ISSN: 0067-2904

Potential Role of TNFα Gene Expression and TNFα / TNFR1 Levels in the Pathogenesis of Hashimoto's Thyroiditis in Iraqi Women

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Received: 17/11/2024 Accepted: 9/4/2025 Published: 30/4/2026

Abstract

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine that promotes inflammation and the development of some autoimmune diseases (AIDs), including Hashimoto's thyroiditis (HT). This research sought to examine TNF-α gene expression and assess serum concentrations of TNF-α and its soluble receptor 1 (sTNFR1) in women with HT. The study group included fifty healthy women and fifty HT women. Serum concentrations TNF-α and TNFR1 levels were evaluated using the sandwich ELISA method. The expression level of TNF-α was quantified using quantitative real-time polymerase chain reaction (qRT-PCR). Our research findings revealed a highly significant increase at $p<0.001$ and a strong positive correlation between TNF-α and TNFR1 ($r=0.401$, $p= 0.004$). This study showed a positive correlation between TNFR1 and Anti-Tg ($r=0.298$, $p=0.036$). The diagnostic value was significant, with an Area Under the ROC Curve (AUC) of 0.896 for TNF-α and 0.848 for TNFR1 at cut-off values of 69.465 and 32.367, respectively. Sensitivity for TNF-α and TNFR1 was 66% and 68%, respectively, while specificity was 100% ($P=0.001$) for both. Additionally, HT women exhibited markedly elevated expression levels of the TNF-α gene compared to HI women ($P<0.046$). This study demonstrates that HT disease (HTD) is strongly associated with higher levels of TNF-α and TNFR1, and increased expression of TNF-α mRNA, suggesting a link with the inflammatory process and disease progression in Iraqi women.

Keywords: Hashimoto’s thyroiditis, TNF-α, TNFR1, TNF-α gene expression, qRT-PCR..

الدور المحتمل لتعبير جين TNF−α ومستويات TNF−α / TNFR1 في تطور مرض التهاب الغدة الدرقية هاشيموتو لدى النساء العراقيات

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الخلاصة

عامل نخر الورم ألفا (TNF−α) هو أحد السيتوكينات المؤيدة للالتهابات والتي تعزز الالتهاب وتطور بعض الأمراض المناعية الذاتية (AIDs)، من ضمنها التهاب الغدة الدرقية هاشيموتو (HT). سعى هذا البحث الى فحص التعبير الجيني TNF−α وتقييم تركيزات TNF−α ومستقبله القابل للذوبان 1 (sTNFR1)

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في المصل لدى النساء المصابات ب HT. شملت مجموعة الدراسة خمسين امرأة سليمة وخمسين امرأة مصابة ب HT. تم تقييم مستويات TNF−α و TNFR1 في المصل باستخدام طريقة ELISA الساندويتش. تم قياس مستوى التعبير الكمي لـ TNF−α بواسطة تفاعل البوليمريز المتسلسل بالزمن الحقيقي (qRT−PCR) كشفت نتائج بحثنا عن زيادة كبيرة للغاية (p<0.001) وارتباط إيجابي قوي بين TNF−α و TNFR1 (r=0.401, p= 0.004). في هذه الدراسة، لاحظنا وجود ارتباط إيجابي بين TNFR1 وAnti−Tg (r=0.298,p=0.036).كانت القيمة التشخيصية مهمة، حيث بلغت المساحة تحت منحنى ROC (AUC) 0.896 لـ TNF−α و0.848 لـ TNFR1 عند قيم حدية 69.465 و32.367 على التوالي. كانت الحساسية لـ TNF−αوTNFR1 66% و 68% على التوالي، بينما كانت الخصوصية 100% (P=0.001)لكليهما. بالإضافة إلى ذلك، أظهرت النساء المصابات HT مستويات تعبير مرتفعة بشكل ملحوظ لجين TNF−α مقارنة بالنساء HI (P<0.046). أظهرت هذه الدراسة أن مرض HTD يرتبط ارتباطًا وثيقًا بمستويات أعلى من TNF−α وTNFR1، وزيادة التعبير عن mRNA لـ TNF−α، مما يشير إلى وجود ارتباط مع العملية الالتهابية وتطور المرض لدى النساء العراقيات.

1. Introduction

The main clinical manifestations of autoimmune thyroid disorders (AITDs) in humans are Hashimoto's thyroiditis (HT) and Graves' disease (GD), defined by hypothyroidism and hyperthyroidism, respectively [1]. The pathophysiology of Hashimoto's thyroiditis disease (HTD) involves the production of thyroid antibodies, namely anti-thyroid peroxidase antibody (TPO-Ab) and antithyroglobulin antibody (Tg-Ab), which results in increasing fibrosis[2]. Tumor necrosis factor-alpha (TNF-α) is a cytokine that has diverse effects on many cell types. It is recognized as a crucial regulator of the inflammatory response. Furthermore, this cytokine is a potent pleiotropic cytokine with multiple cellular activities, implicating it in the pathogenesis of several inflammatory and autoimmune illnesses [3].TNF is a protein composed of (157 amino acids), mostly produced by fibroblasts, macrophages, B and T lymphocytes, natural killer (NK) cells, and dendritic cells, in addition to thyroid epithelial cells, inside the thyroid gland [4, 5]. Two types of TNF-α exist: transmembrane TNF-α (tmTNF-α) and soluble TNF-α (sTNF-α). The first type is the produced precursor form, which necessitates enzymatic processing by TNF-α converting enzyme (TACE) to be liberated as sTNF-α [6]. TNF-α interacts with its receptors, soluble TNF receptor 1 (sTNFR1) and soluble TNF receptor 2 (sTNFR2). The first receptor is expressed in almost all cell types, while the second receptor is
restricted to regulatory T cells, endothelial cells, NK cells, and T lymphocytes [cluster of differentiation 4 (CD4+), cluster of differentiation 8 (CD8+)] [7]. The binding of TNF-α to its two receptors sends molecular signals for biological activities. However, only TNFR1 functions as a death receptor (DR) and has an intracellular death domain through which it triggers cell degeneration and apoptosis. Consequently, in autoimmune thyroid diseases, these biological functions result in the cytotoxic mechanisms that characterize thyroid destruction [8, 9]. Additionally, a strong correlation exists between TNF-α gene expression and the clinical symptoms of autoimmune disorders, including Systemic lupus erythematosus (SLE)[10]. This work aimed to ascertain the function of TNF-alpha and its soluble receptor 1 (sTNFR1) as diagnostic biomarkers for HTD, using the receiver-operating characteristic curve (ROC) to evaluate serum concentrations of TNF-α / TNFR1 and to quantify TNF-α gene expression by quantitative real-time polymerase chain reaction (qRT-PCR) [11].

2. Material and methods

The study group consisted of one hundred females aged between 20-60 years old. It comprised fifty healthy individuals (HI) and fifty patients with HT. All patients were

recruited for nearly eight months from February 2024 to September 2024 at the Azadi Teaching Hospital and some Endocrine Outpatient, especially at the Ochi Medical Complex in Kirkuk Governorate / Iraq. All women to be included in the current study were residents of Kirkuk/Iraq, were older than 20 years and had been diagnosed with HT by a specialized physician (endocrinologist), while the Exclusion criteria in this study were: (children, men, pregnant females, and who were not from Iraq).

2.1. Specimen Collection

Six ml of venous blood samples were collected from selected patients and control subjects. (4.5 ml) was transferred into a gel tube to produce sera by centrifugation at 3000 rpm for about 15 minutes. Sera, after centrifugation, was transferred into Eppendorf tubes to assess thyroid antibodies (TPO-Ab, Tg-Ab), thyroid hormones [free triiodothyronine (fT3), free thyroxine (fT4)] and thyroid stimulating hormone (TSH), levels, by using the (Cobas e 411 analyzer series, Hitachi, Japan) according to the manufacturer's recommendations. Furthermore, serum TNF-α and TNFR1 levels were diagnosed by Elisa (SUNLONG kit / China) [12], and results were measured quantitatively by an (ELISA reader Paramedical, Italy) at (450 nm) absorbance according to the manufacturer's instructions. On the other hand, (1.5 ml) of whole blood in the EDTA tube was kept at - 80 C for RNA extraction. The RNA was extracted using a favor prep blood / cultured cell Total RNA Mini kit - cat NO; FABRK 001 Lot NO: CD719123B15, Germany). RNA samples were quantified by a NanoDrop (Nabi/Korea) spectrophotometer. The extracted RNA concentration ranged from (10.9 –13.8 ng/µl) while RNA purity ranged from (1.87 -2.24), then stored at -20 °C until the moment of use.

2.2. Quantification of TNF-α gene expression

TNF-α gene expression was quantified using quantitative real-time PCR (qRT-PCR) using the Applied GoTaq® 1-Step RT-qPCR Protocol, employing specific primers (provided by Macrogen Company, Korea) for the quantification of TNF-α mRNA. B-globulin served as the housekeeping gene (HKG). The forward primer (FP)and reverse primer (RP) were as detailed below:

TNF-α FP: 5′-CTCTTCTGCCTGCTGCACTTTG -3′ RP: 5′-ATGGGCTACAGGCTTGT CACTC-3′[13]. β-globin FP: 5′-ACACAACTGTGTTCACTAGC-3′ RP: 5′-CAACTTCA TCCACGTTCACC-3′ [14]. Results of (qRT-PCR) were analyzed by the relative quantification of gene expression level fold change (FC) ΔΔCT according to [15]. All cycle threshold (Ct) data obtained by Sacace RT PCR software were calculated via the delta cycle threshold (ΔCt) method, and the values of the patients and controls were compared. The target gene was normalized to an endogenous control (HKG) and relative to the calibrator, which is the target gene in the healthy control group. The FC was calculated for each sample using the following equations:

ΔCt sampΔCt sample = Ct gene - Ct HKG

ΔΔCt sample = ΔCt sample - average ΔCt control group

Fold change sample = $2^{-\Delta\Delta Ct}$ [14, 16].

2.3. Statistical analysis

Data input was conducted using a Microsoft Excel spreadsheet, followed by statistical analysis executed with SPSS version 23. Pearson correlation and Chi-square tests were used to analyze the category data. The significance level was denoted as $P<0.05$, and highly significant was $p<0.001$. A receiver operating characteristic (ROC) curve was used to evaluate the validity of the examined measures in HT women as diagnostic biomarkers and to establish the optimal cut-off value for these parameters. The areas under the curve (AUC)

were considered: Exceptional (1-0.9), Excellent (0.9-0.81), Good (0.8-0.71), Fair (0.7-0.61), and Poor (0.6-0.5). Relative gene expression data were investigated by quantitative real-time PCR, and the CT values produced by Sacace software were evaluated using the ΔΔCT equation described by [15].

3. Results

3.1. Clinical and laboratory manifestations

Table 1 presents the overall manifestations of patients with Hashimoto's thyroiditis and the control group, indicating a high statistical significance in the levels of thyroid antibodies (anti-TPO, anti-Tg), thyroid hormones (fT3, fT4), and TSH in the cohort of women with HTD relative to the healthy women group ($P<0.001$). In addition, eta-squared ($\eta 2$) effect size results for each of the above variables had a large effect (eta square $\eta 2 > 0.14$). Moreover, (76%) of HT patients had a positive family history of thyroid diseases, and the prevalence of psychological stress was elevated among participating patients (62%) in comparison to controls. No significant differences were seen between the HT patients and the HI group regarding age and marital status, with the mean age and standard deviation for HT women being (39.98 ± 11.18) compared to (39.82 ± 11.26) for the HI group. Furthermore, the highest percentage of HT patients was in the age group (41 to 50 years).

Table 1: Characteristics features of HT patients and HI

Characteristic	HT No. (50)	HI No. (50)	P. value
Age /years (Mean ± SD)	39.98 ± 11.18	39.82 ± 11.26	0.943
Age groups			
21 TO 30	8 (16%)	9 (18%)	0.985
31 TO 40	14 (28%)	15 (30%)	
41 TO 50	17 (34%)	16 (32%)	
51 TO 60	11 (22%)	10 (20%)	
Marital status No. (%)			
Unmarried	5/50 (10%)	6/50 (12%)	0.749
Married	45/50 (90 %)	44/50 (88%)	
Family history No. (%)			
YES	38/50 (76%)	5/50 (10%)	0.001**
NO	12/50 (24%)	45/50 (90%)	
Psychological state stress No. (%)			
Psychological state stress	31 /50 (62%)	18/50 (36%)	0.009
No-Psychological state stress	19/50 (38 %)	32/50 (64 %)	
Thyroid antibodies (Mean ± SD)			
Anti-Tg (IU/ml)	207.76± 196.11	38.94± 32.27	0.001**
Anti-TPO (IU/ml)	272.38± 177.79	17.27± 6.75	0.001**
Thyroid hormones (Mean ± SD)			
fT3 [pmol/L]	2.88 ± 0.97	4.61 ± 1.06	0.001**
fT4 [Ng/dl]	0.87±0.22	1.25 ± 0.2	0.001**
TSH [µIU/mL]	12.00± 18.56	2.31 ± 1.22	0.001**

** p-value ≤ 0.001

3.2. Important signs and symptoms of patients with Hashimoto's thyroiditis

The most prominent symptoms of HT women were hair loss (82%), fatigue (72%), dry/rough skin (68 %), joint pain (62%), and depression (52%), while the other symptoms in the woman with HTD such as voice changes, hoarseness, chronic constipation, brittle nails,

forgetfulness, bradycardia, and irregular menstrual periods account for about (32%) (Figure 1).

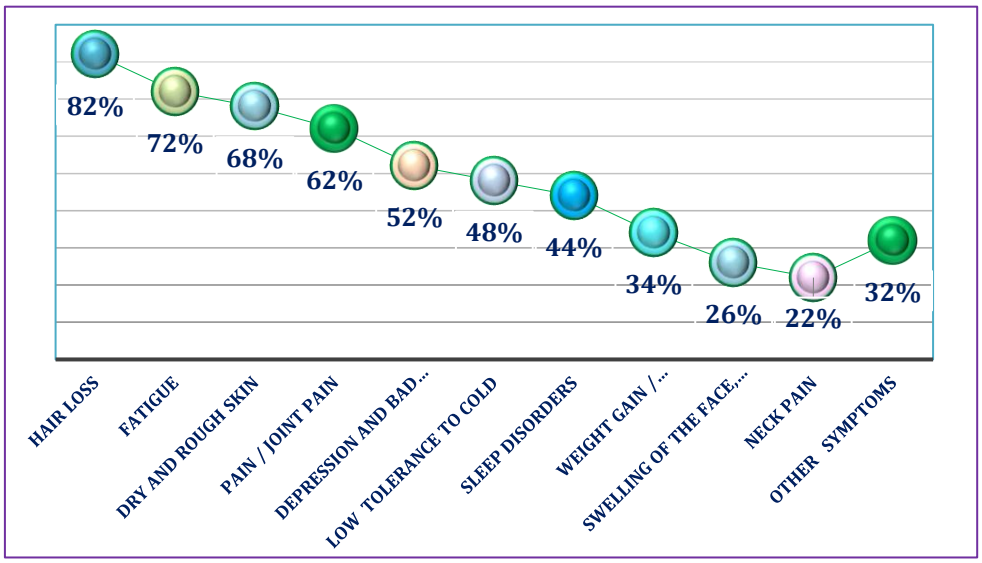

Figure 1: Signs and symptoms associated with HT patients

3.3. TNF-α and TNFR1 levels

Table 2 displays highly significant differences ($p<0.001$) between HT patients and healthy control for TNF-α and TNFR1 (101.63 ± 57.31 pg/ml), (57.70 ± 38.36 pg/ml) for patients and (33.29 ± 5.12 pg/ml), (10.14 ± 2.06 pg/ml) for the controls, respectively.

Table 2: Serum TNF-α and TNFR1 levels in HT and HI

Laboratory parameters	No. (%)	HT (n = 50) (Mean ± SD)	No. (%)	HI (n = 50) (Mean ± SD)	P value
TNF –α (pg/ml)	50 (100%)	101.63 ± 57.31	50 (100%)	33.29± 5.12	0.001**
TNF R1 (pg/ml)		57.70± 38.36		10.14± 2.06	0.001**

3.4. Comparison between TNF- α and TNFR1 and other studied parameters.

Our results demonstrated a strong positive correlation between TNF-α and TNFR1 ($r=0.401$, $p= 0.004$), indicating that the secretion of these cytokines was high in women with HT. Moreover, there were remarkable positive associations between TNFR1 and Anti-Tg ($r=0.298$, $p=0.036$) (Figure 2 A and B).

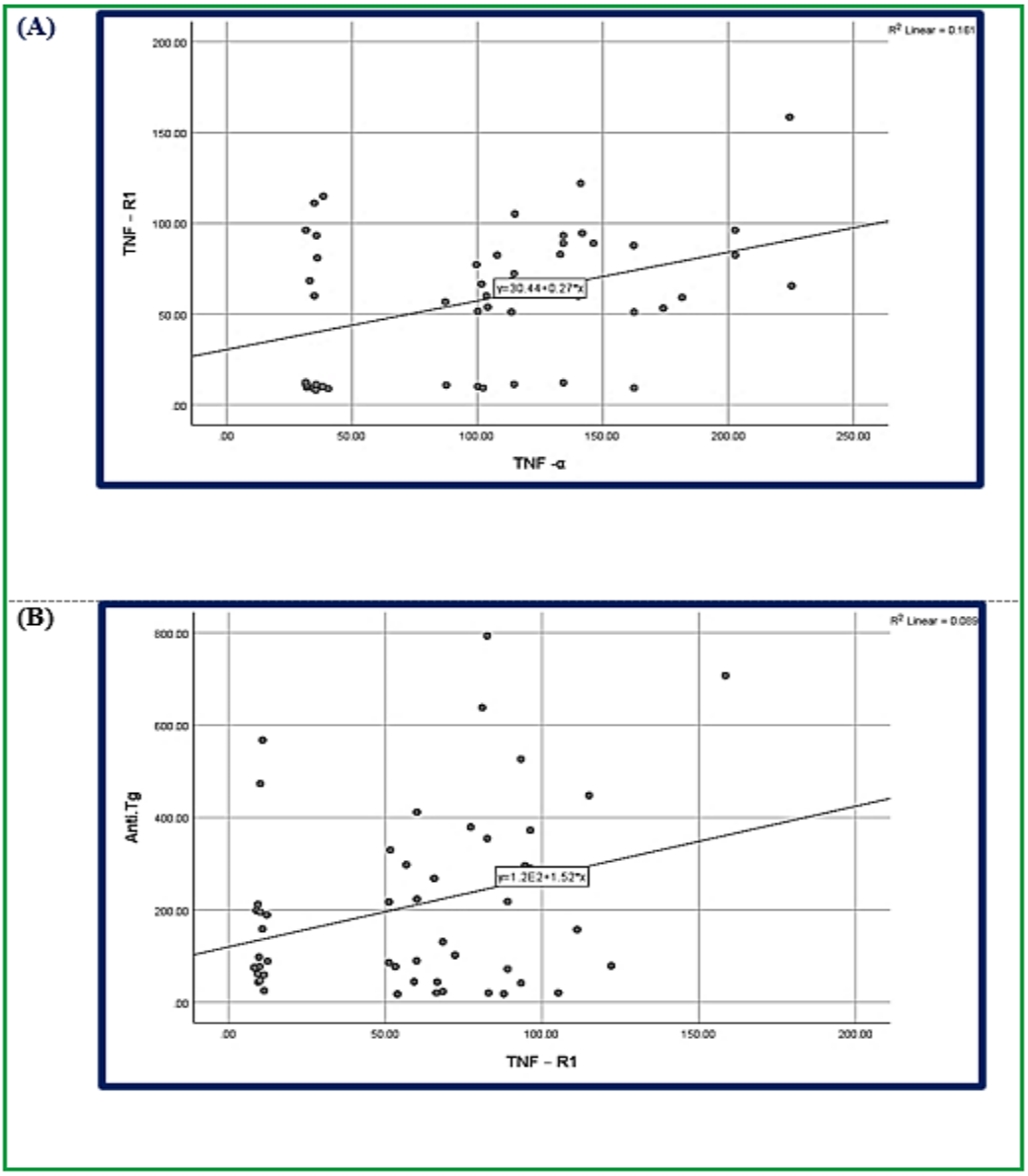

Figure 2: (A) Correlation between TNF-α and TNFR1 among HT patients. (B) Correlation between TNFR1 and Anti-Tg among patients with HT.

While no correlation was observed between TNFR1 with (Anti. TPO, fT3, fT4 and TSH) and TNF- α with (Anti. TPO, Anti. Tg, fT3, fT4 and TSH) (Table 3).

Table 3: Pearson correlation coefficient between TNF- α and TNFR1 with studied parameters

TNFR1	Parameters				
	Anti. TPO	**fT3**		**fT4**	**TSH**
Pearson Correlation	-.078 -	.040		-.089 -	.085
Sig. (2-tailed)	.591	.783		.537	.558
TNF- α	**Parameters**				
	Anti. TPO	**Anti. Tg**	**fT3**	**fT4**	**TSH**
Pearson Correlation	-.015 -	.245	-.006 -	-.227 -	.114
Sig. (2-tailed)	.920	.086	.964	.113	.431

3.5. Receiver-operating characteristic curve (ROC)

The ROC curve was done to assess the validity of TNF-α and TNFR1 in the detection of cases. The curve showed an excellent diagnostic value of a high curve of Area Under the ROC Curve (AUC) (0.896) and (0.848) at a cut-off value of (69.465) and (32.367); Sensitivity was (66%) and (68%) specificity was (100%) for both and AUC for the above-mentioned variables which showed an excellent explanation with a P-value of (0.001) (Table 4 and Figure.3).

Table 4: ROC characteristic curves of TNF– α and TNFR1 in HT diseases

parameters	AUC	Explanation	Cut off	Sensitivity %	Specificity %	P. Value	95% Confidence Interval (CI)
TNF -α	.896	Excellent	69.465	66%	100%	.001	.838 - .954
TNF R1	.848	Excellent	32.367	68%	100%	.001	.772 - .924

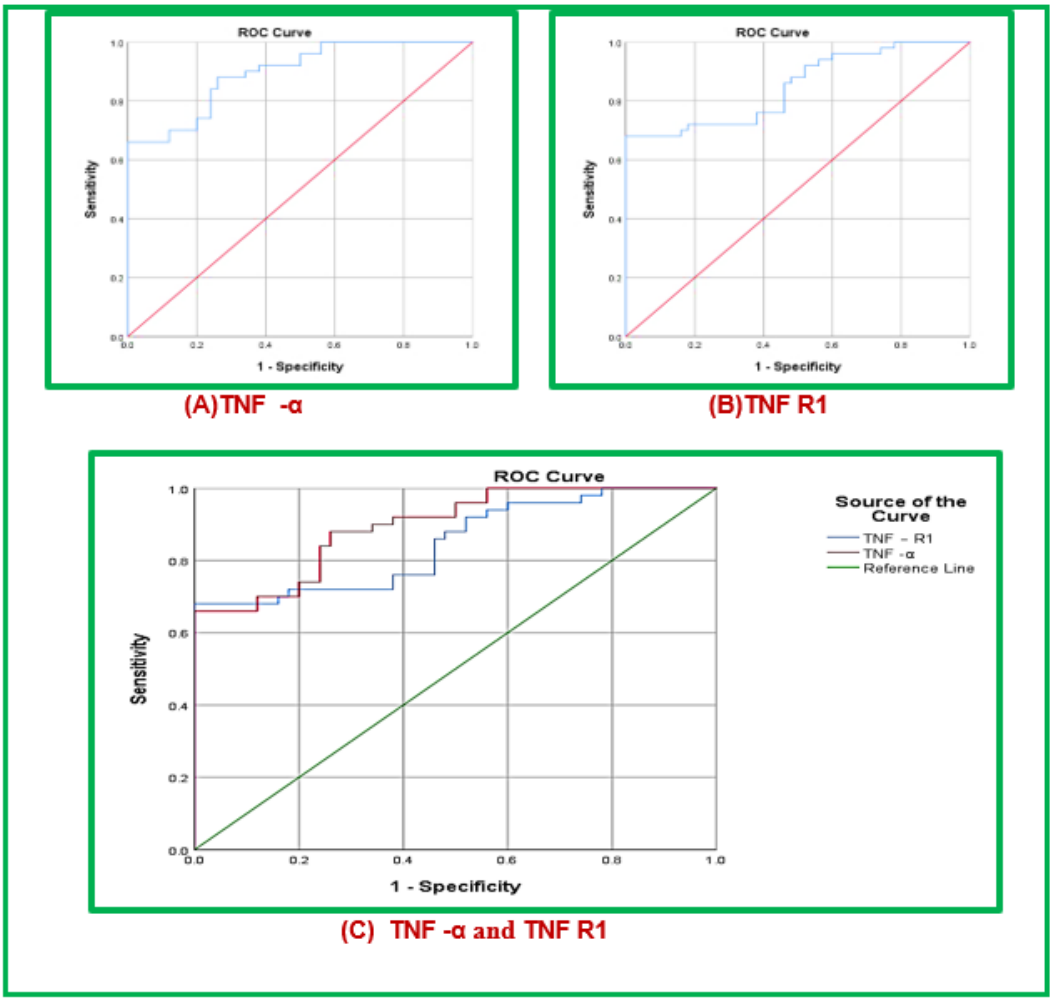

Figure 3: Analysis of the TNF- α and TNFR1 ROC curves

3.6. Altered expression of TNF-α mRNA levels (fold) in women with HT

Figure 4 shows that the expression levels of the TNF-α gene were significantly increased in women with HT. The mean mRNA expression levels of TNF-α in the HT group was 5.19-fold ($p<0.046$) increased compared to the HI group.

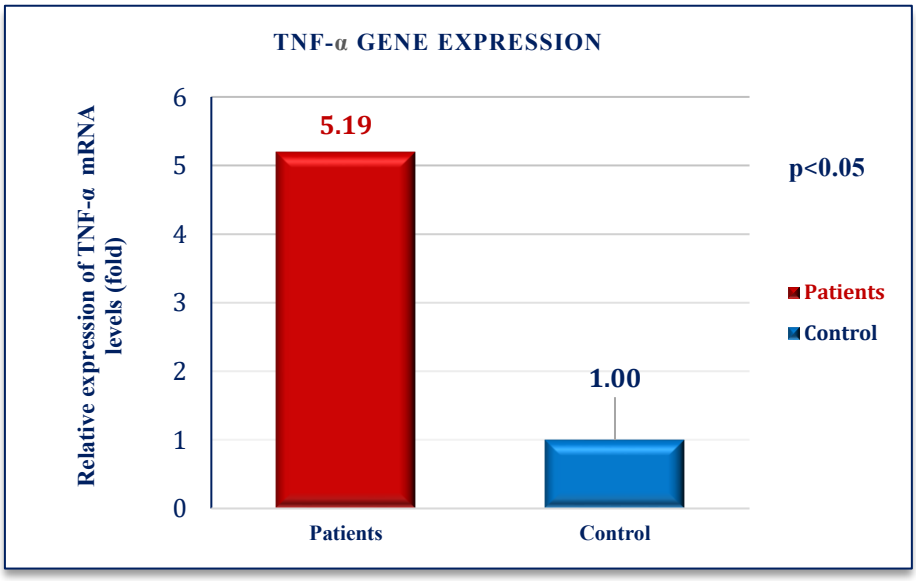

Figure 4: Relative expression of TNF-α mRNA levels (fold) in blood samples of the studied groups

4. Discussion

The pro-inflammatory cytokine TNF-α is involved in the initiation and sustenance of immune responses. It is believed to play a critical role in AITDs [9] and is an extremely powerful mediator in (inflammatory and immunological) reactions [17]. Notably, large amounts of TNF-α affect the growth and differentiation of thyroid cells, which leads to compromised thyroid function and causes damage to these organs [18]. Therefore, TNF is a suggested parameter for detecting thyroid disorders [19]. In this research, we observed Significantly increased TNF-α and TNFR1 in HT patients compared to the healthy controls ($p<0.001$), for both (Table 2). Our findings align well with Hashim and Saeed in Najaf province, Iraq, which indicated that TNF-α levels were considerably elevated in patients with HTD ($p<0.001$) [20]. Other investigations demonstrated significantly higher serum levels of TNF-α in Hashimoto's thyroiditis patients compared to non-Hashimoto's thyroiditis individuals ($p<0.0001$) [19, 21, 22]. Furthermore, It was reported that in hypothyroid patients, serum TNF-α and sTNFR1 levels were significantly elevated compared to healthy individuals, with P values (<0.05) and (<0.01), respectively [23]. In the diagnosis of Hashimoto's disease, we found that patients with HT exhibited significantly elevated levels of anti-thyroid antibodies (Anti-TPO, Anti-Tg) which as indicators in clinical examination and follow-up of patients with AITD [24] and TSH compared to the healthy control group ($p<0.001$), while levels of fT3 and fT4 were lower in women with HT than in healthy women (Table 1). Consequently, patients exhibit significant symptoms, notably hair loss, which occurs in 83.5% of cases, since thyroid hormones are essential for forming and maintaining hair follicles, suggesting that hair loss may indicate thyroid dysfunction [25]. Moreover, hormone regulation significantly influences the modulation of both peripheral and central aspects of fatigue. The processes involved are believed to include controlling the body's metabolic rate and the rate of energy production, regulating a wide array of metabolic activities, activating the hypothalamic-pituitary-adrenal axis, and regulating pro- and anti-

inflammatory cytokine release. Endocrinopathies are thereby linked to self-reported fatigue, lethargy, fatigability, myocardial, and mood disorders [26, 27]. These symptoms align with the results of our research (Figure 1). When studying the correlation between TNF-α and TNFR1 and other variables, there were strong positive correlation coefficients between TNF-α and TNFR1 (p= 0.004) in patients (Figure 2A). TNF-α binds to two surface TNF receptors, TNFR1 and TNFR2, where it binds to the death domain (DD) of TNFR1, which induces apoptosis. Apoptotic signaling via the extrinsic route is initiated when extracellular ligands, including TNF and Fas ligand (Fas-L), bind to the extracellular domain of death receptors (DRs). The order of events in the extrinsic apoptosis process is characterized by the TNF - α / TNFR1 and FasL / Fas Receptore (Fas R) models [10]. Furthermore, the positive correlation between TNFR1 and Anti- Tg (p= 0.036) was recorded in our study in (Figure 2B). As a confirmation, this result is compatible with the study by Gheorghe *et al.* [28], which showed a statistically significant correlation between TgAb and TNFR1 (r=0.42, p=0.016). Regarding the study of the ROC, we noted a high curve of the area under the ROC curve of TNF–α (0.896) with (p<0.001) (Figure 3A and Table 4). Also, both studies by Morawska *et al.* and Kobawala *et al.* reported an evaluation of TNF-α: that recorded AUC (0.840 and 0.827) (p<0:0001) [22, 29]. This may be due to high amounts of releasing of TNF-α from T-cytotoxic cells (CD4+ and CD8+) in HTD [30]. In addition, our result obtained of the ROC curve of TNFR1 showed an excellent diagnostic value of a high curve of AUC (0.848) (p<0.001) (Figure 3B and Table 4). Only a few studies have included the measurements of serum levels of soluble cytokine receptors such as sTNFR1 in autoimmune diseases, while the AUC value of serum sTNFR1 was (0.781) in the systemic lupus erythematosus (SLE) group compared to the control group [31]. On the other hand, our result recorded higher statistical mRNA expression of TNF-α in a patient group rather than the control group (P<0.046) in (Figure 3). The finding of the current study is similar to other previous studies that indicated a high significance of TNF-α mRNA expression (P=0.0049) in thyroid follicular [32] and (P=0.001) in the HT group [33]. TNF-α was shown to increase significantly in Hashimoto disorder patients [21]. Therefore, it may be one of the inflammatory cytokines that have an important pathological role in thyroid dysfunctions [34].

Conclusion

The usefulness of TNF-α as a diagnostic marker for Hashimoto's thyroiditis disease was explored. Where high serum levels of TNF-α and its receptor 1 were revealed by the ROC curve, and high expression of TNF-α mRNA was detected by real-time quantitative PCR. In addition, a strong positive correlation coefficient between serum levels of TNF-α with TNFR1 and sTNFR1 with traditional diagnostic marker Anti-Tg. All these obtained results prove the potential role of TNF-α in the pathogenesis of HTD.

Acknowledgments

The authors are grateful to the female participants who participated in the research, whether they were patients or healthy. We would also like to thank Kirkuk University, Azadi Teaching Hospital of the Iraqi Ministry of Health and Ochi Medical Complex for the facilities provided during the experimental work.

Ethical Certification

The study was approved by the ethical review board of (Azadi Teaching Hospital and the Faculty of Science at the University of Kirkuk. Informed verbal consent and permission were obtained from participants prior to taking blood samples from them. University's Sciences College, Biology Branch approved the study's design. By the document numbered 783 on November 28, 2023.

Conflict of interest statement

The authors declare no conflict of interest.

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