributed to the article and approved the submitted version.

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