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Investigation of Vitamins, Glutathione and Stress Biomarkers in Blood Serum of Patients with Breast Cancer

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Abstract

Breast cancer is a genetic disease caused by the accumulation of mutations in neoplastic cells and is responsible for the highest morbidity and mortality. In this study, blood serum samples from 45 breast cancer patients and 32 healthy individuals (control group) were used. The amounts of vitamins (A, E, β-carotene, C, B_1, B_2, B_3, B_5, B_6, B_9 and B_{12}), glutathione (GSH, GSSG) stress biomarkers (4-HNE, MDA) were determined by HPLC. A significant difference was found between the breast cancer patients and the control group in terms of age and body mass index (BMI) ($p<0.05$). In the breast cancer group, fat-soluble vitamins, A, E and β-carotene decreasing highly significant ($p<0.001$), decreasing water-soluble vitamins was found to be significant ($p<0.05$). No statistically significant difference was observed between the groups that received and did not receive treatment except vitamin B2 ($p> 0.05$) with in the cancer patients. Likewise, no significant difference was observed between the serum vitamin levels of breast cancer patients who breastfeed and not breastfeed, except for vitamins B_2 and B_3 ($p>0.05$). A high level of significant difference was observed between GSH, GSSG, MDA and 4-HNE in the breast cancer patient and control groups ($p<0.001$). There was no significant difference found in GSSG, MDA and HNE values according to the treatment status of breast cancer patients, on the other hand, a significant difference was observed in GSH and GSH/GSSG values ($p<0.05$). While there was no significant difference in GSH, GSSG, MDA and 4-HNE values according to the breastfeeding status of patients, a significant difference was observed in GSH/GSSG values ($p<0.05$).

Keywords: Breast cancer, vitamins, reduced glutathione, oxidised glutathione, 4-hydroxy neoneal, malondialdehyde.

1. INTRODUCTION

Cancer is the uncontrolled and abnormal growth of the body's cells. Arising from a series of molecular events that fundamentally alter the cells' normal properties. The normal control systems which prevent cell overgrowth and invasion of other tissues in cancer cells are disabled. Such altered cells divide and expand in an

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uncontrolled way, and sometimes metastasis by dividing and increasing cells in the presence of signals that normally inhibit cell growth. Cancer cell disorders typically result from mutations in protein-encoding genes that control cell division. With time, more genes are mutated, because the genes that produce the proteins which commonly repair DNA damage do not function normally [1]. Breast is very important for women, growth, development and differentiation take place throughout their lives. The obvious purpose of the breast is to secrete milk for babies [2]. Breast cancer is a genetic disease caused by the accumulation of mutations in neoplastic cells and is responsible for the highest morbidity and mortality [3]. The etiology of breast cancer is multifactorial and several risk factors related to breast cancer may exert their effects by creating an oxidative stress state. Oxidative stress is very effective in the pathogenesis of various chronic diseases such as cancer, diabetes and cardiovascular diseases [4]. Oxidative stress can generally be defined as an oxidant/antioxidant imbalance that can lead to damage. If the amount of reactive species is high and defeats the antioxidant defense mechanisms of the human body, oxidative damage to lipids, proteins or DNA can occur, and this damage is considered to play an important role in the progress of many carcinogenesis-onset diseases, including breast cancer [5]. The measurement of oxidative stress biomarkers in breast cancer patients has a significant interest. There are several markers of oxidative stress, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which have been applied broadly as markers of lipid peroxidation, and protein carbonyl that is the biomarker of protein oxidation mostly used in epidemiological and clinical studies [6]. Cells are preserved from oxidative stress through antioxidant detoxification pathways that are containing non-enzymatic antioxidants like reduced glutathione (GSH) and various antioxidant enzymes. Glutathione has been frequently used as a biomarker of oxidative stress which is a reducing agent that is widely spread in cells. In a patient suffering from breast cancer, it is becoming extremely important to identify possible modifiable factors affecting oxidative stress [4]. In addition, vitamins play an important role as coenzymes or enzymes in many important processes for the proper functioning of the body to necessary for human metabolism. In recent years, due to the large number of studies examining this relationship, vitamins have emerged as important for health and human disease [7, 8], so it is important to know the relationship of vitamins with breast cancer. The study aims to determine the water and fat-soluble vitamins together with the stress biomarkers (MDA, 4-HNE, GSH/GSSG), in the blood serum of women with breast cancer and compare them with the control group.

2. MATERIAL AND METHODS

2.1. Material

In the study, blood samples were taken from a total of 77 individuals aged 20-65 who applied to Hiwa Cancer Hospital between September 2019 and October 2019, 45 with breast cancer patients diagnosed by specialist doctors and 32 healthy controls. Among the 32 of breast cancer patients who did not receive pretreatment, 13 received pretreatment. While 15 of the cancer patients were breastfeeding, 30 did not breastfeed at all. A personal interview was conducted with all patients using a specially designed full history questionnaire with accurate information.

2.2. Exclusion criteria

These criteria include Patients with diabetes mellitus, pre-diabetes, chronic liver or renal disease, and malignancy other than breast cancer and smoking, and alcohol consumption. 5 mL of a blood sample from the individuals was taken into a tube and centrifuged at 3500 rpm for 10 minutes. The

separated serum samples were taken into Eppendorf tubes and transported to the chemistry laboratory of Fırat University by cold chain and stored at -20 °C until analysis. The study was carried out with the approval of the ethics committee of Northern Iraq Hiwa Cancer Hospital dated September-2019 and the approval of the ethics committee at Fırat University with the date and number of 22/10/2019-355284.

2.3. Determination of Body Mass Index (BMI)

Measured height to the nearest 0.5 cm and weight to the nearest 0.1 kg.
BMI is calculated as: BMI = Mass(kg) / [Height m^2] BMI (< 16-18.5) is underweight (18.5-24.9) is normal, (25-29.9) is overweight, (> 30-35) is moderately obese, (> 35-40) is obese severely [9].

2.4. Determination of vitamins A, E, β-carotene and 4-HNE

0.3 mL of serum sample was taken into a tube, 4.0 mL of C_2H_5OH was added, vortexed and centrifuged at 7500 rpm for 10 minutes. Then, the supernatant was transferred and 1.0 mL n-hexane was added, vortexed and centrifuged under the same conditions and the upper n-hexane phase was taken into a glass tube, and this process was repeated twice. The collected n-hexane was dried under a vacuum and the residue in the tube was dissolved in 1.0 mL of methanol and taken into HPLC vials for analysis. Samples were analyzed by HPLC (Shimadzu Prominence-I LC-2030C 3D by PDA detector) using a Supelcosil LC-18 column (25.0 cm x 4.6 mm x 5.0 μm) with a mixture of methanol: acetonitrile: water (63:33: 4.0 v/v) as the mobile phase [10, 11].

2.5. Determination of B, C vitamins, glutathione and MDA

0.7 mL of 0.5 M perchloric acid was added to 0.3 mL of serum sample and vortexed, then the total volume was completed to 3.0 mL by adding distilled water. The mixture was then centrifuged at 7500 rpm for 10 minutes. The supernatant was taken into HPLC vials and B vitamins were analyzed using a Supelcosil LC-18-DB column (150 mm x 4.6 mm ID, 5 μm) [10, 12]. Vitamin C, Glutathione and MDA amounts were determined in HPLC using Utisil-XB-C-8 (25 cm, 4.6 mm ID, 5 μm) column [10, 13]

3. RESULTS AND DISCUSSION

This study included 77 volunteers divided into two groups; The first group consist 45 volunteer patients with breast cancer and the second group consists of 32 healthy individuals from the same demographic region, as the control group. Some characteristics of the groups such as age, body mass index, family history, marital status, parious and breastfeeding are given in Table 1. Differences in breast cancer risk factors are genetic and epidemiological risk factors such as age, family history, childbirth, breastfeeding, and environmental factors. The distribution of demographic characteristics of participants and the relation between identified and suspected factors in the development of breast cancer is presented in Table 1. It reveals that 15% of the patient group were in the age group of 30-39 years, 38% were lies in 40-49 years, 40% of them belong to more than 50 years of age and only 7% of them were <30 years. This indicates that the majority (78%) of breast cancer patients were over 40 years age group. The risk of occurrence of breast cancer increases in middle-aged (after 40 years) and elderly women compared to young women [14]. Human cancer is mainly an age-related disease that involves a person 50 years old or older as our cells change over time and are more susceptible to genetic damage and less able to deal with damage when it occurs.

This problem is thought to be large to a reduction in our immune system's ability to

detect and destroy abnormal cells as they occur, the decreased immune response gives those cells time to develop into potentially lethal cancer [15]. It is well known that breast cancer is associated with weight gain, especially in postmenopausal women as depicted in Table 1. Subjects in both groups were observed to range from normal weight, overweight to obese regardless of their diagnosis. In the present study, the maximum number of cancer patients are in the range of obesity group (44%), 31% are overweight (31%) while the lowest (25%) were in the normal group. The result of this study is confirmed by a direct association between BMI and the risk of the disease developing [16]. Because obesity changes hormone levels in postmenopausal women, such as estrogen, the extra fat cells make estrogen, which can cause hormone receptor-positive breast cancer risk [17]. The distribution of marital status and family history of patients with breast cancer is illustrated in Table 1 that 44% of patients had positive family cancer history. Pal et al. reported that about 3-10% of breast cancers are hereditary cancers. It is estimated that about 85% of these are associated with BRCA1 and BRCA2 mutations. The majority of hereditary breast cancers are accepted as "hereditary breast and ovarian cancer syndrome" due to BRCA1 and BRCA2 mutations [18].

Table 1 General characteristics of all studied groups

Characteristics	**Study groups**		**P-Value**
	Breast cancer (n=45)	**Control (n=32)**	
Age (yrs.) Mean±SD	47.26±11.41	38.81±11.61	0.002[a]
Age distribution (%)			
<30 yrs.	3 (7.0%)	8 (25.0%)	0.113[b]
30-39	8 (15.0%)	7 (22.0%)	
40-49	16 (38.0%)	8 (25%)	
≥50	18(40.0%)	9 (28.0%)	
BMI (kg/m^2) Mean±SD	29.62±5.43	25.43±5.47	0.001[a]
BMI distribution (%)			
Normal weight	11 (25.0%)	18 (56.0%)	0.016[b]
Overweight	14 (31.0%)	7 (22.0%)	
Obese	20(44.0%)	7(22.0%)	
Family history of BC (%)			0.027[b]
Yes	20 (44.0%)	6 (19.0%)	
No	25 (56.0%)	26 (81.0%)	
Marital Status (%)			0.024[b]
Married	36 (80.0%)	17(53.0%)	
single	9 (20.0%)	15 (47.0%)	
Parous (%)			0.353[b]
Parent	18(40.0%)	15(47.0%)	
Nulliparous	27 (60.0%)	17(53.0%)	
Breastfeeding (%)			0.244[b]
Yes	15(33.0%)	14(44.0%)	
No	30(67.0%)	18(56.0%)	

[a] One-way anova test and [b] Pearson Chi-squared test were performed for statistical analyses

Table 2 Amounts of vitamins in breast cancer patients and healthy control groups

Biochemical parameters	Study groups (Mean µg/mL ± SD)		P-Value
	Breast cancer (n=45)	Control (n=32)	
Vitamin A	0.49±0.12	1.89±0.65	0.000**
Vitamin E	4.27±1.04	9.18±4.10	0.000**
β-Carotene	0.54±0.17	0.89±0.20	0.000**
Vitamin C	20.20±4.46	22.70±5.44	0.031*
Vitamin B_1	3.19± 0.83	3.83±1.11	0.007*
Vitamin B_2	3.43±1.10	4.45 ±1.36	0.002*
Vitamin B_3	2.32±0.53	2.63±0.47	0.014*
Vitamin B_5	0.77±0.16	0.85±0.12	0.017*
Vitamin B_6	1.03±0.33	1.26±0.43	0.013*
Vitamin B_9	18.45±7.06	23.34±9.77	0.017*
Vitamin B_{12}	0.12±0.06	0.15±0.06	0.013*

One-way ANOVA test were performed for statistical analyses. *$p<0.05$ was significant and **$p<0.001$was highly significant

Women, especially those who are younger or, who have a child are less likely to get breast cancer. In this study parous of breast cancer patients 60% have a child and 40% of the patient is nulliparous. Women who are single and nulliparous reported a strong elevated risk of breast cancer in most research relative to multi-parous women at the same age. The marital status itself is not a contributing factor for an increased or decreased risk of breast cancer [19]. Breastfeeding among patients with breast cancer is 33% shown in Table 1. It is not clear how breastfeeding decreases the risk of breast cancer. The biological basis for an inverse relation between breastfeeding and breast cancer risk has not been adequately explained. Although various mechanisms have been postulated, one assumption is that lactation causes long-term endogenous hormone changes, possibly decreased estrogen, and increased production of prolactin, which can reduce the cumulative exposure of a woman to estrogen, thus inhibiting the formation or growth of breast cancer cells [20]. The experimental findings of vitamin for the breast cancer patients and healthy control groups are given in Table 2.

In this study, patients with breast cancer had highly significant $p<0.001$ lower serum levels of β-carotene, vitamin A, and vitamin E than healthy control subjects (Table 2). Few studies have investigated the serum levels of vitamins A, E, and β-carotene, in breast cancer patients. In a study conducted by Wald et al. [21] it was shown that low serum levels of vitamins A, E, β-carotene, and retinol precede the development of different cancer types. Results of previous research on vitamin A and E in the serum with breast cancer have provided compelling support for the theory that vitamin A and its precursors prevent mammary carcinogenesis [21, 22]. The findings obtained suggest an association between vitamins and antioxidants and an inverse association with breast cancer. Our research confirms the findings of previous studies that serum concentrations of selected vitamins and carotenoids of patients with other cancers are lowered compared with healthy control groups [23, 24]. Antioxidation functions of β-carotene, vitamins A and E have been reported to reduce DNA damage and maling change [25]. The number of water-soluble vitamins (C, B_1, B_2, B_3, B_5, B_6, B_9 and B_{12}) in cancer patients showed a significant decrease in vitamins compared to the control group ($p<0.05$) (Table 2). Vitamin C acts as a prooxidant, promoting the formation of ROS, such as hydrogen peroxide, hydroxyl radicals, and many others. ROS, generated in response to the high concentration of vitamin C, interacts with critical cellular

molecules and organelles and results in oxidative degradation of these compounds in cancer cells, impairing their viability. Vitamin C acts selectively on tumor cells because they show a decrease of several antioxidant enzymes compared to normal ones [26]. Hussain and Ashafaq [27] stated in their study that the amount of vitamin C in the blood serum of patients with breast cancer is lower than in the control group. Water-soluble vitamins maybe have a complex role in protecting or inhibiting cancer. There are several concerns about the quantities required for their biological functions, and about their beneficial and toxic effects on human health [28]. Vitamin B_1 deficiency has been reported in advanced cancer patients [29]. Vitamin B_2 deficiency has been shown as a risk factor for cancer, but some studies show that riboflavin deficiency increases the risk of cancer in certain regions, while some studies point to a possible weakening effect of riboflavin in the presence of carcinogens [7].

The deficiency of riboflavin has been linked with numerous diseases including cancer and cardiovascular disorders, and there are some indications that the treatment of riboflavin may be effective against oxidative stress-related diseases such as breast cancer [30]. It has been reported that vitamins B_3 and B_6 may be a protective factor against cancer that these vitamins were found to be lower in cancer patients [7, 31]. In a wide range of metabolic functions, vitamin B_5 plays an important role. It is required to form coenzyme A, which is essential for the synthesis of fatty acids, amino acids, steroid hormones, and other significant compounds [32]. Vitamin B_9 is generally reported to be inversely associated with breast cancer risk [33]. Zhang et al. [34] reported that the amount of vitamins B_6 and B_9 in the plasma of patients with breast cancer decreased, but the amount of vitamin B_{12} did not change compared to the control. Vitamins deficiency in the metabolism of patients with breast cancer can be explained in various ways. Reduced levels of vitamins in breast cancer patients can also be due to the eating habit of patients. Lower plasma vitamin levels can be explained by higher oxidative stress caused by lipid peroxidation, and mutations that contribute to higher breast cancer risk [23]. The serum vitamin levels of the groups treated and untreated with breast cancer patients are given in Table 3.

Table 3 Mean concentration of vitamins parameters in breast cancer women considering taking treatments

Biochemical parameters	**Breast cancer patients group (Mean µg/mL ± SD)**		**P-Value**
	Untreated (n=32)	**Treated (n=13)**	
Vitamin A	0.49±0.12	0.48±0.12	0.819
Vitamin E	4.49±1.41	4.14±0.75	0.285
β-Carotene	0.53±0.14	0.55±0.19	0.737
Vitamin C	20.01±2.85	20.30±5.22	0.842
Vitamin B_1	2.88±0.69	3.38±0.87	0.061
Vitamin B_2	2.85±0.68	3.79±1.16	0.007 *
Vitamin B_3	2.29±0.47	2.35±0.58	0.732
Vitamin B_5	0.71±0.12	0.80±0.17	0.054
Vitamin B_6	1.04±0.31	1.01±0.35	0.850
Vitamin B_9	16.74±4.06	19.48±8.27	0.239
Vitamin B_{12}	0.12±0.06	0.11±0.05	0.774

One-way ANOVA test were performed for statistical analyses. *$p<0.05$ was significant and **$p<0.001$was highly significant

The experimental result given in Table 3, shows that there is no statistically significant difference was observed between the patient who has the treatment and has not treatment, except for vitamin B_2, in the blood serum of women with breast cancer ($p > 0.05$). Vitamins levels of patients who have breastfed and didn't breastfed that are illustrated in Table 4. There was no significant difference observed between the serum vitamin levels except for vitamins B_2 and B_3 ($p > 0.05$).

The amounts of glutathione (GSH, GSSG), MDA and 4-HNE in the serum of breast cancer patients and control groups were given in Table 5. A high level of significant difference was observed between the breast cancer patients and the control group ($p<0.001$). The amount of oxidative damage is not only depending on ROS rates but also on the cellular antioxidant defense mechanisms. One of the cellular antioxidant defenses is glutathione that functions as the first line of defence against free radicals. GSH levels decrease significantly ($p<0.001$) in patients with breast cancer compared to the healthy control groups while GSSG levels increased significantly ($p<0.001$). These results were agreeing with the research conducted by Yeh et al. [35] that a low level of GSH and increase GSSG levels in breast cancer patients than in control groups. The ratio of GSH/GSSG may be used as an oxidative stress marker, which is maintained at a high ratio under physiological conditions. GSH is oxidized to GSSG under oxidative stress, and the ratio of GSH/GSSG decreases. In the present study, it was found that the GSH/GSSG ratio in breast cancer patients was significantly ($p<0.05$) lower, compared to control groups. There is a similar report that patient with breast cancer has a lower level of GSH/GSSG ratio in their blood serum [35, 36]. GSH level of serum among participants have a statistically significantly correlating with each of MDA, HNE, and GSSG. GSH has an inverse relationship with oxidative stress due to the antioxidant properties of GSH that is equalized free radical by oxidation to GSSG [37]. It has been reported that while the amount of MDA in the blood of gastric cancer patients increases, GSH levels decrease [38]. The serum GSH, GSSG, MDA and 4-HNE levels of the groups who treated and untreated from patients with breast cancer are given in Table 6.

Table 4 Vitamin concentrations in the serum of women with breast cancer by breastfeeding status

Biochemical parameters	**Breast cancer patients group (Mean µg/mL ± SD)**		**P-Value**
	Have breastfeeding (n=15)	**Didn't have breastfeeding (n=30)**	
Vitamin A	0.51±0.13	0.48±0.12	0.424
Vitamin E	4.42±0.59	4.21±1.17	0.552
β-Carotene	0.59±0.19	0.52±0.17	0.274
Vitamin C	21.19±3.99	19.83±4.64	0.372
Vitamin B_1	2.98 ±0.87	3.26 ±0.82	0.335
Vitamin B_2	2.79±0.68	3.68±1.14	0.021*
Vitamin B_3	1.97±0.29	2.46±0.55	0.008*
Vitamin B_5	0.73±0.13	0.78±0.17	0.336
Vitamin B_6	0.94±0.31	1.06±0.34	0.300
Vitamin B_9	19.11±5.62	18.20±7.61	0.720
Vitamin B_{12}	0.14±0.07	0.11±0.05	0,190

One-way ANOVA test were performed for statistical analyses. *$p<0.05$ was significant and **$p<0.001$was highly significant

Table 5 Glutathione (GSH, GSSG), MDA and 4-HNE amounts in the serum of breast cancer patients and control groups

Biochemical parameters	Study groups (Mean µg/mL ± SD)		P-Value
	Breast cancer (n=45)	Control (n=32)	
GSH	140.55±42.29	262.09±55.94	0.000**
GSSG	57.33±16.44	30.08±7.56	0.000**
GSH/GSSG	3.68±2.38	8.95±1.17	0.000**
MDA	4.91±1.33	0.33±0.06	0.000**
4-HNE	8.99±2.57	4.75±1.91	0.000**

One-way ANOVA test were performed for statistical analyses. *p<0.05 was significant and **p<0.001was highly significant.

Table 6 Mean GSH, GSSG, MDA and 4-HNE concentrations of breast cancer patients by treatment status

Biochemical parameters	Breast cancer patients group (Mean µg/mL ± SD)		P-Value
	Untreated (n=32)	Treated (n=13)	
GSH	88.83±47.30	148.50±73.50	0.006*
GSSG	59.32±41.78	47.22±36.73	0.323
GSH/GSSG	2.55±2.00	4.34±2.33	0.019*
MDA	5.10±1.11	4.79±1.44	0.457
4-HNE	9.33±2.93	8.79±2.37	0.524

One-way ANOVA test were performed for statistical analyses. *p<0.05 was significant and **p<0.001was highly significant

There was no significant difference in GSSG, MDA and HNE values according to the treatment status of breast cancer patients, a significant difference was observed in GSH and GSH/GSSG values (Table 6). GSH, GSSG, MDA and 4-HNE level which classified according to who have breastfeeding and who didn't breastfeed between breast cancer women that are illustrated in Table 7.

There was no significant difference in GSH, GSSG, MDA and 4-HNE values according to the breastfeeding status of patients with breast cancer, a significant difference was observed in GSH/GSSG values (Table 7).

Table 7 Mean concentration of GSH, GSSG, MDA and 4-HNE in women with breast cancer by breastfeeding status

Biochemical parameters	Breast cancer patients' group (Mean µg/mL ± SD)		P-Value
	Have breastfeeding (n=15)	Didn't have breastfeeding (n=30)	
GSH	148.13±39.83	136.20±67.60	0.587
GSSG	39.60±24.45	63.30±38.70	0.067
GSH/GSSG	4.89±2.54	3.21±2.19	0.046 *
MDA	4.85±1.40	5.06±1.18	0.638
4-HNE	8.87±2.50	9.28±2.84	0.649

One-way ANOVA test were performed for statistical analyses. *p<0.05 was significant and **p<0.001was highly significant.

4. CONCLUSION

As a result, it can be concluded that the risk of breast cancer increases with age and BMI increase. There was a decrease in the amount of water and fat-soluble vitamins and GSH in the serum of breast cancer patients, and an increase in the amount of stress markers (MDA, 4-HNE) and GSSG. From these results, it can be said that a serum-based screening test may be useful for the diagnosis of breast cancer. There was a negative association between the participants in the oxidative stress biomarker (4-HNE, MDA, GSSG) and GSH of breast cancer patients. A weak antioxidant protection mechanism might increase oxidative stress in breast cancer patients. The low levels of water-soluble vitamins in breast cancer patients indicate that the demand for B vitamins has been increased for these patients due to accelerated energy metabolism. To validate the change in parameters and determine whether breast cancer patients require supplement B vitamins, more research needs to be done.

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The Declaration of Conflict of Interest/ Common Interest
The authors declared that there are no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' Contribution
Under this title, "First author contributed 40%, second and third authors 25%, and last author10%."

The Declaration of Ethics Committee Approval
The study was carried out with the approval of the ethics committee of Northern Iraq Hiwa Cancer Hospital dated September-2019 and the approval of the ethics committee at Fırat University with the date and number of 22/10/2019-355284.

The Declaration of Research and Publication Ethics
The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

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