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Immunological Investigation of Matrix Metalloproteinases-2 and Tissue Inhibitor of Metalloproteinase-2 in β-Thalassemia Major Patients Infected With Hepatitis C Virus

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Abstract

The levels of Matrix Metalloproteinases-2 (MMP-2) and Tissue Inhibitor of Metalloproteinase-2 (TIMP-2), as well as zinc, iron, and calcium, in patients with β-thalassemia major who undergo repeated blood transfusions, with and without Hepatitis C Virus (HCV) infection, remain unexplored. Many studies have focused on iron overload in β-thalassemia major; however, there is a gap in information regarding the role of MMP-2, both in the presence and absence of HCV infection. The present study focused on the role of MMP-2 and TIMP-2 in β-thalassemia major patients infected with HCV. This cross-sectional observational study included 80 patients suffering from β-thalassemia major; 50 cases had previously been diagnosed as positive for HCV using Enzyme-linked immunosorbent assay (ELISA), and 30 individuals were recorded as negative when using the same technique. Patients aged between 3-48 years had regular blood transfusions and chelation therapy. Sixty healthy individuals were included in this study. Detection of HCV was performed using RT-PCR. ELISA estimated serum levels of MMP-2 and TIMP-2, while calcium, zinc, and iron concentrations were measured using biochemical tests. The receiver operating characteristic (ROC) curve analysis was used to investigate the predictive values of MMP-2 and TIMP-2 for the studied groups. Among ELISA-positive HCV patients, only three individuals (6.0%) showed HCV RNA in their blood samples when RT PCR was used. The mean levels of MMP-2 in HCV-positive and negative β-thalassemia patients were (99.27 ±8.94 and 67.15 ±3.52), respectively, while in the healthy group they were (32.21 ±1.97). The mean levels of TIMP-2 in those patients were (22.91 ±1.54 and 20.29 ±1.22), respectively, and (10.28 ±0.56) for healthy cases. Regarding the mean value of Ca, iron, and Zn, the results were (9.32 ±0.07 for Ca, 341.76 ±11.49 for iron, 67.63 ±3.35 for Zn) and (9.01 ±0.08 for Ca, 284.25 ±8.56 for iron, 59.64 ±3.97 for Zn) in HCV positive and negative β thalassemia patients respectively, while in healthy individuals were (8.71 ±0.07 in Ca, 118.26 ±5.31 in iron, 93.51 ±4.48 in Zn). This study found that infection of β-thalassemia patients with HCV leads to elevated MMP-2 levels, contributing significantly to liver fibrosis through enhanced extracellular matrix breakdown and an inflammatory response. In contrast, TIMP-2, which regulates MMP-2 activity, shows a compensatory increase, reflecting an attempt to balance MMP-2-mediated tissue degradation. This imbalance between MMP-2 and TIMP-2 underscores the accelerated fibrosis seen in these patients.

Keywords: β-Thalassemia major, HCV, MMP-2, TIMP-2, Zinc, PCR, ELISA

التحري المناعي للبروتينات المعدنية-2 ومثبطاتها في النسيج-2 لدى مرضى ثلاسيميا بيتا الكبرى المصابين بفيروس التهاب الكبد نمط ج

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الخلاصة:

لاتزال مستويات البروتينات المعدنية-2 ومثبطاتها في النسيج-2، بالإضافة إلى الزنك والحديد والكالسيوم، غير مستكشفة لدى مرضى الثلاسيميا بيتا الكبرى الذين يخضعون لعمليات نقل دم متكررة، سواء كانوا مصابين بعدوى فيروس التهاب الكبد الوبائي نمط ج ام لا .ركزت العديد من الدراسات على فرط حمل الحديد في الثلاسيميا بيتا الكبرى؛ ومع ذلك، هناك فجوة في المعلومات فيما يتعلق بدور البروتينات المعدنية – 2 ومثبطاتها في النسيج-2، سواء في وجود أو غياب عدوى التهاب الكبد الفيروسي نمط ج. تركز هذه الدراسة على دور البروتينات المعدنية-2 ومثبطاتها في النسيج-2 لدى مرضى الثلاسيميا بيتا الكبرى المصابين بعدوى التهاب الكبد الفيروسي نمط ج وأولئك غير المصابين به .شملت هذه الدراسة الرصدية المقطعية 80 مريضًا يعانون من ثلاسيميا بيتا الكبرى، وقد شُخِّصت 50 حالة سابقًا على أنها إيجابية لفيروس التهاب الكبد الوبائي ج باستخدام تقنية الامتزاز المناعي المرتبط بالإنظيم (الاليزا)، وسُجِّلت نتائج سلبية لـ 30 فردًا باستخدام التقنية ذاتها. خضع المرضى الذين تتراوح أعمارهم بين 3 و48 عامًا لعمليات نقل دم منتظمة وعلاج بالاستخلاب. كما شملت الدراسة 60 فردًا سليمًا. أُجري الكشف الجزيئي عن فيروس التهاب الكبد الوبائي نمط ج باستخدام تفاعل البوليميراز المتسلسل العكسي، وقُدِّرت مستويات البروتينات المعدنية-2 ومثبطاتها في النسيج-2 في مصل الدم باستخدام الاليزا، بينما قيست مستويات الكالسيوم والزنك والحديد باستخدام اختبارات بيوكيميائية. تم استخدام منحنى خاصية تشغيل جهاز الاستقبال للتحري عن القيمة التنبؤية لكل من البروتينات المعدنية-2 ومثبطها في النسيج -2 للمجاميع قيد الدراسة. من بين مرضى التهاب الكبد الوبائي نمط ج الإيجابيين لاختبار الاليزا، أظهر ثلاثة أفراد فقط (6 %) وجود الحمض النووي الريبوزي لفيروس التهاب الكبد الوبائي نمط ج في عينات دمهم عند استخدام تفاعل البوليميراز المتسلسل العكسي. بلغ متوسط مستويات البروتينات المعدنية-2 لدى مرضى بيتا ثلاسيميا الكبرى الإيجابيين والسلبيين (99.27 ± 8.94 و67.15 ± 3.52) على التوالي، بينما بلغ في المجموعة السليمة (32.21 ± 1.97). بينما بلغ متوسط مستويات مثبطات البروتينات المعدنية في النسيج-2 لدى هؤلاء المرضى (22.91 ± 1.54 و20.29 ± 1.22) على التوالي، و(10.28 ± 0.56) لدى الحالات السليمة. وفيما يتعلق بمتوسط قيم الكالسيوم والحديد والزنك، كانت النتائج (9.32 ± 0.07 للكالسيوم، 341.76 ± 11.49 للحديد، 67.63 ± 3.35 للزنك) و (9.01 ± 0.08 للكالسيوم، 284.25 ± 8.56 للحديد، 59.64 ± 3.97 للزنك) في مرضى الثلاسيميا بيتا الكبرى الموجب والسالب لفيروس التهاب الكبد الوبائي نمط ج على التوالي، بينما كانت في الأفراد الأصحاء (8.71 ± 0.07 في الكالسيوم، 118.26 ± 5.31 في الحديد، 93.51 ± 4.48 في الزنك). تكشف هذه الدراسة أن عدوى التهاب الكبد الفيروسي نمط ج لدى مرضى الثلاسيميا بيتا الكبرى تؤدي إلى ارتفاع مستويات البروتينات المعدنية-2، مما يساهم بشكل كبير في تليف الكبد من خلال تعزيز تحلل المادة خارج الخلوية والاستجابة الالتهابية. في المقابل، تظهر مثبطات البروتينات المعدنية في النسيج-2، التي تنظم نشاط البروتينات المعدنية-2، زيادة تعويضية، مما يعكس محاولة لموازنة التحلل النسيجي الذي تسببه البروتيانت المعدنية-2. يبرز هذا الاختلال بين البروتينات المعدنية-2 ومثبطاتها في النسيج-2 التليف المتسارع الذي يُلاحظ في هؤلاء المرضى.

Introduction

Thalassemia is a genetic blood disorder in which the body makes an inaccurate form of hemoglobin. The Greek words "Thalassa" (meaning "sea") and "thalassemia" are derived from the word "Haema" (meaning "blood"). It describes disorders in which the hemoglobin subunits are not synthesized correctly. Hemoglobin is a protein found inside red blood cells that carries oxygen. It comprises two proteins that generate four chains, two

alpha and two beta. Two β-globin genes, located on chromosome 11, and four α-globin genes, located on chromosome 16, synthesize these proteins [1].

Depending on the type of globin that is mutated, there are two types of thalassemia: beta and alpha thalassemia. Whereas β-thalassemia arises from a mutation or damage to both β-globin genes, α-thalassemia caused by damage or alteration to one or more of the four α-globin genes [2]. Additionally, a child with thalassemia major inherits two inaccurate globin genes, one from each parent. In contrast, a child with thalassemia minor inherits a faulty globin gene from a single parent [3, 4]. Patients with thalassemia major typically experience lifelong anemia that starts in early childhood and requires regular blood transfusions due to the abnormalities in their red blood cells. In contrast, patients with minor thalassemia typically exhibit no symptoms and can lead a healthy life without treatment [2].
Thalassemia major is a genetic disorder characterized by severe anemia requiring periodic blood transfusions [5]. However, a transfusion reaction can cause many changes like iron overload, allergic reactions, metabolic complications like hypocalcemia and hypozincemia, the excess volume of fluid in the body, and many other changes that lead to heart, kidney, liver, or splenic failure [6]. Since they get blood transfusions frequently, patients with β-thalassemia major are susceptible to blood-borne viral infections such as Human Immunodeficiency Virus (HIV), hepatitis B, and hepatitis C viruses [7]. Viral hepatitis impacts millions of sufferers globally and has emerged as a significant public health concern [8]. Every hepatotropic virus, such as viruses that cause hepatitis B virus, hepatitis C virus, hepatitis D virus, and hepatitis E virus (HBV, HCV, HDV, and HEV), can result in both acute and chronic infections, impacting millions of people globally, except aside the hepatitis A virus (HAV), which can cause an acute, self-limiting illness that goes away on its own [9].
Iron overload resulting from periodic blood transfusions can cause numerous changes and disrupt the balance and absorption of essential minerals, such as calcium and zinc [10]. Thalassemia patients often experience bone complications that impact calcium metabolism [11]. Iron overload can disrupt the body's calcium balance, potentially lower calcium levels. Furthermore, the iron overload resulting from frequent blood transfusions can interfere with the absorption of other minerals, mainly zinc [12].

However, HCV infection can worsen by affecting and impairing liver function. The liver plays a crucial role in vitamin D metabolism and is essential for calcium absorption. The impaired liver function causes vitamin D deficiency, leading to a decrease in calcium levels. Additionally, the liver plays a crucial role in metabolizing and storing many minerals, including zinc, so impaired liver function leads to altered zinc levels [11]. This imbalance in these minerals causes defects in many biochemical activities and reactions. One of these defects is altered levels of matrix metalloproteinase [12].

The zinc-dependent endopeptidases, known as matrix metalloproteinases (MMPs), are responsible for pathological and physiological tissue remodeling [13]. MMPs degrade multiple substrates not part of the extracellular matrix (ECM) and all its structural elements, like collagen, elastin, and gelatin. MMPs play a key role in many physiological processes, including tissue remodeling, wound healing, and angiogenesis. However, excessive MMP activity can be detected in various pathological conditions, including arthritis, cancer, and cardiovascular diseases [14].

Matrix metalloproteinase-2 (MMP-2), also known as gelatinase A, is a member of the MMP family with specificity for collagen type 4, considered the chief component of basement membranes [15]. The role of MMP-2 is involved in various processes, primarily in

tissue remodeling, cardiovascular disease, and cancer. MMP-2 facilitates the breakdown and rebuilding of tissues during healing and growth. Furthermore, it plays a key role in remodeling blood vessels and heart tissue. Additionally, MMP-2 contributes to tumor invasion and metastasis by breaking down ECM barriers [16].

MMP-2 is activated from its inactive form, Pro-MMP-2, through a complex process involving other inhibitors and enzymes. Regulating the activation of MMP-2 is crucial in both physiological and pathological tissues, and the primary protein that regulates MMP-2 activation is the Tissue Inhibitor of Metalloproteinase-2 (TIMP-2). TIMP-2 is one of the four known TIMPs that inhibit the activity of MMP-2 by binding to it and preventing it from degrading ECM components excessively. Diseases can result from an imbalance between MMPs and TIMPs, which are vital for maintaining balance. However, excessive inhibition may impair normal tissue remodeling and repair, while overactivity of MMP-2 and a lack of sufficient TIMP-2 can facilitate tumor invasion and metastasis [17].

Calcium ions are essential for maintaining the structural integrity and regulation of MMP-2, whereas zinc ions are necessary for the enzyme's catalytic activity [11]. Furthermore, MMP-2 is associated with several thalassemia-related clinical events, including organ damage resulting from iron overload, extramedullary hematopoiesis, tissue remodeling, and bone alterations. Acquiring insight into and focusing on MMP-2 may open up new therapeutic options for thalassemia treatment [12]. The current study aims to determine the prevalence of HCV infections among thalassemia patients and evaluate the serum levels of MMP-2 and TIMP-2 in β-thalassemia patients with and without HCV infection to identify whether these markers may be valuable in these patients.

Materials and Methods

This cross-sectional study was based on a hospital record review and involved 140 samples, comprising 50 β-thalassemia major patients with an ELISA-positive detection for HCV, 30 β-thalassemia major patients with an ELISA-negative detection for HCV, and 60 healthy individuals. These samples were collected from November 2023 to March 2024 at Ibn Al-Baladi Hospital in Baghdad, Iraq.

The blood samples were collected in an EDTA tube with 1 ml of triazole for each 2 ml of whole blood used in HCV molecular detection. The clinical samples that were positive for HCV by ELISA were further tested for HCV detection using Real-Time PCR (RT-PCR) to determine the prevalence of HCV infections among patients with β-thalassemia major. Three ml of blood was dispensed into the gel tube and centrifuged at 3000 rpm for 20 minutes to obtain the serum, then separated into the Eppendorf tubes and stored at -20 ºC until immunological investigations were achieved. Sandwich enzyme immunoassay is the test principle used in estimating the level of MMP-2 and TIMP-2. All collected samples were analyzed using biochemical tests to measure the concentrations of calcium, iron, and zinc [20].

Results and Disc

The study's design was approved on October 28, 2023, by the Research Ethics Committee and Scientific Committee of the University of Baghdad, Department of Biology, College of Science, under reference number CSEC/1023/0071.

Inclusion criteria and exclusion criteria

The inclusion criteria for the patients in this study were limited to patients aged 2-48 years. This age range was selected because the diagnosis of β-thalassemia major is typically determined at this age (after two years) when HbA and HbA2 are produced instead of HbF; therefore, all patients were diagnosed as β-thalassemia major cases using Hb electrophoresis. However, all other types of thalassemia have been excluded.

When MMP-2 levels were reported to be elevated under inflammatory and infectious conditions, healthy control individuals were collected after ensuring that they did not have any conditions by checking their WBC and CRP to exclude cases that showed positive results. The samples were collected correctly; however, all hemolysis samples were excluded because they may have interfered with the spectrophotometer readings, potentially resulting in false positives or false negatives.

Statistical analysis

The SAS (2018) software was utilized to determine the impact of different groups on the study parameters. ANOVA and the least significant difference (LSD) test were used to statistically compare the means. The study employed the chi-square test to compare percentages statistically significantly ($p < 0.05$ and $p < 0.01$). Tables and graphs were used to display each outcome [19]. The receiver operating characteristic (ROC) and area under the ROC curve (AUC) analysis were used to investigate the predictive values of MMP-2 and TIMP-2 for 3 groups (β-thalassemia with positive HCV, β-thalassemia without HCV, and control).

Results and discussion

A total of 80 patients with β-thalassemia major were divided into two groups: the first group, 50 samples with positive HCV ELISA results. The second group: 30 samples with negative HCV ELISA results. The groups were sorted by age using statistical analysis, and the results showed a significant difference between the examined groups ($P \leq 0.05$). The age range of patients with β-thalassemia major was 3-48 years, with a mean age of 22.02 ±1.37 for the first group and 18.80 ±1.72 for the second one, compared to the healthy control group, whose age is nearly the same (5-48), with a mean of 24.45 ±1.23 (Table 1).

Table 1: Distribution of study samples according to age groups in patients and controls

Age group (year)	*Positive No. (%)*	*Negative No. (%)*	*Healthy No. (%)*	*P-value*	X^2
<20 yr.	**21 (42.00%)**	**17 (56.67%)**	**17 (28.33%)**	**0.743 NS**	***0.593***
20-30 yr.	**21 (42.00%)**	**8 (26.57%)**	**26 (43.33%)**	**0.0086 ****	***9.582***
>30 yr.	**8 (16.00%)**	**5 (16.67%)**	**17 (28.33%)**	**0.0109****	***7.882***
Total	**50**	**30**	**60**	**---**	**---**
P-value	**0.0328 ***	**0.0194 ***	**0.255 NS**	**---**	**---**
X^2	**6.389**	**7.261**	**2.037**	**---**	**---**

**** (P≤0.05), ** (P≤0.01)***

Age is considered a standard measure of the severity of β thalassemia major due to many reasons, such as complications over time, bone health, or infection risk that may result from blood transfusion, medications that may play a key role in a weakened immune system, or be associated with frequent hospital visits [20].

Patients with thalassemia may age more quickly and develop consequences like diabetes, heart disease, and other age-related illnesses. The interaction between ordinary aging disorders and thalassemia-related diseases complicates management. Due to persistent iron excess and potential hormone deficiencies, osteoporosis and fractures are more likely,

and bone health deteriorates over time [21]. Furthermore, this study reveals no significant differences between the sexes in the studied groups compared to control cases, as the number of males in the HCV-positive ELISA cases was 26 (52.00%), while the number of females was 24 (48.00%). Additionally, cases with HCV-negative ELISA results comprised 16 males (53.33%) and 14 females (46.67%). Furthermore, the healthy control cases consisted of 31 males (51.67%) and 29 females (48.33%) (Table 2).

Table 2: Distribution of study samples according to sex

Sex	Positive No. (%)	Negative No. (%)	Healthy No. (%)	P-value	X^2
Male	26 (52.00%)	14 (46.67%)	31 (51.67%)	0.0329 *	6.523
Female	24 (48.00%)	16 (53.33%)	29 (48.33%)	0.0405 *	4.783
Total	50	30	60	---	---
P-value	0.778 NS	0.715 NS	0.796 NS	---	---

* (P≤0.05), NS: Non-Significant.

Male β-thalassemia major patients show more severity in symptoms than females for many reasons, such as physiological and hormonal differences. Additionally, multiple studies show that males have more severe iron overload than females, and this is justified simply by menstruation, which involves regular blood loss and is considered a natural protection for females from severe iron overload [22]. Female hormones, such as estrogen, can influence the disease and response to treatment by exerting protective effects against oxidative stress caused by iron overload. Furthermore, female β-thalassemia major patients face a specific challenge in fertility and pregnancy when iron overload can affect reproductive health as well as pregnancy, which requires careful management to minimize the risk for both fetus and mother [23].

Fifty β-thalassemia major patients undergoing weekly blood transfusion and showing positive detection for HCV antibodies by ELISA were molecularly tested for HCV antigens to determine the prevalence of this virus among these patients. It was found that there was a highly significant difference between positive results and negative results. HCV RNA was detected in the plasma of 3 patients using RT-PCR, with a detection rate (6.00%). In contrast, the remaining 47 patients (94.0%) showed no detectable HCV RNA in their plasma (Table 3).

Table 3: Distribution of study samples according to PCR results.

HCV/RT-PCR	**No**	**Percentage (%)**
Positive	**3**	**6.00 %**
Negative	**47**	**94.00 %**
Total	**50**	**100%**
X^2	**---**	**38.720**
P-value	**---**	**0.0001 ******

**** (P≤0.001).**

The periodic blood transfusion process surpasses the risk of HCV infection in these patients. However, all blood samples used to be tested by serological means, such as ELISA, and the infection is likely to occur [24] since HCV infection progresses through several

phases, from the initial stage to severe liver disease. During the initial stages, which precede the window stages, when the body has not yet produced HCV antibodies, it is not possible to detect the viral antibody using the ELISA technique [25]. This could be a serious problem if it can't be treated immediately. Molecular detection of blood units before transfusion is essential and recommended [7]. All those 50 patients had a previous infection of HCV through continuous blood transfusion that showed a positive detection for HCV antibodies by ELISA.

As a response, a medication treatment plan for all patients is necessary to eliminate the viral infection, as direct-acting Antivirals (DAAs) are the antiviral drugs used to reduce the viral load. DAAs are highly effective in treating HCV infections, often leading to a negative PCR result, indicating that the virus has been eradicated. This response is delayed for 12 weeks or more after completing antiviral therapy. Achieving a Sustained Virologic Response (SVR) is regarded as equivalent to a successful treatment, as it demonstrates the eradication of the virus and the absence of its genome in the blood [26]. After the therapy course ended, the HCV RNA remained absent in the patient's plasma and was undetectable by RT-PCR; this case is known as SVR.

The immunological investigation in this study focused on measuring the levels of MMP-2 and TIMP-2. HCV-positive ELISA patients exhibit a significant increase in MMP-2 levels, with a mean of 99.27 ± 8.94. In contrast, HCV-negative ELISA patients exhibit an increase in MMP-2 levels, albeit slightly lower than those in the first group, with a mean of 67.15 ± 3.52. However, the healthy control individuals exhibit different levels of MMP-2 than the patient groups, with a mean of 32.21 ± 1.97, indicating a highly significant difference ($p = 0.0001$) (Table 4).

The presence of HCV infection in thalassemia patients can significantly elevate MMP-2 levels, contributing to liver fibrosis and related complications. Elevated MMP-2 levels can accelerate liver fibrosis, leading to a more rapid progression of liver disease in HCV-infected β-thalassemia patients. This can complicate management and worsen prognosis [32]. Regular monitoring of MMP-2 levels could be useful in assessing liver disease progression in HCV-infected thalassemia patients. MMP-2 inhibitors might be explored as potential therapeutic agents to mitigate liver fibrosis in thalassemia patients with HCV [16]. Several immune response indicators, such as IL-6, IFN, and TNF, in chronic hepatitis C patients demonstrated a highly significant immune response between pre- and post-treatment patients. Additionally, anti-inflammatory cytokines, such as interleukin-10, play a crucial role in protecting the body and promoting immunological tolerance against HCV infection [31, 32].

Table 4: The mean level of MMP-2 in the patients and control groups.

Group	***Mean ±SE***
	MMP-2
G1: Positive	**99.27 ±8.94 a**
G2: Negative	**67.15 ±3.52 b**
G3: Healthy	**32.21 ±1.97 c**
LSD	**16.468 ****
P-value	**0.0001**

This means that the different letters in the same column differed significantly, * (P ≤ 0.005), ** (P ≤ 0.001).

Furthermore, this study revealed another significant difference ($p = 0.0001$) in the levels of TIMP-2 among the tested groups. HCV-positive ELISA Patients show high levels of TIMP-2,

with a mean of 22.91 ± 1.54, and HCV-negative ELISA patients show a very close result to the first group, with a mean of 20.29 ± 1.22. Healthy control individuals have significantly lower levels than the patient groups, with a mean of 10.28 ± 0.56 (Table 5).

Table 5: The mean level of TIMP-2 in patients and control groups.

Group	*Mean ±SE*
	TIMP-2
G1: Positive	**22.91 ±1.54 a**
G2: Negative	**20.29 ±1.22 a**
G3: Healthy	**10.28 ±0.56 b**
LSD	**3.323 ****
P-value	**0.0001**

This means that the different letters in the same column differed significantly, ** (P ≤ 0.001).

Elevated TIMP-2 levels in thalassemia patients with HCV infection indicate a protective response against excessive ECM breakdown and the development of fibrosis [33]. Elevated TIMP-2 levels indicate continued liver fibrosis and an attempt to control ECM turnover [34].

Analysis of the ROC and AUC for MMP-2 and TIMP-2

For group 1 (β-thalassemia major with HCV positive) vs. control, the AUC was 0.927, indicating excellent classification accuracy. At the optimal cutoff of 57.143, sensitivity reached 1.000, ensuring no false negatives, while specificity was 0.633, reflecting moderate false positive rates. Similarly, for group 2 (β thalassemia major without HCV) vs. control, MMP-2 maintained an AUC of 0.866 (good performance), with a cutoff of 54.286, yielding identical sensitivity (1.000) and specificity (0.633). These results suggest MMP-2's utility as a highly sensitive screening tool, albeit with moderate specificity.

TIMP-2 also exhibited strong discriminative power. For group 1 vs. control, the AUC was 0.901, with an optimal threshold of 15.098, achieving perfect sensitivity (1.000) and specificity of 0.617. In group 2 vs. control, TIMP-2's AUC was 0.866, comparable to MMP-2, with a cutoff of 15.074, retaining maximal sensitivity (1.000) and specificity (0.617). While specificity was marginally lower than MMP-2, TIMP-2's balanced sensitivity-specificity profile underscores its potential for confirmatory testing in clinical workflows.

Both biomarkers achieved near-perfect sensitivity, which is critical for minimizing missed diagnoses. However, MMP-2's higher specificity in Group 1 vs. 3 (0.633 vs. TIMP-2's 0.617) suggests a slight superiority in reducing false positives. Clinically, MMP-2's performance aligns with screening applications, whereas TIMP-2's balanced metrics support its use in secondary validation. The consistent AUC values across patient groups (>0.866) further validate their reliability in the tested groups (Table 6).

Table 6: Analysis of the ROC and AUC for MMP-2 and TIMP-2

Biomarker	*Comparison*	*Cutoff*	*AUC*	*Sensitivity*	*Specificity*
MMP-2	**1 vs. 3**	**57.143**	**0.927**	**1.000**	**0.633**
MMP-2	**2 vs. 3**	**54.286**	**0.866**	**1.000**	**0.633**
TIMP-2	**1 vs. 3**	**15.098**	**0.901**	**1.000**	**0.617**
TIMP-2	**2 vs. 3**	**15.074**	**0.866**	**1.000**	**0.617**

MMP-2 and TIMP-2 emerged as high-performing biomarkers for distinguishing thalassemia patients from healthy controls. Their AUC values (>0.866) and perfect sensitivity highlight their diagnostic robustness. While MMP-2's marginally higher specificity may favor its use in initial screenings, TIMP-2's balanced profile supports confirmatory roles.

Additionally, the tested group in this study shows highly significant differences (p = 0.0001) for Ca, iron, and Zn in the means of their concentration (Table 7). Firstly, calcium showed a substantial difference in mean concentration between the tested groups, as HCV-positive ELISA cases have a mean of 9.32 ± 0.07, whereas HCV-negative ELISA cases have a mean of 9.01 ± 0.08. However, healthy cases showed a decrease in their mean of 8.71 ±0.07. To explain this, all cases were investigated to determine whether the individuals were taking calcium supplements. However, all β-thalassemia major patients in this study followed up with preventive measures for bone health, which include a prescription that involves a calcium supplement with a dose of 1,200 mg/day and vitamin D3 with a dose of 5,000-10,000 IU/day.

Table 7: Calcium, iron, and zinc concentrations in the tested group.

Group	*Mean ±SE*		
	Ca (µg/dl)	**Iron (µg/dl)**	**Zn (µg/dl)**
G1: Positive	**9.32 ±0.07 a**	**341.76 ±11.49 a**	**67.63 ±3.35 b**
G2: Negative	**9.01 ±0.08 b**	**284.25 ±8.56 b**	**59.64 ±3.97 b**
G3: Healthy	**8.71 ±0.07 c**	**118.26 ±5.31 c**	**93.51 ±4.48 a**
LSD	**0.227 ****	**27.811 ****	**11.227 ****
P-value	**0.0001**	**0.0001**	**0.0001**

This means having the different letters in the same column differed significantly, where a is the highest mean and so on, where ab is not significantly different from a nor b, ** (P≤0.001).

Iron overload in β-thalassemia major patients can disturb calcium metabolism. Excessive iron can accumulate in various organs, including the bones, thereby interfering with normal calcium homeostasis, a process that requires lifelong monitoring [27]. Iron concentration is more likely to calcium, which shows highly significant differences between the tested groups. HCV-positive ELISA cases have 341.76 ±11.49, while cases with HCV-negative ELISA show 284.25 ±8.56. However, healthy cases have normal iron levels, with a mean of 118.26 ± 5.31. Increased iron levels are considered normal in patients with thalassemia major, as they require periodic blood transfusions throughout their lives, which are necessary for survival. Iron overload causes severe damage to many body tissues. The primary treatment for managing iron overload. Chelators like deferoxamine, deferiprone, and deferasirox bind to excess iron, facilitating its excretion [28].

Finally, zinc levels show significant differences between the tested groups with different perspectives. HCV-positive ELISA cases show a low zinc level with a mean of 67.63 ±3.35, and cases with HCV-negative ELISA appeared to have a mean of 59.64 ±3.97, while healthy controls showed a normal zinc level with a mean of 93.51 ±4.48. Zinc is vital for growth and development; therefore, a deficiency in patients with β-thalassemia major can lead to stunted growth and delayed puberty [29]. Furthermore, zinc deficiency can impair immune function, making patients more susceptible to infections [30]. Patients with β-thalassemia major should be particularly concerned about zinc deficiency due to increased urine excretion, impaired absorption resulting from iron overload, and oxidative stress associated with the condition.

Improving patient outcomes requires handling this issue with possible supplements alongside constant monitoring [31].

Conclusion

In patients with thalassemia major, HCV infection leads to elevated levels of MMP-2, comparable to those of TIMP-2. This elevation reflects the body's response to increased liver fibrosis, as it attempts to regulate tissue degradation and remodeling. Monitoring TIMP-2 levels can provide valuable insights into the severity of liver fibrosis and help guide the management of thalassemia patients with HCV infection. Effective control of MMP-2 and TIMP-2 levels may offer therapeutic benefits, reducing fibrosis progression and improving liver function. Consequently, in future studies, it is necessary to increase the sample size to get more data, thus increasing the reliability of the statistical analysis.

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Data Availability Statement

Upon request, the corresponding author will provide all the data supporting the study's conclusions.

Conflict of interest

The writers declare no conflict of interest.

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