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Assessment of Hematological Parameters, Enzymes Activities, and Oxidative Stress Markers in Salivary and Blood of Algerian Breast Cancer Patients Receiving Chemotherapy

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Abstract

The objective of this study was to evaluate the variation and importance of certain hematological, enzymatic, and oxidative stress markers in women with breast cancer under chemotherapy treatment. Hematological parameters, enzymes' activities, and oxidative stress status were estimated in cancer patients and the sensitivity and specificity of biomarkers (MDA, GSH, Catalase, and ORAC) in blood and saliva were compared between the patient and control groups using receiver operating characteristics (ROC) curve design. Results showed that the erythrocyte line (RBC, HGB, and HCT) significantly decreased ($p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively) and ALP, amylase, and transaminases activities were not significantly changed in breast cancer patients group compared to the control group and the reference values. The results showed that GSH levels in leukocytes, erythrocytes, and saliva, as well as ORAC in serum, were significantly increased in patients with breast cancer compared to healthy controls, whereas MDA level and catalase activity did not change significantly. On the other hand, there was not any significant correlation in GSH levels between erythrocyte, leukocyte, and saliva in patients and control groups. ROC analysis indicated that GSH and ORAC levels are better diagnostic tools than other oxidative markers, suggesting to be used as systematic markers for diagnosis and prognosis of breast cancer.

Key words: Breast cancer, Enzymatic activities, Oxidative stress, ROC analysis

Introduction

Cancer is a group of diseases characterized by abnormal cell growth that can affect other parts of the body (Zhang et al., 2015). Breast cancer has become a major public health problem (Salinas-Martínez at al., 2014). This type of cancer affects and develops in the tissue and mammary cells (Oguntunde et al., 2017) and can occur at any age, especially adult age (CCF, 2015). The WHO estimated more than 9.6 million deaths worldwide in 2018. Lung cancer (2.09 million) and breast cancer (2.09 million) are the most commonly diagnosed cancers. Several types of cancer cause death among which are lung cancer (19.51%), liver cancer (9.08%), and breast cancer (6.96%). In Algeria, breast cancer is the most common type of cancer in women with 11,000 cases/year with a mortality rate of about 2840 cases/year. The epidemiological characteristics of breast cancer seems to differ in developing countries in comparison to developed countries (Atoussi et al., 2018). Among the major causes that may be involved in the initiation and progression of breast cancer is the oxidative stress resulting from the alteration of the balance between antioxidants and pro-oxidants (Gurer-Orhan et al., 2018). Oxidative stress is a physio-pathological state caused by an imbalance between reactive oxygen species (ROS) and their antagonistic antioxidants, which may become mutagenic and thus promote the progression of cancer (Wilmanski et al., 2017). ,However, it has demonstrated that malignant cancer cells show abnormal increase of ROS level with high oxidative stress, which in turn expose these cancer cells more vulnerable to further oxidative stress(Wang et al., 2017) which can lead to cell death, the cellular response to ROS is rather complex: low levels facilitate intracellular signaling, while high levels may cause cell death (Panieri and Santoro, 2016). In many cancer cells, ROS levels are elevated partially due to their higher metabolism rate. Aberrant ROS levels can elicit cancer cell apoptosis and necrosis. Cancer cells have high antioxidant capacity to counteract and scavenge ROS (Shijie et al., 2015). Currently, there is no blood biomarker recommended for the diagnosis or screening of breast cancer. Although, markers including the soluble form of MUC1 protein (CA27.29, CA15-3) and carcinoembryonic antigen (CEA) (Kazarian et al., 2017) are the only indicators accepted at the international level to monitor the stage of the disease (Bayo et al., 2018). To improve the quality of treatment and thus to obtain a high survival rate of cancer it is necessary to detect the disease at an early stage (Siegel et al., 2016). The treatment of breast cancer is based on the diagnostic factors and it is individually or most often multidimensionally, including surgery, hormone therapy, chemotherapy, and radiotherapy (Santa Mina et al., 2017). The aim of the present work was to study the variation and importance of certain hematological, enzymatic, and oxidative stress markers in the diagnosis and prognosis of breast cancer in women under chemotherapy treatment.

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Methods

Study subject

Ethical approval was requested and approved by the Ethics Committee of the Department of Cellular and Molecular Biology, Faculty of Natural Sciences and Life, University of El Oued. Our study was conducted on 36 volunteer women, their age between 25 and 80 years. These women were divided into two groups; a group of 20 healthy control females with the mean age of 42.82±1.20 years old and the other group of 16 patients diagnosed and undergoing treatment for breast cancer, with the mean age of 45.48 ± 1.37 years old. All individuals (patient and control) in this investigation lived in El Oued located in the southeast of Algeria. The social and demographic information of the participants including age, age of marriage, number of children, weight, marital status, job, educational level, and blood group were collected by completing the questionnaires from their medical records or through a direct discussion with the participants.

Inclusion and exclusion criteria

Inclusion criteria for the patient group were having breast cancer, diagnosed clinically for at least three months confirmed by specialist doctors, as well as patients receiving chemotherapy but no other types of chronic disease treatment for at least 30 days. In the control group, the inclusion criteria were healthy people who did not suffer from any chronic or acute diseases and consumed no drugs for at least 30 days. Exclusion criteria were to eliminate the factors, which might affect enzymatic activities and oxidative stress parameters. We excluded all diabetics, arterial hypertension, or any evidence of endocrine disorders in the medical history of the patient and healthy control groups.

Laboratory Investigations

Blood and saliva sampling

Fasting blood samples were collected and transferred into EDTA tubes for oxidative stress markers and in dry tubes after centrifugation at 3000 rpm for 5 min, the serum was removed and retained in other tubes for assay of enzymes activities and ORAC parameters. Serum samples were stored at -20°C until analysis. Blood EDTA tubes contents were centrifuged at 2000rpm for 10min and the plasma was removed. The pellet of EDTA tube was lysis with 50 ml of TBS buffer (EDTA 2.92M; tris 1.21M; pH=7) and incubated in Freezer for 30 min. After incubation, it was centrifuged at 2500 rpm for 10 min, and the obtained supernatant (erythrocyte homogenate) was used for the determination of antioxidant activity. After the separation of erythrocyte, the rest of the EDTA tube contents were centrifuged at 2000rpm for 10min and the plasma was removed. The pellet was washed with lysis buffer and was kept in Freezer for 30 min. After that, it was centrifuged at 2500 rpm for 10 min followed by washing with lysis buffer until the leukocyte pairing and then collect in other tubes to make the dosage of oxidative stress tests (Miller et al., 1988).

In the morning fasting, the saliva was collected from the participants in a dry tube. Then, it was centrifuged at 3000rpm for 10min. The supernatant was collected for protein and oxidative stress assays.

Biochemical and oxidative stress measurements

Serum enzyme markers were measured using commercial kits (Spinreact) (ref: alkaline phosphates-20015, α amylase-20031, GOT-20042, GPT-20046). Saliva protein concentration was determined using the Bradford method (Bradford, 1976). Malondialdehyde (MDA) and reduced glutathione (GSH) were determined in white blood cells (leukocytes), red blood cells (erythrocytes), and saliva using Ohkawa method (Ohkawa et al., 1979) and Ellman's reagent (Sedlak and Lindsay, 1968), respectively. Catalase enzyme activity in white blood cells (leukocytes) and saliva was determined using the Aebi method (Aebi, 1984). The total antioxidant power of the serum, i.e. its Oxygen Radical Absorbance Capacity (ORAC:) was estimated by Blache and Prost (1992) method.

Statistical analysis

The values of the results were expressed as a percentage or an average ± ES (standard deviation). The Student's t-test of independent samples was used to analyze the relationships between different parameters used in the coefficient of Pearson's correlation test. The diagnostic model of breast cancer with several factors was based on Logistic regression analysis. The statistical parameters, which are the area under curves (AUC) and the receiver operating characteristics (ROC) were used to show the potency of a biomarker in the diagnosis of Breast Cancer. Specificity, sensitivity, AUC, and 95% confidence interval (CI) values were calculated. All data in this study were examined by SPSS l7.0 software. $P < 0.05$ indicates a statistically significant difference.

Results

Socioeconomic characteristics of patients

The general data of socioeconomic characteristics of the two groups of subjects included age, age of marriage, number of children, weight, social case, job, educational level, and blood group. These indicators showed no significant differences (as shown in Table 1), $P > 0.05$.

Hematological and Enzymatic markers

The hematological analysis showed that the erythrocyte line (HCT, HGB, RBC) decreased significantly ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively) in patients group compared to the control group and the reference values. Regarding the enzymatic activity, the obtained results did not show any significant variation ($P < 0.05$) of alkaline phosphatase, amylase, and transaminases activities in cancer patients as compared to the control (Table 2)

Oxidative Stress markers

as shown in Table 3, the analysis of the oxidative stress status revealed a significant increase of GSH concentration in leukocytes ($p < 0.01$), saliva ($p < 0,05$), and erythrocytes ($p < 0.001$) as well as a significant increase of serum Oxygen Radical Absorbance Capacity (ORAC) ($p < 0.001$). However, our results showed no significant difference ($P > 0.05$) of MDA level in Leukocyte, erythrocytes, and Saliva as well as catalase activity in leukocytes, and Saliva of the patients group compared to the control.

Sensibility, Specificity and AUC factors of oxidative stress parameters

ROC analysis for MDA level was done as shown in (Fig. 1). The sensitivity of MDA levels in erythrocytes, leukocytes, and saliva was 46.76%, 21.4%, and 30%, respectively. Whereas, the specificity of the MDA level was 60.3% in leukocytes, 45% in erythrocyte, and 57.9% in saliva samples. AUC record of MDA levels were 0.48 (0.263 - 0.697) in leukocytes, 0.439 (0.239 - 0.639) in erythrocytes, and 0.426 (0.211 - 0.642) in saliva (fig. 1 and Table 4), however, no statistically significant was observed ($P > 0.05$).

For testing the sensitivity and specificity of GSH levels at different samples, ROC analysis was done separately in saliva, erythrocytes, and leukocytes (Fig.2a, 2b, 2C and Table 4). The results obtained showed a reduced level of GSH in erythrocytes with 100% (sensitivity), 0.93% (AUC) and 63.2% (specificity), in leukocytes with 85.7 % (sensitivity), 0.87% (AUC) and 50% (specificity) and in saliva with 60 % (sensitivity), 0.732% (AUC) and 73.7% (specificity) and also reduced of ORAC level in serum with 91.7 % (sensitivity), 0.823% (AUC) and 40% (specificity) respectively, which were statistically significant ($P < 0.05$).

In addition, our results showed that there was a law percentage of sensitivity (46.7% and 22.2%), specificity (68.4% and 55.6%), and AUC value (0.55 and 0.47) for catalase activity in leukocytes and Saliva, respectively (fig 3A, 3B and Table 4).

Correlations between analyzed parameters

The result in Figures 4 and 5 represent the correlation between GSH Saliva and GSH Erythrocytes, GSH Saliva and GSH Leukocytes, and GSH Leukocytes and GSH Erythrocytes in both patient and control groups.

There was a significant correlation between GSH Saliva and GSH Leukocytes ($p<0.05$; $R^2= 0.255$) in the control group (figure 4 A), while there was no significant correlation in the patient group. In addition, there was a significant correlation between GSH Leukocytes and Erythrocytes in the patient group ($p<0.05$; $R^2= 0.446$) but there was no significant correlation in the control group. There was no significant correlation between the rest of the correlation tests.

Discussion

Breast cancer is the most dangerous form of cancer in women and it has a very high mortality rate, thus it requires improved methods of screening, diagnosis, and treatment. In this disease, risk factors are multifactorial including obesity, delayed menopause, history of benign breast disease, genetics, and early menarche (Gupta et al., 2012). These factors compromise all cellular mechanisms including cell proliferation, pathways of gene expression regulation, and apoptosis (programmed cell death) (Chandra et al., 2000). Our study showed that RBC, HGB, HCT are significantly decreased in patients with breast cancer as compared to control, which are in agreement with the results of Shrivastava et al., 2017 in which a significant decrease of hemoglobin level and RBC count were observed in women with breast cancer as compared to the control healthy subjects. Blood biomarkers such as serum transaminases, alkaline phosphatase, amylase activities, and serum albumin levels for the systemic inflammatory response are associated with cancer prognosis (Wang et al., 2014). The result of blood enzymes analysis showed no significant change in serum ALP, amylase, and transaminases activities. Chougule et al., (2008) showed elevated transaminases activities in patients with cancer of the head, neck, and cervix compared to healthy individuals. Conversely, Proctor et al. showed a decrease in AST and ALT activities in cancer patients compared to the normal values (Takenaka et al., 2016). Moreover, it has been shown that ALP activity is used to follow bone and liver metastases. Although some investigations have demonstrated a high sensitivity of ALP activity for the detection of bone and metastatic cancers by using total Alkaline Phosphatase or specific iso-enzymes (Prabasheela et al., 2012). Oxidative stress is a state of disequilibrium between pro-oxidant and antioxidant in favor of the first, leading to potential damage in cells (Valenzuela et al., 2003). Under normal physiological conditions, oxidants are neutralized by an enzymatic and non-enzymatic antioxidant defense system. If the free radicals are incompletely eliminated by antioxidants, they will cause an accumulation of ROS. In the effectiveness and insufficiency of the antioxidant defense system are concerned in some pathological conditions induced by ROS (Palipoch and Koomhin, 2015). GSH plays several roles in cellular processes, including apoptosis, cell growth, and defense against oxidative stress, therefore it limits cancer progression (Traverso et al., 2013). In our experimental study, the results showed a significant increase in glutathione levels in erythrocytes, leukocytes, and saliva. Biological biomarkers of saliva and serum samples are useful to some extent for diagnostic purposes for several pathologies (Guerra et al., 2015). This result is in agreement with the study of Javed et al., (2015) that showed a significantly increased level of GSH in patients with breast cancer. A positive correlation was observed between increased GSH synthesis and high levels of cell proliferation in tumors (Obrador et al., 1997). The increase in the level of GSH supports against metastatic invasions of cancer (Reuter et al., 2010). Oxygen Radical Absorption Capacity (ORAC) is used to determine the antioxidant potential in a cell or organ (Waisundara and Hoon, 2013). In our experimental study, the data revealed that the total antioxidant activity (ORAC) increased in the serum of breast cancer patients than in control

women. The results were inconsistent with those observed in the study of Badid et al., (2010) that showed ORAC values were lower in breast cancer patients than in control women. In cells, during oxidative metabolism, reactive oxygen species (ROS) are generated that are subsequently restricted and eliminated by the antioxidant system (Cristiano et al., 1995). The analysis of bioinformatics data and statistical methods are increasingly important in the treatment of a large number of test data in the medical field. The logistic regression test is used to search for risk factors, predictions, and judgments. This statistical test is more commonly used in medicine to predict complications such as predicting the risk of surgical complications of gastric cancer (Zhou et al., 2017) and prediction of prostate cancer (Albitar et al., 2016). In this study, the Logistic regression analysis was evaluated for 4 biochemical markers i.e. GSH, MDA, Catalase, and ORAC in leukocyte, erythrocyte, serum, and saliva. The analysis of the receiver operating characteristic curve (ROC) has been established on different binary methods. The ROC curve was plotted and the area under each curve (AUC) was calculated. A better diagnostic value has been detected when the area under the ROC curve is large. Turkan et al. (2015) used the ROC curve to evaluate the diagnostic value of Signal Peptide-Cub-Epidermal growth factor domain-containing protein 1 (SCUBE1) for breast cancer. Zhang et al. (2015) used logistic regression and the ROC curve to analyze serum markers for the diagnostic Primary-sclerosing-cholangitis (PSC). This study showed that oxidative stress marker GSH in leukocyte, erythrocyte, and saliva have a significant correlation with breast cancer, which can serve as a sensitive indicator of a cancer diagnosis. This is also consistent with the study of El-Sharabasy et al., (1993). Glutathione (GSH) is an antioxidant that contributes to the non-enzymatic defense system against free radical-induced oxidative stress (Galano and Alvarez-Idaboy, 2011). It is a tripeptide (Gly, Glu, Cys) widely distributed in all cells, tissues, and organs. GSH has a sulfhydryl (SH) group that can interact with free radicals, which reduces their deleterious effect. These elements (GSH) can also contribute as a cofactor of several enzymes in the enzymatic detoxification phenomenon against reactive oxygen species (Derouiche et al., 2017). The high sensitivity of GSH in leukocytes is a very sensitive marker to oxidative stress, which can detect a high level of GSH in white blood cells. On the other hand, several researchers have reported that high levels of GSH could be a significant predictor of poor response to chemotherapy in several types of cancers in humans. In addition, lymphocyte activity requires GSH in an immune response to multiply in order to kill cancer cells (Lomaestro and Malone, 1995). Reduced Glutathione directly limits free radicals that are essential for antitumor activity. Our results showed no significant correlation in GSH level between saliva, leukocyte, and erythrocyte, which is inconsistent with the results of Öngöz Dede et al., (2016), who found a significant positive correlation in GSH levels between saliva, plasma, and gingival cervical fluid in obese and normal-weight individuals. Our results indicated that the mechanism of GSH mobilization is different in red blood cells, saliva, and white blood cells and therefore there is no correlation between GSH concentrations at different localization in the organism.

In Conclusion, Our results showed that GSH in leukocytes, erythrocytes, and saliva are at the top of the list as a diagnostic biomarker of breast cancer. It is followed by ORAC in serum, which could serve as a sensitive marker of breast cancer. Finally, saliva is as equally reliable sample as blood and hematological parameters, GSH, MDA, and catalase may be used as cost-effective diagnostic biomarkers for various diseases especially cancer.

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Table 1. Socioeconomic characteristics of control and patients with breast cancer.

		Control (n=20)	Patients N=16)
Age (ys)		42.82±1.20	45.48± 1.37
Age of marriage (ys)		20.532±0.575	21.957±0.720
Number of children		5.087±0.387	4.630±0.427
Body Weight (kg)		73.40±1.67	72.48±2.07
Social case	Married%	88	86
	Single%	6	10
	Divorced%	4	2
	Widow%	2	2
Job	Worker%	16	16
	Housewife %	84	84
Educational level	Illiterate%	12	14
	Primary%	14	36
	Medium%	30	30
	High School%	32	14
	High education%	12	6
Blood group	A%	20.40	34
	B%	30	15.90
	O%	48.97	47.72
	AB%	0	2.27

Table 2. Serum enzymes activities in patients and control groups.

Parameters	Reference values	Control N=20	Patients N=16	P *value*
Serum ALP (UI/l)	50-300	57.60 ±3.23	62.27±7.19	0.527
Serum Amylase (UI/I)	10-90	44.70±3.53	36.33±4.61	0,091
Serum GPT (UI/I)	5-40	24.85±3.38	29.31±5.94	0,464
Serum GOT (UI/I)	5-40	24.65±2.01	48.4±20.9	0,274

Values are mean± SEM.

Table 3. Reduce glutathione (GSH) concentration, Malondialdehyde (MDA) level, and catalase activity in Leukocytes, Erythrocytes, saliva, and serum ORAC in patients and control groups.

Parameters		Control N=20	Patients N=16	P *value*
Leukocyte	GSH (µmol/mgHb)	2.261±0.480	17.96±5.11	0.008
	MDA (nmol/mg Hb)	106.74±8.34	107.5±19.3	0.968
	Catalase (UI/g Hb)	3.884±0.959	5.37±1.50	0.435
Erythrocytes	GSH (µmol/mgHb)	8.528±0.925	16.48±1.53	0.000
	MDA (nmol/mg Hb)	16.94±1.69	14.71±1.58	0.520
Saliva	GSH (mmol/mg pr)	0.425±154	0.844±0.197	0.046
	MDA (µmol/mg pr)	9.95±1.50	8.11±1.20	0.159
	Catalase (UI/mg pr)	2.231±0.518	2.93±1.36	0.622
Serum	ORAC (UI)	12.020±0.913	15.619±0.542	0.000

The results are presented by mean± SEM.

Table 4. AUC, Sensitivity and Specificity of oxidative stress parameters

Parameters		Sensitivity %	Specificity %	AUC	CI_{95}	P *value*
MDA	Leukocytes	46.73	60	0.480	0.263 - 0.697	0.841
	Erythrocytes	21.4	45.0	0.439	0.239 - 0.639	0.552
	Saliva	30.0	57.9	0.426	0.211 - 0.642	0.521
GSH	Leukocytes	100	63.2	0.930	0.850 - 1.000	0.000
	Erythrocytes	85.7	50.0	0.875	0.754 - 0.996	0.000
	Saliva	60.0	73.7	0.732	0.544 - 0.919	0.044
Catalase	Leukocytes	46.7	68.4	0.551	0.353 - 0.749	0.615
	Saliva	22.2	55.6	0.471	0.230 - 0.711	0.802
ORAC	Serum	91.7	40.0	0.823	0.666 - 0.980	0.003

AUC = Area under the ROC curve, CI=Confidence Interval, P=Significance level.

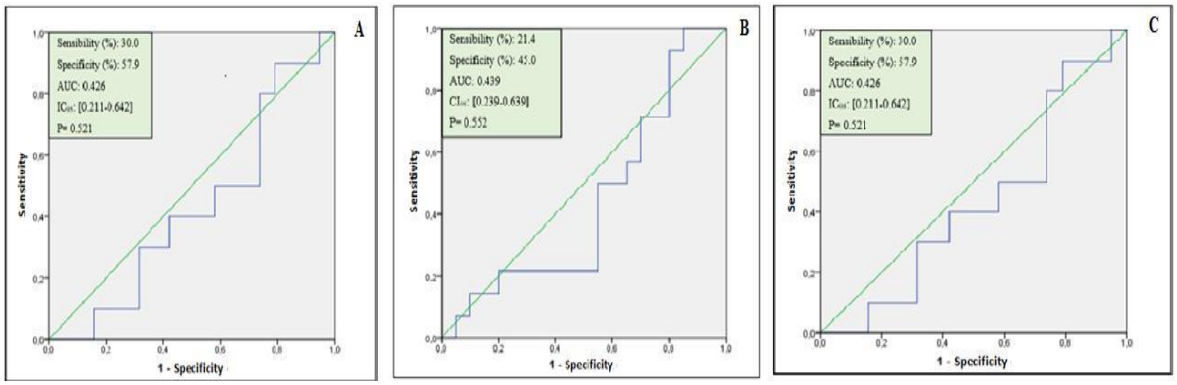

Figure 1: Curve ROC for MDA level in leukocytes (A), Erythrocytes (B), and saliva (C)

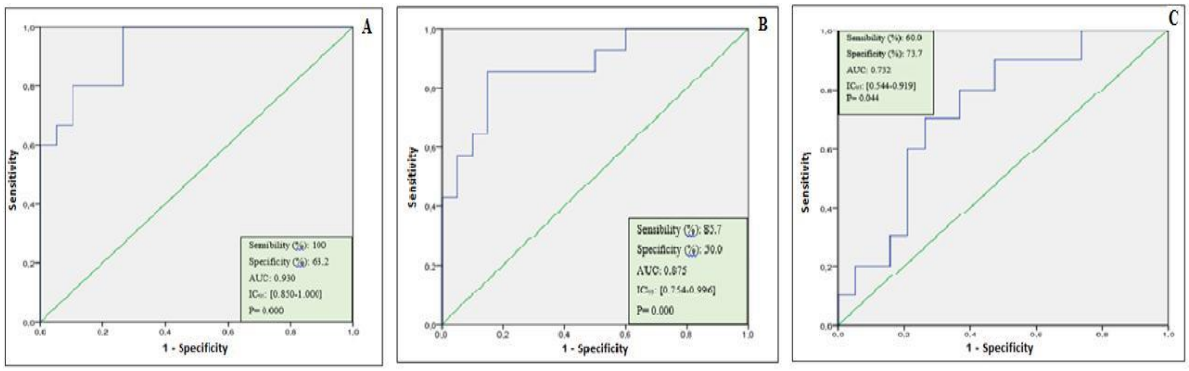

Figure 2: Curve ROC for GSH level in leukocytes (A), Erythrocytes (b), and saliva (c)

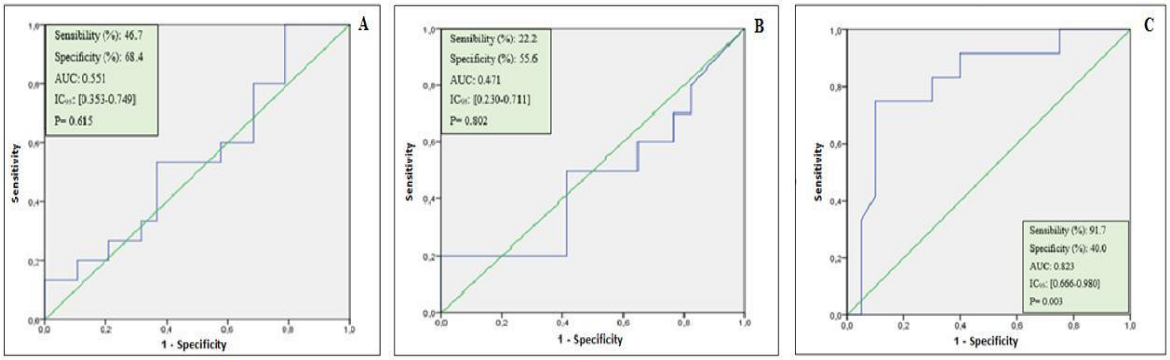

Figure 3: Curve ROC for Catalase activity in leukocytes (A), saliva (B), and serum ORAC (C)

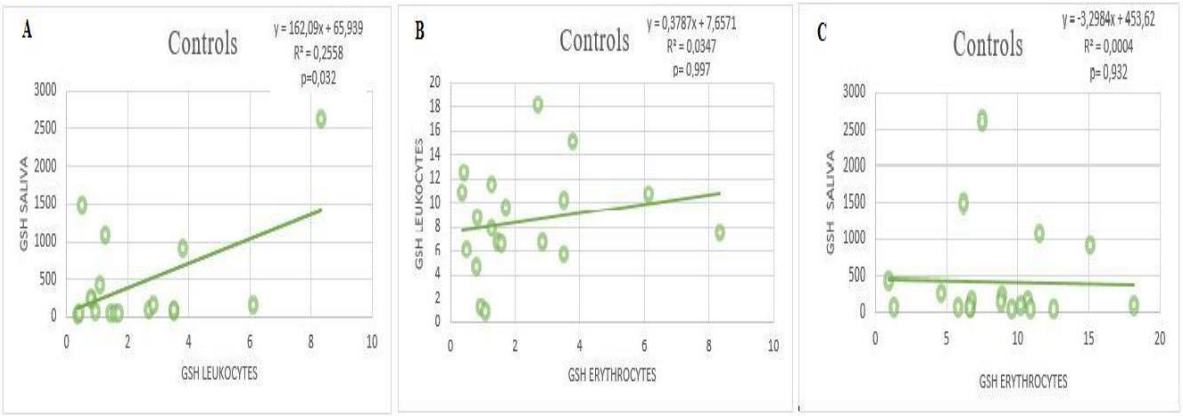

Figure 4. Correlation between GSH level in Saliva and Leukocytes (**A),** in Leukocytes, Erythrocytes (**B)** and in Saliva and Erythrocytes (**C)** for control groups

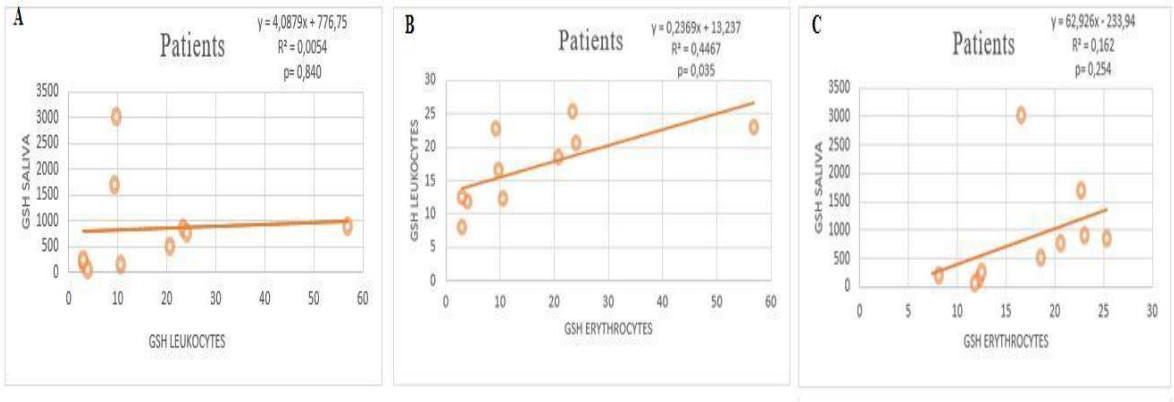

Figure 5. Correlation between GSH level in Saliva and Leukocytes (A), in Leukocytes and Erythrocytes (B), and in Saliva and Erythrocytes (C) for patient groups