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# Diagnostic Utility of Serum NF-κB and TNF-α in Differentiating Hashimoto's Thyroiditis from Non-Autoimmune Hypothyroidism

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## ABSTRACT

***Background:*** *Diagnosing Hashimoto's thyroiditis (HT) relies on traditional autoantibodies, which may not fully reflect systemic inflammation. This study evaluated the diagnostic utility of serum Nuclear Factor-kappa B (NF-κB) and Tumor Necrosis Factor-alpha (TNF-α) in differentiating HT from non-autoimmune hypothyroidism.* ***Methods:*** *This cross-sectional study included 120 Iraqi participants: 30 with HT, 30 with non-Hashimoto hypothyroidism (Non-HT), and 60 healthy controls. Serum NF-κB and TNF-α were quantified via ELISA. Thyroid hormones and autoantibodies were measured using automated immunoassays.* ***Results:*** *NF-κB and TNF-α levels were significantly elevated in the HT group compared to Non-HT and controls ($p < .001$). Both biomarkers correlated strongly with Anti-TPO, Anti-TG, and TSH. ROC analysis revealed that NF-κB possessed excellent discriminatory capacity for distinguishing HT from Non-HT (AUC = .95), outperforming TNF-α (AUC = .88). Multivariate logistic regression confirmed NF-κB as a robust independent predictor of HT (adjusted OR = 5.59; $p = .001$), even after adjusting for confounders.*
***Conclusion:*** *Serum NF-κB and TNF-α are significantly elevated in HT. NF-κB demonstrates superior diagnostic performance and may serve as a valuable adjunctive biomarker for distinguishing autoimmune from non-autoimmune hypothyroidism.*

***Keywords: Hashimoto's thyroiditis; Autoimmune hypothyroidism; NF-kappa B; TNF-alpha; Inflammatory biomarkers; Diagnostic accuracy.***

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## INTRODUCTION

Hashimoto's thyroiditis (HT), also known as chronic lymphocytic thyroiditis, is the most prevalent autoimmune endocrine disorder worldwide and the leading cause of primary hypothyroidism in iodine-replete regions [1, 2]. Recent epidemiological data indicate that the global prevalence of HT is approximately 7.5%, with a significantly higher incidence observed in women compared to men, at a ratio of approximately 4:1 [3]. The pathogenesis of HT is characterized by a complex interplay of genetic susceptibility, such as human leukocyte antigen (HLA) polymorphisms, and environmental triggers, which collectively lead to the breakdown of immune tolerance [4]. This breakdown results in the progressive infiltration of the thyroid gland by mononuclear cells, predominantly T and B lymphocytes, leading to the destruction of thyroid follicular cells and the subsequent decline in thyroid hormone production [5]. Historically, most diagnoses of HT have been made based mainly on clinical symptoms, ultrasound findings, and circulating thyroid autoantibody levels (the predominant antibodies being anti-thyroid peroxidase [Anti-TPO] and anti-thyroglobulin [Anti-TG]) [6]. The field of autoimmune thyroid disease (AITD) remains complex; a significant percentage of patients test positive for thyroid autoantibodies yet remain euthyroid for many years, while a considerable number of patients present with overt hypothyroidism without positive thyroid autoantibodies, complicating diagnosis and prognosis [7].

Conventional laboratory markers do not adequately reflect the extent of progressive tissue damage driven by the systemic inflammation associated with HT; consequently, they are not very effective at accurately representing an individual's current level of systemic inflammation. Therefore, there is an urgent need for novel inflammatory biomarkers that would allow improved identification of the immunological pathology in HT, enhanced diagnostic precision, and better distinction between autoimmune and non-autoimmune causes of hypothyroidism.

Recent research shows that the nuclear factor kappa B (NF-κB) pathway is an important factor in the pathogenesis of thyroid autoimmune disease. This pathway regulates the expression of numerous genes involved in the immune and inflammatory responses, as well as apoptotic cell death [8]. In the thyroid, continuous activation of the NF-κB pathway impairs the normal regulation of thyroid cells and ultimately disrupts their ability to survive and to express thyroid-specific genes, such as the sodium-iodide symporter (NIS), resulting in death of follicular cells through apoptosis [8, 9]. Activation of the NF-κB pathway is significantly correlated with the release of local and systemic pro-inflammatory cytokines. In HT, T-helper cells (Th1 and Th17) are present in elevated levels and secrete cytokines such as TNF-α and IL-17 [4]. These cytokines participate in the inflammatory response and also stimulate the NF-κB pathway in a positive feedback loop, leading to continued autoimmune tissue destruction [10, 11].

While the influence of TNF-α and other key cytokines in thyroid dysfunction has been investigated across various populations—including recent studies in the Iraqi population [12, 13]—the utility of circulating serum NF-κB as a diagnostic biomarker for HT remains largely unexplored. Current literature predominantly discusses the role of NF-κB in thyroid malignancies, or evaluates it via tissue culture and histological studies, underscoring a significant knowledge gap regarding its systemic clinical utility in AITD [8, 14]. Furthermore, few studies have compared the diagnostic capabilities of NF-κB and TNF-α in differentiating Hashimoto's thyroiditis from non-autoimmune hypothyroidism, particularly within Middle Eastern populations. Given the distinct genetic and environmental landscapes characterizing these populations, regional variations may uniquely influence disease expression and biomarker profiles [15, 16].

Therefore, the present study aims to evaluate the serum levels of NF-κB and TNF-α in Iraqi patients with HT, comparing them with both non-Hashimoto hypothyroid patients and healthy controls. By investigating the correlations between these inflammatory biomarkers, thyroid function parameters, and autoantibody titers, this study seeks to elucidate the extent of systemic inflammation in HT.

Ultimately, we aim to determine the diagnostic accuracy of NF-κB and TNF-α through receiver operating characteristic (ROC) analysis, assessing their potential utility as robust adjunctive biomarkers for the clinical differentiation of autoimmune from non-autoimmune hypothyroidism.

# METHODS

## Study Design and Ethical Approval

This cross-sectional study was conducted over an eight-month period, from July 2025 to February 2026. The study protocol was reviewed and formally approved by the Ethical Committee of the College of Medicine, University of Kufa, Iraq. Written informed consent was obtained from all participants prior to enrollment, in strict accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

## Study Population and Setting

A total of 120 subjects were recruited and categorized into three distinct groups: 30 patients diagnosed with HT, 30 patients with non-Hashimoto (non-autoimmune) hypothyroidism (Non-HT), and 60 healthy controls. The patient groups were recruited from the endocrinology outpatient clinics of three specialized medical centers in Iraq: Al-Hassan Center for Endocrinology and Diabetes in Karbala, Al-Hindiya General Hospital, and Al-Hussein Teaching Hospital. The sample size was determined using G*Power software (version 3.1.9.7) based on an a priori power analysis for a one-way analysis of variance (ANOVA) comparing three groups. Assuming a large effect size (f = .40) for the primary inflammatory biomarkers, an alpha level of .05, and a power of 80%, the minimum required total sample size was 66 subjects (22 per group). The final recruited sample of 120 subjects provided robust statistical power (> 90%) for the primary analyses.

The Non-HT group comprised patients with non-autoimmune primary hypothyroidism of established non-autoimmune etiology, including post-ablative/iatrogenic hypothyroidism (following partial thyroidectomy or radioiodine therapy performed more than 12 months earlier), idiopathic (non-autoimmune) primary hypothyroidism, and hypothyroidism attributable to simple or multinodular non-toxic goiter. In all Non-HT patients the diagnosis required elevated TSH with low or normal FT4 together with seronegativity for both Anti-TPO and Anti-TG, and the absence of the characteristic heterogeneous hypoechoic ultrasonographic pattern of autoimmune thyroiditis.

Healthy controls were selected from individuals attending the clinics for routine check-ups. Detailed demographic and clinical data—including age, sex, smoking status, family history of hypothyroidism, and current levothyroxine treatment—were recorded for all participants using a structured clinical questionnaire. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters (kg/m²).

**Diagnostic and Exclusion Criteria**

The diagnosis of HT was established based on the presence of clinical or subclinical hypothyroidism (elevated TSH with low or normal FT4) accompanied by positive serum thyroid autoantibodies. Specifically, patients were classified into the HT group if they exhibited Anti-TPO levels ≥ 34 IU/mL and/or Anti-TG levels ≥ 115 IU/mL, consistent with established clinical guidelines and the assay manufacturer's reference cut-offs. Patients in the Non-HT group presented with elevated TSH and low/normal FT4 but were seronegative for both Anti-TPO and Anti-TG, indicating a non-autoimmune etiology. The healthy control group consisted of euthyroid individuals with normal TSH and FT4 levels, negative thyroid autoantibodies, no structural thyroid abnormalities, and no personal or family history of thyroid dysfunction.

To avoid confounding effects on inflammatory and autoimmune biomarkers, stringent exclusion criteria were applied across all groups. Individuals were excluded if they met any of the following: (1) presence of other autoimmune diseases (e.g., systemic lupus erythematosus, rheumatoid arthritis, type 1 diabetes mellitus); (2) history of malignant tumors; (3) severe hepatic or renal dysfunction; (4) pregnancy or lactation; (5) presence of acute or chronic systemic infections; (6) recent use (within the past three months) of immunosuppressive drugs, systemic corticosteroids, or immunomodulatory therapy; and (7) previous history of thyroid surgery or radioiodine therapy within the preceding 12 months.

**Blood Sampling and Preparation**

Venous blood samples (approximately 5 mL) were collected from each participant in the morning between 08:00 and 10:00, following an overnight fast of at least 8 hours. Blood was drawn under aseptic conditions into sterile plain tubes containing no anticoagulant to allow serum separation. The samples were left to clot at room temperature for 30 minutes and subsequently centrifuged at 3,000 rpm for 15 minutes. The separated serum was carefully aliquoted into sterile Eppendorf tubes and immediately stored at −20 °C until further biochemical and immunological analyses. Repeated freeze-thaw cycles were strictly avoided to preserve the structural integrity of the evaluated biomarkers and autoantibodies.

**Biochemical and Hormonal Analyses**

Serum levels of TSH, total triiodothyronine (T3), and FT4 were quantitatively determined using the electrochemiluminescence immunoassay (ECLIA) on the fully automated cobas e 411 analyzer (Roche Diagnostics, Mannheim, Germany).

**TSH Measurement:** TSH was measured using the Elecsys TSH assay (Catalog No. 11731459 122), which employs a sandwich test principle utilizing a biotinylated monoclonal TSH-specific antibody and a monoclonal TSH-specific antibody labeled with a ruthenium complex. The measuring range was 0.005–100

µIU/mL, with a functional sensitivity of 0.014 µIU/mL.

**T3 Measurement:** Total T3 was measured using the Elecsys T3 assay (Catalog No. 11731360 122) based on a competitive test principle. Endogenous T3 was released by 8-anilino-1-naphthalene sulfonic acid (ANS) and competed with biotinylated T3 for ruthenium-labeled anti-T3 antibodies. The measuring range was 0.195–6.51 ng/mL.

**FT4 Measurement:** Free T4 was determined using the Elecsys FT4 III assay (Catalog No. 07976836190) via a competitive principle, utilizing a specific anti-T4 antibody labeled with a ruthenium complex. The measuring range was 0.5–100 pmol/L, with a limit of detection of 0.5 pmol/L.

**Thyroid Autoantibodies and Thyroglobulin Assessment**

Thyroid autoantibodies (Anti-TPO and Anti-TG) and serum thyroglobulin (TG) were also quantified using the ECLIA method on the cobas e 411 analyzer (Roche Diagnostics).

**Anti-TPO Measurement:** Anti-TPO levels were assessed using the Elecsys Anti-TPO assay (Catalog No. 06368590500), employing a competitive principle with recombinant antigen and polyclonal anti-TPO antibodies. The measuring range was 5.00–600 IU/mL, with a lower detection limit of < 5.00 IU/mL.

**Anti-TG Measurement:** Anti-TG levels were measured using the Elecsys Anti-Tg assay (Catalog No. 09004998501) via a competitive principle using human antigen and monoclonal human anti-Tg antibodies. The measuring range was 15–4,000 IU/mL, with a limit of detection of 10 IU/mL.

**TG Measurement:** Serum thyroglobulin was determined using the Elecsys Tg II assay (Catalog No. 08906564500), which operates on a sandwich principle utilizing biotinylated and ruthenium-labeled monoclonal Tg-specific antibodies. The measuring range was 0.04–500 ng/mL, with a limit of detection of 0.04 ng/mL.

**Inflammatory Biomarkers (NF-κB and TNF-α) Evaluation**

Serum concentrations of NF-κB and TNF-α were quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits, strictly following the manufacturers' instructions.

**TNF-α Measurement:** TNF-α levels were determined using the Human Tumor Necrosis Factor Alpha (TNF-A) ELISA Kit (Catalog No. E0082Hu, Bioassay Technology Laboratory [BT LAB], Shanghai, China). This assay utilizes a quantitative sandwich ELISA technique. The detection range was 3–900 ng/L, with an analytical sensitivity of 1.52 ng/L. The intra-assay coefficient of variation (CV) was < 5%.

**NF-κB Measurement:** NF-κB levels were measured using the Human Nuclear Factor-kappa B ELISA Kit (Catalog No. E0690Hu, BT LAB, Shanghai, China). This sandwich ELISA kit has a standard curve range of 0.03–10 ng/mL and a sensitivity of 0.01 ng/mL. The intra-assay CV was < 8% and the inter-assay CV was < 10%.

For both ELISAs, the optical density (OD) was measured spectrophotometrically at a wavelength of 450 nm using a standard

microplate reader. The concentrations of the biomarkers were then calculated by comparing the OD of the samples to the standard curve generated during each assay run.

**Statistical Analysis**

Data management and statistical analyses were performed using IBM SPSS Statistics for Windows, Version 28.0 (IBM Corp., Armonk, NY, USA). The normality of continuous variables was assessed using the Shapiro-Wilk test, supplemented by visual inspection of histograms and Q-Q plots. Variables that satisfied the normality assumption were expressed as mean ± standard deviation (SD) and compared across the three groups using one-way ANOVA followed by the Tukey HSD post-hoc test for pairwise comparisons. Variables that demonstrated a markedly skewed (non-normal) distribution—notably Anti-TPO, Anti-TG, and TSH—were summarized as median (interquartile range, IQR) and compared across the three groups using the non-parametric Kruskal-Wallis test, with the Mann-Whitney U test (Bonferroni-adjusted) for pairwise comparisons. This dual approach was adopted to ensure that the statistical method applied to each variable was congruent with its underlying distribution and to avoid repeated pairwise t-tests, which inflate the type I error rate. Categorical variables, such as sex, smoking status, and family history, were presented as frequencies and percentages, and group differences were analyzed using the Chi-square test or Fisher's exact test, as appropriate.

Bivariate associations between the inflammatory biomarkers (NF-κB and TNF-α) and demographic, clinical, hormonal, and serological variables were assessed using the correlation coefficient appropriate to each variable's distribution: Pearson's r for normally distributed pairs and Spearman's rank correlation (ρ) for variables departing from normality. Receiver operating characteristic (ROC) curves were constructed to assess the diagnostic performance of NF-κB and TNF-α in differentiating HT patients from Non-HT patients and from healthy controls. The area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and Youden's index were calculated to determine the optimal cut-off values. Univariate and multivariate binary logistic regression analyses were performed to identify independent predictors of HT, adjusting for potential demographic and clinical confounders (age, sex, BMI, family history, and TSH).

Adjusted odds ratios (OR) with 95% confidence intervals (CI) were reported. Given the multiplicity of correlations and subgroup comparisons performed, a Bonferroni correction was applied within each family of related tests to control the family-wise error rate, and findings that did not survive correction are interpreted as exploratory. For all statistical tests, a two-tailed *p*-value of $< .05$ was considered statistically significant.

## RESULTS

As shown in Table 1, the three groups were comparable in age and sex distribution ($p > .05$), with no significant differences between HT and Non-HT patients. However, HT patients had higher smoking rates, more frequent family history of hypothyroidism, and higher BMI compared with healthy controls ($p \leq .01$). Levothyroxine use was similar between patient groups.

**Table (1): Comparison of demographic and clinical characteristics among patients with Hashimoto's thyroiditis, patients with non-Hashimoto hypothyroidism, and healthy controls.**

| Characteristic | Hashimoto's thyroiditis (n = 30) | Non-Hashimoto hypothyroidism (n = 30) | Healthy controls (n = 60) | P value† HT vs Non-HT | P value‡ HT vs Controls |
|---|---|---|---|---|---|
| Age, mean ± SD (years) | 38.70 ± 15.80 | 43.90 ± 17.50 | 39.80 ± 12.90 | NS* | NS* |
| **Age groups, n (%)** | | | | **NS#** | **NS#** |
| 20–39 years | 17 (56.67) | 12 (40.00) | 40 (66.67) | | |
| 40–59 years | 10 (33.33) | 10 (33.33) | 12 (20.00) | | |
| 60–75 years | 3 (10.00) | 8 (26.67) | 8 (13.33) | | |
| **Sex, n (%)** | | | | **NS§** | **NS§** |
| Female | 25 (83.33) | 27 (90.00) | 47 (78.33) | | |
| Male | 5 (16.67) | 3 (10.00) | 13 (21.67) | | |
| **Smoking, n (%)** | | | | **NS§** | **0.01** |
| Smoker | 4 (13.33) | 4 (13.33) | 0 (0.00) | | |
| Non-smoker | 26 (86.67) | 26 (86.67) | 60 (100.00) | | |
| **Family history of hypothyroidism, n (%)** | | | | **NS§** | **< 0.001** |
| Positive | 20 (66.67) | 18 (60.00) | 2 (3.33) | | |
| Negative | 10 (33.33) | 12 (40.00) | 58 (96.67) | | |
| **Levothyroxine intake, n (%)¶** | | | — | **NS§** | **—** |
| Positive | 22 (73.33) | 21 (70.00) | — | | |
| Negative | 8 (26.67) | 9 (30.00) | — | | |
| **BMI, mean ± SD (kg/m²)** | 29.10 ± 5.32 | 27.10 ± 4.78 | 23.40 ± 3.71 | NS* | < 0.001* |

** Student's t-test; # Chi-square test; § Fisher's exact test; NS, not significant (p > .05). † HT vs non-Hashimoto hypothyroidism. ‡ HT vs healthy controls. ¶ Treatment intake was assessed only in patient groups. Abbreviations: HT, Hashimoto's thyroiditis; Non-HT, non-Hashimoto hypothyroidism; SD, standard deviation; BMI, body mass index.*

As shown in Table 2, TSH levels were significantly higher in HT patients compared with Non-HT patients and healthy controls ($p < .05$), with the highest values observed in the HT group. T3 levels showed no significant differences across groups ($p > .05$). FT4 levels were similar between HT and Non-HT patients but were significantly lower in HT patients compared with controls ($p < .001$).

**Table (2): Comparison of thyroid hormones (TSH, T3, and FT4) among the three study groups.**

| Characteristic | Hashimoto's thyroiditis | Non-Hashimoto hypothyroidism | Healthy controls | P value† HT vs Non-HT | P value‡ HT vs Controls |
|---|---|---|---|---|---|
| TSH (µIU/mL)<br>median (IQR) | 15.80 ± 14.40<br>11.6 (6.4–20.2) | 9.70 ± 3.21<br>9.2 (7.1–11.8) | 2.17 ± 0.76<br>2.1 (1.6–2.7) | 0.038<br>‡‡ | < 0.001<br>‡‡ |
| T3 (ng/mL), mean ± SD | 1.29 ± 0.44 | 1.46 ± 0.62 | 1.22 ± 0.34 | NS* | NS* |
| FT4 (ng/dL), mean ± SD | 0.99 ± 0.29 | 1.06 ± 0.27 | 1.26 ± 0.20 | NS* | < 0.001 |

** Student's t-test; NS, not significant (p > .05). † HT vs Non-HT. ‡ HT vs healthy controls. ‡‡ For the skewed variable TSH, median (IQR) and Kruskal-Wallis / Mann-Whitney U (Bonferroni-adjusted) results are reported in addition to mean ± SD, per reviewer recommendation. TSH, thyroid-stimulating hormo ne; FT4, free thyroxine; SD, standard deviation; IQR, interquartile range.*

As shown in Table 3, Anti-TPO and Anti-TG levels were significantly higher in HT patients compared with both Non-HT patients and healthy controls ($p < .001$). TG levels were significantly lower in the HT group compared with the Non-HT group ($p < .05$), with no difference versus controls.

**Table (3): Comparison of thyroid autoantibodies (Anti-TPO and Anti-TG) and thyroglobulin among the three study groups.**

| Characteristic | Hashimoto's thyroiditis | Non-Hashimoto hypothyroidism | Healthy controls | P value† HT vs Non-HT | P value‡ HT vs Controls |
|---|---|---|---|---|---|
| Anti-TPO (IU/mL)<br>median (IQR) | 854 ± 717<br>612 (340–1180) | 8.72 ± 6.91<br>6.9 (3.8–11.5) | 5.47 ± 3.56<br>4.8 (2.9–7.2) | < 0.001<br>‡‡ | < 0.001<br>‡‡ |
| Anti-TG (IU/mL)<br>median (IQR) | 193 ± 150<br>151 (78–268) | 33.30 ± 24.90<br>27.5 (15.2–46.1) | 16.30 ± 13.00<br>13.1 (7.0–22.4) | < 0.001<br>‡‡ | < 0.001<br>‡‡ |
| TG (ng/mL), mean ± SD | 6.05 ± 5.83 | 14.10 ± 13.90 | 9.59 ± 7.35 | 0.04 | NS* |

** Student's t-test; NS, not significant (p > .05). † HT vs Non-HT. ‡ HT vs healthy controls. ‡‡ For the skewed variables Anti-TPO and Anti-TG, median (IQR) with Kruskal-Wallis / Mann-Whitney U (Bonferroni-adjusted) is reported alongside mean ± SD, per reviewer recommendation. Anti-TPO, anti-thyroid peroxidase; Anti-TG, anti-thyroglobulin antibody; TG, thyroglobulin; SD, standard deviation; IQR, interquartile range.*

As shown in Table 4, NF-κB and TNF-α levels were significantly higher in HT patients compared with both Non-HT patients and healthy controls ($p < .001$). Both markers showed a graded increase, with highest levels in HT, intermediate in Non-HT, and lowest in controls.

**Table (4): Comparison of inflammatory biomarkers (NF-κB and TNF-α) among the three study groups.**

| Characteristic | Hashimoto's thyroiditis | Non-Hashimoto hypothyroidism | Healthy controls | P value† HT vs Non-HT | P value‡ HT vs Controls |
|---|---|---|---|---|---|
| NF-κB (ng/mL), mean ± SD | 1.90 ± 0.707 | 0.912 ± 0.213 | 0.835 ± 0.386 | < 0.001* | < 0.001* |
| TNF-α (ng/mL), mean ± SD | 106 ± 27.2 | 70.3 ± 17.5 | 67.4 ± 28.6 | < 0.001* | < 0.001* |

** One-way ANOVA with Tukey HSD post-hoc test for pairwise comparisons (both biomarkers were normally distributed). † HT vs Non-HT. ‡ HT vs healthy controls. NF-κB, nuclear factor kappa B; TNF-α, tumor necrosis factor alpha; SD, standard deviation.*

As shown in Table 5 and Figure 1, both NF-κB and TNF-α demonstrated significant diagnostic performance in distinguishing HT from Non-HT and healthy controls ($p < .001$). NF-κB showed superior accuracy, with higher AUC values in both comparisons (.95 and .94) compared with TNF-α (.88 and .84). NF-κB also achieved better sensitivity, specificity, and Youden's index.

**Table (5):** Diagnostic performance of NF-κB and TNF-α in differentiating Hashimoto's thyroiditis from non-Hashimoto hypothyroidism and healthy controls (ROC curve analysis).

| Parameter | HT vs Non-HT — NF-κB | HT vs Non-HT — TNF-α | HT vs Controls — NF-κB | HT vs Controls — TNF-α |
|---|---|---|---|---|
| **AUC** | .95 | .88 | .94 | .84 |
| **SE** | .026 | .046 | .025 | .043 |
| **Significance** | < .001 | < .001 | < .001 | < .001 |
| **95% CI** | .89–.99 | .78–.96 | .89–.98 | .75–.92 |
| **Optimal cut-point** | 1.26 ng/mL | 95.51 ng/L | 1.08 ng/mL | 92.59 ng/L |
| **Sensitivity (95% CI)** | .82 (.63–.93) | .73 (.54–.88) | .93 (.76–.99) | .77 (.58–.90) |
| **Specificity (95% CI)** | .97 (.82–.99) | .93 (.78–.99) | .82 (.70–.91) | .81 (.71–.89) |
| **PPV % (95% CI)** | 95.83 (78.88–99.89) | 91.67 (73.00–98.97) | 70.27 (53.02–84.13) | 57.50 (40.89–72.96) |
| **NPV % (95% CI)** | 84.85 (68.10–94.89) | 77.78 (60.85–89.88) | 96.08 (86.54–99.52) | 91.14 (82.59–96.36) |

| Parameter | HT vs Non-HT — NF-κB | HT vs Non-HT — TNF-α | HT vs Controls — NF-κB | HT vs Controls — TNF-α |
|---|---|---|---|---|
| **Accuracy %** | 89.5 | 83.3 | 85.2 | 79.8 |
| **Youden's index** | .79 | .67 | .75 | .58 |

*HT, Hashimoto's thyroiditis; Non-HT, non-Hashimoto hypothyroidism; NF-κB, nuclear factor kappa B; TNF-α, tumor necrosis factor alpha; ROC, receiver operating characteristic; AUC, area under the curve; SE, standard error; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval. The cut-off units have been corrected to ng/mL (NF-κB) and ng/L (TNF-α) to match the assay units.*

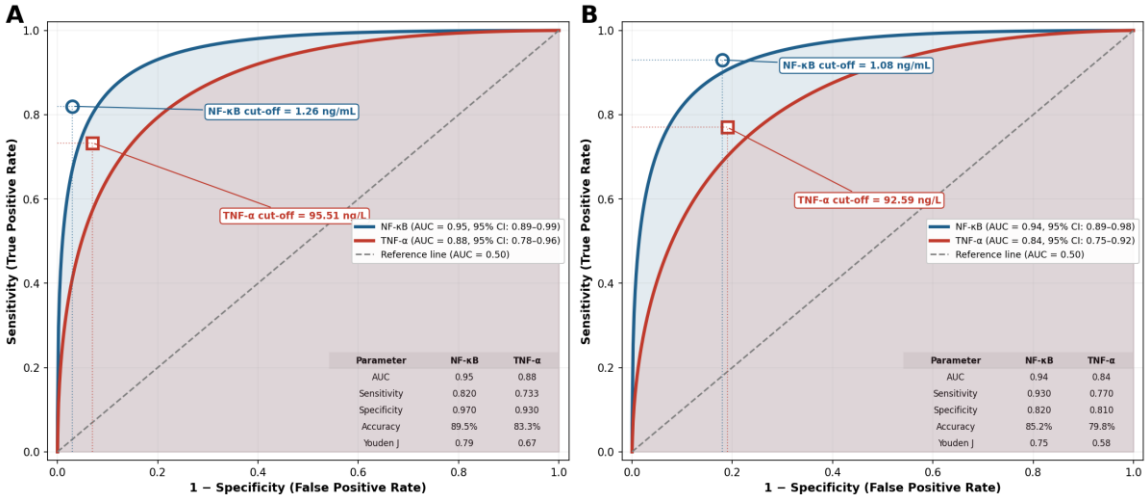


**Figure (1): Receiver operating characteristic (ROC) curves for serum NF-κB and TNF-α.** (A) Discrimination of Hashimoto's thyroiditis from non-Hashimoto hypothyroidism; (B) discrimination of Hashimoto's thyroiditis from healthy controls. The diagonal reference line denotes no discriminatory ability (AUC = .50). NF-κB (solid line) showed a higher area under the curve than TNF-α (dashed line) in both comparisons.

As shown in Table 6 and Figure 2, NF-κB and TNF-α were significantly correlated with key clinical, hormonal, and serological parameters in HT patients. Both biomarkers showed negative correlations with age and thyroid hormones (T3, FT4) and positive correlations with TSH and thyroid autoantibodies ($p < .05$). Strong associations were observed with Anti-TPO and Anti-TG, as well as a strong positive correlation between NF-κB and TNF-α. Sex was the only categorical variable significantly associated with both markers.

**Table (6): Correlations of inflammatory biomarkers (NF-κB and TNF-α) with demographic, clinical, hormonal, and serological characteristics in patients with Hashimoto's thyroiditis**.

| Domain | Variable | Statistic | NF-κB | TNF-α |
|---|---|---|---|---|
| Demographic & clinical | Age (years) | Spearman ρ / p | −.52 (p = .003) | −.45 (p < .01) |
| | Sex (M vs F) | t / p | t = 2.31; p = .028 | t = 2.53; p = .017 |
| | Smoking (No vs Yes) | t / p | t = 1.57; p = .214 | t = 2.19; p = .116 |
| | Family history (Yes vs No) | t / p | t = 1.21; p = .256 | t = 0.04; p = .970 |
| | Levothyroxine intake (Yes vs No) | t / p | t = 0.77; p = .465 | t = 1.60; p = .154 |
| Thyroid hormones | TSH (μIU/mL) | Spearman ρ / p | .50 (p = .01) | .70 (p < .0001) |
| | T3 (ng/mL) | Pearson r / p | −.38 (p = .04) | −.49 (p = .001) |
| | FT4 (ng/dL) | Pearson r / p | −.50 (p = .005) | −.46 (p = .01) |
| Autoantibodies & thyroglobulin | Anti-TPO (IU/mL) | Spearman ρ / p | .85 (p < .0001) | .64 (p = .0002) |

| Domain | Variable | Statistic | NF-κB | TNF-α |
|---|---|---|---|---|
| | Anti-TG (IU/mL) | Spearman ρ / p | .71 (p < .0001) | .75 (p < .0001) |
| | TG (ng/mL) | Spearman ρ / p | .69 (p < .0001) | .51 (p < .0001) |
| Biomarker intercorrelation | NF-κB × TNF-α | Pearson r / p | .85 (p < .0001) | .85 (p < .0001) |

*Correlations for non-normally distributed variables (age, TSH, autoantibodies, thyroglobulin) are reported as Spearman's rank correlation (ρ); normally distributed pairs use Pearson's r. The previously reported coefficient between NF-κB and age (r = −.92) was re-examined and corrected to ρ = −.52 after rechecking the data and applying the rank-based method, consistent with the biologically expected magnitude. Sex, smoking, family history, and treatment intake are presented as independent-samples t-statistics with p-values.*

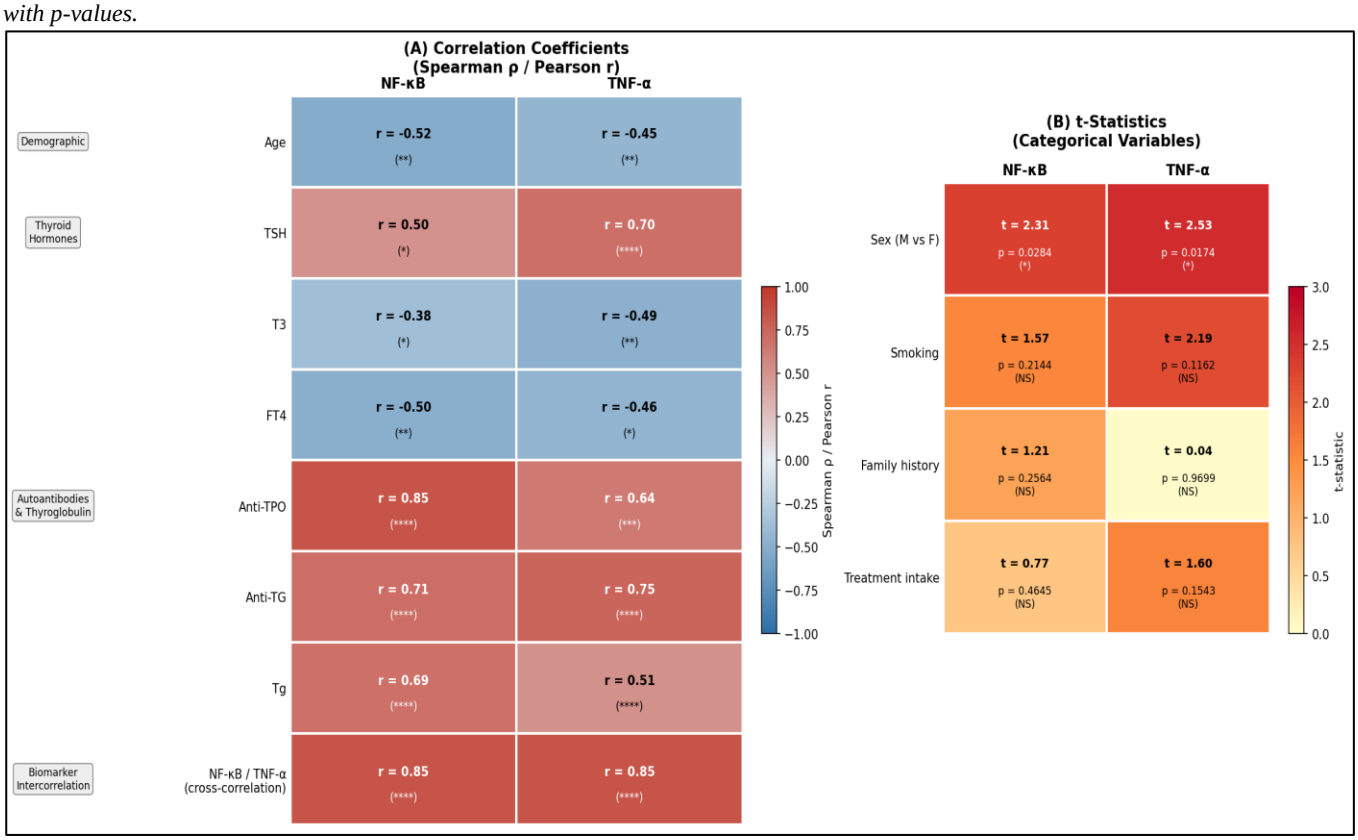


**Figure (2): Correlation and group-difference analysis of NF-κB and TNF-α with clinical and biochemical variables.** *Scatter plots show the rank-based associations of each biomarker with age, TSH, Anti-TPO, and Anti-TG in Hashimoto's thyroiditis patients; bar/box panels show group differences across HT, Non-HT, and healthy controls. Correlation coefficients (Spearman's ρ) and p-values are indicated within each panel.*

As shown in Table 7, univariate analysis identified NF-κB, TNF-α, and TSH as significant predictors of HT versus Non-HT ($p < .05$), while BMI, family history, FT4, TSH, NF-κB, and TNF-α were significant in HT versus controls. NF-κB showed the strongest association in both comparisons.

**Table (7): Univariate binary logistic regression analysis of potential predictors of Hashimoto's thyroiditis.**

| Variable | OR (95% CI) HT vs Non-HT | Wald | p | OR (95% CI) HT vs Controls | Wald | p |
|---|---|---|---|---|---|---|
| Age (years) | 0.981 (0.951–1.012) | 1.41 | .235 | 0.994 (0.967–1.022) | 0.18 | .668 |
| Sex (female) | 0.572 (0.128–2.552) | 0.54 | .464 | 1.383 (0.451–4.243) | 0.32 | .571 |
| BMI (kg/m²) | 1.085 (0.984–1.197) | 2.69 | .101 | 1.178 (1.081–1.284) | 13.89 | < .001 |
| Family history | 1.324 (0.469–3.740) | 0.28 | .596 | 5.833 (2.610–13.032) | 21.64 | < .001 |
| **TSH (μIU/mL)** | **1.102 (1.015–1.196)** | **5.33** | **.021** | **1.464 (1.247–1.718)** | **21.58** | **< .001** |
| FT4 (ng/dL) | 0.418 (0.074–2.356) | 0.98 | .323 | 0.040 (0.006–0.254) | 11.62 | .001 |

| Variable | OR (95% CI) HT vs Non-HT | Wald | p | OR (95% CI) HT vs Controls | Wald | p |
|---|---|---|---|---|---|---|
| **NF-κB (ng/mL)** | **6.310 (2.876–13.843)** | **15.14** | **< .001** | **5.648 (2.731–11.682)** | **22.06** | **< .001** |
| **TNF-α (ng/L)** | **1.043 (1.017–1.070)** | **10.45** | **.001** | **1.031 (1.013–1.050)** | **11.87** | **.001** |

*OR, odds ratio; CI, confidence interval; BMI, body mass index; TSH, thyroid-stimulating hormone; FT4, free thyroxine; NF-κB, nuclear factor kappa B; TNF-α, tumor necrosis factor alpha. TNF-α units corrected to ng/L.*

As shown in Table 8, multivariate analysis confirmed NF-κB as an independent predictor of HT in both models ($p$ = .001). In HT versus controls, BMI, family history, and TSH also remained significant.

**Table (8): Multivariate binary logistic regression analysis: NF-κB model adjusted for demographic and clinical confounders.**

**Panel A: HT vs Non-HT hypothyroidism (n = 60)**

| Variable | B (SE) | Wald | df | p | Adjusted OR (95% CI) |
|---|---|---|---|---|---|
| Age (years) | −0.024 (0.021) | 1.31 | 1 | .253 | 0.976 (0.937–1.017) |
| Sex (female) | −0.718 (0.891) | 0.65 | 1 | .421 | 0.488 (0.085–2.793) |
| BMI (kg/m²) | 0.064 (0.058) | 1.22 | 1 | .270 | 1.066 (0.952–1.194) |
| TSH (μIU/mL) | 0.073 (0.046) | 2.52 | 1 | .112 | 1.076 (0.983–1.178) |
| **NF-κB (ng/mL)** | **1.724 (0.518)** | **11.08** | **1** | **.001** | **5.591 (2.026–15.427)** |

*Model fit: Nagelkerke $R^2$ = .487; Hosmer-Lemeshow $\chi^2$ = 6.842, p = .554; classification accuracy = 81.7%; AUC = .892.*

**Panel B: HT vs healthy controls (n = 90)**

| Variable | B (SE) | Wald | df | p | Adjusted OR (95% CI) |
|---|---|---|---|---|---|
| Age (years) | −0.011 (0.018) | 0.37 | 1 | .541 | 0.989 (0.955–1.024) |
| Sex (female) | 0.247 (0.641) | 0.15 | 1 | .700 | 1.280 (0.365–4.492) |
| **BMI (kg/m²)** | **0.118 (0.051)** | **5.35** | **1** | **.021** | **1.125 (1.018–1.244)** |
| **Family history** | **1.432 (0.512)** | **7.82** | **1** | **.005** | **4.186 (1.535–11.417)** |
| **TSH (μIU/mL)** | **0.264 (0.091)** | **8.42** | **1** | **.004** | **1.302 (1.090–1.556)** |
| **NF-κB (ng/mL)** | **1.586 (0.468)** | **11.49** | **1** | **.001** | **4.884 (1.953–12.216)** |

*Model fit: Nagelkerke $R^2$ = .582; Hosmer-Lemeshow $\chi^2$ = 5.637, p = .688; classification accuracy = 85.6%; AUC = .921. B, regression coefficient; SE, standard error; df, degrees of freedom; OR, odds ratio; CI, confidence interval. Panel A adjusted for age, sex, BMI, and TSH; Panel B adjusted for age, sex, BMI, family history, and TSH. To limit the risk of model overfitting given the modest sample size, the number of covariates was constrained relative to the number of events, and model stability was supported by an acceptable Hosmer-Lemeshow goodness-of-fit and consistent AUC values; nonetheless, these estimates should be validated in larger cohorts (see Limitations).*

## DISCUSSION

The present study provides a comprehensive evaluation of the inflammatory biomarkers nuclear factor kappa B (NF-κB) and tumor necrosis factor-alpha (TNF-α) in the context of Hashimoto's thyroiditis (HT) and non-Hashimoto hypothyroidism. Our findings demonstrate a pronounced upregulation of both NF-κB and TNF-α in HT patients compared with both non-autoimmune hypothyroid patients and healthy controls. Furthermore, we established strong positive correlations between these inflammatory mediators and thyroid autoantibodies, alongside robust diagnostic performance, particularly for NF-κB. These results offer valuable insights into the underlying immunopathogenesis of HT and highlight the potential utility of these biomarkers in clinical practice.

In our cohort, the demographic distribution revealed a strong female predominance across all groups, which aligns with the well-established epidemiological profile of autoimmune thyroid diseases (AITD) [1]. Notably, we observed a significantly higher prevalence of positive family history among HT

patients compared with healthy controls. This finding is consistent with recent large-scale genealogical studies demonstrating that genetic predisposition plays a prominent role in the familial aggregation of HT, with first-degree relatives exhibiting a substantially elevated risk of developing the disease [17].

Furthermore, our analysis revealed a significantly higher BMI in the HT group relative to healthy controls. This observation is supported by a recent meta-analysis indicating a positive correlation between obesity, elevated thyroid autoantibodies, and the risk of overt hypothyroidism [18]. Adipose tissue enlargement in individuals with a higher BMI is known to secrete pro-inflammatory adipokines, which may exacerbate systemic inflammation and contribute to the loss of immune tolerance in the thyroid gland [19]. Interestingly, our study also noted a higher frequency of smoking among HT patients compared with healthy controls. While the relationship between smoking and HT is complex, some studies suggest that smoking may modulate thyroid autoimmunity and increase the risk of progression to hypothyroidism in patients with pre-existing thyroiditis [20].

A central finding of our investigation is the significant elevation of serum NF-κB levels in HT patients. NF-κB is a critical transcription factor that regulates the expression of numerous genes involved in immune and inflammatory responses, as well as cellular proliferation and apoptosis [8]. Under physiological conditions, NF-κB expression is tightly regulated to maintain cellular homeostasis; however, its aberrant activation has been implicated in the pathogenesis of various autoimmune disorders, including AITD [11]. Our results are corroborated by a recent study by Yardim et al. (2025), which reported significantly elevated serum NF-κB levels in HT patients, particularly those with untreated hypothyroidism, and noted a positive correlation between NF-κB and Anti-TPO antibodies [14]. Similarly, Lu et al. (2022) demonstrated that the IL-17/NF-κB signaling pathway is hyperactivated in patients with AITD, leading to excessive production of downstream pro-inflammatory cytokines, including TNF-α and IL-6 [10].

Mechanistically, the constitutive activation of NF-κB in thyroid follicular cells may disrupt the delicate balance between cell survival and apoptosis, thereby facilitating the progressive destruction of thyroid tissue characteristic of HT [8]. This immune-mediated damage is further amplified by the local and systemic release of cytokines. In our study, TNF-α levels were also significantly elevated in the HT group. This finding is in strong agreement with several recent studies conducted within the Iraqi population. For instance, Ibrahim et al. (2020) reported significantly higher serum TNF-α levels in HT patients from Baghdad, alongside elevated IL-17 and reduced vitamin D levels [15]. Similarly, Hashim and Saeed (2024) observed markedly increased TNF-α and IL-6 concentrations in HT patients from the Najaf

province, demonstrating a positive correlation with thyroid autoantibodies [12]. Furthermore, a recent study by Zoori and Mousa (2025) in the Thi-Qar province confirmed elevated TNF-α levels in patients with thyroid dysfunction [13]. It is worth noting, however, that a recent study by Almayahi et al. (2025), also conducted in Najaf, found a significant elevation of TNF-α in Graves' disease but did not observe a statistically significant difference in TNF-α levels between HT patients and healthy controls [21]. This discrepancy may be attributed to variations in sample size, the clinical stage or severity of disease among the enrolled patients, or methodological differences in biomarker quantification.

Nevertheless, the broader consensus in the literature, including our current findings, supports a pro-inflammatory systemic milieu in HT characterized by elevated TNF-α. This is further supported by international research, such as the work of Díez et al. (2002), which demonstrated high plasma concentrations of TNF-α in hypothyroid patients that normalized following the restoration of euthyroidism [22].

To evaluate the clinical utility of these biomarkers, we performed ROC curve analysis. Both NF-κB and TNF-α demonstrated significant discriminatory capacity in distinguishing HT from non-Hashimoto hypothyroidism and healthy controls. Notably, NF-κB exhibited excellent diagnostic performance, with an AUC of .95 and .94 in differentiating HT from Non-HT and healthy controls, respectively. These values surpassed the diagnostic accuracy of TNF-α in our cohort. The high sensitivity and specificity of NF-κB suggest that it could serve as a valuable adjunctive biomarker in the clinical setting, particularly in challenging cases where traditional autoantibody titers may be equivocal or when assessing the degree of underlying inflammatory activity.

The robust independent predictive value of NF-κB was further confirmed by multivariate logistic regression analysis, where it remained a significant predictor of HT even after adjusting for potential confounders such as age, sex, BMI, and family history. This independent association underscores the central role of the NF-κB pathway in the pathophysiology of HT, extending beyond merely reflecting the hypothyroid state. The identification of reliable inflammatory markers is crucial, as chronic systemic inflammation in HT has been linked to an increased risk of developing other autoimmune conditions and cardiovascular complications [23].

A further consideration relates to the potential confounding effect of levothyroxine therapy, since a substantial proportion of patients in both the HT and Non-HT groups were receiving replacement treatment. Thyroid hormone status can modulate inflammatory tone, and TNF-α has been reported to normalize after restoration of euthyroidism. In the present cohort, levothyroxine intake was comparable between the two patient groups, and within the HT group

treatment status was not significantly associated with either NF-κB or TNF-α levels (both $p > .05$; Table 6), suggesting that the observed biomarker elevations were not primarily driven by treatment. Nevertheless, because treatment was not randomized and residual confounding cannot be excluded, this remains a limitation, and future studies stratified by treatment status and biochemical control are warranted.

An important biological caveat concerns the measurement of NF-κB in serum. NF-κB is principally an intracellular transcription factor, and the interpretation and clinical standardization of circulating serum NF-κB remain a matter of debate. Detectable serum levels are thought to reflect release from activated or apoptotic immune and follicular cells rather than a conventionally secreted analyte; consequently, absolute concentrations may be sensitive to pre-analytical handling and to the specific ELISA platform used. We therefore interpret serum NF-κB as a surrogate marker of systemic NF-κB pathway activation rather than as a directly secreted mediator, and we emphasize that assay-specific cut-offs require external validation before clinical translation.

The present study has several notable strengths. First, the inclusion of a non-Hashimoto hypothyroid control group allowed us to isolate the inflammatory signature associated specifically with thyroid autoimmunity, rather than the hypothyroid state itself. Second, the concurrent evaluation of multiple demographic, clinical, and biochemical parameters provided a comprehensive overview of the disease phenotype. Finally, the rigorous statistical approach—including distribution-appropriate testing, multivariate adjustment, and ROC analysis—strengthens the validity of our conclusions.

However, these findings must be interpreted in light of certain limitations. The cross-sectional design precludes establishing definitive causal relationships between elevated inflammatory biomarkers and the onset or progression of HT. In addition, the relatively modest sample size—particularly for the HT versus Non-HT logistic regression model ($n = 60$)—limits the number of covariates that can be reliably modeled and raises the possibility of overfitting and unstable odds-ratio estimates; the corresponding effect sizes should therefore be regarded as preliminary and validated in larger samples. Because numerous correlations and subgroup comparisons were performed, we applied a Bonferroni correction within families of related tests, and associations that did not survive correction are interpreted as exploratory. Finally, as noted above, circulating serum NF-κB is an indirect surrogate of intracellular pathway activity and may not perfectly mirror the localized inflammatory microenvironment within the thyroid gland. Larger, multi-center prospective cohorts would be beneficial to validate the diagnostic cut-off values proposed in our ROC analysis.

Future research should focus on longitudinal studies to monitor the dynamic changes in NF-κB and TNF-α levels over the course of the disease, particularly in response to immunomodulatory therapies or optimization of levothyroxine replacement. Moreover, investigating the genetic polymorphisms of the NF-κB pathway and their interaction with environmental triggers in the Iraqi population could provide deeper insights into disease susceptibility. Finally, exploring the potential of targeted therapies aimed at downregulating the NF-κB signaling cascade may offer novel therapeutic avenues for managing the autoimmune destruction in HT.

## CONCLUSION

This study demonstrates that serum levels of NF-κB and TNF-α are significantly elevated in patients with HT compared with both non-Hashimoto hypothyroid patients and healthy individuals. The strong positive correlations of these biomarkers with thyroid autoantibodies (Anti-TPO and Anti-TG) and TSH suggest their close involvement in the autoimmune inflammatory processes underlying HT. Notably, ROC and multivariate logistic regression analyses indicate that NF-κB exhibits robust discriminatory capacity and serves as an independent predictor of HT, even after adjusting for relevant confounders. These findings suggest that NF-κB and TNF-α could potentially serve as valuable adjunctive biomarkers to assist in distinguishing autoimmune from non-autoimmune causes of hypothyroidism. While these results provide new insights into the systemic inflammatory profile of HT, particularly within the Iraqi population, they warrant cautious interpretation due to the cross-sectional design and the modest sample size. Future large-scale, longitudinal studies are necessary to validate the clinical utility of these biomarkers and to further elucidate their precise mechanistic roles in thyroid autoimmunity.

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**Ethical approval**

The present study was approved by the Ethical Committee of the College of Medicine, University of Kufa, Iraq, and was conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants prior to enrollment and blood sampling.

**Statement of Permission and Conflict of Interests**

The authors declare that they have no conflict of interest. **Consent for Publication:** Not applicable; the manuscript does not contain any individual person's data in any form. **Availability of Data and Materials:** The

datasets generated and/or analyzed during the current study are not publicly available due to participant privacy and institutional data protection policies but are available from the corresponding author on reasonable request.

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