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Study of the effectiveness of prolidase with iron status in people with thalassemia in Anbar Governorate

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Abstract:

Thalassemia is a common genetic disease that is transmitted from parents to children. Many people still do not follow the recommended health instructions, especially before marriage, as early examination is an important procedure to raise people's knowledge level for early detection of the disease. The current study aimed to conduct a biochemical study and measure iron status among thalassemia patients in Anbar Governorate who were diagnosed as thalassemia patients by doctors and study its effectiveness with the prolidase enzyme. The study was divided into two groups according to gender, males and females, aged between (5-20) years, with thalassemia, where each group included (30) samples A total of (60) samples from patients with thalassemia. The study included healthy individuals, numbering (30) people (males and females) aged (5-20) years, who were used as a control group. We discussed the impact of iron on the prolidase enzyme, which was shown to be highly effective when exposed to this disease (thalassemia). Therefore, we can rely on the estimation of the high effectiveness of this enzyme as a biomarker of the beginning of the deterioration of the health status of people with thalassemia, thus avoiding reaching an advanced stage of thalassemia. The levels of iron, ferritin, and transferrin were measured. The study showed a significant increase in the levels of iron and ferritin at the probability level ($P \leq 0.01$) in patients compared to the control group. While the transferrin level showed a significant decrease at the probability level ($P \leq 0.01$) compared to the control group. Statistical corelation relationships were studied using the PERSON equation between the prolidase enzyme and the variables, where the relationship was positive (direct) between the prolidase enzyme and (iron and ferritin). While the relationship was negative (inverse) between the prolidase enzyme and (transferrin).

دراسة فعالية إنزيم البروليداز مع حالة الحديد لدى الأشخاص المصابين بالثلاسيميا في محافظة الأنبار

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مستخلص:

الثلاسيميا مرض وراثي شائع ينتقل من الآباء إلى الأبناء. لا يزال الكثير من الناس لا يتبعون التعليمات الصحية الموصى بها، وخاصة قبل الزواج، حيث يُعد الفحص المبكر إجراءً مهمًا لرفع مستوى معرفة الناس بالكشف المبكر عن المرض. هدفت الدراسة الحالية إلى إجراء دراسة بيوكيميائية وقياس حالة الحديد لدى مرضى الثلاسيميا في محافظة الأنبار الذين تم تشخيصهم على أنهم مرضى ثلاسيميا من قبل الأطباء ودراسة فعاليته مع إنزيم البروليداز. قُسِّمت الدراسة إلى مجموعتين حسب الجنس، ذكور وإناث، تتراوح أعمارهم بين (5–20) عامًا، مصابين بالثلاسيميا، حيث ضمت كل مجموعة (30) عينة بإجمالي (60) عينة من مرضى الثلاسيميا. شملت الدراسة أفرادًا أصحاء، عددهم (30) شخصًا (ذكور وإناث) تتراوح أعمارهم بين (5–20) عامًا، والذين تم استخدامهم كمجموعة ضابطة. ناقشنا تأثير الحديد على إنزيم البروليداز، والذي ثبتت فعاليته العالية عند التعرض لهذا المرض (الثلاسيميا). لذلك، يمكننا الاعتماد على تقدير الفعالية العالية لهذا الإنزيم كمؤشر حيوي لبداية تدهور الحالة الصحية للأشخاص المصابين بالثلاسيميا، وبالتالي تجنب الوصول إلى مرحلة متقدمة من الثلاسيميا. تم قياس مستويات الحديد والفيريتين والترانسفيرين. أظهرت الدراسة زيادة كبيرة في مستويات الحديد والفيريتين عند مستوى الاحتمال (P0.01) لدى المرضى مقارنة بالمجموعة الضابطة. بينما أظهر مستوى الترانسفيرين انخفاضًا كبيرًا عند مستوى الاحتمال ($P \leq 0.01$) مقارنة بالمجموعة الضابطة. تمت دراسة علاقات الارتباط الإحصائية باستخدام معادلة PERSON بين إنزيم البروليداز والمتغيرات، حيث كانت العلاقة موجبة (مباشرة) بين إنزيم البروليداز و(الحديد والفيريتين). بينما كانت العلاقة سالبة (عكسية) بين إنزيم البروليداز و(الترانسفيرين).

1. Introduction:

1. 1. Thalassemia: Thalassemia is a common hereditary hemolytic disease that occurs due to the absence or decrease in the synthesis of one of the globin chains (α-globin, β-globin), which leads to a decrease in the level of hemoglobin in red blood cells (erythrocyte) due to a genetic mutation or deletion of certain parts of the main genes when at least one of the parents is a carrier of this disease or affected by it so that they can transmit it to the next generation [(1)].Therefore, thalassemia is considered one of the blood diseases that are inherited across generations, leading to the production of abnormal hemoglobin as a result of a defect in the composition of one or more of the globin chains (alpha and beta) that are found in hemoglobin. In normal cases, the globin chains (alpha and beta) are produced equally, but in the case of thalassemia disorder, the rate of production of the alpha and beta globin chains is not equal, which leads to an increase in one chain compared to the other chain, and as a result, a defect occurs in the composition of hemoglobin in an abnormal manner [(2)].

1.2. Prolidase: Prolidase enzyme has the classification number E.C.3.4.19.9 and is one of the enzymes that catalyze the hydrolysis of the basic material of dipeptides that contain proline or hydroxyproline derivatives at the terminal end of the alpha carbon to produce free proline, as it plays an important role in the final stages of the demolition of proteins rich in amino acids (proline and hydroxyproline) such as the demolition of collagen [(3)]. Prolidase enzyme is found in red blood cells and white blood cells, and abnormal activity of the enzyme has been observed in liver disorders and osteoporosis, as the activity of this enzyme can be used as an indicator to detect different types of thalassemia accompanied by a decrease in collagen deposition [(4)] . Prolidase enzyme depends on manganese near the active site as a catalyst for the activation process in the cleavage of dipeptides to break the bond present in the Glycyl-L-proline compound to produce two compounds Glysine and L-proline, as it requires special conditions of temperature ranging between (35-55 C°) and acidity ranging between (6-8) and a narrow range in terms of the specificity of the substrate to work

on the decomposition of a small number of terminal peptide bonds. Its importance lies in the liberation and recycling of proline in the protein systems of bacterial enzymes [5]. Glycine L-proline is considered the best substrate for the prolidase enzyme, which has been found in microorganisms as well as in several types of mammalian tissues to be purified by several sources such as human red blood cells [6].

1. 3. Iron: Iron is an essential element in the body due to its great importance as a vital component of every living organism. Many metabolic processes such as oxygen transport, neurotransmitter synthesis and electron transport require iron. Therefore, the concentration of iron in the body's tissues must be strictly regulated to avoid the formation of reactive oxygen species (ROS) that are a natural product of Fenton reactions because excess amounts of iron can cause tissue damage [7].

1. 4. Transferrin: It is a glycoprotein consisting of (679) amino acids found in blood plasma with a molecular weight of (79KD) used to transport iron safely from the bloodstream to various tissues such as the liver, spleen and bone marrow to store iron in the form of ferritin [8].

1. 5. Ferritin: A globular protein with a molecular weight of (450-500 KD) that represents the storage form of iron. It is mainly found in the liver and spleen. It is used in recycling iron to form blood. It is essential for iron balance and participates in a wide range of physiological and pathological processes [9]. It contains 24 subunits of chains that form a complex to carry up to 4500 atoms. The normal level of ferritin in males ranges between (12-300 ng\ml) while in females it ranges between (12-150 ng\ml) [10].

2. Materials and methods of work:

2.1.Blood sample collection

Blood samples of (60) thalassemia patients were collected from males and females aged between (5-20) years in Ramadi Teaching Hospital / Anbar Health Department / Thalassemia Unit / from patients clinically diagnosed by specialist doctors, where (5 ml) of venous blood was drawn from each patient and stored in gel tubes to be separated by a centrifuge to obtain serum for the purpose of measuring biochem-

ical variables. Then (30) samples were collected for healthy individuals to be used as a control group.

2.2. Determination of prolidase activity

The effectiveness of the enzyme Prolidase was evaluated through its catalytic action on its substrate, leading to the hydrolysis of the dipeptide glycine-proline (Gly-l-pro) as the enzyme's substrate, resulting in the free amino acids (Gly and pro). The amount of liberated proline is measured colorimetrically after its reaction with ninhydrin at a wavelength of 515 nm, based on proline being used as a standard substance [11],[12].

Procedure:

1. Serum Dilution: Serum was diluted by adding (250 µl) of the dilution solution to (50 µl) of serum, then the mixture was incubated for 24 hours at a temperature of 37°C to prepare the diluted serum for addition, with (100 µl) added to the control samples.

2. Preparation of the three test tubes: The following test tubes were prepared according to Table:

Reagent	Test	Control
Substrate solution	100µl	..……
Diluted serum solution	100µl	µl100

The tubes were incubated for 30 minutes at a temperature of 37°C. The reaction was then stopped by adding 1 ml of a 0.45 mol/L TCA solution. After stopping the reaction, the tubes were placed in a centrifuge for 5 minutes at a speed of 2000 RPM, and then 500 µl of the supernatant was withdrawn from both the test and control tubes

3. Preparation of the four test tubes: Four test tubes were prepared as shown in the following

Solutions	control	Sample(test)	Standard	Blank
Chinard Detector	1 ml	1 ml	1 ml	1 ml
Glacial Acetic Acid	1 ml	1 ml	1 ml	1 ml
Clear solution	0.5 ml	0.5 ml	…….	---------
Standard Proline Solution	------	-----	0.5 ml	----------
Stop Solution	--------	------	-------	0.5 ml

The four tubes were incubated for (10 minutes) at a temperature of (90°C) in a water bath to complete the formation of the colored complex, after calibrating the device with a blank solution. The absorbance was then measured at a wavelength of (515 nm).

Calculation:

The Prolidase Activity in the blood samples under study was calculated based on the following equation [13].

Prolidase Activity =

$$\frac{\text{Abs of test} - Abs\ control}{\text{Abs } of\ standard} \times 2.4 \times [S]$$

2.3. Determine the level of iron in the blood.

After separating the iron Fe^{+3} bound to transferrin in an acidic medium, ascorbic acid reduces the iron from Fe^{+3} to Fe^{+2}, which leads to a reaction between Fe^{+2} and the compound (Ferro Zine), after which a colored complex is formed between them, the absorbance (color intensity) of which is measured at a wavelength of (580) nm, where the absorbance is directly proportional to the amount of iron in the sample, so that the concentration of iron is determined by measuring the increase in absorbance, and thiourea is added to the solutions to prevent the interference of copper [14].

Procedure: The reducing solution R1 was mixed with the colored solution R2 in a ratio of 1:50 and left for five minutes at room temperature. Then the absorbance of the samples and the standard solution was measured at a wavelength of (580nm) against the reagent blank to find the first and second absorbances. according to Table:

Additives used to find the first absorbance A1

Tubes / Solution	Blank	Sample	Standard
Distil water	200 µL	……..	…….
Sample	…….	200 µL	……
Standard	…….	…….	200 µL
Working Reagent	1000 µL	1000 µL	1000 µL

Additions used to find the second absorbance A2

Tube / Solution	Blank	Sample	Standard
Distil water	25 µL	……..	…….
Sample	…….	25 µL	……
Standard	…….	…….	25 µL
Working Reagent	1000 µL	1000 µL	1000 µL

Calculation: The iron concentration was calculated by applying the following equation [14]. Where the standard iron concentration is (200 µg/dL).

$$\text{Iron Conc.}(\mu g/dl) = \frac{(A\,2 - A1)\text{Sample}}{(A2 - A1)\text{standard}} \text{ x Concentration of Standard}$$

2.4. Determination of ferritin level in blood:

The ferritin concentration was estimated for patients with thalassemia and healthy individuals using the enzyme-linked immunosorbent assay (ELISA) method.

2.5. Determination of transferrin concentration:

Anti-transferrin antibodies are mixed with samples containing transferrin to form insoluble complexes that cause a change in absorbance depending on the concentration of transferrin in the patient's sample. Transferrin forms a precipitate in the serum that is determined as a turbidity by measuring the absorbance at a wavelength of (340) nm[15] .

Procedure: The reagents used to determine the concentration of (TF) in the serum were: the first reagent (PEG 5%, NaCl 25mmol\l), the second reagent (Anti-human transferrin, NaCl 100mmol\l), and the third reagent (Standard 200 µg/dL).

R1 was mixed with R2 in a ratio of 5:1 and left for 3 minutes at room temperature. Then the first and second absorbances were found against Planck's reagent to determine the transferrin concentration.

Additives used to find the first and second absorbances

Reagent R1	**800(µL)**
Sample or Calibrator10 (µL)	10 µL
The process of mixing and measuring the absorbance (A1) is done after adding the sample, then we put the solutions	
Reagent R2	200(µL)
The mixing process is done and the absorbance (A2) of the standard solution and the sample is measured 3 minutes after addition	

Calculation: The transferrin concentration was calculated by applying the following equation [15].

$$\text{Transferrin (g/L)} = \frac{(A2 - A1)\text{Sample}}{(A2 - A1)\text{standard}} \times \text{Concentration of Standard}$$

3. Discussion results:

The levels of iron status and prolidase enzyme activity were estimated in thalassemia patients and control groups according to gender as shown in Table (3-1). Comparisons between patient groups and control groups showed a significant increase in the concentrations of variables (IRON, Ferritin, PD) while (TF) levels showed a significant decrease at the probability level ($P \leq 0.01$) compared to control groups.

Table (3-1): Iron status levels and prolidase activity of thalassemia patients compared to the control group by gender

Parameters	Mean ±S.D						P - Value
	Patients group (n=80)			Control group(n=50)			
	Male	Female	Total	Male	Female	Total	
Prolidase (IU/L)	800.715.78 ±	796.06± 9.88	798.385±7.83	239.05 ± 25.11	229.07± 3.42	234.06± 14.26	0.001**
IRON (µg/dl))	288.78±57.89	278.93± 66.13	283.85 ± 62.01	163±4.44	158.53± 5.81	160.76± 5.12	0.001**
Ferritin (ng/mL)	2608.75±1815.73	3381.4± 2003.1	2994.5 ± 1909.41	106.52± 52.99	104.3± 43.05	105.41 ± 48.02	0.001**
TF (µg/dl))	262.39±98.86	246.03± 32.82	254.21 ± 65.84	320.64± 22.59	326± 12.08	323. ± 12.335	0.001**

3.1 . Estimation of prolidase activity in blood serum of the groups under study

Table (3-1) showed that the level of total prolidase and the level of prolidase in males and females increased significantly at a probability value ($P \leq 0.001$) in people with thalassemia compared to the level of total prolidase and the level of prolidase in males and females in healthy people who were used as a control group, as shown in Figure (3-1) below.

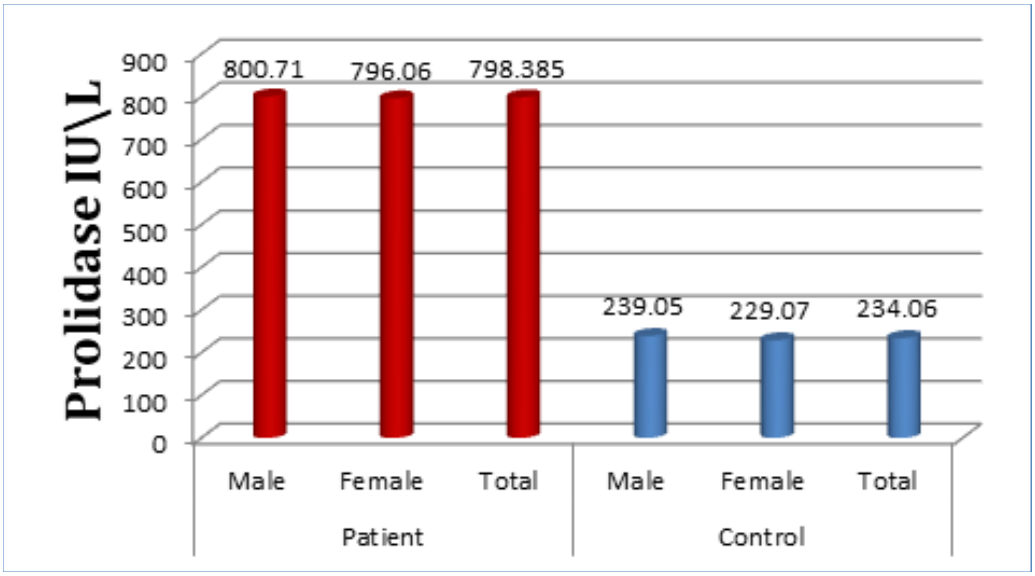

Figure (3-1): Comparison between different groups in the level of prolidase.

The results of the current study are consistent with the findings of (Cakmak, Alpay), (Belli, Seyda) and their group [16], [17]. The reason for the high activity of the prolidase enzyme can be attributed to a defect in collagen recycling, which causes many cases and development of thalassemia. The prolidase enzyme can play a role in metabolic disorders in people with thalassemia. The reason for the increased activity of the prolidase enzyme is attributed to a disruption in collagen turnover, which causes many disease conditions and disease progression. The prolidase enzyme may have a role in metabolic disorders in individuals with thalassemia, as collagen is a major extracellular component found in various tissues, including red blood cells. Prolidase (Iminodipeptidase) is a homogeneous enzyme that releases proline and hydroxyproline from polypeptides and is involved in collagen turnover and the remodeling and growth of cells in the body. The activity of the prolidase enzyme is a determining factor in the biological regulation of collagen, and the breakdown of tissue barriers is stimulated by protein-degrading enzymes. Therefore, inhibiting the enzyme's activity may be considered a therapeutic target for patients with thalassemia[18] .

3.1 . Iron concentration levels in blood serum of the groups under study

Table (3-1) showed that the level of total iron concentration and iron concentration in males and females increased significantly at the probability level of $P \leq 0.01$ in people with thalassemia compared to the level of total iron concentration and iron concentration in males and females in healthy people who were used as a control group. As shown in the following figure (3-2).

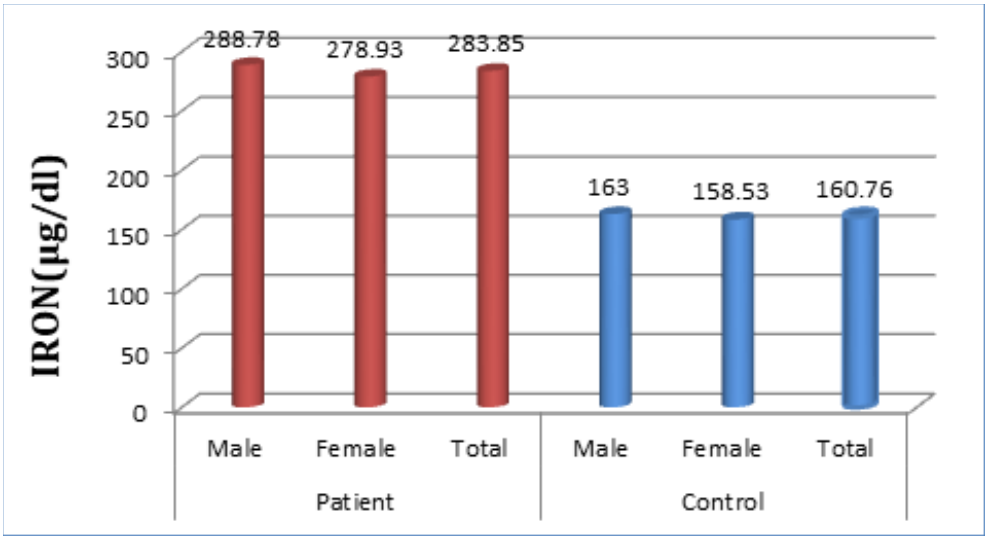

Figure (3-2): Comparison between different groups in iron level

The results of the current study on total iron concentration and iron concentration in males and females are consistent with the study conducted by researcher (Smesam) [19] . and researcher (Numan) [20] . and their group, where they found in their research results that there is a significant increase in total iron concentration in patients with thalassemia compared to total iron concentration in the blood serum of healthy individuals (control group). The reason for the high level of total iron in thalassemia patients is due to repeated blood transfusions, which leads to excessive absorption of iron and increased iron concentration in the serum. The results shown in Table (3-1) indicate that there is a significant and significant difference ($P \leq 0.01$) in the iron concentration between patients with thalassemia and healthy individuals, as many stud-

ies show similar results for the iron concentration in the human body in patients with thalassemia, where their iron concentration is higher than the iron concentration in healthy individuals, which is consistent with the current study, as the increase in iron concentration in patients indicates iron overload, and this increase in concentration occurs as a result of thalassemia dependent on blood transfusion (TDT), rapid disintegration of red blood cells, and increased absorption of iron in the digestive system [21].

3.2. Serum ferritin concentration levels of the groups under study

Table (3-1) showed that the total ferritin concentration and the ferritin concentration in males and females increased significantly at the probability level of $P \leq 0.01$ in people with thalassemia compared to the total ferritin concentration and the ferritin concentration in males and females in healthy people who were used as a control group, as shown in the following figure (3-3).

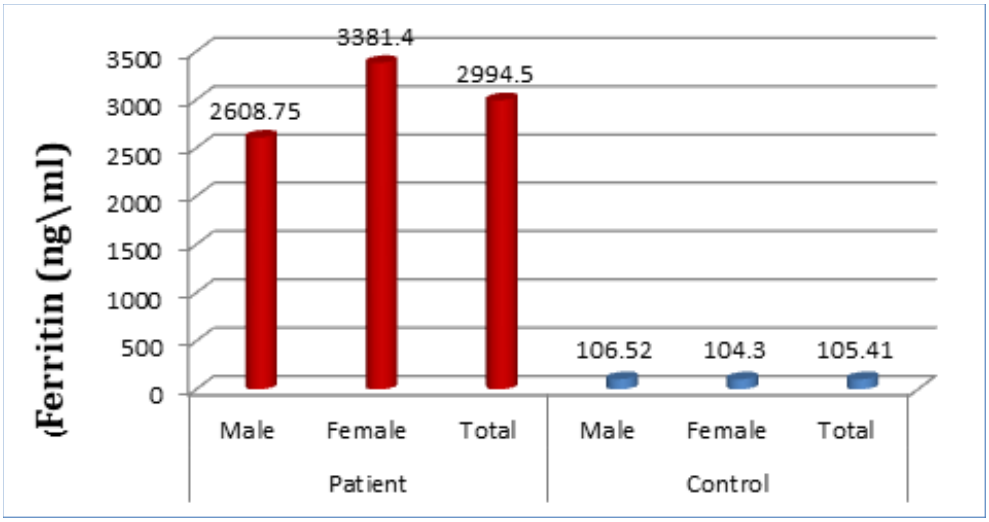

Figure (3-3): Comparison between different groups in terms of ferritin levels.

Table (3-1) showed that the total ferritin concentration and the ferritin concentration in males and females increased significantly at the probability level of $P \leq 0.01$ in people with thalassemia compared to the ferritin concentration level in healthy people who were used as a control group, as shown

in Figure (3-3). The high ferritin concentration in the body of thalassemia patients is attributed to the hemolysis of red blood cells and repeated blood transfusions. Therefore, the results of the current study agree with what the researcher (Eshagh) [22].who found that there is a significant increase in the ferritin concentration in the serum of thalassemia patients compared to the ferritin concentration in healthy people. The reason for the increase in ferritin concentration in the serum of thalassemia patients is due to the formation of ineffective red blood cells and repeated blood transfusions that lead to an increase in iron, as each unit of red blood cells transferred contains about (250) mg of iron While the body cannot secrete more than (1) mg of iron per day [22] .

3.3 . Transferrin levels in blood serum of the groups under study

Table (3-1) showed that the concentration of total (TF) level and (TF) level in males and females decreased significantly at the probability level of $P \leq 0.01$ in people with thalassemia compared to the total (TF) level and (TF) level in males and females in healthy people who were used as a control group. As shown in the following figure (3-4).

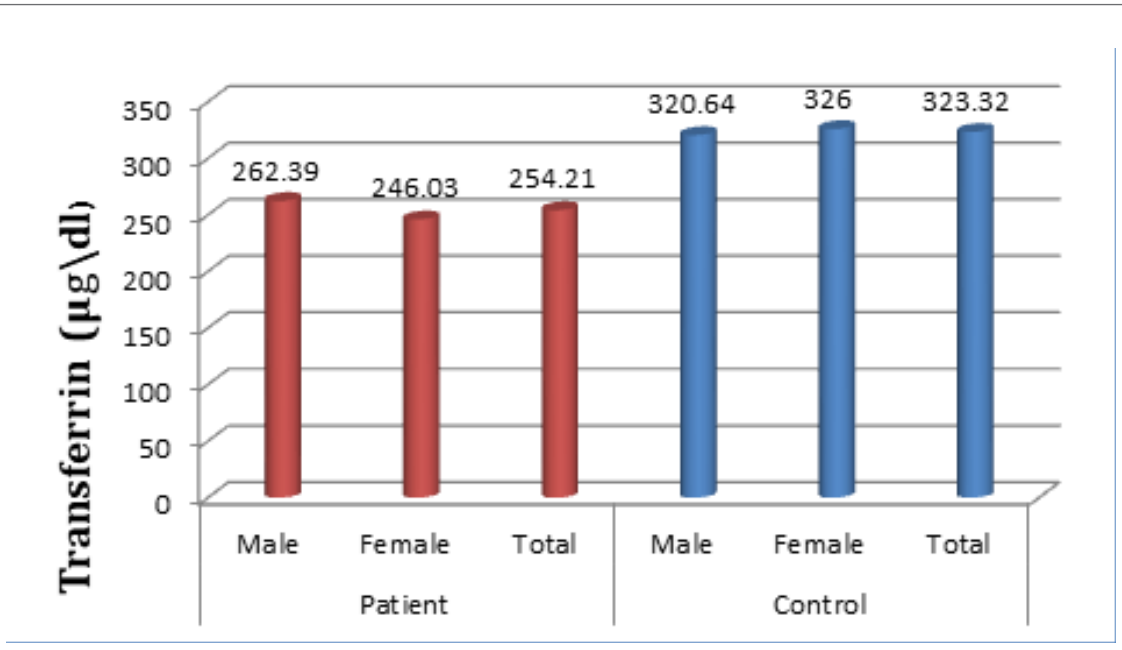

Figure (3-4): Comparison between different groups in the level of Transferrin (TF).

Table (3-1) showed that the total (TF) level and the (TF) level in males and females decreased significantly at a probability level of $P \leq 0.01$ in individuals with thalassemia compared to the (TF) level in healthy individuals who were used as a control group, as shown in Figure (3-4). The decrease in the (TF) level in the body of thalassemia patients occurs due to the state of iron overload and hemolysis of red blood cells, which leads to the accumulation of iron in the blood serum of individuals with thalassemia, which causes a decrease in the (TF) level, as the results of the current study agree with the results of the study of (Onyekwere) and his group [23]. Those who indicated the presence of a decrease in the concentration of (TF) due to the state of iron overload in the serum of patients with thalassemia as a result of hemolysis due to oxidative damage in these patients, and they also attributed the decrease in the level of (TF) in the blood serum to the expansion of the red blood cell marrow that occurs to compensate for the observed anemia. The results of the current study do not agree with regard to the concentration of (TF) with many studies, including the study conducted by researcher (Al-Hakeim) [24] . where he indicated the presence of an increase in the level of transferrin in patients with thalassemia compared to the control group (healthy people), and that the reason for the increase in the level of (TF) in the serum of patients with thalassemia is due to a defect in the globin chain, which leads to the formation of ineffective red blood cells, i.e., not carrying iron, which stimulates the body to produce more transferrin to help the body compensate for the deficiency that occurred in the concentration of iron.

3.5. Correlations between prolidase activity and iron status

3.5.1. Correlation between prolidase activity and iron level

The results indicate that there is a positive relationship between the activity of Prolidase and iron in the serum of people with thalassemia, as the value of the correlation coefficient was ($r = 0.370$), as shown in Figure (3-5) below.

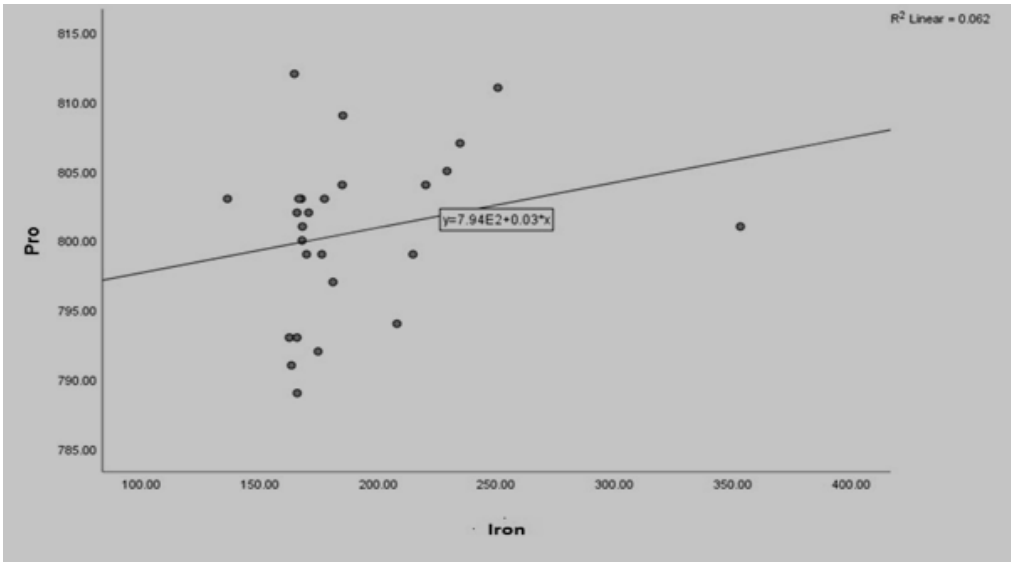

Figure (3-5): The correlation between the activity of the prolidase and the iron level in the serum of thalassemia patients.

3.5.2. Correlation between the activity of the prolidase and the level of ferritin:

The results indicate that there is a positive relationship between the activity of Prolidase and ferritin in the serum of people with thalassemia, as the value of the correlation coefficient was (r = 0.237), as shown in Figure (3-6) below

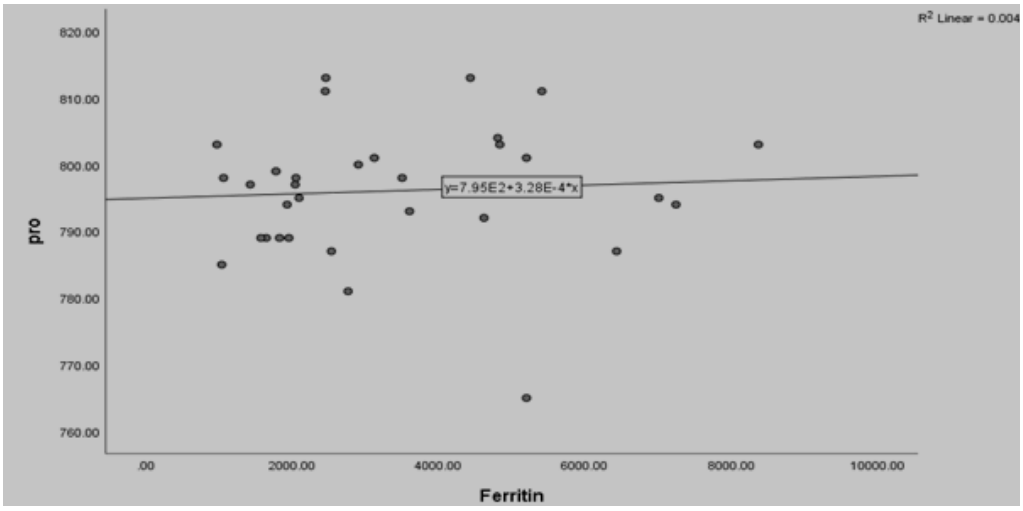

Figure (3-6): Correlation between the activity of the prolidase and the level of ferritin in the serum of thalassemia patients.

. 3.5.3. Correlation between the activity of the prolidase and the level of transferrin:

The results indicate that there is a negative relationship between the activity of the prolidase and transferrin in the serum of people with thalassemia, as the value of the correlation coefficient was (r = - 0.102), as shown in Figure (3-7) below.

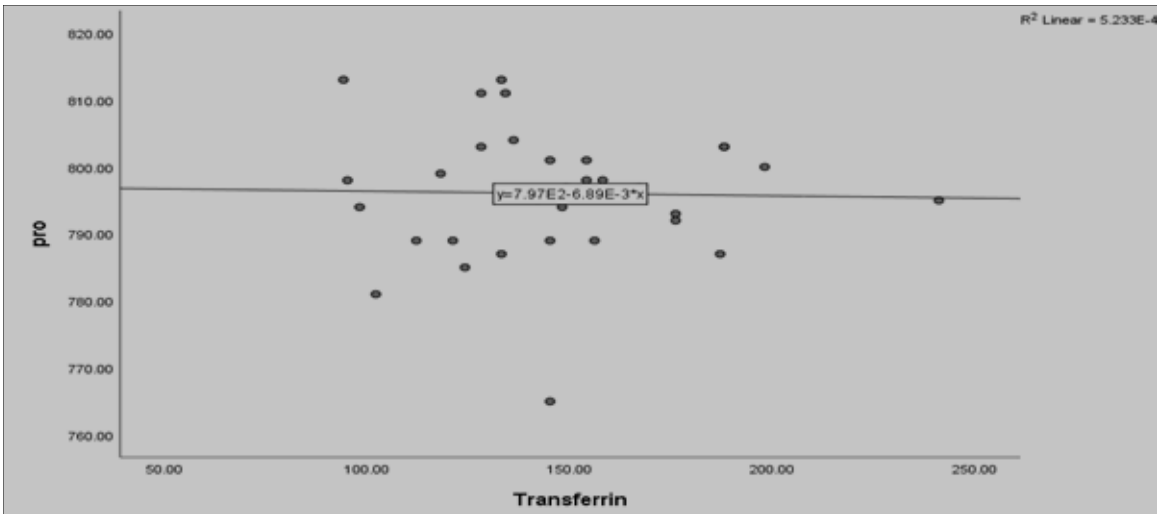

Figure (3-7): Correlation between the activity of the prolidase and the level of transferrin in the serum of thalassemia patients.

4. Conclusions and Recommendations

4.1. Conclusions

1- Measuring the level of the prolidase can be used as a preliminary indicator to identify people with thalassemia.

2 - Thalassemia patients suffer from high levels of iron and ferritin, with a severe decrease in hemoglobin concentration and a decrease in transferrin levels, in thalassemia patients compared to healthy people.

3.We can conclude that the significant increase in the levels of the studied biochemical indicators may be due to some diseases such as kidney, heart and liver diseases, which are also one of the characteristics of thalassemia patients.

Recommendations 4.2

1- Study the effect of cytokines such as (IL-12, IL-1β, IL-6, TNF-α, and IL-23) in thalassemia patients.

2- Premarital genetic screening as a major strategy for preventing genetic disorders such as thalassemia and other blood diseases.

3- Evaluation of the role of prolidase enzyme in diabetic patients, hypertensive patients and various disease conditions.

4- Conducting a kinetic study of the effect of stimulants and inhibitors on the activity of the prolidase enzyme purified from the blood serum of people with thalassemia.

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