

Evaluation of the Level and Polymorphism of the Osteocalcin Gene in Patients with Rheumatoid Arthritis

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ABSTRACT

The hormone is known as the bone protein γ -carboxyglutamic acid. The hormone belongs to the family of proteins that depend on vitamin K, as this vitamin is a catalyst in the formation of (γ -carboxylation) that determines its attraction to the bone matrix and calcium, and this contributes to the formation of bones. Moreover, the non-carboxylated form of the hormone that exerts hormonal properties and is also involved in glucose and energy metabolism, stimulates osteoblasts to secrete insulin directly and indirectly from the pancreas through the synthesis of glucagon-like-peptide 1 GLP-1. From the small intestine this enhances insulin sensitivity in fat cells and muscle cells. This property may have an effect on bone mineralization and may lead to a change in bone quality and an increased risk of fractures.

The aim of the study is to evaluate the level of osteocalcin in patients with rheumatoid arthritis in comparison with the healthy ones and its relationship with the enzymes (GSH, CP, SOD), and to detect the gene encoding the hormone osteocalcin in the disease and to find the relationship between the hormone osteocalcin and vital variables.

Blood samples were collected from (30) healthy people (15) males and (15) females, their ages ranged from (25-60) years and (30) patients from (19) males and (11) females in a hospital Salah Al-Din General and Al-Sharqat General Hospital, and the analyzes were measured in the central laboratory of the hospital, which were examined by the specialist doctor.

This study showed that the levels of OC in the blood serum of people with rheumatoid arthritis is statistically higher compared to the healthy ones, and a significant decrease in the level of GSH for patients compared with the healthy ones, and a significant increase in the level of CP and the level of SOD for patients compared with the healthy ones, and there is a positive correlation between the level of OC and the level of CP. And a negative relationship between OC level, GSH level and SOD level. It was revealed that there were significant differences at the level of probability ($P < 0.01$) of repeating the allele for the OC gene between the group of patients and the healthy ones, where the value of the repeating of the mutated C allele increased and the level of the normal T allele decreased within the group of patients compared to the healthy ones.

Keywords- rheumatoid arthritis, osteocalcin gene, glutathione, ceruloplasmin.

I. INTRODUCTION

Many researchers rely on measuring the level of activity of hormones and enzymes in both normal and pathological conditions, and benefit from them in diagnosing and treating diseases ⁽¹⁾. There are many hormones that have a very important diagnostic role in the clinical follow-up of diseases of the organism that have been used in the diagnosis and follow-up of diseases ⁽²⁾, so recent studies indicate that increased

activity of the hormone oxytocalcin is one of the biomarkers used in the diagnosis of patients with rheumatoid arthritis⁽³⁾. The glucose metabolic pathway is regulated by the participation of the hormone osteocalcin, which is secreted by the skeleton, for example, during bone formation during childhood, the continuous remodeling of the skeleton in adults, as well as the healing of fractures during injury. This clearly indicates that the bones require a large amount of energy to perform their vital functions, and this is evidence of

The regulation of acetocalcin in the balance of glucose in the blood⁽⁴⁾. Osteocalcin is recognized by the Bone Gamma-Carboxyglutamate Protein (BGLAP) gene and its receptors include GPRC6A, GPR158 and GPR37. These genes are among the most important genetic risk factors for rheumatoid arthritis⁽⁵⁾. Where if there is a genetic defect in the hormone osteocalcin in patients, they are five times more likely to develop rheumatoid arthritis than those who do not suffer from it⁽⁶⁾, because genetic factors contribute by 60% to the occurrence of rheumatoid arthritis⁽⁷⁾. It was found that oxidative stress plays an important role in the metabolic pathways of the body, leading to an imbalance in metabolism and a lack of control in the detoxification of the active oxygen species ROS⁽⁸⁾, and that high levels of glucose in the blood increases the stimulation of free radicals easily, through the non-enzymatic oxidation of glucose followed by Oxidation of glycoproteins⁽⁹⁾, this leads to weakening the body's defense system and making it unable to confront the active classes of oxygen and as a result of an imbalance between antioxidants and the active classes of oxygen ROS, cellular damage occurs in enzymes and organelles, leading to increased lipid peroxidation and cell damage⁽¹⁰⁾. Molecular studies were carried out to find out the genetic variants associated with RA patients, the genetic material (DNA) was isolated and an attempt was made to detect the genetic mutation of the osteocalcin gene at the rs1800247 site by polymerase chain reaction (PCR) using Tetra-Amplification Refractory Mutation System Polymerase Chain Reaction (Tetra). -ARMS-PCR⁽¹¹⁾.

II. MATERIALS AND METHODS OF WORK

2.1 Sample collection and blood sample preparation

The study included (60) samples (30) of patients with rheumatoid arthritis and (30) of healthy people (control group) from Shirqat General Hospital and Salah El-Din General Hospital, for the period from November 2021 until March 2022, their ages ranged between (25- 60), (5) ml of venous blood was drawn from each person suffering from arthritis and a healthy person, and the blood was divided into two parts:

1- The first section (3.5) ml was collected in normal test tubes and left to coagulate until the serum is separated from it using a centrifuge at a speed of 3000 revolutions per minute for a period of 10 minutes, and then kept the separated serum under ultra-freezing until it is used in serological tests.

2- The second section (1) ml was kept in test tubes containing CADS (Citric Acid Dextrose Solution), an anticoagulant, which consists of the following substances:

A- Citric acid 0.48% W/V.

B- Sodium Citrate 1.32% W/V.

C- Dextrose 1.47% W/V.

It was added at a ratio of 1/6 where 1 ml of blood was mixed with 175 µl of the solution and the blood was kept under freezing until used for DNA extraction.

2.2. Blood tests

2.2.1 Determination of the activity of superoxide dismutase enzyme

The activity of superoxide dismutase was estimated using the Modified Photochemical Nitroblue Tetrazolium (NBT) method. This method included the use of sodium cyanide as a peroxidase inhibitor. This method is based on the indirect estimation of the activity of the enzyme SOD through the appearance of a change in the optical density of formazine formed by O₂ reduction. For the dye nitrobutyrate (NBT), which in turn is generated by irradiation of blood serum, (as the decrease in the optical density of formazine indicates an increase in the activity of the enzyme SOD).

2.2.2 Determination of glutathione concentration in blood serum

Serum glutathione level was measured using the Ellman reagent method.

2.2.3 Determination of ceruloplasmin in serum

The mode of activity of ceruloplasmin depends on the oxidation of paraphenylene diamine (PPD) to a blue-violet solution, and the rate of formation of the product depends on the concentration of ceruloplasmin in the blood serum.

2.2.4 Determination of the level of oxtocalcin (OC)

Osteocalcin is measured according to the kit supplied by Sunlong Biotech (Sunlong Biotech Co.,Ltd).

2.2.5 DNA extraction steps

DNA extraction was carry out using DNA extraction kit from Geneaid compant.

2.2.6 Electrophoresis on agarose gel

Genomic DNA samples and PCR product were detected using agarose gel electrophoresis.

2.2.7 Statistical Analysis

The results were analyzed in this study using the statistical analysis program (SPSS- Statistical Package for the Social Sciences) using the completely randomized design method (CRD) through a (t-test) to analyze the variance between two groups at the level of probability ($P \leq 0.05$) and the simple linear correlation coefficient was found to find the relationship between the variables under study.

III. RESULTS AND DISCUSSION

The study was conducted on (60) blood samples, (30) samples from people with rheumatoid arthritis (11 males and 19 females), and (30) samples from healthy people as a control group (15 males and 15 females), and the ages of the affected and healthy people ranged from (25) -60) years old, as shown in the table

Table 1.1: Distribution of samples under study

Healthy		Patients	
N=30		N=30	
Females	Male	Females	Male
15	15	19	11

3.1 Osteocalcin level

The results showed that the average standard deviation of the level of osteocalcin was (0.877 ± 0.241)

mg/L in the sera of patients with rheumatoid arthritis, while it was (0.275 ± 0.631) in the sera of healthy subjects as shown in the table.

Table 1.2: The average standard deviation of the level of osteocalcin in the serum of the samples under study

Variable	The mean standard deviation of healthy subjects	Mean standard deviation of patients with rheumatoid arthritis
Osteocalcin mg/L	0.631 ± 0.275	0.877 ± 0.241

The results show a significant difference in the level of osteocalcin at the probability level ($p \leq 0.01$) in the blood serum of patients with rheumatoid arthritis compared to the control group, as shown in Figure (1-1).

The results showed that the mean standard deviation of the level of osteocalcin in males was (0.0604 ± 1.0095) mg/l for those with rheumatoid arthritis, and it was (0.2437 ± 1.2318) mg/l for females at the probability level $P \leq 0.01$, as in the table

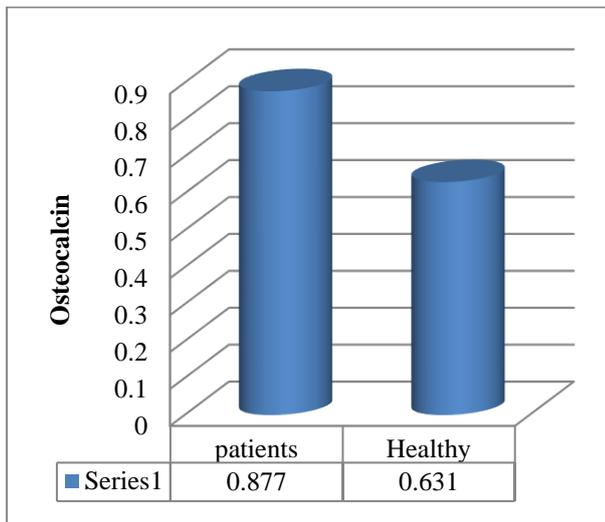


Figure 1.1: The level of osteocalcin in the serum of the samples under study

These results are consistent with many studies, namely that the level of osteocalcin in the serum of patients is higher than that of the healthy group, and it was found that bone formation in patients with rheumatoid arthritis leads to high blood sugar, and also to the function of osteoblasts and reduces the production of osteocalcin⁽¹²⁾, Another study in which the levels of osteocalcin in the blood serum of patients were analyzed and explained that high blood sugar prevents the work of osteoblasts and thus production decreases and leads to the secretion of osteocalcin in blood sugar, and also to an increase in the production of reactive oxygen species (ROS) responsible for bone marrow aging. And another study found that the level of osteocalcin was within the normal limits in the sera of patients compared to healthy ones⁽¹³⁾.

Table 1.3: The average standard deviation of osteocalcin for patient samples when categorized by gender

Variable	Rheumatoid Arthritis patients	
	Male	Female
Osteocalcin	1.0095 ± 0.0604	1.2318 ± 0.2437

It is clear from the above table that there is a highly significant difference in the level of osteocalcin at the probability level ($p < 0.01$) in the sera of male patients compared to female patients with rheumatoid arthritis, as in the figure.

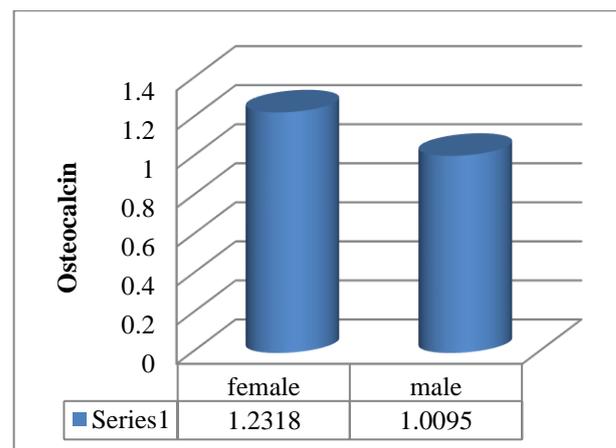


Figure 1.2 The level of osteocalcin in the serum of patients (males and females)

3.2 Level of Glutathione

The results showed that the average standard deviation of the level of glutathione in the sera of the group of patients with rheumatoid arthritis was mg/l

(11.482 ± 0.917), while it was (12.204 ± 0.110) mg/l in the sera of healthy patients as shown in the table.

Table 1.4: The average standard deviation of the level of glutathione in the serum of the samples under study

Variable	The mean standard deviation of healthy subjects	Mean standard deviation of patients with rheumatoid arthritis
GSH mg/L	12.204±0.110	11.482±0.917

It is evident from the above table that there was a significant decrease in the level of glutathione at the probability level of $P \leq 0.01$ in the sera of infected patients compared to the control group, as shown in the figure.

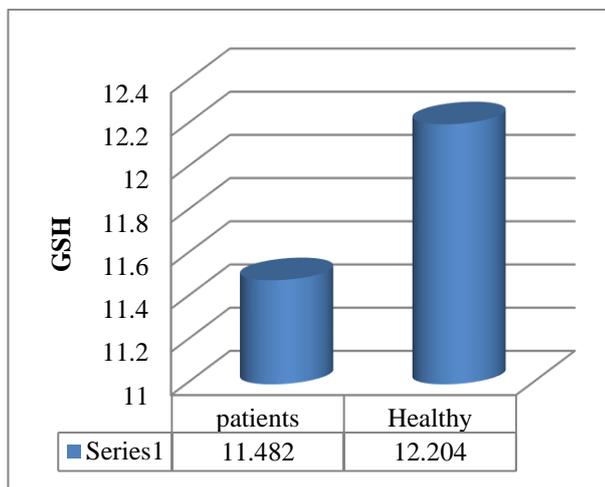


Figure 1.3: The level of glutathione in the serum of the samples under study

These results are consistent with several studies that indicated a significant decrease in the level of glutathione in the serum of patients with rheumatoid arthritis. To the inactive form GSSG Glutathione Disulfide⁽¹⁴⁾, the thiol group in the synthesis of glutathione is a good reducing agent because it blows hydrogen easily because of the weak bond between sulfur and hydrogen (S-H) compared to the strength of the bond between hydrogen and carbon (C-H), so it is responsible for protecting membranes Cellular damage from free radicals, and one of the reasons for the low level of glutathione is a deficiency in the raw materials for its construction, especially the coenzyme (reduced form) nicotinic amide adenine dinucleotide phosphate NADPH resulting from the pentaphosphate sugar pathway to obtain the active form of glutathione from the inactive form through an enzyme Glutathione Reductase GRd⁽¹⁵⁾⁽¹⁶⁾. Other studies indicated that the cause of the low level of GSH is the occurrence of oxidative stress caused by hyperglycemia in patients

with rheumatoid arthritis and type 2 diabetes. Oxidative stress also leads to a decrease in glutathione⁽¹⁷⁾.

Table (1-5) shows the average standard deviation of glutathione in the sera of male patients with rheumatoid arthritis (11.694±0.455) mg/l and for females (11.359±0.392) mg/l.

Table 1.5: Mean and standard deviation of glutathione for patient samples when categorized by gender

Variable	Rheumatoid Arthritis patients	
	Male	Female
GSH	11.694±0.455	11.359±0.392

The above results show that there are no significant differences in the level of glutathione at the probability level of $P \leq 0.05$ in the sera of male patients compared to females, as shown in the figure.

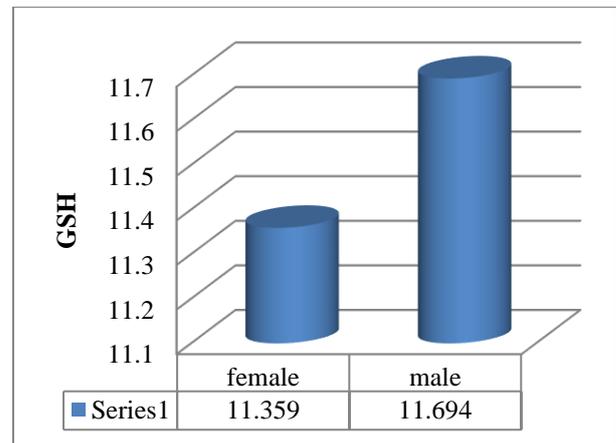


Figure 1.4: The level of glutathione in the serum of rheumatoid arthritis patients (male and female)

This study agrees with the studies that indicated that there was no significant difference in the level of glutathione between male and female patients with rheumatoid arthritis, as the level of GSH may not be affected by sex in the case of these diseases⁽¹⁸⁾.

3.3 Ceruloplasmin level

The mean standard deviation of ceruloplasmin in the group of patients with rheumatoid arthritis was (0.271±0.066) mg/l, while it was (0.254±0.048) mg/l for the control group.

Table 1.6: average standard deviation of ceruloplasmin level in sera of samples under study

Variable	The mean standard deviation of healthy subjects	Mean standard deviation of patients with rheumatoid arthritis
CP mg/L	0.254±0.048	0.271±0.066

The above results show that there is a significant difference in the level of ceruloplasmin in the sera of patients with rheumatoid arthritis compared to the healthy group at the probability level of $P \leq 0.01$, as in the figure

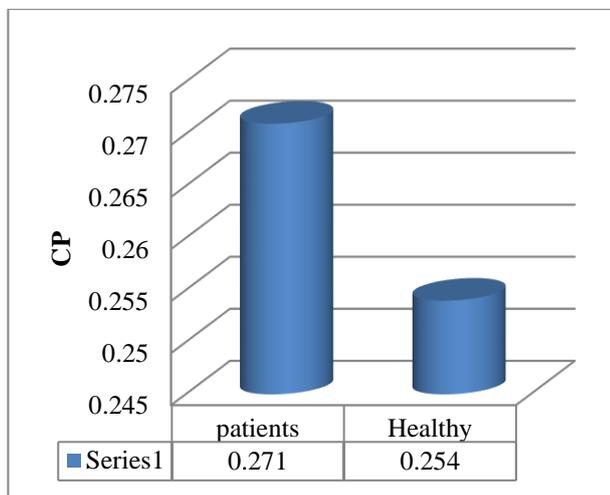


Figure 1.5: The level of ceruloplasmin in the serum of the samples under study

Our results are consistent with previous studies^{(19) (20)}, which indicated that there was a significant increase in the level of ceruloplasmin in the sera of patients with rheumatoid arthritis compared to healthy ones, and they indicated that the reason for this increase is due to high fat, or to the increase in the concentration of free radicals resulting from Fat peroxidation in patients, which leads to an increase in the production of ceruloplasmin to reduce or limit free radicals because it controls iron ions and prevents it from being freely present in cells, because if it is found in free form, it reacts with hydrogen peroxide H_2O_2 to form two molecules of OH^\cdot to form free radicals that it attacks cell components and leads to damage to that cell, while another study indicated that the level of ceruloplasmin decreased significantly in infected patients compared to healthy patients⁽²¹⁾.

Table (1-7) shows the average standard deviation of ceruloplasmin in the sera of male patients with rheumatoid arthritis (0.262 ± 0.0167) mg/l and for females (0.276 ± 0.0322) mg/l.

Table 1.7: Mean and standard deviation of ceruloplasmin for patient samples when categorized by gender

Variable	Rheumatoid Arthritis patients	
	Male	Female
CP	0.262 ± 0.0167	0.276 ± 0.0322

The above results indicate that ceruloplasmin levels have no significant difference at the probability

level of $P \leq 0.05$ in the sera of male patients compared to female patients, as shown in the figure.

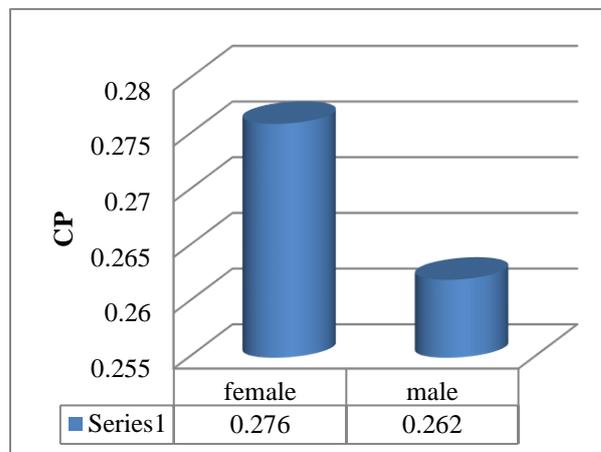


Figure 1.6: The level of ceruloplasmin in the serum of rheumatoid arthritis patients (males and females)

3.4 Superoxide Dismutase Level

The mean standard deviation of (SOD) in the group of patients with rheumatoid arthritis (11.822 ± 1.390) mg/l, while it was ($10,485 \pm 0.852$) mg/l for the control group.

Table 1.8: Is the average standard deviation of the level of superoxide dismutase in the sera of the samples under study

Variable	The mean standard deviation of healthy subjects	Mean standard deviation of patients with rheumatoid arthritis
SOD mg/L	10.485 ± 0.852	11.822 ± 1.390

The above results show a significant increase in the level of superoxide dismutase in the sera of patients with rheumatoid arthritis compared to the healthy group, at the probability level $P \leq 0.01$, as in the figure

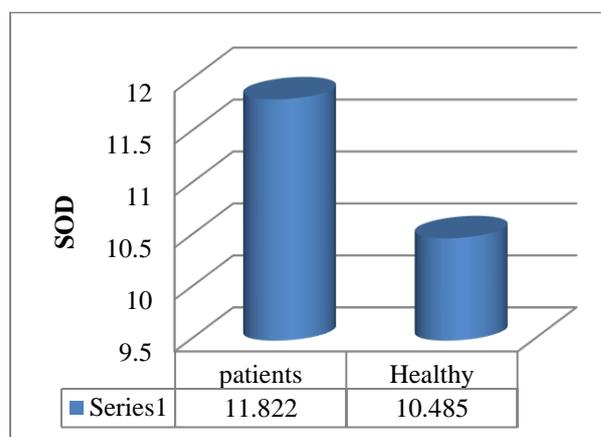


Figure 1.7: The level of superoxide dismutase in the serum of the samples under study

Our results are consistent with the study that found that the level of superoxide dismutase in patients with rheumatoid arthritis was significantly higher compared to the healthy group, which was attributed to the deterioration of antioxidant enzymes due to free radicals during the detoxification process, that the enzymatic and non-enzymatic antioxidant systems are less effective. In patients with rheumatoid arthritis, patients are thus exposed to oxidative stress, and he summarized the process as that these changes are due to efforts to reduce lipid peroxidation in the blood in order to reduce tissue damage⁽²²⁾.

Mean standard deviation of superoxide dismutase in sera of male patients with rheumatoid arthritis (13.088±0.818)mg/l and for females (10.931±0.532)mg/l

Table 1.9: Is the average standard deviation of superoxide dismutase for patient samples when categorized by gender

Variable	Rheumatoid Arthritis patients	
SOD	Male	Female
	13.088±0.818	10.931±0.532

The above results indicate that the levels of superoxide dismutase in patients with rheumatoid arthritis have a significant increase in the sera of males compared to females at the probability level of $P \leq 0.05$, as shown in the figure.

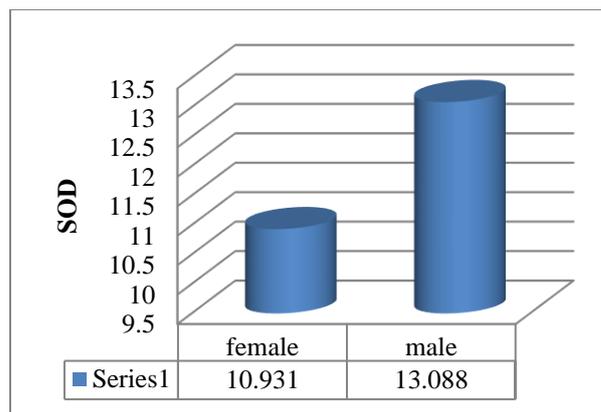


Figure 1.8 Superoxide dismutase level in sera of rheumatoid arthritis patients (males and females)

3.5 Evaluation of the relationship between the level of osteocalcin hormones and biochemical variables in patients with rheumatoid arthritis

The relationship between the level of osteocalcin and the biochemical parameters measured for patients with rheumatoid arthritis was studied, in order to clarify the nature of the relationship between the level of the two hormones and biochemical parameters.

Table 1.10: Correlation coefficient of osteocalcin with biochemical variables for patients with rheumatoid arthritis

(mg/l) Variable	R	Relationship Type
GSH	-0.006912	Negative Relationship
CP	0.533366	Positive Relationship
SOD	-0.396927	Negative Relationship

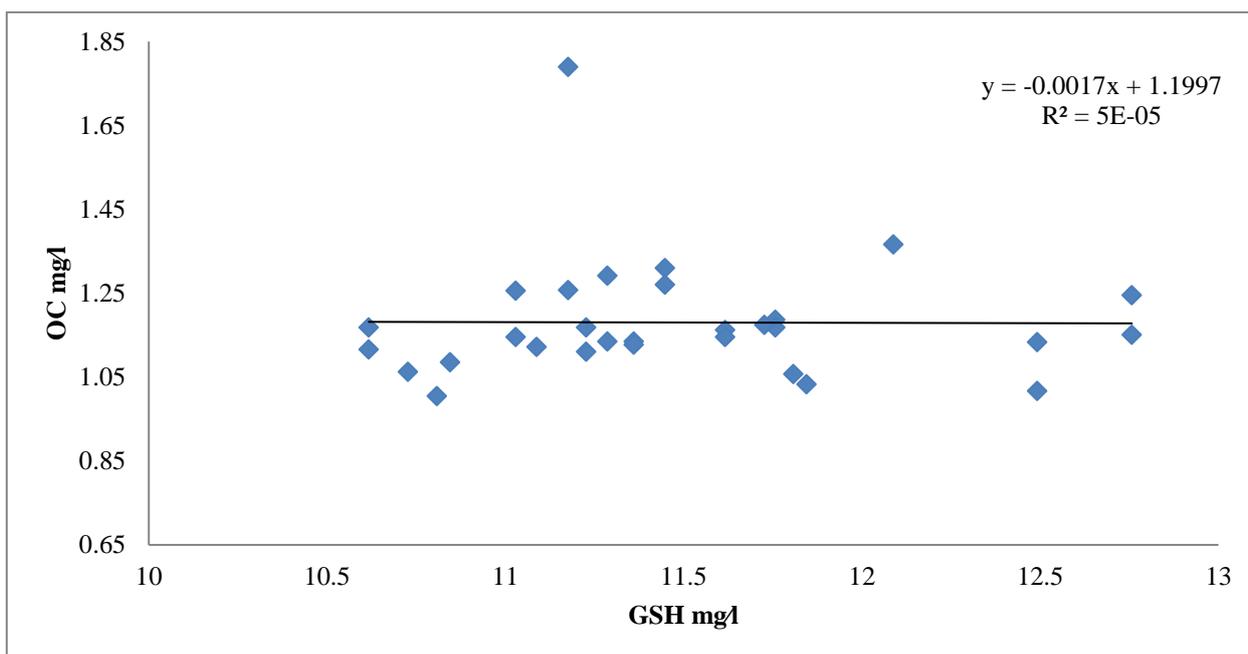


Figure 1.9: Correlation coefficient between osteocalcin level and glutathione level in rheumatoid arthritis patients

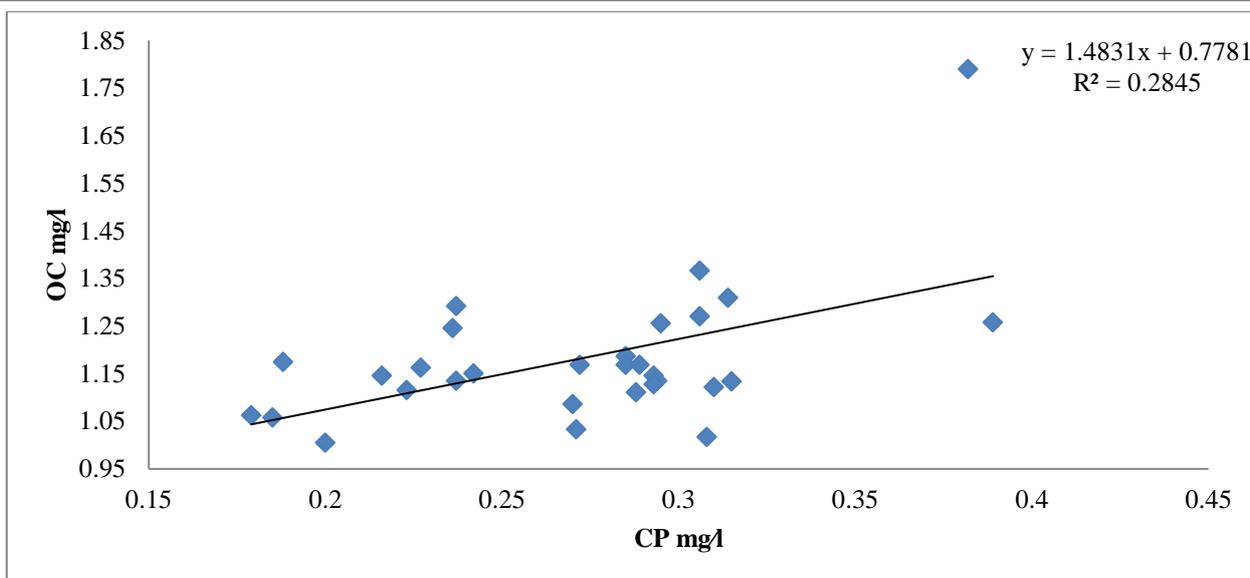


Figure 1.10: Correlation coefficient between osteocalcin level and ceruloplasmin level in rheumatoid arthritis patients

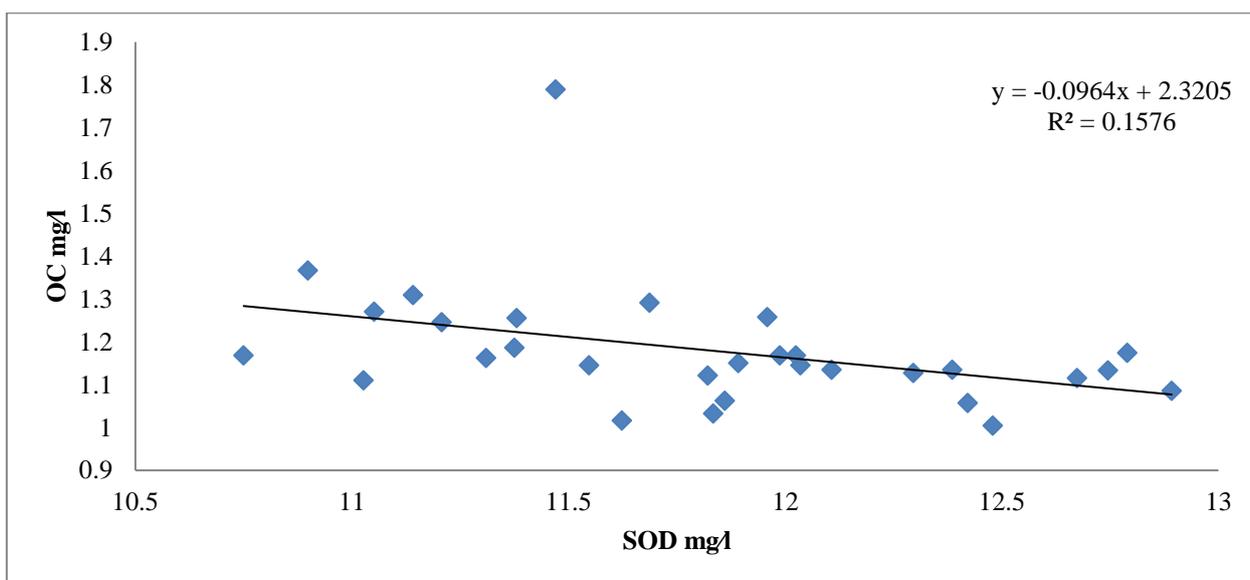


Figure 1.11: Correlation coefficient between the level of osteocalcin and the level of superoxide dismutase in patients with rheumatoid arthritis

IV. MOLECULAR FINDINGS

4.1 DNA extraction

The DNA was extracted as a first step to isolate the target segment of the osteocalcin gene and then by using the polymerase chain reaction (P.C.R) by (buffered solutions) method (extraction)⁽²³⁾.

4.1.1 Polymorphism of the osteocalcin gene at the rs1800247. locus

Electrotransfer of PCR-ARMS results for osteocalcin gene for rs1800247 showed three genotypes (TT, CT, CC) as shown in the figure.

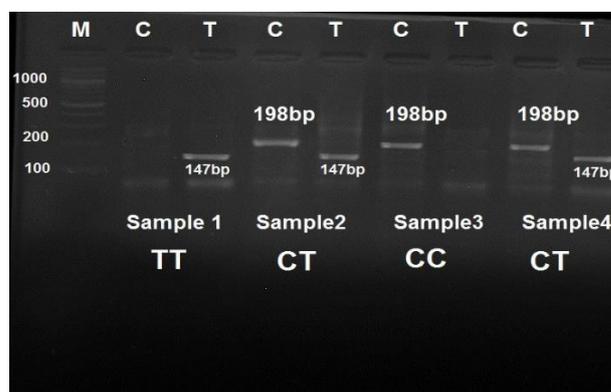


Figure 1.12: Results of the electrophoresis of the osteocalcin gene

The results of PCR-ARMS migration of the osteocalcin gene for the site rs1800247 for the group of patients with rheumatoid arthritis. The homozygous mutated genotype CC (0) did not appear in the group of patients, and in the healthy group, the number of

observed for the TT genotype was (8), the number observed for the heterozygous TC genotype (5), and the number observed for the homozygous mutant CC genotype was (2).

Table 1.11: Comparison of the frequency of genotypes and alleles of osteocalcin gene for locus rs1800247 for patients with rheumatoid arthritis with healthy controls

Genotypes	Patients No. (30)		Control No. (15)		OR	(95% CI)	P value
	No.	%	No.	%			
TT	23	76.67	8	53.33	1 Ref.	-	-
CT	7	23.33	5	33.33	0.487	0.119 - 1.977	0.314
CC	0	0	2	13.33	0.072	0.003 - 1.664	0.1007
Alleles	No.	%	No.	%	OR	(95% CI)	P value
T	53	88.33	21	70	1 Ref.	-	0.0007*
C	7	11.67	9	30	0.117	0.034 - 0.404	

As shown in the table, the normal TT genotype was (76.67) and (53.33) for patients and healthy subjects, respectively, and the heterozygous TC genotype was (23.33) and (33.33) for patients and healthy subjects, respectively, and the percentage of the CC mutant genotype was (0) and (13.33) for patients and healthy subjects, respectively, and the OR value for individuals carrying the TC genotype (0.487) at a probability level (0.314) and there were no significant differences when comparing rheumatoid arthritis patients with healthy subjects, and the OR value for individuals carrying the CC genotype was (0.072) when Probability level (0.1007) and not significant. The results also showed that the frequency of the normal T allele is (88.33) for patients versus (70) for the healthy, and the frequency of the mutant C allele is (11.67) for patients versus (30) for the healthy. The OR in the ratio of the frequency of the mutant allele C to the normal allele T was (0.117) with a significant difference when comparing the group of patients and healthy people at a probability level (0.0007), and this indicates that the mutant allele C works to protect against infection with this disease.

The results of this study agreed with other studies, the rs1800247 polymorphism of the osteocalcin gene was studied in patients with tumors and bone fractures, and it was found that the level of osteocalcin in the blood was within the normal range in both groups

(patients and healthy ones), and also showed that the rs1800247 polymorphism of the osteocalcin gene did not show any effect on the level of osteocalcin. Osteocalcin in the group of patients with bone tumors and fractures⁽²⁴⁾.

Another study examined the polymorphism of the osteocalcin gene rs1800247, and the presence of the C/T polymorphism was determined in the osteocalcin gene in (northern Poland). genetic reasons⁽²⁵⁾.

While a study differed when it evaluated the relationship of daily boron intake and osteoporosis by osteocalcin, and found that osteocalcin levels in the serum of patients were significantly higher than the healthy group at the probability level ($P \leq 0.05$), and the correlation between the level of osteocalcin in the blood and the polymorphism rs1800247 was not significant at the probability level. ($P \geq 0.05$) and that the level of osteocalcin in the normal genotype was much higher compared to the heterozygous genotype and the mutant genotype⁽²⁶⁾.

In order to study the effect of the normal T allele and the mutant C allele within the homozygous and heterozygous normal phenotypes of the rs1800247 gene on the levels of (osteocalcin, osteopontin, superoxide dismutase, ceruloplasmin, and glutathione) according to the genotype of the rheumatoid arthritis group, as shown in Table.

Table 1.12: Comparison between (GSH, CP, SOD, OC) according to the genotypes of the osteocalcin gene rs1800247 polymorphism for a group of rheumatoid arthritis patients.

Parameter	TT (No. 23) Mean ± SD	TC (No. 7) Mean ± SD	P value
OC	0.8576±0.0756	0.7674±0.0622	$P \leq 0.01$
OPN	6.5681±0.3888	5.411±0.2981	$P \leq 0.01$
SOD	12.879±0.523	7.9199±0.0893	$P \leq 0.01$
CP	0.2801±0.0087	0.2706±0.0103	$P \leq 0.01$
GSH	11.5064±.00117	11.4734±0.0151	$P \leq 0.01$

The results show that according to the genotype of the osteocalcin gene at the rs1800247 locus, there are highly significant differences in the levels of the hormone acetocalcin, as the mean of the normal genotype was TT (0.8576 ± 0.0756) compared to the mean of the heterogeneous genotype TC (0.7674 ± 0.0622) at the probability level of $P \leq 0.01$.

There were highly significant differences in the levels of osteopontin hormone, as the mean of the heterozygous TC genotype decreased (5.411 ± 0.2981) compared to the mean of the normal TT genotype (6.5681 ± 0.3888) at the probability level of $P \leq 0.01$.

As for the levels of antioxidants (SOD, CP, GSH) according to the osteocalcin gene genotype showed highly significant differences in their levels, as their averages for the TT genotype were (12.879 ± 0.523) (0.2801 ± 0.0087) (11.5064 ± 0.0117) compared to the TC genotype (7.9199 ± 0.0893) (0.2706 ± 0.0103) (11.4734 ± 0.0151) respectively at the probability level of $P \leq 0.01$, and the results show that all the normal TT genotypes for the two hormones and antioxidants were higher compared to the heterozygous TC genotypes, and this indicates that the polymorphism of the osteocalcin gene rs1800247 No effect was shown on hormonal levels (OPN, OC) and levels of antioxidants in patients with rheumatoid arthritis.

V. CONCLUSIONS

- 1- An increase in the level of osteocalcin hormone in patients with rheumatoid arthritis compared to the control group.
- 2- The level of glutathione decreased in patients with rheumatoid arthritis compared to the control group.
- 3- A significant increase in the level of ceruloplasmin in patients with rheumatoid arthritis compared with the control group.
- 4- High level of superoxide dismutase in patients with rheumatoid arthritis compared with the control group.
- 5- There is a positive correlation between OC level and CP level and a negative relationship between OC level, GSH level and SOD.
- 6- There are significant differences at the probability level ($P < 0.01$) in the frequency of the allele for the osteocalcin gene between the group of patients and the healthy ones, as the value of the mutated C allele frequency increases and the level of the normal T allele decreases within the group of patients compared with the healthy group.

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