

Erythrocyte Membrane Fatty Acid Composition in Premenopausal Patients with Iron Deficiency Anemia

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Abstract: Iron deficiency anemia (IDA) is one of the most common nutritional disorders in the world. In the present study, we evaluated erythrocyte membrane fatty acid composition in premenopausal patients with IDA. Blood samples of 102 premenopausal women and 88 healthy control subjects were collected. After the erythrocytes were separated from the blood samples, the membrane lipids were carefully extracted, and the various membrane fatty acids were measured by gas chromatography (GC). Statistical analyses were performed with the SPSS software program. We used blood ferritin concentration <15 ng/mL as cut-off for the diagnosis of IDA. The five most abundant individual fatty acids obtained were palmitic acid (16:0), oleic acid (18:1, n-9c), linoleic acid (18:2, n-6c), stearic acid (18:0), and erucic acid (C22:1, n-9c). These compounds constituted about 87% of the total membrane fatty acids in patients with IDA, and 79% of the total membrane fatty acids in the control group. Compared with control subjects, case patients had higher percentages of palmitic acid (29.9% case versus 25.3% control), oleic acid (16.8% case versus 15.1% control), and stearic acid (13.5% case versus 10.5% control), and lower percentages of erucic acid (11.5% case versus 13.6% control) and linoleic acid (15.2% case versus 15.4% control) in their erythrocyte membranes. In conclusion, the total-erythrocyte-membrane saturated fatty acid (SFA) composition in premenopausal women with IDA was found to be higher than that in the control group; however, the total-erythrocyte-membrane unsaturated fatty acid (UFA) composition in premenopausal women with IDA was found to be lower than that in the control group. The differences in these values were statistically significant.

Key words: anemia, iron deficiency, erythrocyte membrane, fatty acid, premenopausal patients

1 INTRODUCTION

Organisms contain iron in the ferrous (Fe^{2+}) or the ferric states (Fe^{3+}) in their blood¹. Serum iron content is dependent on age, sex, geographic region, race, and socio-economic status². The iron concentration in the blood of adults is approximately 2 to 4 g; this iron is located in hemoglobin, myoglobin, ferritin, transferrin, and some enzymes. Iron-containing enzymes are involved in numerous metabolic pathways, including those related to protein and lipid metabolism^{3, 4}. Iron is associated with fatty acid desaturase enzymes, which play an integral role in fatty acid synthesis.

According to World Health Organization (WHO), one of the most common nutritional disorders in the world is iron deficiency anemia (IDA), prevalent in both developed as well as developing countries⁵. Iron deficiency may occur at any stage of the lifecycle; however, women are the most

commonly affected because of their high iron requirements to maintain reproductive function, additionally, during pregnancy, and lactation. Iron deficiencies are associated with decreased general health and increased exhaustion. When iron deficient women were supplemented with iron or advised to consume a diet rich in bioavailable sources of iron, their iron status tended to improve⁶. The status of iron in individuals is known to influence fatty acid metabolism in the plasma and their composition in cell membranes⁷. In view of the extensive involvement of iron in metabolism, it is not surprising that iron deficiency results

Abbreviations: IDA: Iron deficiency anemia, UFA: Unsaturated fatty acids, PUFA: Polyunsaturated fatty acids, ROS: Reactive oxygen species, BHT: Butylated hydroxytoluene, FAME: Fatty acid methyl esters, GC: Gas chromatography, FID: Flame ionization detector, SFA: Saturated fatty acid, MUFA: Mono unsaturated fatty acid, GR: Glutathione reductase, MDA: Malondialdehyde

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in a series of adverse effects. The most common symptoms of iron deficiency are anemia and diminished work capacity^{3,4)}.

Unsaturated fatty acids (UFA) are known to help increase membrane fluidity⁸⁾. Loss of polyunsaturated fatty acids (PUFA) results in excessive membrane rigidity, and can alter the conformation and functioning of proteins, receptors, and ion channels^{7,9)}. IDA causes increased production of reactive oxygen species (ROS), especially free radicals in the red blood cells^{3,10)}. High levels of ROS and free radicals in erythrocytes can lead to damage of the antioxidant defense system. This leads to lipid peroxidation and increased cellular damage¹¹⁾.

Fatty acid compositions in erythrocyte membranes have been previously studied in some disorders; such as type 1 diabetes, depressive patients, cognitive decline, and schizophrenia^{8, 11–13)}; however, our literature search showed that those in premenopausal patients with IDA had not yet been reported. Therefore, the objective of this study was to evaluate the erythrocyte membrane fatty acid composition in premenopausal patients with IDA. We also studied the serum glutathione reductase (GR) activity, which is one of the indicators of oxidative stress. GR enzyme activity and its connection with the erythrocyte membrane fatty acid levels in patients and healthy subjects have been discussed; in particular, the relationship with UFA in premenopausal patients with IDA was analyzed.

2 MATERIALS AND METHODS

2.1 Chemicals

Methanol, chloroform, hexane, n-heptane, boron trifluoride (BF₃), and potassium chloride (KCl) were obtained from Merck. All other chemicals were analytical grade and obtained from Sigma-Aldrich Company.

2.2 Study Design

The study protocol was approved by the Medical Ethics Committee of Erzincan University (Mengucek Gazi Training and Research Hospital School, Erzincan, Turkey). Written informed consent was obtained from all participants.

Between February and June 2012, this cross-sectional study was performed in 102 premenopausal women with IDA (mean age: 39.3 ± 12.1 years) and 88 healthy control subjects (premenopausal women; mean age: 35.1 ± 13.1 years).

We evaluated each patient (age range: 18 to 55 years) for the presence of IDA, and their medical records were reviewed to assess them for enrollment into the study. For IDA diagnosis, concentration of blood ferritin < 15 ng/mL was accepted as the cut off inclusion criteria¹⁴⁾. Exclusion criteria included anemia other than iron deficiency, pregnancy, blood-transfused patients in the last 6 months, doc-

umented coronary artery disease, congestive heart failure, active and chronic infection, and autoimmune disease. Hundred and two premenopausal women with IDA were included in the study. Healthy, age and sex-matched premenopausal individuals referred from outpatient clinics of the Internal Medicine Department of Erzincan University (*n* = 88) were taken as control subjects. They had to meet the same inclusion and exclusion criteria as the case patients.

2.3 Blood Sampling and Laboratory Analyses

Venous blood samples for biochemical analyses were collected from the 102 patients with IDA and from the 88 healthy subjects. All biochemical analyses, including those for the hemogram, hematocrit, and serum ferritin levels, were undertaken using an oxidase-based technique by the Roche/Hitachi Modular System (Mannheim, Germany) in the Central Biochemistry Laboratory of the Erzincan University Mengucek Gazi Training and Research Hospital.

2.4 Preparation of the Hemolysate

Erythrocytes were purified from fresh human blood obtained from the Blood Centre of Erzincan Hospital. The blood samples (2 mL) were centrifuged at 2,250 × *g* for 15 min; the serum and buffy coat were removed. The packed red cells were washed three times with KCl (0.16 M) and hemolyzed with an equal volume of ice-cold water, and then centrifuged (4°C, 10,000 × *g* for 30 min) to remove the blood cell ghosts and intact cells¹⁵⁾.

2.5 Measurement of Glutathione Reductase (GR) Activity

Beutler's method was slightly modified for the measurement of enzymatic activity¹⁵⁾. One enzyme unit was defined as the oxidation of 1 μmol NADPH per min under the assay conditions, at 25°C.

2.6 Extraction of Erythrocyte Membrane for Lipids

Total lipids were extracted from erythrocyte membranes through a modification of the Folch method¹⁶⁾. An aliquot of the membrane suspension (500 μL) was mixed with 2 mL of methanol containing 50 μL of butylated hydroxytoluene (BHT) solution (1 mg/mL ethanol) as antioxidant, and shaken vigorously for 40 seconds. Chloroform-methanol (2/1; 1 mL) was added while shaking the mixture for an additional 20 seconds. The extract was washed with 1 mL of 0.9% NaCl. After the mixture separated into two phases, the chloroform layer was transferred into a tube and evaporated to dryness under nitrogen. The residue containing membrane lipids was obtained for preparation of fatty acid methyl esters.

2.7 GC-FID Condition and Analysis of Fatty Acids

Membrane lipids were saponified by standard procedures according to the Standard IUPAC methods¹⁷⁾. Fatty acids

were esterified by 10% (v/v) BF_3 -MeOH as reagent. The fatty acid methyl esters (FAME) of total lipids were obtained by transmethylation¹⁸⁾. Gas chromatography (GC) analyses were performed using a Perkin Elmer Clarus 500 Series GC system, in split mode, 50:1, equipped with a flame ionization detector (FID) equipped with a TR-FAME (Thermo Scientific) apolar capillary column (30 m $\times$ 0.25 mm ID and 0.25 μm film thickness). Helium (0.5 mL/min) was used as the carrier gas. The injector temperature was set at 250°C and the FID was operated at 260°C. An initial column oven temperature of 100°C was elevated to 220°C at a rate of 2°C/min and held for 0 min. Identification of fatty acid components was accomplished by comparison of their retention times with those of authentic standards (Supelco 37 Comp. Fatty acid Mix, 18919). The relative peak area percentages of compounds were calculated on the basis of FID data.

2.8 Statistical Analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (Windows version 21.0: SPSS). Data have been reported as mean $\pm$ standard error mean. Statistical differences between the two groups for parametric data were analyzed using Student t-test. For obtaining nonparametric data, the Mann-Whitney U-test and Kruskal-Wallis test were used. $p < 0.05$ was considered significant for all tests.

3 RESULTS

The baseline characteristics of the 102 patients with IDA and the 88 healthy subjects are shown in Table 1. There were no age differences between patients with IDA and the healthy subjects. The indicator of lipid peroxidation was the presence of malondialdehyde (MDA) and antioxidant enzymes, including GR, in the serum. In this study, GR activity was measured to evaluate lipid peroxidation. We found that patients with IDA had significantly lower levels of hemoglobin, hematocrit, and serum ferritin (Table 1). To our knowledge, this is the first study to evaluate the fatty acid composition of erythrocyte membranes in premeno-

pausal patients with IDA.

We found 31 individual fatty acids in the erythrocyte membranes of both case and control groups (Table 2). In both groups, the five most abundant individual fatty acids were palmitic acid (16:0), oleic acid (18:1, n-9c), linoleic acid (18:2, n-6c), stearic acid (18:0), and erucic acid (C22:1, n-9c). These compounds constitute about 87% of total membrane fatty acids in patients with IDA, and 79% of total membrane fatty acids in the control group. Compared with control subjects, case subjects had higher percentages of palmitic acid (29.9% case versus 25.3% control), oleic acid (16.8% case versus 15.1% control), stearic acid (13.5% case versus 10.5% control), and lower percentages of erucic acid (11.5% case versus 13.6% control) and linoleic acid (15.2% case versus 15.4% control) (Table 2).

Figure 1 shows the mean percentages of saturated and unsaturated fatty acid composition of erythrocyte membranes in both case and control groups. Total saturated fatty acid (SFA) content in the red blood cell membranes of premenopausal patients with IDA (45.45%) was found to be higher than that in the control group (39.98%).

Total UFA content in the red blood cell membranes of premenopausal patients with IDA (54.49%) was found to be lower than that in the control (59.79%) group. The differences in the values of UFA content obtained were statistically significant ($p < 0.05$) between the two groups (Fig. 1 and Table 2). Although the total mono-unsaturated fatty acid (MUFA) content in the erythrocyte membranes of premenopausal patients with IDA (33.02%) was considerably lower than that in the control group (39.53%) ($p < 0.05$), the total PUFA content was similar in both the groups and the results were not statistically significant ($p > 0.05$).

The total omega-3 (n-3) fatty acid level in erythrocyte membranes of subjects in the case group (3.95%) was higher than that in subjects of the control group (3.59%); the differences in n-3 levels between the two groups were not statistically significant (Fig. 2). The total omega-6 (n-6) fatty acid level in erythrocyte membranes of the case group subjects (20.58%) was lower than that in the control group subjects (22.77%); the differences in n-6 levels between the two groups were significant statistically (Fig. 2 and Table 2).

Table 1 Demographic and laboratory results of patients (IDA) and control groups³¹⁾.

Parameters	Patients with IDA (n=102)	Control (n=88)	P value*
Age (years)	39.3 $\pm$ 12.1	35.1 $\pm$ 13.1	0.083
Hemoglobin (mg/dL)	9.4 $\pm$ 1.5	13.9 $\pm$ 1.3	<0.001
Hematocrit (%)	30.7 $\pm$ 4.1	42.3 $\pm$ 3.53	<0.001
Ferritin (ng/mL)	3.60 $\pm$ 2.7	53.5 $\pm$ 41.7	<0.001
Glutathion Reductase (U)	3.16 $\pm$ 3.34	1.11 $\pm$ 3.05	<0.001

*Student t-test

Table 2 Percentage of fatty acids composition of erythrocyte membrane of patient (IDA) and control. (ns: not significant).

Systematic Name	Common Name	Case (IDA) (n:102)		Control (n: 88)		Significant (<i>p</i>)
		Mean	SEM	Mean	SEM	
C4:0	Butyric acid	0.22	0.06	0.49	0.06	ns
C6:0	Caproic Acid	0.19	0.06	0.05	0.02	ns
C8:0	Caprylic Acid	Nd		0.58	0.20	0.002
C12:0	Lauric Acid	0.32	0.27	0.36	0.33	ns
C14:0	Myristic Acid	0.20	0.03	0.23	0.04	ns
C14:1	Myristoleic Acid	Nd		0.19	0.09	0.03
C15:0	Pentadecanoic Acid	0.20	0.02	0.92	0.29	0.01
C15:1(n-10)	Pentadecenoic Acid	3.06	0.83	6.11	0.72	0.01
C16:00	Palmitic Acid	29.91	0.54	25.37	0.55	0.001
C16:1(n-7c)	Palmitoleic Acid	0.46	0.04	0.78	0.08	0.001
C17:0	Heptadecanoic Acid	0.48	0.05	0.54	0.09	ns
C18:0	Stearic Acid	13.53	0.33	10.59	0.34	0.001
C18:1(n-9c)	Oleic Acid	16.83	0.35	15.10	0.34	0.05
C18:1 (n-9t)	Elaidic Acid	0.82	0.04	0.81	0.11	ns
C18:2 (n-6c)	Linoleic Acid	15.29	0.55	15.39	0.61	ns
C18:2 (n-6t)	Linolelaidic Acid	0.21	0.08	0.24	0.08	ns
C18:3 (n-6c)	γ -Linolenic Acid	0.63	0.23	0.01	0.01	0.01
C18:3 (n-3c)	α -Linolenic Acid	0.56	0.03	0.49	0.03	ns
C20:0	Arachidic Acid	0.09	0.08	0.24	0.08	0.02
C20:2(n-6c)	Eicosadienoic Acid	1.33	0.26	1.08	0.42	ns
C20:3(n-6c)	Eicosatrienoic Acid	0.5	0.17	0.01	0.01	ns
C20:3(n-3c)	Eicosatrienoic Acid	1.07	0.11	1.01	0.10	ns
C20:4(n-6c)	Arachidonic Acid	Nd		0.30	0.11	0.01
C20:5(n-3c)	Eicosapentaenoic Acid	0.34	0.15	1.02	0.34	ns
C21:0	Heneicosanoic Acid	0.03	0.03	Nd		0.25
C22:0	Behenic Acid	0.10	0.08	0.03	0.02	ns
C22:1(n-9c)	Erucic Acid	11.54	0.41	13.06	0.58	0.03
C22:2(n-6c)	Docosadienoic Acid	0.16	0.10	0.06	0.05	ns
C22:6(n-3c)	Docosahexaenoic Acid	1.98	0.20	1.52	0.18	ns
C24:0	Lignoceric Acid	0.19	0.08	0.14	0.10	ns
C24:1	Nervonic Acid	0.41	0.15	1.02	0.34	0.05
Σ SFA		45.45	0.69	39.98	0.83	0.01
Σ USFA		54.49	0.68	59.79	0.77	0.01
Σ MUFA		33.02	0.66	39.53	0.83	0.01
Σ PUFA		21.47	0.79	20.25	0.80	0.06
$\Sigma \omega$ -3 (n-3)		3.95	0.55	3.59	0.46	ns
$\Sigma \omega$ -6 (n-6)		20.58	0.90	22.77	1.06	0.05

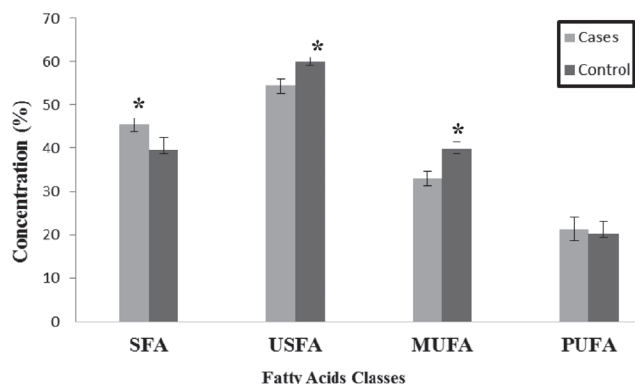


Fig. 1 Concentration of saturated fatty acid and unsaturated fatty acid of erythrocyte membrane of patient with IDA and control (*: The differences are significant statistically).

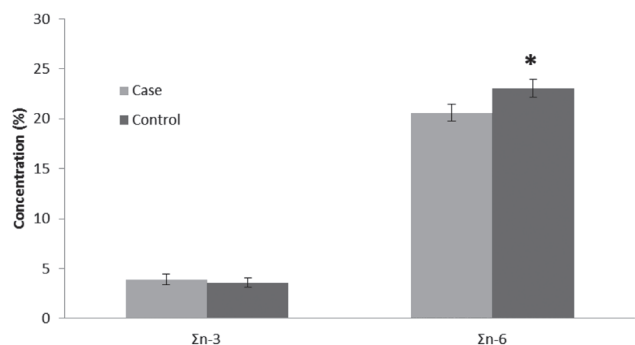


Fig. 2 Concentration of total n-3 and total n-6 of erythrocyte membrane of patient (IDA) and control (*: The differences are significant statistically).

We compared the activity of GR between the patient and the healthy groups (Table 1), and found that the plasma levels of GR were significantly higher in patients with IDA than in the healthy subjects (3.16 ± 3.34 and 1.11 ± 3.05 , respectively; $p < 0.001$).

4 DISCUSSION

4.1 Saturated Fatty Acids

Concentration of palmitic acid (C16:0) in the erythrocyte membranes was found to be 29.91% in the case group subjects and 25.37% in subjects of the control group. The differences of palmitic acid concentration in between the groups were significant statistically ($p = 0.001$) (Table 2). Our results for palmitic acid concentrations were in agreement¹⁹⁾ with those reported in some previous studies, as well as higher^{20, 21)} and lower²²⁾ than those reported in other studies.

Stearic acid (C18:0) concentration was found to be 13.53% in the erythrocyte membranes from the case group

and 10.59% in that from the control group. Stearic acid concentration in this study was lower^{19, 20, 22–24)} as well as in agreement^{26, 27)} to those reported in previous studies, and additionally, they were higher than that reported by Yilmaz *et al.*²⁵⁾.

Other SFA in the erythrocyte membranes had concentrations lower than 1% individually. Therefore, these fatty acids were not meaningful when discussed individually.

4.2 Unsaturated Fatty Acids

Oleic acid (C18:1) concentration in the erythrocyte membranes was found to be 16.83% in subjects of the case group and 15.10% in the control group subjects. The differences of oleic acid concentrations in between the groups were significant statistically ($p = 0.05$) (Table 2). Oleic acid concentrations reported in this study were in agreement^{19, 20, 23, 26)}, as well as higher^{22, 24, 27)} and lower²⁵⁾ than those reported in some previous studies.

Linoleic acid (C18:2) concentration was found to be 15.29% in the erythrocyte membranes in subjects of the case group and 15.39% in those of the control group. The differences in concentrations in between the groups were not significant statistically ($p = 0.89$) (Table 2). Linoleic acid concentrations reported in this study were in agreement^{19, 28)}, as well as higher^{13, 19, 20, 22–24, 27, 29)} and lower^{25, 26)} than those reported in previous studies.

Erucic acid (C22:1) concentration was found to be 11.54% in the erythrocyte membranes of the case group subjects and 13.06% in those of the control group. The differences in concentrations between the groups were significant statistically ($p = 0.03$) (Table 2). Our results for erucic acid concentrations were higher than those reported a previous study²⁷⁾.

Docosahexaenoic acid (C22:6) concentration in the erythrocyte membranes was found to be 1.98% in subjects of the case group and 1.52% in control group subjects. The differences in concentrations between the groups were not significant statistically ($p = 0.09$) (Table 2). Docosahexaenoic acid concentrations reported in this study were in agreement with some previous reports¹³⁾ as well as lower than that reported in other studies^{19, 22, 23, 27, 29, 30)}.

Eicosadienoic acid (C20:2) concentration in erythrocyte membranes was found to be 1.33% in subjects of the case group and 1.08% in the control group subjects. The differences in concentrations between the groups were not significant statistically ($p = 0.61$) (Table 2). Eicosadienoic acid concentration in this study was higher than that reported in some previous studies^{19, 20, 23, 27, 29)} and lower than that reported in another study²²⁾.

Lipid peroxidation is a unique mechanism of oxidative injury in biological membranes, which is triggered and promoted by different radical and non-radical members of the ROS, or occurs through the catalytic decomposition of pre-formed lipid hydroperoxides in tissues by several agents.

Under oxidative stress, the levels of lipid peroxidation products and antioxidant enzymes increase. In our study, plasma GR levels were found to be higher in premenopausal patients with IDA compared to that in healthy subjects³¹⁾. GR catalyzes oxidized glutathione (GSSG) to glutathione (GSH) by using NADPH. GSH also plays an important role in the conversion of Fe^{+2} to Fe^{+3} ³³⁾. A high GSH/GSSG ratio is essential for protection against oxidative stress. Erythrocytes of patients with IDA are more sensitive to agents that are able to induce oxidation of unsaturated fatty acids³²⁾.

While total omega-3 (n-3) levels in the erythrocyte membrane of the patient group were found to be higher than that in the control group, the total n-6 levels in patients were lower than that in the control group. This is because there was greater oxidation of n-6 fatty acids in the patient group.

In conclusion, the total SFA composition in erythrocyte membranes of premenopausal women with IDA was found to be higher than that in the healthy subjects; however, the total UFA composition in erythrocyte membranes of premenopausal women with IDA was found to be lower than that in the healthy subjects. This may be because of increased levels of ROS. According to the results, it can be confirmed that increased serum GR activity reduced UFA concentrations in the erythrocyte membranes of premenopausal women with IDA. Hence, further *in vivo* experimental studies are required to establish this finding.

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