Libyan Journal of Medicine

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/zljm20

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To cite this article: Nevena Vidović, Marija Knez, Marija Ranić, Fathia Faid, Dragana Jović, Marija Glibetic & Marija Takić (2023) Comparative assessment of erythrocyte sphingolipid levels as potential cardiovascular health markers in women from Libya and Serbia: a small-scale study, Libyan Journal of Medicine, 18:1, 2264568, DOI: 10.1080/19932820.2023.2264568

To link to this article: https://doi.org/10.1080/19932820.2023.2264568

Published online: 07 Oct 2023.

LIBYAN JOURNAL OF MEDICINE
2023, VOL. 18, 2264568
https://doi.org/10.1080/19932820.2023.2264568

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ORIGINAL ARTICLE

Comparative assessment of erythrocyte sphingolipid levels as potential cardiovascular health markers in women from Libya and Serbia: a small-scale study

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ABSTRACT

Aim: Cardiovascular diseases (CVDs) represent the major cause of morbidity and mortality worldwide including Libya, where they account for 43% of all deaths. Sphingolipids are involved in the pathology of numerous diseases including cardiovascular diseases and are proposed as potential biomarkers of cardiovascular health that could be more effective compared to traditional clinical biomarkers. The aim of this study was to determine the sphingolipid content in the erythrocyte membrane of Libyan migrant and Serbian resident women. In addition, to examine if sphingolipid levels could be used as a novel indicator of cardiovascular risk, we evaluated possible correlations with some well-established biomarkers of cardiovascular health.

Materials and Methods: A total of 13 Libyan and 15 Serbian healthy women participated in the study. The high-performance version thin-layer chromatography (HPTLC) using the image analysis tool JustTLC was applied for quantification of erythrocytes' sphingolipids.

Results: Lower mean values of erythrocytes' sphingolipids and cholesterol concentrations were found in the group of Libyan emigrants compared to Serbian resident women. Besides, in this group of apparently healthy women ($n = 28$), the sphingolipid content of erythrocytes was inversely related to the Omega-3 index (r =-0.492, $p = 0.008$) and directly linked to vitamin D status ($r = 0.433$, $p = 0.021$) and membrane cholesterol levels ($r = 0.474$, $p = 0.011$).

Conclusion: The erythrocytes' sphingolipid levels should be measured/assessed as an additional biomarker of CV health, by applying a simple and routine method. Still, further investigation in a larger population-specific context is warranted.

ARTICLE HISTORY
Received 13 June 2023
Accepted 25 September 2023

KEYWORDS
Cardiovascular health; Libyan women; membrane cholesterol; Omega-3 index; sphingolipids; vitamin D

1. Introduction

As the World Health Organization (WHO) estimated, cardiovascular diseases (CVDs) accounted for 17.9 million deaths globally in 2019, which represented 32% of all deaths, while strokes and heart attacks were responsible for 85% of these deaths [1]. According to another source, in 2017, CVDs were responsible for 17.8 million deaths worldwide, corresponding to 330 million years of life lost and an additional 35.6 million years lived with disability [2]. Cardiovascular diseases remain a major burden, it is also the leading cause of illness and death in Libya, contributing to 43% of all deaths in year 2014 [3]. Similarly, according to the 2017-year statistics, CVD accounted for 45% and 37% of all deaths in Europe and European Union, respectively [4]. According to a recent study, in 2019 CVD represented the major cause of death in Serbia accounting for 51.8%, while 17.7% died from ischemic heart disease [5]. Considering that cardiovascular diseases are the main cause of morbidity and mortality in their country, the Libyan Cardiac Society recently recommended that cardiovascular diseases should be given high priority in the health system to develop a program for efficient prevention, early diagnosis, and treatment [6].

Sphingolipids are identified as potential biomarkers of cardiovascular health that could be more effective compared to traditional clinical biomarkers such as serum LDL cholesterol and triglyceride levels. This is based on the observation that nearly every single sphingolipid from this class of compounds measured in patients with coronary artery disease was significantly increased compared to the values in the control population [7]. As demonstrated over the years, sphingolipids are involved in a variety of diseases, especially cardiovascular diseases (CVD) with the balance between the two main sphingolipids ceramide and sphingosine-1-phosphate being disturbed in myocardial infarction, and stroke [8]. At the same time, in some genetic disorders, disturbed sphingolipid metabolism leads to abnormal accumulation of

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sphingolipids in many types of tissues contributing to cardiovascular complications [9,10]. As suggested by Choi et al. (2021) circulating ceramide and total sphingolipid levels in humans have emerged as biomarkers that can predict cardiometabolic complications, such as coronary artery disease, heart failure, and diabetes [11]. Monitoring sphingolipid levels and their alterations could enable better assessment of progression and severity of cardiovascular disease, and thus, these biomarkers could serve as targets for potential future therapeutic approaches [12]. A limited number of studies investigated phospholipid fractions and sphingolipid content in erythrocyte membranes, although they may reflect the lipid profile over a long period of time. In two studies investigating phospholipid composition of erythrocyte membranes, the ratio of sphingolipids to phosphatidylethanolamine was increased in Alzheimer's disease [13] and schizophrenia patients [14]. Lower eicosapentaenoic fatty acid (EPA) plus docosahexaenoic fatty acid (DHA) to arachidonic fatty acid ratio in erythrocyte sphingomyelin (of the sphingolipid class) was correlated with worse depressive symptoms in patients with coronary artery disease [15].

Some other factors such as vitamin D status [16], Omega-3 index, defined as the sum of two major omega-3 fatty acids, EPA and DHA [17], and blood cholesterol levels are also found to be associated with cardiovascular health [18]. Besides, serum cholesterol, content of cholesterol in erythrocyte membranes can serve as an indicator of cardiovascular health [19]. Membrane cholesterol levels may be elevated in patients with type 2 diabetes, linking diabetes and associated cardiovascular complications [20]. Also, the cholesterol content of erythrocyte membranes has been identified as a marker of atheromatous growth in patients with coronary disease and it has been suggested as a novel marker of clinical instability [21]. Membrane cholesterol levels are not only considered a risk factor for cardiovascular disease but may also form strong bonds with sphingolipids in membranes [22,23].

In our previous work, we investigated vitamin D status and some related parameters (like blood lipids and magnesium levels) in small groups of Libyan migrants and women residing in Serbia [24]. We found that the average vitamin D intake was below the proposed dietary reference values in both groups accompanied by its low serum level. More favorable cardio-metabolic status was found in the selected group of Libyan women, as their serum cholesterol levels were lower, and the content of omega-3 fatty polyunsaturated fatty acids (PUFA), as well as Omega-3 index was higher in erythrocytes compared to the group of Serbian women.

Taking into account these facts, the aim of our study was to determine sphingolipid levels in the erythrocyte membrane of Libyan migrant and Serbian women. In addition, we wanted to evaluate if sphingolipid level could be considered as a novel indicator of cardiovascular risk, by analyzing its possible correlations with well-established biomarkers of cardiovascular health.

2. Methods

2.1. Participants and study design

A total of 13 Libyan emigrant and 15 Serbian healthy women participated in the study. Participants' general characteristics and study design were described in detail previously [24]. Written informed consent was obtained from all participants prior to the enrolment, and the study was undertaken according to the Helsinki Declaration 1983 principles (approved by the Clinical Hospital Centre Zemun, Belgrade, Serbia, E0120/2017 and E0123/2017).

2.2. Total lipid extraction and gas chromatography analysis of erythrocytes' phospholipids fatty acids

Total lipid extracts were prepared from erythrocytes using the organic solvents, chloroform, and isopropanol (7:11, by volume) [25]. The analysis of fatty acids is described in detail in the previous study [24]. Omega-3 Index was calculated as the sum of eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) percentages of totally detected fatty acids.

2.3. Separation of phospholipids

Solutions of individual phospholipids phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, and sphingomyelin (Sigma-Aldrich, Germany) were prepared in chloroform:methanol 2:1 (v/v). The concentrations of sphingomyelin used for the calibration curve were as follows: 0.1, 0.13, 0.2, 0.25, 0,333, 0.4, 0.5 mg/ml. Erythrocytes' samples for HPTLC analysis were prepared from total lipid extracts. They were evaporated in nitrogen steam, and the residue was dissolved in the same organic solvents' mixture (extracts from 100 µl of erythrocytes were dissolved in 20 µl of mixture).

The original commercial ALUGRAM sheets SIL G/UV 254 plates (Macherey-Nagel, Germany) were cut to dimensions 10 × 10 cm. The developing chamber was commercial glassware, and the saturation time was 1 h. The sample volumes of 5 µl were applied onto HPTLC plates using micro syringes as 0.7 cm bands. A chloroform:ethyl-acetate:n-propanol:methanol:0.25% aqueous potassium chloride mixture (25:25:25:13:9, v/v/v/v/v) [26] was used as the mobile phase for separation of phospholipids that run for about 45 min. The plates were slowly dried in the air for 15 min. The HPTLC plates were then stained using modified copper(II) sulphate reagent as described previously [27]. Briefly, the

developed dried plates were immersed in the reagent for 6 s and 10% copper(II) sulphate pentahydrate in methanol with the addition of 8 ml of ortho-phosphoric and 8 ml of sulfuric acid per 200 ml of staining solution. Then, it was dried with cold air using the hairdryer for the 30 s and after that heated at 140°C in oven for 30 min. The HPTLC plates were stained using methanol with 8 ml of sulfuric acid added in 200 ml of solvent using the same staining procedure as for the modified copper(II) sulphate reagent [27].

2.4. Scanning of TLC plates and digital image analysis with densitometric evaluation

Images of stained HTLC plates were made using a flatbed scanner (HP ScanJet 3800) and were stored as TIFF images. A free online available software, JustTLC (JustTLC Version 4.6, Sweday, Lund, Sweden), was employed as a tool for the analysis of TLC and 1D gel images.

2.5. Determination of cholesterol in erythrocyte membrane lipid extracts

Cholesterol concentrations were determined in total lipid extracts of erythrocytes by Liebermann–Burchard reaction [28].

2.6. Statistical analysis

The normal distribution of data was tested by the Shapiro–Wilk test, and data were presented as mean ± (SD) for the normally distributed variables. The independent sample t-test was applied for comparisons between the groups of Libyan and Serbian apparently healthy women. Associations of sphingolipids and other analyzed parameters were evaluated by Pearson's correlation analysis. All data analyses were performed using the SPSS statistical software (ver. 20.0) and p values of <0.05 were considered statistically significant.

3. Results

3.1. Separation of phospholipids

The most abundant phospholipids in the erythrocyte membrane were sphingolipids, phosphatidyl-choline, phosphatidyl-serine, and phosphatidylethanolamine. Their effective separation was achieved in one dimension on un-modified HPTLC silica gel 60 with chloroform: n-propanol: ethyl acetate: methanol: 0.25% aqueous potassium chloride (25:25:25:13:9, v/v/v/v/v) as shown in Figure 1.

The obtained retention factor (Rf) values for erythrocytes' phospholipids were as follows: 0.160 for sphingomyelin, 0.240 for phosphatydilcholine, 0.280 phosphatidylserine, and 0.480 for phosphatidylet hanolamine.

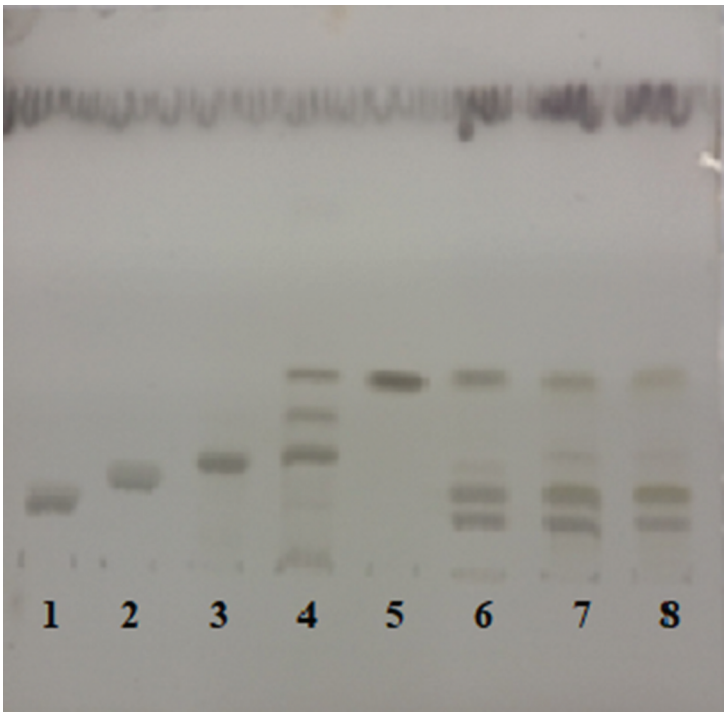

Figure 1. Separation of phospholipids on un-modified HPTLC silica gel 60 plates; 1: sphingomyelin, 2: phosphatydilcholine, 3: phosphatidylserine, 4: phosphatydilethanolamine, 5, 6, 7, 8: total lipid extracts of erythrocytes phosphatidylserine.

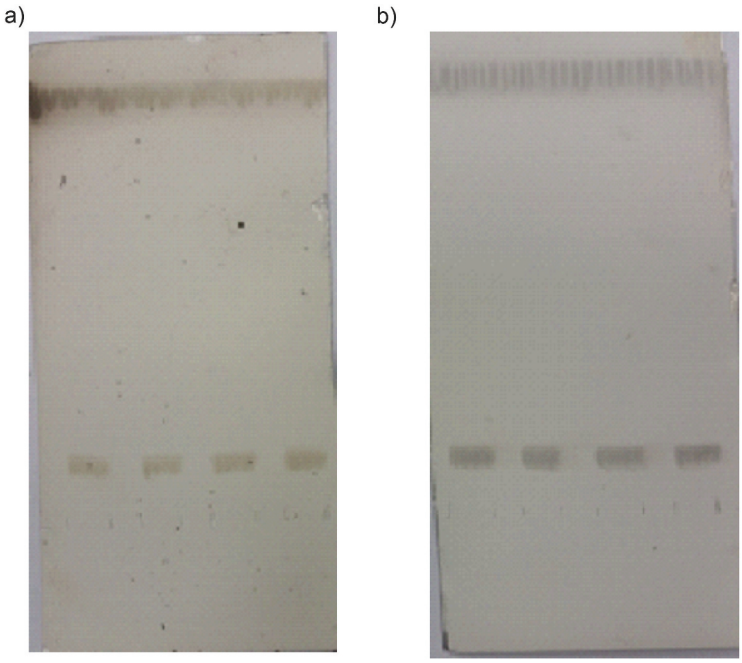

Figure 2. Staining of HPTLC plates with a) modified copper (II) sulphate reagent b) sulfuric acid in methanol, sample 2.5 µg of sphingomyelin standard quadruplicate.

3.2. Staining of HPTLC plates with sulfuric acid in methanol and modified copper reagent

The staining of plates with two different reagents is shown in Figure 2. Modified copper(II) reagent gave a higher response for sphingomyelin in comparison to two staining methods, and analysis of plates using the Just Quantify program showed that the response was 1.727 times higher ($p < 0.001$) for modified copper(II) reagent staining compared to staining with sulfuric acid in methanol (each sample was stained six times with both methods).

3.3. Erythrocytes' sphingolipids and cholesterol content

Differences in erythrocyte levels of Omega-3 Index, sphingolipids, and cholesterol levels between groups of Libyan and Serbian women are shown in Figure 3. Libyan women had significantly higher Omega-3 Index values and lower levels of erythrocytes' sphingolipids and cholesterol.

3.4. Association between erythrocytes' sphingolipids and cardio-metabolic risk factors

Correlations between sphingolipids with serum vitamin D level, Omega-3 index, and membrane cholesterol are presented in Figure 4. We observed a significant negative association among erythrocyte sphingolipids content and Omega-3 index of erythrocytes (r =-0.492, p = 0.008). At the same time, positive correlations between sphingolipids with vitamin D status (r = 0.433, p = 0.021) and membrane cholesterol (r = 0.474, p = 0.011) were found. Sphingolipids were not significantly associated with serum total, LDL-, and HDL-cholesterol levels (data not shown).

4. Discussion

In the present study, the levels of sphingolipids and cholesterol in erythrocytes differed significantly between the groups of Libyan and Serbian women, with lower levels found in the first group. Accordingly, Libyan women had significantly higher Omega-3 Index values. In addition, significant correlations were found between the level of sphingolipids in erythrocytes and some already known cardiovascular risk factors such as membrane cholesterol, omega-3 index, and vitamin D, suggesting that sphingolipids in erythrocytes could be used as an additional marker of cardiovascular disease. This is an important finding as CVD represents the major cause of disability and mortality worldwide, and the number of people suffering from CVD increases.

The lower cholesterol and sphingolipid levels in the red blood cell membrane observed in Libyan women suggest a lower cardiovascular risk in this group, which agrees with the data of our previous study in which lower serum cholesterol levels were found in the group of Libyan women compared with Serbian women [24]. The association between blood cholesterol and CVDs is well established, and lowering of serum low-density lipoprotein (LDL)-cholesterol is identified as a primary target of dietary and pharmaceutical therapies [29]. At the same time, an inverse

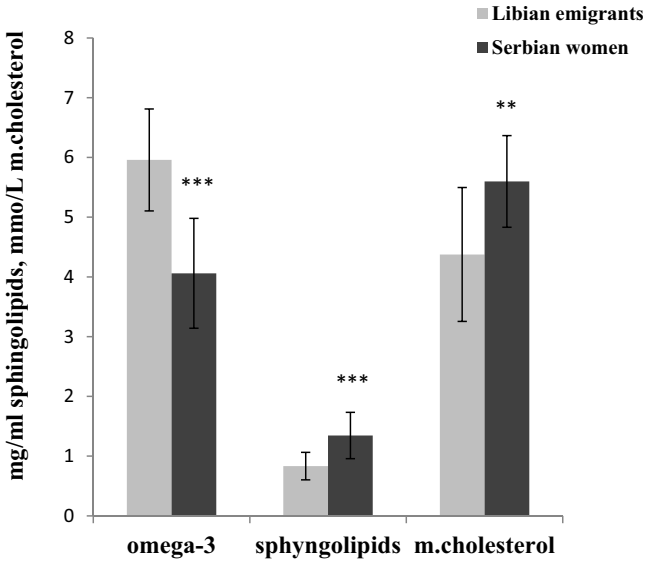

Figure 3. Erythrocyte's content of sphingolipids and cholesterol in Libyan emigrants and resident women in Serbia; $^{**}p < 0.01$, $^{***}p < 0.001$; data presented as mean ± (SD).

association was seen between the risk factors for heart disease and concentrations of high-density lipoprotein (HDL)-cholesterol levels [18]. Besides serum cholesterol, measurement of membrane cholesterol has become increasingly popular in the last few decades, as the mechanisms that lead to increased cholesterol levels in erythrocyte membranes have been intensively investigated. Membrane sphingomyelin shows a molecular attraction for cholesterol and seems to play an important role in the regulation of unesterified cholesterol 'trapping' within membranes [22,23]. Moreover, the erythrocytes cholesterol levels were higher in acute compared to stable coronary artery disease patients confirming its role as a risk factor in cardiovascular diseases [21]. We found a positive correlation between the erythrocytes' sphingolipids and membrane cholesterol levels, with no statistically significant associations with total, LDL- and HDL-cholesterol. As a recent review article has underlined, the growing number of studies emphasizes the role of sphingolipids in CVD pathophysiology, suggesting that sphingolipid levels could serve as valuable indicator of cardiovascular health in clinical practice [30].

Furthermore, there is an association between vitamin D deficiency and an increased risk of CVD, including hypertension, heart failure, and atherosclerosis [16]. In our previous study, dietary intake and status of vitamin D in Libyan migrant and Serbian resident women was below recommended levels for this population group [24]. Interestingly, we found a direct correlation between serum vitamin D status and erythrocytes' sphingolipid levels in this study. This was in line with previously detected lower vitamin D level in group of Libyan women and in the present study reported lower sphingolipid levels in the same group of women. Obtained results are in accordance with the findings of two recent studies that explored the relationship between the serum sphingolipids and vitamin D status. Al-Daghri and al. (2019) reported that total serum sphingomyelins that comprise about 78% of total serum sphingolipids were lower in normal weight and obese vitamin D deficient hyperlipemic individuals [31]. At the same time, Chen et al. (2020) found that vitamin D_3 supplementation increased serum levels of stearoyl-ceramide and stearoyl-sphingomyelin in a dose-dependent fashion among overweight/obese African Americans [32]. The results of these two studies suggest that vitamin D status is related to serum sphingolipid levels, but to our knowledge there are no literature data for sphingolipids in erythrocytes. Additionally, studies investigating the mechanisms of vitamin D3 action suggested its role in the sphingolipid signaling pathways. More precisely, treatment of cells with 1,25-dihydroxyvitamin D3 could activate sphingomyelin turnover and increase the level of sphingolipid metabolite ceramide [33]. Based on these results, we hypothesized that vitamin D might play a role in sphingolipid metabolism and also alter the sphingolipid content of erythrocytes.

Omega-3 Index, defined as the sum of EPA and DHA percentage in total lipids, has been associated with health conditions such as acute coronary syndrome, coronary disease, and sudden cardiac arrest. The proposed cut-points for Omega-3 Index values are <4% for 'high risk' and >8% for 'low risk' for cardiovascular disease supported by findings for fatal coronary heart disease [17]. In this study, we found a statistically significant inverse correlation between the erythrocytes' total sphingolipids and

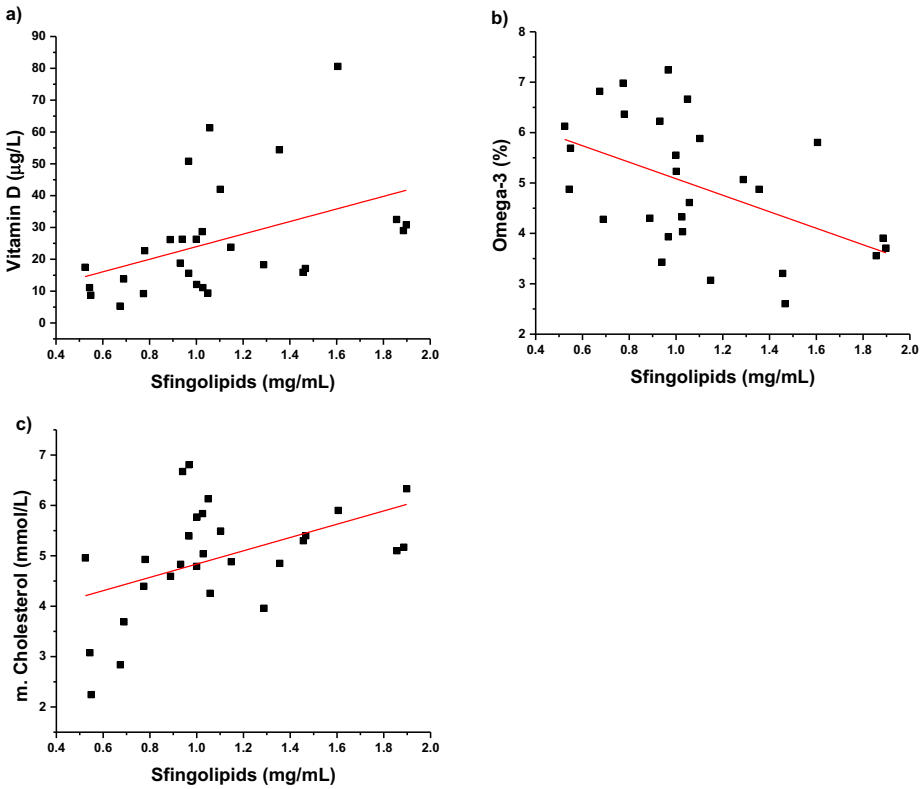

Figure 4. Correlations between sphingolipids and A) vitamin D (r = 0.433, p = 0.021), B) Omega-3 index (*r* =-0.492, p = 0.008) and C) membrane cholesterol (r = 0.474, p = 0.011).

Omega-3 Index. This is consistent with the observed lower sphingolipid levels in the group of Libyan women and the previously reported higher omega-3 index in the same group [24]. This is consistent with the results of several recent studies that have demonstrated a relationship between omega-3 status and sphingolipids in serum as well as in tissues – liver and muscle [34–36]. Duran et al. (2019) reported that omega-3 PUFAs dietary supplementation in individuals with type 2 diabetes leads to a reduction of sphingosine levels in the plasma [34]. The authors also demonstrated improvement of neuropathic pain symptoms in patients after a dietary treatment with omega-3 PUFAs and hypothesized that this was achieved by reversing lipotoxicity-induced sphingolipid-metabolite overload [34]. Omega-3 PUFA supplementation contributed to a positive change in triglyceridemia and glycemia, suggested to be induced through the regulation of hepatic and muscle sphingolipid synthesis [35]. Ferchaud-Roucher et al. (2020) found that omega-3 PUFA led to improvements in apoB100 metabolism and correlated with changes in sphingolipids [36].

In the present study, the HPTLC technique using the image analysis tool JustTLC (JustTLC Version 4.6, Sweday, Lund, Sweden) for quantification of erythrocytes' sphingolipids was applied. It is important to mention that this technique does not involve complicated set-up, so it could be easily implemented in any laboratory for routine analysis. As the effective separation of main erythrocytes' phospholipids was achieved in one dimension, this method allows rapid analysis of a large number of samples. Using Bitman et al. (1990) mobile phase good separation of sphingolipids from other main erythrocytes' phospholipid fractions was obtained [26]. Also, staining the plates with a 10% copper sulphate reagent in methanol gives a higher response to sphingolipids compared to staining with sulphuric acid in methanol.

The data obtained in this study, although tested in a small group of women, confirm the original hypothesis about the potential use of sphingolipid levels as an indicator of cardiovascular health and its relationship to some of the already known cardiovascular risk factors. In addition, we have shown that sphingolipids can be separated and quantified by an already established method that provides reliable and rapid results, making it suitable for routine analysis of a large number of samples. Regardless of small sample size, we included subjects from populations that have been shown to have a high incidence of cardiovascular disease and mortality. Although our data suggest that sphingolipid levels in erythrocytes could be used as a promising biomarker of cardiovascular health, further investigation in larger population cohorts is certainly warranted.

5. Conclusion

In the present study, which was, according to our knowledge, the first to examine sphingolipid levels of erythrocyte membranes of Libyan and Serbian women, we found significant association of erythrocytes sphingolipid levels with certain biomarkers of cardiovascular health, the Omega-3 Index, membrane cholesterol, and vitamin D levels. In addition, significantly lower sphingolipid and membrane cholesterol levels in erythrocytes were found in the group of Libyan women, which was consistent with the previously reported lower cardiovascular risk of this group compared with the group of Serbian residents. Considering the obtained results, sphingolipid level in erythrocytes could be an additional biomarker of CV health which can be determined by a simple and routine method. Further studies in a broader population-specific context are needed to examine the full efficacy of this newly proposed biomarker in different population groups and in different settings.

Acknowledgments

This work was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (contract number 451-03-47/2023-01/200015).

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The author(s) reported there is no funding associated with the work featured in this article.

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