Research Article

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Investigation of erythrocyte membrane lipid profile, oxidative stress and DNA damage parameters in patients with chronic lymphoid leukemia

Kronik lenfoid lösemili hastalarda eritrosit membran lipid profili, oksidatif stres ve DNA hasarı parametrelerinin araştırılması

Bahadır Karakula, Gülsüm Akgün Çağlıyan, Başak Ünver Koluman, Mehmet Nuri Başer, Melek Tunç Ata, Fatih Altıntaş, Emine Kılıç Toprak, Ayşegül Çört

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Abstract

Purpose: Chronic lymphocytic leukemia (CLL) is the most seen type of leukemia in adults. There are few biomarkes that are used for better understanding how oxidative stress is involved in the pathophysiology of hematologic malignency. We aimed to evaulate oxidative stress, DNA damage and erythrocyte membrane lipid profile in CLL patients in this study. The study is included 38 CLL patients and 38 age-sex matched controls.

Materials and method: Total oxidant status (TOS), total antioxidant status (TAS), oxidative stress index (OSI), DNA damage examination with Comet assay, serum 8-OHdG measurement and gas chromatographic analysis were performed between the case and control groups.

Results: It was observed that TOS and OSI values were higher in the case group than in the control group (p=0.014 and p=0.022, respectively). DNA damage measured by Comet method was found to be increased in the case group (p<0.05). Although erythrocyte membrane fatty acid levels were found to be decreased in the case group compared to the control group, no statistically significant difference was found (p=0.641).

Conclusion: It has been shown that CLL patients had higher oxidant capasity as consequence. Oxidative stress and DNA damage are increased in CLL patients in this study. It is early to evaulate on erythrocyte membrane lipid profile in CLL patients. However, the study can be ligthed the way future studies on the subject.

Keywords: Oxidative stress, DNA damage, erythrocyte membrane lipid profile, chronic lymphocytic leukemia.

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Öz

Amaç: Kronik lenfositik lösemi (KLL), erişkinlerde en sık görülen lösemi türüdür. Hematolojik malignite patofizyolojisinde oksidatif stresin nasıl rol oynadığını daha iyi anlamak için kullanılan az sayıda biyobelirteç vardır. Bu çalışmada KLL hastalarında oksidatif stres, DNA hasarı ve eritrosit membran lipit profilini değerlendirmeyi amaçladık. Çalışmaya 38 KLL hastası ve yaş-cinsiyet uyumlu 38 kontrol dahil edildi.

Gereç ve yöntem: Olgu ve kontrol grupları arasında total oksidan durum (TOS), toplam antioksidan durum (TAS), oksidatif stres indeksi (OSI), Comet assay ile DNA hasarı incelemesi, serum 8-OHdG ölçümü ve gaz kromatografik analizi yapıldı.

Bulgular: TOS ve OSI değerlerinin olgu grubunda kontrol grubuna göre daha yüksek olduğu görüldü (sırasıyla p=0,014 ve p=0,022). Comet yöntemiyle ölçülen DNA hasarının olgu grubunda arttığı belirlendi (p<0,05). Olgu grubunda eritrosit membran yağ asidi düzeylerinde kontrol grubuna göre azalma saptanmasına rağmen istatistiksel olarak anlamlı fark bulunamadı (p=0.641).

Sonuç: Sonuç olarak KLL hastalarının oksidan kapasitesinin daha yüksek olduğu gösterilmiştir. Çalışmamızda KLL hastalarında oksidatif stres ve DNA hasarı artmıştır. KLL hastalarında eritrosit membran lipid profilini değerlendirmek için henüz erkendir. Ancak çalışmamız konuyla ilgili gelecekte yapılacak çalışmalara ışık tutabilir.

Bahadır Karakula, M.D. Tavas State Hospital Internal Medicine Clinic, Denizli, Türkiye, e-mail: drbahadr79@gmail.com (https://orcid.org/0009-0008-3611-3193)

Gülsüm Akgün Çağlıyan, Assoc. Prof. Pamukkale University Faculty of Medicine, Department of Hematology, Denizli, Türkiye, e-mail: drgulsumakgun@yahoo.com (https://orcid.org/0000-0002-2073-1949) (Corresponding Author, Contributed Equally)

Başak Ünver Koluman, Asst. Prof. Pamukkale University Faculty of Medicine, Department of Hematolog, Denizli, Türkiye, e-mail: basakunver@yahoo.com (https://orcid.org/0000-0003-1106-5021)

Mehmet Nuri Başer, M.D. Adnan Menderes University Faculty of Medicine, Department of Medical Oncology, Aydın, Türkiye, e-mail: mehmetnuribaser@gmail.com (https://orcid.org/0000-0003-1809-5581)

Melek Tunç Ata, Ph.D. Pamukkale University Faculty of Medicine Department of Physiology, Denizli, Türkiye, e-mail: melekt@pau.edu.tr (https://orcid.org/0000-0002-0384-2356)

Fatih Altıntaş, M.D. Pamukkale University Faculty of Medicine Department of Physiology, Denizli, Türkiye, e-mail: faltintas@pau.edu.tr (https://orcid.org/0000-0001-8779-0110)

Emine Kılıç Toprak, Assoc. Prof. Pamukkale University Faculty of Medicine Department of Physiology, Denizli, Türkiye, e-mail: ektoprak@pau.edu.tr (https://orcid.org/0000-0002-8795-0185)

Ayşegül Çört, Prof. Dr. Pamukkale University Faculty of Medicine Department of Biochemistry, Denizli, Türkiye, e-mail: aysegulcort@gmail.com (https://orcid.org/0000-0001-8946-7173)

Anahtar kelimeler: Oksidatif stress, DNA hasarı, eritrosit membran lipid profili, kronik lenfositik lösemi.

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Introduction

CLL is a slowly progressive, monoclonal lymphoproliferative disorder characterized by increased production and accumulation of mature but immunologically dysfunctional B lymphocytes [1]. Oxidative stress can be defined as too much oxidant exposure or inadequate antioxidant systems. Oxidative stress causes many structural and functional changes in DNA, proteins and lipids [2]. Irreversible accumulation of oxidative damage leads to structural and functional disorders at cell, tissue and organ levels. It is known to tumor cells have altered antioxidant systems and increased formation of reactive oxygen radicals. In general, high lipid peroxidation and various DNA lesions have been detected in most neoplastic tissues [3]. Therefore, in our study, we investigated to the oxidative status, DNA damage and erythrocyte membrane lipid profile in newly diagnosed CLL patients.

Materials and methods

We informed all participants about the study processes and the consent form. Our study was performed with 38 newly diagnosed CLL patients and 38 age-sex matched controls. The effect size obtained in the reference study was strong (d=1.96). As a result of the power analysis we conducted by assuming that we could obtain a lower effect size (d=0.6) based on the results in the reference study, it was calculated that 80% power could be obtained at 95% confidence level when at least 64 people (at least 32 people for each group) were included in the study. For CLL diagnosis, the 2018 International Workshop on Chronic Lymphocytic Leukemia (iwCLL) update was used. The patient group was included participants without a history of comorbidities such as diabetes, hypertension, renal failure, cerebrovascular disease, gastrointestinal disease, coronary artery disease, chronic liver disease, hematologic or solid organ malignancy. The control group was selected from participants who were age-sex-matched and had similar characteristics to the patient group. The control group had no known history of disease and drug use. A commercial kit was used for TOS, TAS measurement. The Comet assay was performed after leukocyte isolation from venous blood to measure DNA damage. Head length (HL), tail length (TL), head density (HI) and tail density (TI) were used for statistical analysis as parameters of DNA damage. Details of the methods used are explained below.

Leukocyte isolation from blood samples

Peripheral venous blood was drawn in the morning on an empty stomach. We collected blood samples in 10 mL vacuum tubes containing K3EDTA (Vacusera, Türkiye) and separated lymphocytes using Histopaque-1077 (Sigma Aldrich, Inc., St Louis, Mo, USA). We diluted the blood 1:1 with phosphate buffered saline (PBS, Life Technologies, Rockville, MD, USA) and transferred directly into a Leucosep tube (Greiner Bio-One, Austria). We then centrifuged at 800 g and room temperature for 15 minutes. We removed the Buffy coatings and washed them twice with PBS.

Serum isolation from blood samples

In the morning, 8 milliliters of peripheral venous blood were collected on an empty stomach. Blood samples were collected in vacuum gel tubes (Vacusera, Türkiye). We separated the samples from cellular fragments by centrifugation at 7260 rpm for 6 minutes at room temperature. We aliquoted the serum samples and stored them at -80°C until analysis.

Total oxidant level measurement

The principle of measurement is based on the conversion of the ferrous ion chelator complex formed from oxidants in the sample to ferric ion, which reacts with the chromogen in an acidic environment, causing an increase in absorbance. The increase in absorbance seen spectrophotometrically is directly proportional to the oxidant molecules in the sample. TOS was determined in homogenates of serum samples obtained at the end of the experiment using a commercial kit (Rel Assay Diagnostic, Gaziantep, Türkiye). The intensity of the color,

which is related to the amount of oxidants (lipids, proteins, etc.) present in the sample, was measured spectrophotometrically with an ELISA reader at a wavelength of 492 nm. Results were expressed per µmol H_2O_2 equivalent/L.

Total antioxidant level measurement

The measurement principle is based on the fact that antioxidants in the sample convert the blue-green 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical into colorless reduced ABTS. The change in absorbance is directly proportional to the level of antioxidants. TAS was measured using a commercial kit (Rel Assay Diagnostic, Gaziantep, Türkiye). 405 nm wavelength was evaluated spectrophotometrically with an ELISA reader. Results were expressed per mmol Trolox equivalent/L.

Oxidative stress index measurement

Another parameter indicating the level of oxidative stress is the Oxidative Stress Index (OSI), which is obtained by calculation. This index was calculated with the following formula using TAS and TOS values:

OSI= TOS (µmol H_2O_2 Eqv/L) / TAS (mmol Trolox Eqv/L) X 100

DNA damage analysis with comet assay

Five ml of anticoagulated blood from the experimental groups was taken into a tube and diluted 1:1 with PBS. This 10 ml of diluted blood was transferred to another tube containing 3 ml of Ficol-1077 and centrifuged at 400 g for 20 minutes. The pellet obtained by centrifugation was washed 2-3 times with 1 ml RPMI and counted with a hemocytometer and adjusted as ^{2x104} cells per 100 µL. From the lymphocyte suspension prepared in this way, 80 µL was taken and resuspended with 100 µl of "Low melting" agarose (LMA, BioShop, Canada) prepared with 0.5% $^{Ca+2}$ and $^{Mg+2-free}$ PBS at 37°C. This LMA + cell mixture was poured in a thin layer onto a slide previously coated with 1% "normal melting" agarose (NMA, Sigma, USA) and kept on ice for 30 minutes, followed by a third layer of 70 µl of 0.5% LMA and kept on ice for 10 minutes. The slide was then treated with cold lysis binding buffer with a pH of 10 at 40°C for 60 minutes to remove cellular proteins. After lysis, the slides were transferred to horizontal gel electrophoresis (BIO-RAD, California, USA) and incubated in freshly prepared alkaline electrophoresis buffer for 30 minutes. At the end of this period, electrophoresis was performed at 25 V, 300 mA for 30 minutes at the same temperature. After electrophoresis, the slides were washed 3 times for 5 minutes at 4°C with neutralization buffer (0.4M Tris-HCl, pH 7.5) to remove alkaline and detergents. Following neutralization, the slides were stained with 60 µl ethidium bromide (2µl/ml) and examined under fluorescence microscopy and possible DNA damage was assessed using the «Comet assay IV System (AutoComet)» software (Perceptive Instruments, United Kingdom). Damage assessment was expressed as HL (µm), TL (µm), HI (percentage of DNA in head, % H-DNA) and TI (percentage of DNA in tail, % T-DNA) using a software.

Serum 8-OHdG measurement

Reactive oxygen radicals produce more than 20 oxidative base damage products in DNA. Among the damaged bases, 8-OHdG is the most sensitive and most common marker of oxidative DNA damage. Its level can be measured in leukocytes or urine. Serum 8-OHdG level was measured by ELISA method using Elabscience 8-OHdG (8-Hydroxydeoxyguanosine) ELISA Kit (E-EL-0028) at Pamukkale University Faculty of Medicine Physiology Laboratory.

Gas chromatographic analysis

This analysis was conducted at Pamukkale University Advanced Technology Application and Research Center Laboratories. The instrument used for Gas Chromatographic (GC) analysis was equipped with a flame ionization detector and aRtx-2330 column (90% biscyanopropyl-10% phenylcyanopropyl polysiloxane capillary column; 60 m, 0.25 mm i.d., 0.20-mm film thickness). The heat application was started at 160°C for 55 minutes, increased by 5°C per minute and kept at 195°C for 10 minutes and then increased by 10°C per minute to 250°C. The constant pressure was applied at 29 psi and the temperature was started at 150°C and increased by 8°C per minute to 250°C after 1 minute. The constant pressure mode was selected as 13 psi and all fatty acids and isomers were determined by GC analysis. Validation was done by comparison with references. Fatty acid content of cell membranes was given as percentages.

This study was approved by the Pamukkale University Non-Interventional Clinical Research Ethics Committee (date: 31.05.2021, issue: 60116787-020-56364). We conducted all procedures involving human participants in accordance with the ethical standards of institutional and/or national research committees and the Declaration of Helsinki.

Statistical analysis

Mean, standard deviation, median, minimum, maximum, frequency and ratio values were used in descriptive statistics of the data. The distribution of variables was measured using the Kolmogorov-Smirnov test. Independent sample t test and Mann-Whitney U test were used to analyze quantitative independent data. Chi-square test was used in the analysis of qualitative independent data. Spearman correlation analysis was used for correlation analysis. Statistical Package for Social Sciences (SPSS) version 28.0 (IBM SPSS Statistics Inc., Chicago, IL) software was used in the analysis. In all analyses, $p<0.05$ was considered statistically significant at 95% confidence interval in each analysis.

Results

Age and gender distribution were not significantly different between the case and control groups ($p=0.680$ *and* $p=0.488$, respectively) (Table 1). TOS and OSI values were significantly higher in the case group than in the control group ($p=0.014$ and $p=0.022$, respectively). TAS value between case and control group did not show a significant difference ($p=0.798$) (Table 2). Tail length, tail intensity, tail moment and tail migration value in case group was significantly higher than the control group ($p=0.002$, $p=0.003$, $p=0.003$, $p=0.005$ and $p=0.000$, respectively). Head intensity value in the case group was significantly lower than the control group ($p=0.003$). Head length value between case and control group did not show a significant difference ($p=0.670$) (Table 3). 8-OhdG value between case and control group did not show a significant difference ($p=0.809$) (Table 4). Hexadecanoate, Nonadecanoate, Octadecanoate, Tetradecanoate values, which are monounsaturated fatty acids in erythrocyte membrane, did not show a significant difference between the case and control groups ($p=0.733$) (Table 5).

Table 1. Intergroup analysis of age and gender data

		Control Group			Case Group			*p* value	
		Mean±ss n - %		Median	Mean±ss n - %		Median		
Age		66.9±10.9		66.5	65.9±11.3		66.0	0.680	t (t=0.414)
Gender	**Woman**	18	47.4%		15	39.5%		0.488	X^2 (x^2=0.482)
	Male	20	52.6%		23	60.5%			

t independent sample t test, X^2 Chi-square test, Mean: Mean, ss: Standard Deviation
Age and gender distribution were not significantly different between the case and control groups ($p>0.05$)

Table 2. Intergroup analysis of oxidative stress parameters

	Control Group		Case Group		*p* value	
	Mean±ss	Median	Mean±ss	Median		
TOS (µmol H2O2 equivalent/l)	6.3±1.7	6.3	8.5±3.6	8.5	0.014*	m (z=-2.462)
TAS (mmol Trolox equivalent/l)	1.07±0.30	1.05	1.05±0.33	1.06	0.798	t (t=0.257)
OSI (arbitrary unit, A.U)	648.3±311.2	583.9	970.6±888.1	802.0	0.022*	m (z=-2.296)

t independent sample t test, m Mann-whitney u test, Mean: Mean, ss: Standard Deviation
TOS: Total Oxidant Level, TAS: Total Antioxidant Level, OSI: Oxidative Stress Index
TOS and OSI values were significantly higher in the case group than in the control group ($p=0.014$ and $p=0.022$, respectively)
TAS between case and control group value did not show a significant difference ($p>0.05$)

Table 3. Intergroup analysis of DNA damage parameters by comet method

	Control Group		Case Group		*p* value	
	Mean±ss	**Median**	**Mean±ss**	**Median**		
Head Length (µm)	29.3±2.3	29.0	29.0±2.0	28.8	0.670	m (z=-0.426)
Head Intensity (%)	83.5±9.1	85.9	76.4±11.3	76.2	0.003*	m (z=-2.997)
Tail Length (µm)	24.4±6.0	22.9	28.3±5.8	27.9	0.002*	m (z=-3.132)
Tail Intensity (%)	16.5±9.1	14.1	23.6±11.3	23.8	0.003*	m (z=-2.997)
Tail Moment	2.6±2.0	1.9	3.9±2.3	3.5	0.005*	m (z=-2.826)
Tail Migration	9.7±5.9	7.4	13.9±5.5	13.6	0.000*	m (z=-3.766)

m Mann-whitney u test Mean: Mean ss: Standard Deviation
Tail length, tail intensity, tail moment and tail migration in case group value was significantly higher than the control group (p=0.002, p=0.003, p=0.003, p=0.005 and p=0.000, respectively) Head intensity in the case group value was significantly lower than the control group (p=0.003) Head length between case and control group value did not show a significant difference (p>0.05)

Table 4. Intergroup Analysis of 8-OHdG levels

	Control Group		Case Group		*p* value	
	Mean±ss	**Median**	**Mean±ss**	**Median**		
8-OHdG (ng/ml)	10.0±8.4	7.7	11.3±15.5	6.3	0.809	m (z=-0.242)

m Mann-whitney u test Mean: Mean ss: Standard Deviation,
8-OHdG between case and control group value did not show a significant difference (p>0.05)

Table 5. Intergroup analysis of erythrocyte membrane fatty acids

Monounsaturated Fatty Acids	**Control Group**		**Case Group**		*p* value	
Hekzadekanoat	6	30.0%	6	27.3%	0.733	X^2 (x^2=1.284)
Nonadecanoate	2	10.0%	5	22.7%		
Octadecanoate	6	30.0%	6	27.3%		
Tetradecanoate	6	30.0%	5	22.7%		
	Control Group		**Case Group**			
	Mean±ss / n-%	**Median**	**Mean±ss / n-%**	**Median**		
Percentage of Area (%) - FAME	30.0±27.9	30.0	27.3±30.0	15.9	0.641	m (z=-0.466)

m Mann-whitney u test Mean: Mean ss: Standard Deviation
Hexadecanoate, Nonadecanoate, Octadecanoate, Tetradecanoate values, which are monounsaturated fatty acids in erythrocyte membrane, did not show a significant difference between the case and control groups (p>0.05)

Discussion

CLL is the most common adult leukemia in western countries [4]. The incidence increases with age, it is about 2 times more common in men than in women [5]. In etiologic studies, the relationship between environmental, chemical and radiation exposure, diet, virus infection and autoimmune diseases and CLL development has not been proven [6]. In a study comparing the first-degree relatives of the case and control groups, it was shown that the risk was 8.5 times higher in the relatives of the case group, suggesting a genetic tendency. It has been shown in previous studies that CLL is a disease susceptible to changes in antioxidant enzymes and oxidative stress, and that there is a dominant oxidative stress in these patients [7, 8]. In this study, we investigated the presence of DNA damage and erythrocyte membrane lipid profile in lymphocytes isolated by comet

method in addition to systemic oxidative stress parameters in CLL patients. With the data obtained, we aimed to make a scientific contribution to the pathogenesis of CLL and future treatments.

We used some biomarkers in our study. Total oxidative status and total antioxidant status were measured and information was provided about the total status rather than individual parameters. TOS and OSI values of the case group in our study were significantly higher than the control group (p=0.014 and p=0.022, respectively). TAS value was not significantly different between the case and control groups (p=0.798). When similar studies in the literature are evaluated together, there are some limitations to the comparison such as evaluation of oxidative stress with different parameters, different oxidative stress measurement kits, differences in age and gender distribution, variability of disease stages and heterogeneity between groups. However, as a common result; oxidative stress parameters are increased and antioxidant parameters are decreased in CLL patients [9-13]. We think that the different results obtained in oxidative level measurements in the body may be due to the difference in the measurement kit and the oxidative parameter measured and the measurement method. Because methods including colorimetric, fluorescence or chemiluminescence can be used to measure oxidative status [14, 15]. In our study, the oxidative status caused by CLL was evaluated and the parameter differences between TAS, TOS, OSI, serum 8-OHdG level and the level of DNA damage by comet method were also revealed. The markers reflecting the oxidant status are also contradictory among themselves. The fact that it is not known which method of oxidative stress measurement is more specific for CLL and the lack of superiority studies of the methods can be considered among the limiting reasons of our study. In this respect, it is seen that there is a need for further research and the establishment of measurement parameters to be established with international standards in determining the indicators of oxidant level.

Like the oxidative processes that proteins, carbohydrates and lipids undergo, DNA is also affected despite its stable structure. Due to DNA repair processes, oxidative damage can be detected even in healthy individuals. In DNA, 8-OHdG, the most well-known of the base mutations, is formed by the interaction of the hydroxy radical at the 8th position of the guanine base. 8-OHdG is one of the oxidative base damage products of ROS on DNA. 8-OHdG level in serum and urine is evaluated as a marker of oxidative stress. ROS have short half-lives and therefore direct in vivo measurement of ROS is difficult. Currently, 8-OHdG is one of the few ROS-mediated products used to assess oxidative stress on DNA instead of ROS itself. In this study, in addition to the total oxidative stress in the body, we aimed to show the effects of CLL on DNA with ROS products and to contribute to the studies on DNA damage repair mechanism by measuring 8-OHdG level. In our study, there was no difference in 8-OHdG levels between the case and control groups (6.3 vs. 7.7 ng/ml, p=0.809). When other studies on the same subject in the literature are examined, it is observed that 8-OHdG levels in blood and urine are increased in case groups [15, 16]. In the results of our study, the lack of difference in the comparison of 8-OHdG levels, which is an indicator of the total oxidant status in the body and the effect of oxidant levels on DNA, between the groups may have been due to the uncertainty of the amount of oxidant that may cause damage to DNA, the lack of a certain measurement standardization, the experimental conditions and the differences in the commercial kits used.

Comet assay, also known as single cell gel electrophoresis, is a simple method for measuring DNA damage and repair in eukaryotic cells. Damage to DNA can be assessed using the comet assay, which combines the effects of genotoxic, cytotoxic, and oxidative stress. It is an economical, reliable, and fast technique. The shape, size, and amount of DNA in the ‘comet’ play an important role in determining the level of damage. In this assay, DNA damage parameters are measured by measuring tail length, density and momentum, and head length and density. The study of these parameters is done using the relevant software. Depending on the degree of damage, DNA breaks move from the nucleus to the periphery and elongate from the center to the edge. As a result, cells with increased damage develop a comet appearance [17]. In our study, DNA damage was measured by comet

method and values were recorded as head length, tail length, head intensity, tail intensity, tail moment, tail migration. Tail length, tail intensity, tail moment and tail migration values were significantly higher in the case group than in the control group (28.3±5.8 vs. 24.4±6.0 μm, p=0.002; 23.6±11.3 vs. 16.5±9.1%, p=0.003; 3.9±2.3 vs. 2.6±2.0, p=0.005; and 13.9±5.5 vs. 9.7±5.9, p=0.000). The head intensity value was significantly lower in the case group than in the control group (76.4±11.3 vs. 83.5±9.1%, p=0.003). There was no significant difference in head length between the case and control groups (29.0±2.0 vs. 29.3±2.3 μm, p=0.670). Therefore, DNA damage is increased in CLL patients compared to the control group. Similar results have been obtained in other studies on CLL patients using the comet method [18].

In our study, four different monounsaturated fatty acids (MUFA) in erythrocyte membrane were examined and the percentages of each fatty acid were given. Among the erythrocyte membrane lipids analyzed, nonadecanoate was found to be higher in the case group (22.7%) compared to the control group (10.0%) (p=0.733). Hexadecanoate was found to be high in the control group (30.0%) and decreased in the case group (27.3%) (p=0.733). Octadecanoate was also found to be high in the control group (30.0%) and low in the case group (27.3%) (p=0.733). Tetradecanoate levels were found to be high in the control group (30.0%) and decreased in the case group (22.7%) (p=0.733). When the MUFAs included in the study were compared between the case and control groups, a decrease was observed in the case group (15.9%) compared to the control group (30%) (p=0.641). When the literature was reviewed, no other study investigating erythrocyte membrane lipid profile in CLL patients was found. Our study is the first to examine this issue. The lack of statistically significant difference between our results may be related to the small number of samples in which membrane lipids were evaluated, and the length of the disease process in the case group may also affect this situation. For these reasons, we believe that further research in larger sample groups is needed.

In conclusion, despite the limitations of our study, we demonstrated that oxidative stress and DNA damage are increased in CLL patients. We believe that this study will be instructive for future studies on erythrocyte membrane lipid profile in CLL patients with a larger sample size.

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