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The Association of TIMP-1 Serum Level and Genetic Polymorphisms in Type II Diabetes Mellitus Patients: A Novel Case-Control Study

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Abstract

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia due to decreased insulin secretion, deficient insulin action, or both. DM includes type 1 and type 2. Type 2 diabetes mellitus (T2DM) is primarily caused by insulin resistance, decreased insulin secretion, and increased glucose production. Tissue inhibitors of metalloproteinase-1 (TIMP-1) play a crucial role in extracellular matrix remodeling, which is essential for maintaining skin integrity and promoting wound healing. This study aimed to investigate the association between TIMP-1 genetic variants and their serum levels with the susceptibility to the disease and the development of skin problems in T2DM patients. This study, conducted from November 2023 to April 2024, involved 83 T2DM patients and 75 healthy controls, aiming to investigate the TIMP-1 serum level and determine TIMP-1 single nucleotide polymorphisms (SNPs) rs4898 and rs5953060 frequencies. Results showed higher TIMP-1 levels in the T2DM group (1.83±0.04 *vs.* 1.66±0.03pg/ml, respectively). Genotyping revealed an increased percentage of TC, CC genotypes, and *C* allele frequencies for rs4898, and CC genotype and *C* allele for rs5953060 in the T2DM group (42.17%, 33.73%, and 55.0% for rs4898, 24.10%, and 44.0% for rs5953060). Pairwise linkage disequilibrium analysis between the two SNPs demonstrated that rs4898 had a strong LD with rs5953060 (D'=0.95). Haplotype reconstruction yielded three common two-SNP haplotypes in T2DM versus controls, with none reaching statistical significance (CG:42.6% *vs.* 38.0% TG:52.2% *vs.* 54.0%, and TC:3.6% *vs.* 8.0%, respectively, all P>0.05). Although the TC haplotype showed a non-significant protective trend. In conclusion, TIMP-1 serum levels and the TC, CC/*C* of rs4898, and CC/*C* of rs5953060 may be risk factors for skin lesions and susceptibility to T2DM.

Keywords: Type 2 diabetes mellitus, TIMP-1, SNPs, rs4898, rs5953060, PCR, DNA sequencing, LD, haplotype, skin lesions, diabetic foot.

علاقة مستوى الانزيم المعدني مثبط الأنسجة–1 في المصل والتغاير الوراثي في مرضى داء السكري النمط الثاني: دراسة مرضى – سيطرة جديدة

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الخلاصه

داء السكري هو اضطراب ايضي يتميز بارتفاع سكر الدم المزمن الناتج عن انخفاض افراز الانسولين، نقص في عمل الانسولين، أو كليهما. داء السكري يشمل النمطين الأول و، داء السكري النمط الثاني يحدث بشكل أساسي نتيجة مقاومة الانسولين، وانخفاض افرازه، وارتفاع انتاج الكلوكوز. تلعب الانزيمات المعدنية المثبطة للأنسجة (TIMP−1) دورا مهما في إعادة تشكيل المصفوفة خارج الخلية، وهو أمر ضروري لسلامة الجلد والتئام الجروح. هدفت هذه الدراسة الى استقصاء العلاقة بين المتغيرات الجينية لـ TIMP−1 ومستواه في المصل من جهة أخرى، وقابلية الإصابة بالآفات الجلدية عند مرضى السكري النمط الثاني من جهة أخرى. أجريت الدراسة في الفترة من تشرين الثاني 2023 إلى نيسان 2024، شملت الدراسة 83 مريضا بداء السكري النمط الثاني و75 فردا سليما. أظهرت النتائج ارتفاعا معنويا في متوسط مستويات TIMP−1 في مجموعة المرضى مقارنة بمجموعة السيطرة الصحية ()). كشف التحليل الجيني عن زيادة في النسب المئوية للأنماط الوراثية TC وCC وتردد الأليل *C* للموقع rs4898 (على التوالي)، كذلك النمط الوراثي CC وتردد الأليل *C* للموقع rs5953060 (على التوالي). أظهر اختبار الاختلال الارتباطي الثنائي LD بين الموقعين ارتباطا قويا (D` = 0.95). أفضى إعادة بناء الأنماط الوراثية الى تحديد 3 أنماط شائعة مكوّنة من موقعين مختلفين لتغاير النيكليوتيدة المفردة في مرضى السكري النمط الثاني مقارنة بمجموعة السيطرة الصحية، ولم يصل أي منها الى دلالة إحصائية (النمط GC بنسبة 42.6% مقابل 38.0%، النمط TG بنسبة 52.2% مقابل 54.0%، والنمط TC بنسبة 3.6% مقابل 8.0%، قيمة الاحتمالية لجميع الأنماط كانت أكبر من مستوى الدلالةالاحصائية 0.05). مع ذلك، أظهر النمط TC اتجاها وقائيا غير ذي دلالة إحصائية. الاستنتاجات، تشير نتائجنا الى ان ارتفاع مستويات TIMP−1 في المصل، الى جانب الأنماط الوراثية TC وCC والأليل *C* للموقع rs4898 والنمط الوراثي CC والأليل *C* للموقع rs5953060، قد تشكل عوامل خطر للإصابة بالآفات الجلدية وزيادة القابلية للإصابة بداء السكري النمط الثاني.

1. Introduction

Diabetes mellitus (DM), a set of metabolic disorders, is characterized by chronic hyperglycemia and is caused by either decreased insulin secretion, deficient insulin action, or both [1, 2]. DM generally falls into two categories: type 1 and type 2. Type 1 is mostly caused by a complete lack of insulin [3], but type 2 diabetes, or T2DM, is the most prevalent type of the disease and is brought on by a loss of insulin sensitivity in the target tissue such as the adipose, skeletal, and hepatic tissues, also is primarily caused by variable degrees of insulin resistance, decreased insulin secretion, and high glucose production [4]. Other forms of diabetes are very rare and may result from a single gene mutation; one such variety is gestational diabetes, which appears during pregnancy [5]. Diabetes cannot be cured. However, with the correct care and lifestyle modifications, individuals can live longer than those who are healthy [6]. Diabetes can cause dermatological issues in 80% of patients, affecting their quality of life, especially when those did not follow a healthy lifestyle, leading to an increase in the death percentage [7]. Skin lesions have a substantial negative influence on patients' quality of life and are frequently used as markers of systemic vascular problems and metabolic abnormalities in T2DM [8].

Zinc-dependent endopeptidases are a group of enzymes that play a crucial homeostatic function in extracellular signaling and remodeling [9]. The production of these enzymes typically results from the activation of wounded cells (epithelial, fibroblast, and vascular endothelial cells) as well as inflammatory cells (macrophages and neutrophils) [10]. These primary enzymes are responsible for breaking down the extracellular matrix (ECM). One example of zinc-dependent endopeptidases is matrix metalloproteinases (MMPs), which are

capable of breaking down nearly every component of the ECM [11]. They have the ability to cleave the peptide bonds found in most ECM components, including tight junction proteins, fibronectin, laminin, proteoglycans, and different forms of collagen [12]. The disruption of ECM by MMPs is known to affect several physiological processes, including embryo implantation, healing of wounds, and remodeling of tissues, and also pathophysiological processes in many conditions, such as cancer, rheumatoid arthritis, genetic disorders, chronic renal disease, aging, and cardiovascular diseases [13, 14].

In contrast, the tissue inhibitors of matrix metalloproteinases (TIMPs) family include four conserved proteins (TIMP-1 – 4) that consist of 184–194 amino acids with a molecular weight of around 21 kDa; all TIMPs are soluble in the ECM, except TIMP-3, which is attached to the matrix. The function of TIMPs is to inhibit and regulate MMPs activity and perform MMP-independent tasks, which are endogenous protein regulators [15]. Nonetheless, TIMP-1's biological roles extend beyond its inherent participation in ECM turnover. TIMP-1 has come to light in recent years as a multifunctional protein that, even in the absence of MMP inhibitory activity, is capable of regulating a variety of cellular processes, such as cell division, apoptosis, stem cell differentiation, and neutrophil granulocyte homeostasis [16, 17]. The majority of body fluids and tissues contain the soluble protein TIMP-1, which is discharged into the extracellular space. When subjected to external stimuli such as phorbol esters, serum, cytokines (IL-6 and IL-1), and various growth factors, cells upregulate the expression of TIMP-1, which in turn modulates ECM remodeling and inflammatory signaling [18]. In the context of T2DM, elevated TIMP-1 levels have been associated with vascular complications and insulin resistance, suggesting its involvement in the pathophysiological remodeling of diabetic tissues [18–20]. TIMP-1 plays a dual role in the development of skin lesions in T2DM through its regulation of ECM remodeling and its genetic variability. At the protein level, the elevated TIMP-1 expression in diabetic patients may disrupt the balance with MMP-9, leading to impaired collagen turnover, delayed wound healing, and fibrotic skin changes [19]. Genetically, polymorphisms in the TIMP-1 may influence susceptibility to diabetic complications by altering TIMP-1 expression or function [20, 21]. For this purpose, the present study aimed to investigate the association between TIMP-1 genetic variants and their serum levels with the susceptibility to the disease and the development of skin problems in T2DM patients.

2. Materials and methods

This study was approved by Ethical Committee of Biotechnology Department, College of Science, University of Baghdad with the reference number (CSEC/0124/0008) and 83 patients clinically diagnosed with T2DM (44 females and 39 males), whose ages ranged from 35 to 79, and 75 non-diabetic healthy volunteers (37 females and 38 males), whose ages ranged from 35 to 79, considered a healthy control group. The blood samples were collected from individuals who visited Al-Kadhimiya Teaching Hospital, Yarmouk Teaching Hospital, and City Medical Center in Baghdad city. The demographical information of each patient and control was documented via a questionnaire under the supervision of the consultant. The exclusion criteria of the present study included T1DM, TIIDM without skin lesions, and T2DM patients with a disease duration of less than five years. Healthy people without diabetes or other endocrine issues made up the control group.

Samples' collection

A volume of venous blood sample (5 ml) was dropped from both the patients and the healthy control groups. Each sample was divided into two parts, 2 ml of dropped blood was transferred to a sterile EDTA tube for molecular experiments, and the residual 3ml was transferred to a gel tube, to allow the blood to clot at room temperature for 15 minutes; then

centrifuged at 3000 rpm for 10 minutes to obtain the serum, which was divided into two 0.5 ml aliquots in sterile tightly closed Eppendorf tubes, then stored at -20°C until assayed for subsequent biochemical and immunological analysis.

TIMP-1 serum level assessment

The sandwich ELISA kit, provided by Al-Shkairate establishment of medical supply (Jordan), was designed for the quantitative detection of TIMP-1 in the serum of the studied groups. The detection rate of the kit (Catalogue No: RDEEH0294) was 31.25 – 2000 pg/ml, and the sensitivity was <18.75 pg/ml, while the intra-assay: CV <8% and inter-assay: CV <10%.

Genomic DNA extraction, PCR amplification and Sanger sequencing

The FavorPrep™ Genomic DNA Extraction Mini Kit (Taiwan) was used to extract the whole genomic DNA from the frozen blood. The concentration and purity of the extracted DNA were measured using a Nanodrop at a 260/280 nm wavelength. Primers used for the genotyping of TIMP-1 were newly designed using SnapGene software and then verified using the NCBI and UCSC In-Silico PCR websites [22, 23]. Macrogen DNA Company supplied the primers as a lyophilized product to amplify the targeted region of the TIMP-1 genes according to the instructions of the manufacturing company through dissolving it with the appropriate volume of nuclease-free water, to get a final concentration of 100 pmol/µl, then stored at –20°C. The primers' sequences, conditions, and PCR product size are illustrated in Table 1.
All PCR reactions were amplified in a thermal cycler machine (Bioer Gene Explorer). The mixture of reaction was prepared as follows: 12.5µl from OneTaq® 2X Master Mix provided by (Biolabs, England), 1µl (10 pmol) from each primer, 4 µl of template genomic DNA (50 - 100 ng/µL), and nuclease-free water were added to accomplish a total volume of 25 µl. The PCR thermal conditions are illustrated in Table 1. The gel electrophoresis technique was applied to visualize the amplified bands for each sample on a 1.5% agarose gel pre-stained with Red Safe stain® (Intron, South Korea) based on the size of the resulting bands through using the Universal DNA ladder (Transgen, China). The amplicons were sent to Macrogen DNA company (South Korea) for sequencing the amplified fragments using the Sanger sequencing technique and analyzing the DNA sequences to determine the resulting genetic variants of the targeted region.

Table 1: The primers' sequencing of TIMP-1, conditions and amplicon size

Primers sequence (5'->-3')	PCR Conditions	PCR Product size
Forward primer **CAGTAAAAGAGACACTTCCCC** **Reverse primer** **ACAAGGGAGAATTAGAGGGAC**	-An Initial denaturation at 94 C° for 3 min. -Then,35 cycles, each cycle consisted of - denaturation at 94C° for 30s. - annealing at 51 C° for 45s. - extension at 72C° for 35s. -A final extension 72C° for 10 min.	753bp

Statistical Analysis

The effect size and power of the samples were calculated using the G*Power program version 3.1.9.4 [24]. All statistical analyses were conducted using IBM SPSS version 29.0. Normality, homogeneity, and randomisation were checked for the continuous variables. Continuous variables were presented as means and standard error, while categorical variables were expressed as numbers and percentages. Significant differences between two continuous variables were determined using an independent t-test and an ANOVA table (Duncan test) for multiple comparisons. WinepiP version 11.65 and an online Hardy-Weinberg equilibrium calculator [25] were used to calculate the odds ratio, 95% confidence intervals, Bonferroni

corrected probability, and the frequencies of the genotypes and alleles. P values <0.05 were considered statistically significant. Bonferroni correction was used to determine the yielding threshold of 0.0125, resulting from dividing the α-level probability (0.05) by 4 (allele frequency + the three genotype frequency comparisons). So, the corrected P-value (Pc) was calculated as the P-value multiplied by 4, and any Pc-value < 0.0125 was deemed significant. Linkage disequilibrium (LD) and haplotype construction were analyzed using SHEsis Plus online software [26]. The LD coefficient (D') was employed to represent the LD between SNPs.

3. Results

The present study included 150 samples, totalling 75 blood samples collected from patients with T2DM who had foot skin lesions, compared with 75 individuals as healthy control group. Table 2 presents a comprehensive comparison of attributes between T2DM patients and a control group, focusing on parameters such as age, sex, occupation, smoking habits, diabetes history, and disease duration.

The results in Table 3 showed the levels of TIMP-1 in the sera of the studied groups. There was a significant increase in the level of TIMP-1 in the sera of the T2DM group compared to the healthy control group ($P < 0.05$). Additionally, the results in Table 4 show the distribution of TIMP-1 serum levels according to the age groups of the T2DM group. The results showed that no significant differences appeared among the compared age groups ($P > 0.05$), but patients in the 45–49 years age group had the highest level of TIMP-1.

Additionally, the results in Table 5 show the distribution of TIMP-1 serum levels according to the patients' occupations. The results showed no significant differences ($P > 0.05$) appeared among the patients' jobs. The housewives' and retired patients had the highest level of TIMP-1. In addition, the results in Table 5 showed the distribution of TIMP-1 serum levels according to the patients' smoking status and patients' family history status, respectively. For the smoking status, the results showed a non-significant increased level ($P > 0.05$) of TIMP-1 in the non-smokers' patients compared to the smokers' patients. Similarly, for the family history status, the results showed a non-significant increase in TIMP-1 levels ($P > 0.05$) in patients with a negative family history compared to those with a positive family history.

Table 2: The demographic data of the studied groups

The demographic data		Patients' group (n=75)	Control group (n= 75)	P-value
Age mean ± SE (years)	Total	55.83 ± 1.02	55.13 ± 1.0	0.626
	♂	55.59 ± 1.76	55.89 ± 1.72	0.882
	♀	56.05 ± 1.14	54.35 ± 0.99	0.400
	P-value	0.818	0.459	
Sex frequency (%)	♂	39 (47.0)	38 (50.7)	0.213
	♀	44 (53.0)	37 (49.3)	
	Total	83 (100.0)	75 (100.0)	
Occupations frequency (%)	Employees	31 (37.3)	25 (33.3)	0.006
	Not-employees	3 (3.6)	15 (20.0)	
	Housewives	41 (49.4)	25 (33.3)	
	Retired	8 (9.6)	10 (13.3)	
Smoking status frequency (%)	Smokers	34 (41.0)	0 (0.0)	3.9×10^{-10}
	Non-smokers	49 (59.0)	75 (100.0)	
Family history frequency (%)	Yes	46 (55.4)	0 (0.0)	1.9×10^{-14}
	No	37 (44.6)	75 (100.0)	
Disease duration period (years)	1 – 10	18 (21.7)	-	Uncountable
	11 – 20	43 (51.8)	-	
	21 – 30	21 (25.3)	-	
	31 – 40	1 (1.2)	-	

Table 3: The mean and SE of TIMP-1 levels between the studied groups

Groups	TIMP-1 serum level mean ± SE (pg/ml)	P-value
Patients' group	1.83 ± 0.04	**0.002**
Control group	1.66 ± 0.03	

Table 4: The mean and SE of TIMP-1 levels in the patients group according to the age groups

Aged groups (years)	TIMP-1 (pg/ml)
35 - 39	1.81 ± 0.08 ^{A}
40 - 44	1.70 ± 0.06 ^{A}
45 - 49	**1.82 ± 0.06** ^{A}
50 - 54	1.72 ± 0.09 ^{A}
55 - 59	1.79 ± 0.07 ^{A}
60 - 64	1.72 ± 0.08 ^{A}
65 – 69	1.77 ± 0.10 ^{A}
70 – 75	1.69 ± 0.09 ^{A}
ANOVA table (Duncan test): the similar letters referred to non-significant differences (P > 0.05) among the compared aged groups	

Table 5: The mean and SE of TIMP-1 levels in the patients group according to the patients' occupations, smoking status, and family history status.

Jobs	TIMP-1 (pg/ml)
Employees	1.81 ± 0.08 ^{A}
Not-employees	1.74 ± 0.21 ^{A}
Housewives	1.86 ± 0.06 ^{A}
Retired	1.85 ± 0.09 ^{A}
Smoking status	
Smokers	1.74 ± 0.08
Non-smokers	1.90 ± 0.05
P-value	0.065
Smoking status	
Smokers	1.74 ± 0.08
Non-smokers	1.90 ± 0.05
P-value	0.065
ANOVA table (Duncan test): the similar letters referred to non-significant differences (P > 0.05) among the compared jobs	

The results in Tables 6 and 7 showed the distribution of TIMP-1 serum levels according to the patients' disease duration, foot lesions, and diabetic foot status, respectively. For disease duration, the results of TIMP-1 showed non-significant differences (P > 0.05) among the compared periods. The patients with a disease duration of 31–40 had the highest level of TIMP-1. In addition, the results showed non-significant increases in TIMP-1 serum levels in the patients with a positive foot lesion status compared to those with a negative foot lesion status. In contrast, the results showed a non-significant increase (P > 0.05) in the level of TIMP-1 in the patients with positive diabetic foot compared to those with negative diabetic foot.

Table 6: The mean and SE of TIMP-1 levels in the patients group according to the patients' disease duration

Disease duration periods	TIMP-1 (pg/ml)
1 – 10 years	1.70 ± 0.14 ^{A}
11 – 20 years	1.89 ± 0.05 ^{A}
21 – 30 years	1.87 ± 0.05 ^{A}
31 – 40 years	0.93 ^{A}
ANOVA table (Duncan test): the similar letters referred to non-significant differences (P > 0.05) among the compared disease duration periods	

Table 7: The mean and SE of TIMP-1 levels in the patients group according to the patients' foot lesions and diabetic foot status

Foot lesions	TIMP-1 (pg/ml)
Yes	1.87 ± 0.04
No	1.72 ± 0.13
P-value	0.165
Diabetic foot status	
Yes	1.91 ± 0.08
No	1.79 ± 0.05
P-value	0.191

Molecular analysis

This study involved the extraction of DNA from frozen blood samples of individuals with T2DM and healthy controls using the FavorPrep ™ Genomic DNA Extraction kit. The results showed that the DNA concentration was adequate for PCR amplification.

The genetic polymorphisms of TIMP-1 SNPs rs4898

Using the Eppendorf thermocycler, the target region of the TIMP-1 gene SNPs rs4898 was amplified according to the program described in Table 1. The PCR product (2.5 µL) was electrophoresed on a 1.5% agarose gel at 80 volts for 80 minutes, together with a universal DNA ladder (100-1500 bp). The gel was then visualised using an ultraviolet transilluminator. The presence of 753 bps bands indicated amplification of the TIMP-1 gene rs4898, as appeared in Figure 1. All PCR products were stored at –20°C, and the PCR product samples were genotyped with the Sanger sequencing technique

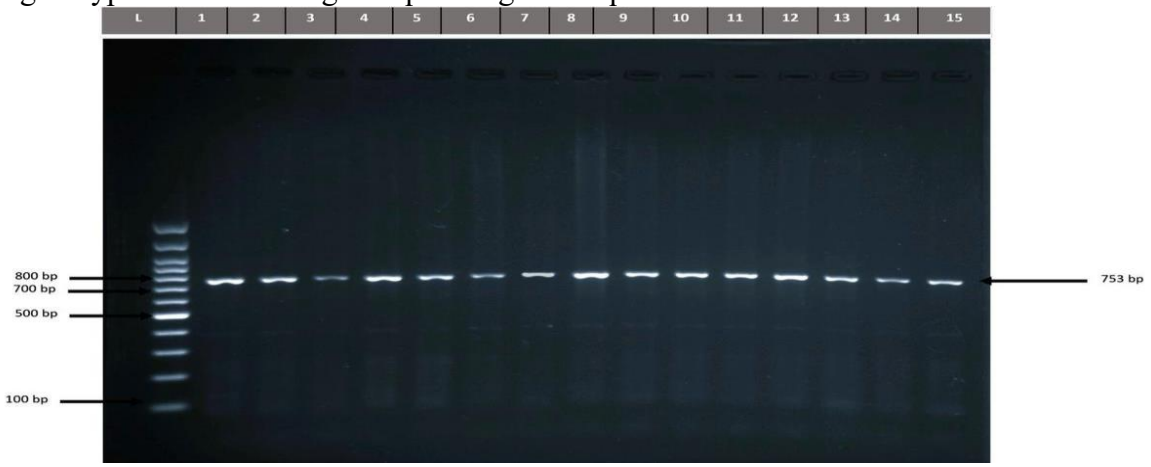

Figure 1: TIMP-1 SNPs rs4898 gel electrophoresis using a 100-volt electric current on a 1.5% agarose gel stained with red safe dye. The amplified PCR product has a molecular size of 753 bp. Lane L has a 50 bp DNA ladder; the numbers represent the examined samples.

The PCR results were analysed using Sanger sequencing on an Illumina automated DNA sequencing instrument, manufactured by Macrogen Corporation (South Korea). To identify TIMP-1 SNPs by aligning them with previously published DNA sequences from the National Center for Biotechnology Information (NCBI). The obtained sequencing results were next subjected to analysis using Geneious Prime software.

The TIMP-1 SNPs were studied through the PCR technique and DNA sequencing. Two SNPs appeared for the amplified fragment of the TIMP-1 gene, rs4898 and rs5953060. For TIMP-1 rs4898, two alleles appeared (*T*, *C*) corresponding to three genotypes (TT, TC, and CC). Figures 2 and 3 refer to the DNA sequence of TIMP-1 SNP rs4898 and rs5953060. The results in Table 8 referred to the compatibility of TIMP-1 SNPs rs4898 results with the Hardy-Weinberg equilibrium. Hardy-Weinberg equilibrium tests showed that the observed distribution for both TIMP-1 SNPs rs4898 (*P*-HWE= 0.1755 for T2DM and 0.6938 for the healthy controls) did not deviate significantly from equilibrium, and it matched the expected distribution. This consistency indicates reliable genotyping and argues against major population stratification or systematic genotyping errors at this locus. Also, the results in Table 9 showed a significant decrease in the percentage of TT genotype and *T* allele in the T2DM group compared to the healthy controls (24.10% *vs.* 54.67%, OR: 0.26, *Pc*: 5.0 x 10^{-5} and 45.0% *vs.* 73.0%, OR: 0.30, *Pc*: 2.5x10^{-7}, respectively). Also, the results showed a significant increased percentage of CC genotype and *C* allele in the T2DM group compared to the healthy controls (33.73% *vs.* 80%, OR: 5.85, *Pc*: 4.2 x 10^{-5}, and 55.0% *vs.* 27.0%, OR: 3.34, *Pc*: 2.5 x 10^{-7}, respectively). While the TC genotype frequency appeared to has a non-significantly increased percentage in the T2DM group compared to the healthy controls (42.17% *vs.* 37.33%, OR: 1.22, *Pc:* 0.518). The high values of OR referred to the role of the CC genotype and *C* allele as an attractive fraction for disease susceptibility, with values of 28.0% and 38.4%, respectively. The low values of OR in the TT genotype and *T* allele referred to the role of these genotypes and alleles as protective factors against the disease (40.3% and 51.4%, respectively).

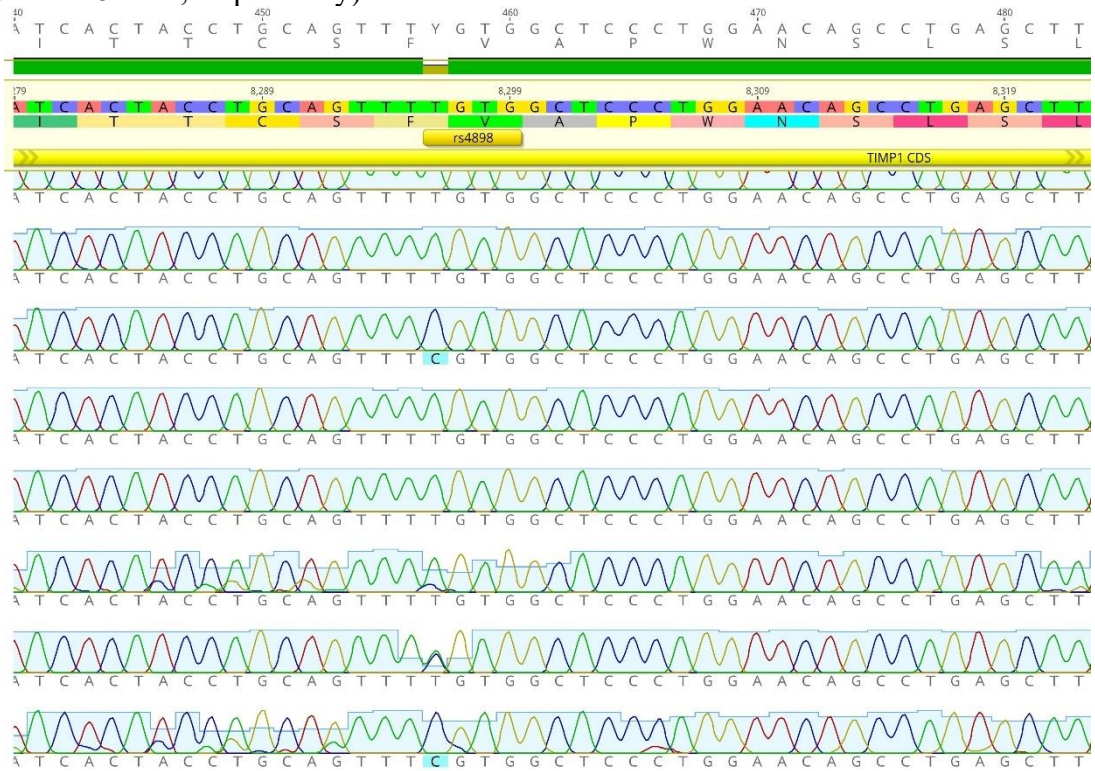

Figure 2: The DNA sequence of the TIMP-1 gene SNPs rs4898

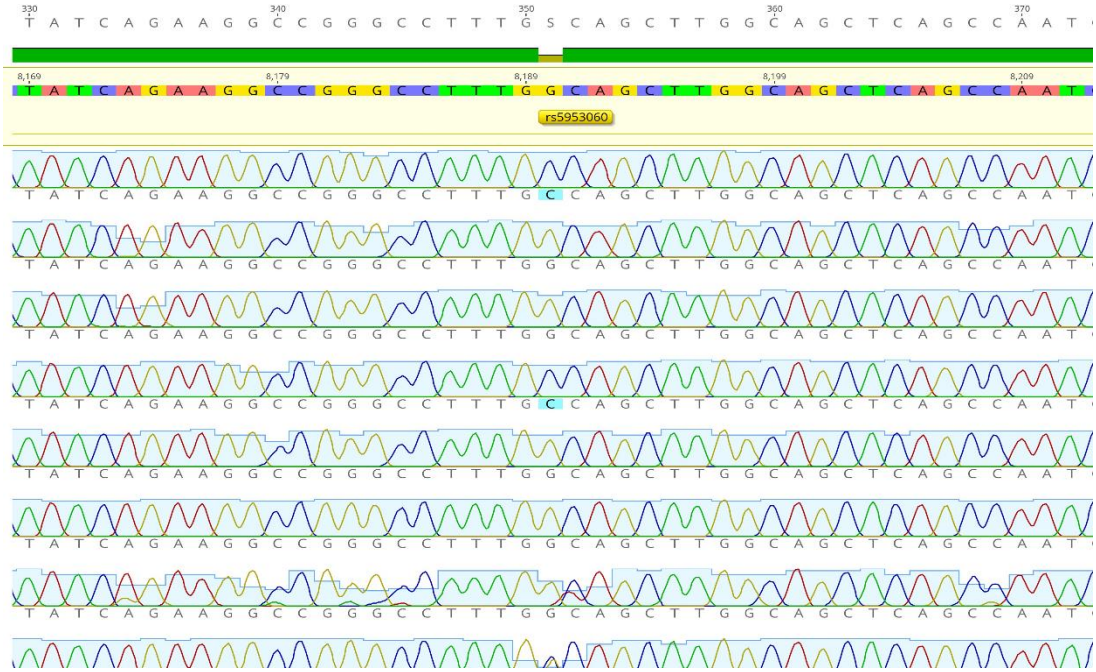

Figure 3: The DNA sequence of the TIMP-1 gene SNPs rs5953060

Table 8: The TIMP-1 SNP rs4898 genotyping frequencies and Hardy Weinberg compatibility in the patients' group compared to controls

Genotyping frequencies of TIMP-1 rs4898	Patients group (n=83)		Control group (n=75)	
	Observed	Expected	Observed	Expected
TT	20 (24.10)	16.94 (20.41)	41 (54.67)	40.33 (53.78)
TC	35 (42.17)	41.11 (49.54)	28 (37.33)	29.33 (39.11)
CC	28 (33.73)	24.94 (30.05)	6 (8.0)	5.33 (7.11)
Total	83 (100.0)	83 (100.0)	75 (100.0)	75 (100.0)
***P*-HWE**	0.1755		0.6938	

P-HWE: Hardy-Weinberg equilibrium probability

Table 9: The TIMP-1 SNP rs4898 genotypes and allele frequencies in the patients' group compared to controls

Genotyping and alleles frequencies of TIMP-1 rs4898	Patients group (n=83)	Control group (n=75)	OR (95% CI)	AF or PF	*P*-value	*Pc*-value
T	75 (45.0)	110 (73.0)	0.30 (0.19-0.48)	51.4	$4.2x10^{-7}$	$2.5x10^{-7}$
C	91 (55.0)	40 (27.0)	3.34 (2.08-5.35)	38.4	$4.2x10^{-7}$	$2.5x10^{-7}$
TT	20 (24.10)	41 (54.67)	0.26 (0.13-0.52)	40.3	$9.0x10^{-5}$	$5.0x10^{-5}$
TC	35 (42.17)	28 (37.33)	1.22 (0.65-2.31)	7.7	0.626	0.518
CC	28 (33.73)	6 (8.0)	5.85 (2.28-15.05)	28.0	$8.0x10^{-5}$	$4.2x10^{-5}$
Total	83 (100.0)	75 (100.0)				

OR: odd ratio, 95%CI: 95% confidence intervals, AF: attractive fraction, PF: protective fraction, *P*-value: Fishers' exact probability, *Pc*-value: Bonferroni corrected probability

In addition, the results in Table 10 observed the distribution of TIMP-1 serum level according to the TIMP-1 SNPs rs4898. The results showed a significantly increased level of TIMP-1 for

the TC genotype compared to the TT genotype in the T2DM group. No significant differences were observed when comparing the CC genotype with other genotypes for T2DM, nor among all genotypes of the healthy control group. In addition, there was a significantly increased level of TIMP-1 in the TC genotype among both the T2DM and healthy control groups.

Table 10: The mean and SE of TIMP-1 levels according to genotypes of TIMP-1 SNPs rs4898 and rs5953060 in the patients' group compared to the controls.

TIMP-1 SNPs rs4898	TIMP-1 mean ± SE (pg/ml)		P-value
	Patients group	Control group	
TT	1.77 ± 0.09 ^{B}	1.65 ± 0.05 ^{A}	0.124
TC	1.97 ± 0.04 ^{A}	1.67 ± 0.05 ^{A}	0.002
CC	1.79 ± 0.07 ^{AB}	1.69 ± 0.07 ^{A}	0.527
Duncan test: the similar letters referred to a non-significant difference (P > 0.05) between the compared genotypes in the same group			

Additionally, a second SNP was identified through the alignment and comparison of the amplified DNA sequence with the NCBI genome sequence. TIMP-1 rs5953060 appeared with the rs4898, and two alleles were recorded (*G*, *C*) that corresponded to three genotypes (GG, GC, and CC). The results in Table 11 showed the compatibility of TIMP-1 SNP rs5953060 frequencies with the Hardy-Weinberg equilibrium. Hardy-Weinberg equilibrium tests showed that the observed distribution for TIMP-1 SNPs rs5953060 (*P*-HWE= 0.0785 for T2DM and 0.7326 for healthy controls) did not deviate significantly from equilibrium, and it matched the expected distribution. This consistency indicates reliable genotyping and argues against major population stratification or systematic genotyping errors at this locus. Also, the results in Table 12 showed a significantly increased percentage of CC genotype and *C* allele in the T2DM group compared to the healthy control group (24.10% *vs.* 10.67%, OR: 2.66, *Pc*: 0.023, and 44.0% *vs.* 31.0%, OR: 1.72, *Pc*: 0.021, respectively). There was a non-significant decrease in the percentage of the GG and GC genotypes in the T2DM group compared to the healthy control group (36.14% *vs.* 48.0%, OR: 0.61, *Pc*: 0.110, and 39.76% *vs.* 41.33%, OR: 0.94, *Pc*: 0.749, respectively). In contrast, a significantly decreased percentage of the *G* allele was observed in the T2DM group compared to the healthy control group (56.0% *vs.* 69.0%, OR: 0.58, *Pc:* 0.021). In addition, the high values of OR referred to the role of this genotype and allele (CC and *C*) as an attractive fraction in the disease susceptibility (15.0% and 18.4%, respectively), while the low values referred to the role of these genotypes and allele (GG, GC and *G*) as a protective fraction from the disease infection (18.6%, 2.6% and 28.7%, respectively).

Table 11: The TIMP-1 SNP rs5953060 genotyping frequencies and Hardy-Weinberg compatibility in the patients' group compared to controls

Genotyping frequencies of TIMP-1 rs5953060	Patients group (n=83)		Control group (n=75)	
	Observed	Expected	Observed	Expected
GG	30 (36.14)	26.05 (31.39)	36 (48.0)	35.36 (47.15)
GC	33 (39.76)	40.90 (49.27)	31 (41.33)	32.27 (43.03)
CC	20 (24.10)	16.05 (19.34)	8 (10.67)	7.36 (9.82)
Total	83 (100.0)	83 (100.0)	75 (100.0)	75 (100.0)
***P*-HWE**	0.0785		0.7326	

P-HWE: Hardy-Weinberg equilibrium probability

Table 12: The TIMP-1 SNP rs5953060 genotypes and allele frequencies in the patients' group compared to controls

Genotyping and allele frequencies of TIMP-1 rs5953060	Patients group (n=83)	Control group (n=75)	OR (95% CI)	AF or PF	*P*-value	*Pc*-value
G	93 (56.0)	103 (69.0)	0.58 (0.37-0.92)	28.7	0.027	0.021
C	73 (44.0)	47 (31.0)	1.72 (1.09-2.72)	18.4	0.027	0.021
GG	30 (36.14)	36 (48.0)	0.61 (0.33-1.15)	18.6	0.148	0.110
GC	33 (39.76)	31 (41.33)	0.94 (0.50-1.76)	2.6	0.872	0.749
CC	20 (24.10)	8 (10.67)	2.66 (1.10-6.43)	15.0	0.036	0.023
Total	83 (100.0)	75 (100.0)				

OR: odd ratio, 95%CI: 95% confidence intervals, AF: attractive fraction, PF: protective fraction, *P*-value: Fishers' exact probability, *Pc*-value: Bonferroni corrected probability

As such, the results in Table 13 showed the distribution of TIMP-1 serum levels according to the genotyping of TIMP-1 SNP rs5953060. There is a significantly increased level of TIMP-1 in the GC genotype compared to the GG and CC genotypes in the T2DM group, and no significant difference was detected between the GG and CC genotypes in the T2DM. Also, no significant differences were recorded among the compared genotypes (GG, GC, and CC) in the healthy control groups. A significantly increased level was observed between the T2DM and the healthy control groups for the GC genotype.

Table 13: The mean and SE of TIMP-1 levels according to genotypes of TIMP-1 SNPs rs5953060 in the patients' group compared to the controls**.**

TIMP-1 SNPs rs5953060	TIMP-1 mean ± SE (pg/ml)		Probability
	Patients group	Control group	
GG	1.77 ± 0.10 ^{B}	1.64 ± 0.05 ^{A}	0.135
GC	1.99 ± 0.04 ^{A}	1.67 ± 0.05 ^{A}	0.001
CC	1.79 ± 0.06 ^{B}	1.70 ± 0.06 ^{A}	0.509

Duncan test: the similar letters referred to a non-significant difference (P > 0.05) between the compared genotypes in the same group

Pairwise LD analysis between the two TIMP-1 gene SNPs demonstrated that rs4898 had a strong LD with rs5953060 (D' = 0.95) (Figure 4). In terms of haplotypes (in order: rs4898 and rs5953060), the frequency of the CC haplotype was non-significantly increased in the T2DM group compared to the controls (42.6% *vs*. 38.0%, OR: 1.213, 95% CI: 0.624~2.357, *p*: 0.617 (Table 14).

While, TG and TC haplotypes were non-significantly decreased in T2DM group compared to the controls (52.2% *vs*. 54.0%, OR: 0.93, 95% CI: 0.485~1.782, *p*: 0.869; and 3.6% *vs*. 8.0%, OR: 0.438, 95% CI: 0.112~1.705, *p*: 0.253, respectively) (Table 14). None of these associations reached statistical significance.

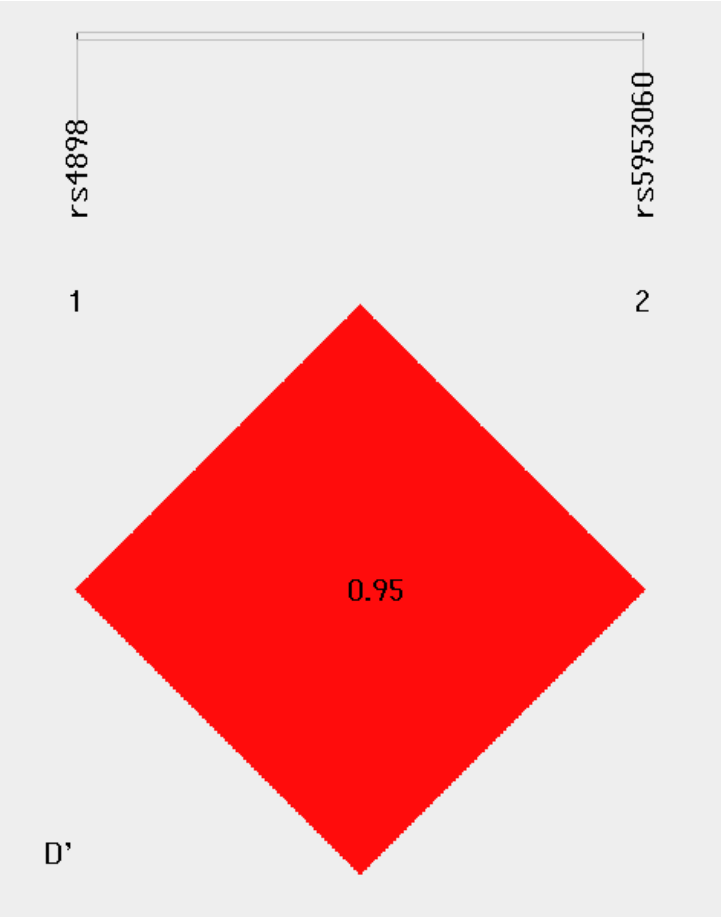

Figure 4: The pairwise linkage disequilibrium (LD) map illustrates the two SNPs (s4898 and rs5953060) for the TIMP-1 gene, as genotypes using the SHEsis Plus online software. The LD between these SNP pairs is quantified by the D' value, where values near zero signify no LD, and values close to 1.0 indicate complete LD. The red-colored squares depict varying degrees of LD, with darker shades representing stronger LD.

Table 14: Haplotype analysis of TIMP-1 gene SNPs rs4898 and 5953060 between the T2DM and control groups

Haplotypes	T2DM freq. (%)	Control freq. (%)	P-value	OR (95% CI)
CG	58 (42.6)	19 (38.0)	0.617	1.213 (0.624~2.357)
TG	71 (52.2)	27 (54.0)	0.869	0.93 (0.485~1.782)
TC	5 (3.6)	4 (8.0)	0.253	0.438 (0.112~1.705)

P-value: two-tailed Fisher's exact probability, OR: odds ratio, 95% CI: 95% confidence intervals

4. Discussion

The findings in Table 2 align with a previous Iraqi study conducted on Iraqi patients with T2DM by Kryst *et al.,* [27], which demonstrated elevated serum levels of TIMP-1 in the patient groups compared to the control group, thereby validating the inflammatory condition in these cohorts. Also, in patients with T1DM, increased levels of MMPs and TIMP-1 have been documented, correlating with hyperglycemia, chronic inflammation, and endothelial dysfunction [28]. Another study by Fabrikantov *et al.,* [29] found that TIMP-1 is independently linked to cardiac diastolic dysfunction in individuals with T2DM, and is independently linked to cardiac diastolic dysfunction in individuals with T2DM.

The present results in Table 4 regarding TIMP-1 serum levels according to the patients' age groups are incompatible with a previous study done by Shi *et al.*, which reported that the levels of TIMP-1 in individuals with T2DM exhibit significant variation among age groups, especially concerning sequelae such as diabetic retinopathy and nephropathy. The variation in TIMP-1 levels among different age groups in individuals with T2DM is related to several complications, such as diabetic retinopathy (DR) and nephropathy. For age-related changes in ECM remodeling, TIMP-1 levels show significant age-related variation in individuals with T2DM, particularly in the context of diabetic retinopathy and nephropathy. This variation can be attributed to age-related changes in extracellular matrix modeling, heightened inflammation and oxidative stress, and the cumulative metabolic burden of long-standing diabetes. Additionally, TIMP-1 plays a dual role, acting as a protective factor against early vascular damage, but becoming dysregulated with disease progression and ageing [19]. Also, the elderly persons displayed increased matrix metalloproteinase-9 (MMP-9) levels, which correlate with the severity of diabetic problems, although TIMP-1 levels generally diminished in these individuals [18]. The imbalance is likely due to impaired feedback control in the MMP/TIMP system, cellular senescence that reduces TIMP-1 synthesis, chronic inflammation, and oxidative stress that suppresses its expression. Additionally, prolonged diabetes duration in older adults may exhaust compensatory mechanisms, contributing to vascular damage and worsening complications such as diabetic retinopathy and nephropathy [18].

Zaki *et al*., [30] demonstrated that the levels of TIMP-1 were considerably reduced in patients with T2DM and diabetic nephropathy compared to those with T2DM alone. However, the elevated MMP-9 levels and reduced TIMP-1 are linked to complications in older T2DM patients.

Additionally, Ismael *et al.,* and Kiani *et al.,* indicated that hereditary variables and lifestyle decisions, including smoking, may significantly influence the expression of this biochemical marker [31, 32]. Although the results in Table 5 indicated no significant variations of TIMP-1 levels according to employment position, other research underscores the relevance of this biomarker in diverse health conditions [33]. The absence of direct comparisons in the current literature between job status and this biomarker highlights the need for further studies to investigate potential relationships. Job-related factors, including stress and environmental exposure, may indirectly affect TIMP-1 and MMPs levels; however, specialized studies are required to elucidate these associations.

TIMP-1 levels in T2DM patients showed different associations with disease duration and complications, as reported in a previous study by Nehring *et al*., [34]. Also, the levels of TIMP-1 were markedly elevated in patients with a history of T2DM exceeding 10 years. In addition, Yong and Feng revealed that TIMP-1 levels were markedly elevated in T2DM patients with Diabetic Peripheral Neuropathy (DPN) relative to those without, suggesting a correlation between TIMP-1 and neurovascular damage, and the duration of diabetes was longer in the DPN group, further supporting the link between TIMP-1 levels and disease severity [35].

Also, the study done by Ghanem *et al*., which examined diabetics with varied foot ulcer problems, found that diabetic foot ulcers have higher plasma MMP-9 and lower TIMP-1 levels, indicating a proteolytic imbalance that slowed the healing, which is contradicted by the current investigations' data, which shows diabetic foot ulcer patients had slightly higher TIMP-1 levels, but this difference is not statistically significant [36]. Importantly, Ghanem and colleagues studied patients with long-standing, non-healing ulcers, where excessive ECM breakdown may overwhelm TIMP-1 production, whereas our cohort primarily included early-

stage foot lesions; in early wounds, TIMP-1 upregulation is part of the acute reparative response, which could underlie our observed non-significant upward trend. Wang showed that serum levels of TIMP-1 were elevated in T2DM patients with extracranial artery stenosis, potentially indicating aberrant extracellular matrix (ECM) metabolism [37].

The present investigations of TIMP-1 SNP rs4898 genotyping were inconsistent with Çelik *et al*., findings, who reported that the TIMP-1 SNPs rs4898 were not significantly associated with the disease [38]. To our knowledge, there is no study regarding the relationship between the genetic variants of TIMP-1 polymorphisms and T2DM. While the present investigations were partially compatible with Çelik *et al*., who reported that the levels of TIMP-1 were significantly higher in T2DM compared to the healthy controls and in the TC genotype compared to other genotypes [38]. Beyond our Iraqi cohort and the Turkish analysis by Çelik *et al*., there are no published studies to date that specifically test the association of TIMP-1 rs4898 with T2DM in other ethnic populations. Most studies focused on non-diabetic traits like aseptic loosening after hip arthroplasty in Chinese Han subjects [39], or cancer risk in Taiwanese's [40]. Although the level of TIMP-1 appeared similarly in individuals with TC genotype in both T2DM and control groups, the present findings indicate that diabetic milieu uniquely amplifies TIMP-1 expression in TC genotype is not more common in T2DM, the pathological environment associated with diabetes and appears to interact with the TC genotype, leading to elevate TIMP-1 serum levels in patients compared to controls. This suggests a gene-environment interaction where the diabetic context particularly enhances TIMP-1 expression in subjects with the TC genotype, thereby contributing to the observed differences in TIMP-1 levels.

For the GG and CC genotype frequencies of TIMP-1 SNPs rs5953060 in Table 13, although the levels of TIMP-1 in patients are slightly higher than those in the controls, these differences may reflect inherent inter-individual variability rather than a specific genotype–driven effect under diabetic conditions. Therefore, the present results suggest that the observed influence of diabetes on TIMP-1 levels is more pronounced in subjects with the GC genotype. This finding underlines a gene-environment interaction that is selective rather than uniform across all genotypes.

To our knowledge, this study is the first study that deals with the interaction between the genetic polymorphisms and the serum levels of TIMP-1 SNPs rs5953030 and the susceptibility of DM, and there is no recent study regarding the relationship between the genetic variants of TIMP-1 polymorphisms and T2DM. Regarding disease association investigations, TIMP-1 SNPs rs4898 and rs5953060 have not been thoroughly investigated, and only a few observations have been reported. Nehring *et al*., investigations referred to the more frequent frequency of *T* allele of TIMP-1 rs4898 in patients with colonic diverticulosis than in the healthy controls [34].

Regarding the fact that the TIMP-1 SNP rs4898 is located within TIMP-1's C-terminal domain, which is responsible for docking onto MMP-9, we hypothesize that the *C* allele may disrupt a critical hydrogen bond network or local charge distribution, finely weakening inhibitory binding to MMP-9 [41]. Meanwhile, TIMP-1 SNP rs5953060 resides near an exon-intron boundary, or in the 3′ UTR, where the C variant could influence mRNA splicing or stability, yielding altered TIMP-1 isoform levels.

The results of haplotype analysis confirmed strong LD between TIMP-1 SNPs rs4898 and rs5953060 (D′ = 0.95), and no combined allele blocks were significantly associated with T2DM risk in this cohort. The CG and TG haplotypes should have nearly identical frequencies in T2DM and control groups, indicating that they likely do not modulate disease susceptibility. The low frequency of the TC haplotype displayed a non-significant protective

trend (OR = 0.44), but small counts (5 carriers in T2DM, and 4 in controls) yielded wide confidence intervals, limiting interpretability. Overall, these data suggested that, despite the high LD, the individual SNP effect on TIMP-1 expression may outweigh any additional influence of multi-allelic haplotypes. Larger studies will be needed to clarify whether rare haplotypes, such as TC, truly confer an altered risk.

5. Conclusions

Based on the findings of this study, it was demonstrated that T2DM patients exhibit significantly elevated TIMP-1 serum levels compared to healthy controls. The TIMP-1 genetic variants TC and CC genotypes, along with the *C* allele of rs4898, as well as the CC genotype and *C* allele of rs5953060, are associated with increased disease susceptibility. These findings not only suggest that TIMP-1 may play a role in the ECM modeling underlying diabetic skin complications, but also hint at a gene-environment interaction wherein the diabetic milieu may exacerbate the functional effects of these polymorphisms. Haplotype reconstruction of TIMP-1 rs4898 and rs5953060 yielded three common two-SNP haplotypes (CG, TG, and TC), none of which reached statistical significance. However, the rare TC haplotype showed a non-significant protective trend. Although the present study provides novel insight, limitations such as a relatively models sample size and the lack of functional assays necessitate further research. Future studies should aim to elucidate the molecular mechanisms by which TIMP-1 variants influence diabetic pathology and assess their potential utility as biomarkers for earlier detection and risk stratification in T2DM patients. Also, to determine whether the strong *C* allele risk observed in this Iraqi study is generalizable or population-specific.

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Declaration of competing interest

The authors declare no relevant financial or non-financial interests.

Conflict of interest

The authors declare there are no conflicts of interest to disclose.

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