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ORIGINAL PAPER

Serum Bruton's tyrosine kinase and NF-κB in Hashimoto's thyroiditis – a case-control study of potential diagnostic biomarkers

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ABSTRACT

Introduction and aim. Hashimoto's thyroiditis (HT) is the leading cause of hypothyroidism, yet its diagnosis relies on markers with known limitations. Bruton's tyrosine kinase (Btk) and nuclear factor kappa B (NF-κB) are implicated in B-cell-mediated autoimmunity. This study aimed to explore the serum levels and preliminary diagnostic potential of Btk and NF-κB in Iraqi patients with HT.

Material and methods. In this case-control study, 30 HT patients, 30 non-autoimmune hypothyroidism patients, and 60 healthy controls were enrolled from three centers in Karbala, Iraq. HT was defined by elevated TSH (>4.2 μIU/mL), reduced fT4 (<0.93 ng/dL), and positive anti-TPO and/or anti-Tg, whereas non-autoimmune hypothyroidism was defined by elevated TSH and low fT4 with negative thyroid autoantibodies. Serum Btk and NF-κB were measured by enzyme-linked immunosorbent assay. Thyroid hormones and autoantibodies were determined by electrochemiluminescence immunoassay. Receiver operating characteristic (ROC) analysis was performed to assess preliminary diagnostic accuracy for distinguishing HT from non-autoimmune hypothyroidism and healthy controls.

Results. Serum Btk and NF-κB levels were significantly elevated in HT patients compared to both control groups (Btk: 7.81±0.98 vs. 7.00±0.77 vs. 6.10±1.60 ng/mL; NF-κB: 1.90±0.71 vs. 0.91±0.21 vs. 0.84±0.39 ng/mL, $p<0.001$). In ROC analysis, NF-κB showed an area under the curve (AUC) of 0.95 (95% CI: 0.89–0.99) for discriminating HT from non-autoimmune hypothyroidism. Both biomarkers correlated positively with anti-TPO, anti-Tg, and TSH, and negatively with fT4.

Conclusion. Serum Btk and NF-κB were elevated in HT and showed preliminary associations with autoimmune activity. NF-κB, in particular, demonstrated promising initial diagnostic performance. These exploratory findings require validation in larger, independent cohorts.

Keywords. aortic stiffness, autonomic recovery, carotid–femoral pulse wave velocity, heart rate recovery, heart rate variability, submaximal aerobic exercise

Introduction

Hashimoto's thyroiditis (HT), also known as chronic lymphocytic thyroiditis, is the most common autoimmune thyroid disease and the leading cause of hypothyroidism in iodine-sufficient regions worldwide.[1,2]

As one of the most prevalent organ-specific autoimmune disorders, HT affects an estimated 5–10% of the global population, with a striking predilection for women, who are diagnosed five to ten times more frequently than men, particularly between the ages of 45 and 65.[3,4]

The disease is characterized by a progressive breakdown of self-tolerance to thyroid antigens, leading to a chronic inflammatory state within the thyroid gland. This autoimmune assault is mediated by both cellular and humoral immune responses, involving extensive infiltration of the thyroid parenchyma by T and B

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lymphocytes and the production of characteristic autoantibodies against thyroid peroxidase (anti-TPO) and thyroglobulin (anti-Tg).[5,6] Ultimately, this sustained inflammation results in the apoptotic destruction of thyroid follicular cells, culminating in glandular failure and clinical hypothyroidism.[3]

The pathogenesis of HT is complex and multifactorial, arising from a sophisticated interplay of genetic susceptibility and environmental triggers.[5] The immune response in HT involves a diverse array of immune cells, including Th1, Th2, Th17, and regulatory T (Treg) cells, which orchestrate the inflammatory cascade.[3]

Systemic inflammatory markers such as the neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) have been proposed as accessible indicators of immune/inflammatory activity in Hashimoto's thyroiditis.[7] Furthermore, HT is associated with various inflammatory indices such as the DeRitis score, red cell distribution width, uric acid to HDL cholesterol ratio, HALP and SII indexes, and C-reactive protein to lymphocyte count ratio.[8-13] Central to this process is the activation of B lymphocytes, which not only differentiate into plasma cells to produce thyroid autoantibodies but also function as potent antigen-presenting cells, further perpetuating the autoimmune cycle by activating autoreactive T cells.[14]

Given the pivotal role of B cells and the strong inflammatory component in the pathogenesis of HT, key signaling molecules that govern their activation and function have emerged as areas of investigation. One such molecule is Bruton's tyrosine kinase (Btk), a non-receptor tyrosine kinase that serves as a component of the B-cell receptor (BCR) signaling pathway.[15] Btk is also strongly associated with inflammation.[16] Upon BCR engagement, Btk is activated and initiates a downstream signaling cascade that is essential for B-cell proliferation, differentiation, survival, and antibody production.[17] Dysregulation of Btk signaling has been implicated in various B-cell malignancies and a growing number of autoimmune diseases, making it a subject of therapeutic interest.[16,18]

A crucial downstream effector of the Btk-mediated signaling cascade is the nuclear factor kappa B (NF-κB) family of transcription factors.[19] NF-κB is a master regulator of the immune and inflammatory responses, controlling the expression of hundreds of genes involved in lymphocyte development, activation, and cell survival.[20,21] The NF-κB pathway is intrinsically linked to inflammation and is activated in numerous conditions.[22] The canonical pathway of NF-κB activation is initiated by various stimuli, including BCR signaling, and typically involves the phosphorylation and degradation of the inhibitor of κB (IκB), allowing the p50/p65 NF-κB heterodimer to translocate to the nucleus and drive gene expression.[23] A direct mechanistic link has been established wherein Btk is required for BCR-induced NF-κB activation, in part through the direct phosphorylation of IκB-α by Btk.[24] Given that deregulated NF-κB activity is a hallmark of many chronic inflammatory and autoimmune conditions, the Btk/NF-κB signaling axis represents a highly relevant pathway in the immunopathology of HT.[20,21,23]

Despite the high prevalence of HT, its diagnosis still relies primarily on the measurement of serum TSH and thyroid autoantibodies (anti-TPO and anti-Tg). While these markers are valuable, they have recognized limitations; for instance, a subset of patients may be seronegative, and antibody titers do not always correlate with disease severity or progression.[6,25] This underscores the need for investigation of additional biomarkers that may reflect the underlying molecular pathology.[26,27]

Aim

This study was designed to investigate the serum levels of Btk and NF-κB in Iraqi patients with Hashimoto's thyroiditis and to explore their potential as diagnostic biomarkers in a preliminary, exploratory analysis. We hypothesized that the levels of these two key signaling molecules would be significantly elevated in HT patients compared to patients with non-autoimmune hypothyroidism and healthy controls, reflecting the underlying B-cell activation and inflammation central to the disease.

Material and methods

Study design and ethical approval

This case-control study was conducted from July 10, 2025, to February 10, 2026. The study protocol was reviewed and approved by the Research Ethics Committee at the College of Medicine, University of Kufa, Najaf, Iraq (Approval No.: 4540/2025). All procedures involving human participants were performed in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments. Written informed consent was obtained from all individual participants prior to enrollment.

Study population and participant recruitment

A total of 120 participants were enrolled in this study using a consecutive sampling method from three clinical centers in Karbala Governorate, Iraq: the Al-Hassan Center for Endocrinology and Diabetes, Al-Hindiya General Hospital, and Al-Hussein Teaching Hospital. Patients attending the outpatient endocrinology clinics at these centers during the study period who met the inclusion criteria were invited to participate. Healthy controls were recruited from hospital staff and accompanying people at the same centers who volunteered after screening confirmed euthyroid status and negative

autoantibody results. Participants were divided into three groups:

Hashimoto's thyroiditis (HT) group (n=30): Patients diagnosed with HT based on clinical and biochemical criteria, including elevated serum thyroid-stimulating hormone (TSH) levels (>4.2 μIU/mL), decreased free thyroxine (fT4) levels (<0.93 ng/dL), and the presence of high titers of thyroid autoantibodies (anti-TPO and/or anti-Tg).[6]

Non-Hashimoto's hypothyroidism (non-HT) group (n=30): Patients with primary hypothyroidism characterized by elevated TSH and low fT4 levels but with negative results for both anti-TPO and anti-Tg autoantibodies, thereby excluding an autoimmune etiology.

Healthy controls (n=60): Euthyroid, apparently healthy individuals with no personal or family history of thyroid disease or other autoimmune disorders, with normal thyroid function tests and negative thyroid autoantibody status.

Exclusion criteria for all groups included pregnancy, a history of thyroid surgery or radioiodine therapy, acute or chronic inflammatory diseases, severe systemic illness, malignancy, and the use of medications known to interfere with thyroid function or immune status (e.g., amiodarone, lithium, corticosteroids).

It should be noted that a proportion of patients in both the HT group (73.33%) and the non-HT group (70.00%) were receiving levothyroxine replacement therapy at the time of enrollment. As this was a cross-sectional observational study, treatment was not discontinued for ethical reasons. The potential influence of levothyroxine on the measured biomarkers is addressed in the limitations section.

For each participant, demographic data including age, sex, smoking status, and family history of thyroid disease were collected using a structured questionnaire. Anthropometric measurements, including height and weight, were taken to calculate the body mass index (BMI), which was classified according to World Health Organization criteria.[28]

Blood sample processing

From each participant, 5 mL of venous blood was collected under aseptic conditions into a plain gel-separator tube between 08:00 and 10:00 AM after an overnight fast. The blood was allowed to clot at room temperature for 30 minutes and then centrifuged at 3000 rpm for 15 minutes. The resulting serum was carefully separated, aliquoted into sterile labeled Eppendorf tubes, and stored at −20°C until the time of analysis. The interval from phlebotomy to serum freezing was approximately 45–60 minutes. All samples were analyzed within four weeks of collection to ensure analyte stability. Repeated freeze–thaw cycles were avoided.

Hormonal and autoantibody analysis

Serum levels of TSH, T3, fT4, anti-TPO, anti-Tg, and thyroglobulin (Tg) were quantitatively determined using a fully automated Cobas e 411 immunoassay analyzer (Roche Diagnostics, Mannheim, Germany). This system utilizes electrochemiluminescence immunoassay (ECLIA) technology. The specific commercial kits used were: TSH (Elecsys TSH, REF 07027921190), T3 (Elecsys T3, REF 07027956190), fT4 (Elecsys FT4 III, REF 06368611190), anti-TPO (Elecsys Anti-TPO, REF 06368590190), anti-Tg (Elecsys Anti-Tg, REF 09004998160), and Tg (Elecsys Tg II, REF 08906564190). All assays were performed according to the manufacturer's instructions. The instrument was calibrated, and quality control materials were run daily to ensure accuracy and precision.

Biomarker analysis by ELISA

Serum concentrations of Btk and NF-κB were measured using quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kits from Bioassay Technology Laboratory (BT LAB, Shanghai, China). The Human Btk ELISA Kit (Cat. No.: E0708Hu; detection range: 0.05–35 ng/mL; sensitivity: 0.02 ng/mL; intra-assay CV <8%; inter-assay CV <10%) and the Human NF-κB ELISA Kit (Cat. No.: E0690Hu; detection range: 0.03–10 ng/mL; sensitivity: 0.01 ng/mL; intra-assay CV <8%; inter-assay CV <10%) were used. These kits measure total protein concentrations in serum. Additional technical details, including lot numbers and calibration data, are provided in the Supplementary Material.

All ELISA measurements were performed in duplicate, and the mean value was used for analysis. Laboratory personnel performing the ELISA assays were blinded to the clinical group assignment of the samples. The optical density was measured at 450 nm using a microplate reader, and biomarker concentrations were determined by interpolation from a standard curve.

Statistical analysis

All statistical analyses were performed using SPSS version 25.0 (IBM Corp., Armonk, NY, USA). Data were expressed as mean±standard deviation (SD) for continuous variables and as numbers (n) and percentages (%) for categorical variables. For non-normally distributed variables (e.g., TSH, anti-TPO, anti-Tg), median and interquartile range (IQR) were additionally reported. The normality of data distribution was assessed using the Shapiro–Wilk test.

For normally distributed continuous variables, differences between two independent groups were analyzed using the independent-samples Student's t-test. For comparisons among the three groups, one-way analysis of variance (ANOVA) was performed, followed by the Tukey honestly significant difference (HSD) post-

hoc test for pairwise comparisons. For variables that did not meet the assumption of normality, the Mann–Whitney U test was used for two-group comparisons and the Kruskal–Wallis H test for three-group comparisons. A global p-value across the three groups was reported first, followed by post-hoc pairwise comparisons. Categorical data were compared using the chi-square ($\chi 2$) test or Fisher's exact test, as appropriate.

Spearman's rank correlation coefficient (ρ) was used to assess relationships between continuous variables, as this method is robust to non-normality, outliers, and non-linear relationships. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the preliminary diagnostic performance of Btk and NF-κB. The area under the curve (AUC), sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and optimal cutoff values (determined by the Youden index) were calculated. A combined biomarker model was also evaluated using predicted probabilities from logistic regression. Cohen's d effect sizes were calculated for between-group comparisons. Multivariable logistic regression was performed to assess the independent association of Btk and NF-κB with HT status after adjusting for potential confounders. Given the exploratory nature of this study and the relatively small sample size, no formal correction for multiple comparisons was applied; however, this is acknowledged as a limitation. A two-tailed p-value of <0.05 was considered statistically significant for all tests.

Results

Table 1 summarizes the baseline demographic and clinical characteristics across the three groups (HT, n=30; non-HT, n=30; controls, n=60). Mean age and the distribution of age categories were comparable between groups (p>0.05), and sex distribution did not differ significantly, with females predominating in all groups (p>0.05). Smoking status was similar between HT and non-HT (p>0.05) but differed between HT and controls, as all controls were non-smokers compared with 13.3% smokers in HT (p=0.01). A family history of hypothyroidism was markedly higher in HT than controls (66.7% vs. 3.3%, p<0.001), while no significant difference was observed between HT and non-HT (p>0.05). Levothyroxine use did not differ significantly between HT and non-HT (p>0.05).

Table 1A. Potential confounding factors and analytical adjustments

Confounder	Measured/Reported	Adjusted in analysis
Age	Yes	Yes (logistic regression)
BMI	Yes	Yes (logistic regression)
Smoking status	Yes	No (acknowledged as limitation)
Levothyroxine use	Yes	No (acknowledged as limitation)
Iodine status	No	No (not collected)

Table 1B. Demographic and clinical characteristics of Hashimoto's thyroiditis patients compared with non-Hashimoto's hypothyroidism patients and healthy controls*

Characteristic	HT (n=30)	Non-HT (n=30)	Controls (n=60)	p#	Post-hoc
Age (years), mean±SD	38.70±15.80	43.90±17.50	39.8±12.9	NS	–
Sex, female n (%)	25 (83.33)	27 (90.00)	47 (78.33)	NS	–
Sex, male n (%)	5 (16.67)	3 (10.00)	13 (21.67)		
Smoking, n (%)	4 (13.33)	4 (13.33)	0 (0)	0.01	HT vs. C: 0.01
Family history (+), n (%)	20 (66.67)	18 (60.00)	2 (3.33)	<0.001	HT vs. C: <0.001
Levothyroxine use, n (%)	22 (73.33)	21 (70.00)	–	NS	–
Age 20–39, n	17	12	40	NS	–
Age 40–59, n	10	10	12		
Age 60–75, n	3	8	8		

* # – Kruskal–Wallis or chi-square across three groups, Post-hoc – pairwise comparisons, NS – not significant (p>0.05), C – controls

Table 2 compares BMI and simplified BMI categories across groups. Mean BMI was significantly higher in HT (29.1±5.32 kg/m²) than controls (23.4±3.71 kg/m²; p<0.001), while HT and non-HT did not differ significantly (p>0.05). Overweight and obesity comprised 80% of HT patients, whereas most controls had normal BMI.

Table 2. Comparison of BMI and simplified BMI classification in Hashimoto's thyroiditis patients versus non-Hashimoto's hypothyroidism patients and healthy controls*

Characteristic	HT (n=30)	Non-HT (n=30)	Controls (n=60)	p#	Post-hoc
BMI (kg/m²), mean±SD	29.1±5.32	27.10±4.78	23.4±3.71	<0.001	HT vs. C: <0.001
Underweight, n	0	2	10	<0.001	HT vs. C: <0.001
Normal, n	6	5	32		
Overweight/Obese, n	24	23	18		

* # – Kruskal–Wallis or chi-square across three groups, Post-hoc – pairwise comparisons, NS – not significant (p>0.05), C – controls, BMI categories collapsed into underweight, normal, and overweight/obese to avoid sparse cell violations in chi-square analysis

Thyroid function and autoantibodies

Table 3 details the thyroid function tests and autoantibody profiles. Both HT and non-HT groups exhibited elevated TSH relative to controls (global p<0.001). TSH showed a median (IQR) of 10.50 (6.80–19.25) μIU/mL in HT, 9.10 (7.20–12.30) μIU/mL in non-HT, and 2.05 (1.60–2.70) μIU/mL in controls. Anti-TPO was markedly higher in HT (854±717 IU/mL) than in non-HT (8.72±6.91 IU/mL) and controls (5.47±3.56 IU/mL) (both p<0.001). Anti-Tg was also significantly higher in HT (193±150 IU/mL) versus non-HT (33.3±24.9 IU/mL) and controls (16.3±13.0 IU/mL) (both p<0.001).

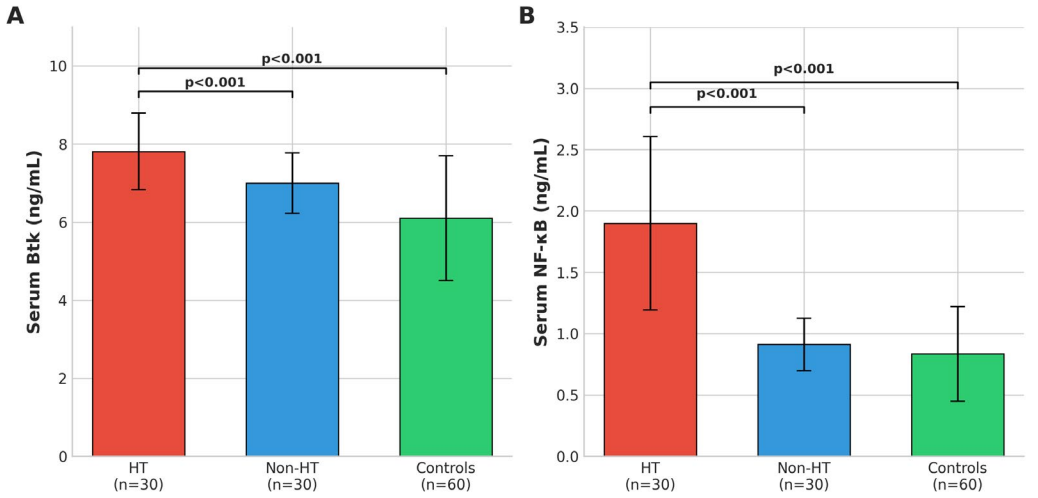

Fig. 1. Comparison of serum Bruton's tyrosine kinase (Btk) and nuclear factor kappa B (NF-κB) levels among Hashimoto's thyroiditis patients, non-Hashimoto's hypothyroidism patients, and healthy controls, A: Serum Btk levels, B: Serum NF-κB levels, data are expressed as mean±SD, ***p<0.001

Table 3. Thyroid function tests and autoantibody profiles in Hashimoto's thyroiditis patients compared with non-Hashimoto's hypothyroidism patients and healthy controls

Parameter	HT (n=30)	Non-HT (n=30)	Controls (n=60)	p#	Post-hoc
TSH (μIU/mL), mean±SD	15.80±14.4	9.70±3.21	2.17±0.76	<0.001	HT vs. C: <0.001; HT vs. nHT: 0.038
TSH, median (IQR)	10.50 (6.80–19.25)	9.10 (7.20–12.30)	2.05 (1.60–2.70)		
T3 (ng/mL)	1.29±0.44	1.46±0.62	1.22±0.34	NS	–
fT4 (ng/dL)	0.99±0.29	1.06±0.27	1.26±0.20	<0.001	HT vs. C: <0.001
Anti-TPO (IU/mL), mean±SD	854±717	8.72±6.91	5.47±3.56	<0.001	HT vs. nHT: <0.001; HT vs. C: <0.001
Anti-TPO, median (IQR)	620 (280–1200)	6.50 (3.80–12.40)	4.20 (2.50–7.80)		
Anti-Tg (IU/mL)	193±150	33.3±24.90	16.30±13.0	<0.001	HT vs. nHT: <0.001; HT vs. C: <0.001

* # – ANOVA or Kruskal–Wallis, Post-hoc – pairwise comparisons, NS – not significant (p>0.05), C – controls, median (IQR) reported for non-normally distributed variables, nHT – non-HT

Table 4. Serum levels of Bruton's tyrosine kinase (Btk) and nuclear factor kappa B (NF-κB) across study groups*

Parameter	HT (n=30)	Non-HT (n=30)	Controls (n=60)	p#	Post-hoc
Btk (ng/mL)	7.81±0.98	7.00±0.77	6.10±1.60	<0.001	HT vs. nHT: <0.001; HT vs. C: <0.001
NF-κB (ng/mL)	1.90±0.707	0.912±0.213	0.835±0.386	<0.001	HT vs. nHT: <0.001; HT vs. C: <0.001

* # – ANOVA across three groups

Serum Btk and NF-κB levels

Table 4 shows significant elevation of both Btk and NF-κB in HT. Serum Btk was higher in HT (7.81±0.98 ng/mL) than non-HT (7.00±0.77 ng/mL; p<0.001) and controls (6.10±1.60 ng/mL; p<0.001). Serum NF-κB was also elevated in HT (1.90±0.707 ng/mL) versus non-HT (0.912±0.213 ng/mL; p<0.001) and controls (0.835±0.386 ng/mL; p<0.001). Cohen's d effect sizes were large for both biomarkers: Btk (HT vs. non-HT: d=0.92; HT vs. controls: d=1.29) and NF-κB (HT vs. non-HT: d=1.89; HT vs. controls: d=1.87) (Figure 4).

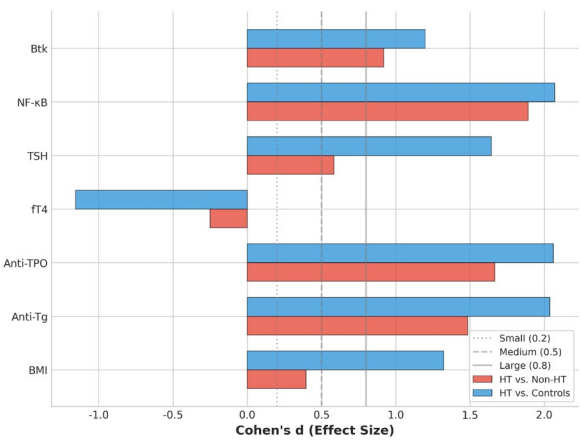

Fig. 2. Forest plot of Cohen's d effect sizes for clinical parameters and biomarkers comparing Hashimoto's thyroiditis (HT) patients to non-HT patients and healthy controls, error bars represent 95% confidence intervals

Correlations between biomarkers and clinical parameters

Table 5 shows that, within HT patients, both Btk and NF-κB correlated with disease-related hormonal and autoimmune markers using Spearman's rank correlation coefficient (ρ). Both biomarkers were negatively correlated with age (Btk: ρ=−0.59, p<0.001; NF-κB: ρ=−0.89, p<0.001). Both biomarkers correlated positively with TSH (Btk: ρ=0.45, p=0.013; NF-κB: ρ=0.47,

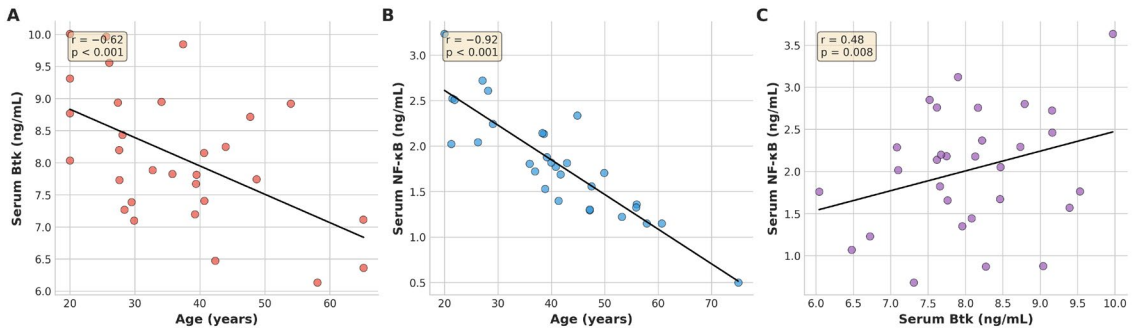

Fig. 3. Correlation analyses of serum Btk and NF-κB with age and their inter-biomarker relationship in Hashimoto's thyroiditis patients (n=30), A: Btk vs. age, B: NF-κB vs. age, C: Btk vs. NF-κB, Spearman correlation coefficients (ρ) and p-values are shown

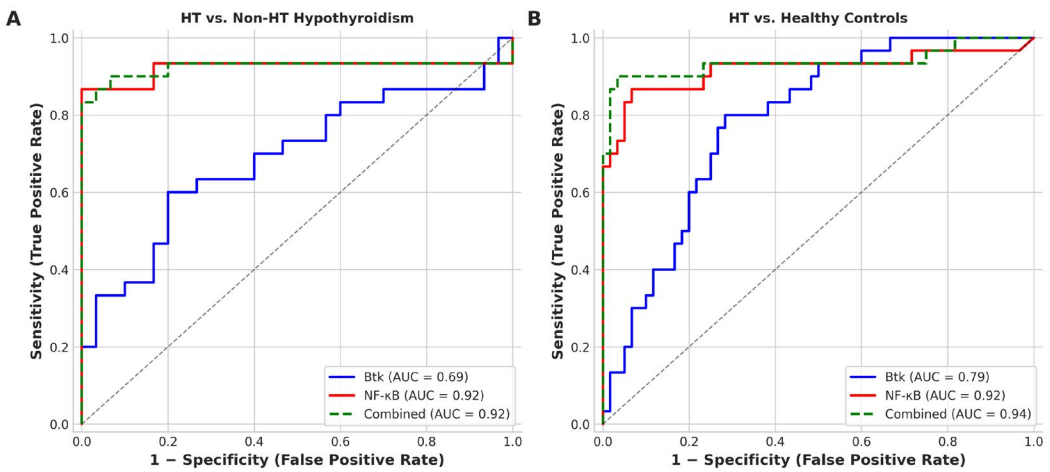

Fig. 4. Receiver operating characteristic (ROC) curves for serum Btk and NF-κB in discriminating Hashimoto's thyroiditis from non-Hashimoto's hypothyroidism and healthy controls, A: HT vs. Non-HT, B: HT vs. Controls, the combined model (Btk + NF-κB) is shown in green, AUC – area under the curve

p=0.009) and negatively with fT4 (all p<0.05). Strong positive correlations were observed with anti-TPO and anti-Tg (all p<0.001). Btk correlated positively with NF-κB (ρ=0.45, p=0.013) (Figure 3).

Table 5. Spearman's rank correlations between Btk, NF-κB, and clinical parameters in HT patients (n=30)

Variable	Btk (ρ)	Btk (P)	NF-κB (ρ)	NF-κB (P)
Age (years)	−0.59	<0.001	−0.89	<0.001
BMI (kg/m^2)	0.12	NS	0.08	NS
TSH (μIU/mL)	0.45	0.013	0.47	0.009
T3 (ng/mL)	−0.42	0.021	−0.35	0.058
fT4 (ng/dL)	−0.34	0.066	−0.47	0.009
Anti-TPO (IU/mL)	0.72	<0.001	0.82	<0.001
Anti-Tg (IU/mL)	0.63	<0.001	0.68	<0.001
Tg (ng/mL)	0.78	<0.001	0.66	<0.001
Btk vs. NF-κB	0.45	0.013	0.45	0.013

Spearman's rank correlation coefficient (ρ), NS – not significant (p>0.05)

Diagnostic performance of Btk and NF-κB

To determine whether Btk and NF-κB were independently associated with HT, a multivariable logistic regression analysis was performed (Table 6). Even after adjusting for age, BMI, TSH, fT4, anti-TPO, and anti-Tg, both Btk (β=0.563, p<0.05) and NF-κB (β=1.106, p<0.05) remained significant independent predictors of HT.

Table 6. Multivariable logistic regression analysis predicting Hashimoto's thyroiditis status (HT vs. non-HT)*

Predictor	Coefficient (β)	p
Age	−0.037	NS
BMI	0.037	NS
TSH	1.094	<0.05
fT4	−0.074	NS
Anti-TPO	1.478	<0.05
Anti-Tg	1.509	<0.05
Btk	0.563	<0.05
NF-κB	1.106	<0.05

* NS – not significant (p>0.05), dependent variable: HT status (1=HT, 0=non-HT)

ROC curve analysis was performed to evaluate the preliminary diagnostic performance of Btk and NF-κB (Table 7, Figure 4). For distinguishing HT from non-HT patients, NF-κB achieved an AUC of 0.95 (95% CI: 0.89–0.99; p<0.001) at a cutoff point of 1.26 ng/mL, with 82% sensitivity and 97% specificity. Btk yielded an AUC

of 0.75 (95% CI: 0.62–0.87; p=0.001) at a cutoff point of 7.62 ng/mL, with 62.1% sensitivity and 80% specificity. A combined model incorporating both Btk and NF-κB improved the diagnostic accuracy to an AUC of 0.94 (95% CI: 0.88–0.99), with 90.0% sensitivity and 93.3% specificity. For HT versus controls, NF-κB achieved an AUC of 0.94 (95% CI: 0.89–0.98; p<0.001). The combined model for HT vs. controls yielded an AUC of 0.91 (95% CI: 0.85–0.98).

Table 7. Receiver operating characteristic (ROC) analysis of Btk and NF-κB for discriminating Hashimoto's thyroiditis*

Comparison/ Biomarker	AUC (95% CI)	Cutoff point	Sensitivity (%)	Specificity (%)	p
HT vs. Non-HT					
Btk	0.75 (0.62–0.87)	>7.62 ng/mL	62.1	80.0	0.001
NF-κB	0.95 (0.89–0.99)	>1.26 ng/mL	82.0	97.0	<0.001
Combined (Btk + NF-κB)	0.94 (0.88–0.99)	—	90.0	93.3	<0.001
HT vs. Controls					
Btk	0.82 (0.73–0.91)	>7.12 ng/mL	83.0	75.0	<0.001
NF-κB	0.94 (0.89–0.98)	>1.08 ng/mL	93.0	82.0	<0.001

* AUC – area under the curve, CI – confidence interval, Cutoff points determined by Youden index, combined model based on predicted probabilities from logistic regression

Discussion

This study investigated the serum levels of Btk and NF-κB in an Iraqi cohort of patients with HT,, and healthy controls. Our results reveal a significant association of both Btk and NF-κB with the autoimmune process of the disease. Furthermore, preliminary ROC analysis indicated initial diagnostic potential for these biomarkers, particularly NF-κB, in distinguishing HT from both non-autoimmune hypothyroidism and healthy individuals. These findings, when contextualized with the existing literature, provide insights into the molecular mechanisms driving HT and suggest avenues for further investigation.

The central finding of our study is the elevation of serum Btk and NF-κB in HT patients. This aligns with emerging evidence implicating the Btk/NF-κB signaling axis in various autoimmune disorders.[16,29] A recent bioinformatic and clinical validation study by Liu et al. identified Btk as a key immune-related biomarker in HT, demonstrating its increased expression in peripheral blood mononuclear cells and a significant positive correlation with thyroid autoantibodies.[30] Our results provide supporting evidence at the serum protein level in a distinct ethnic population. Similarly, the elevated NF-κB levels in our HT cohort are consistent with a recent Turkish study by Yardim et al., which reported on serum NF-κB levels in HT patients and found a positive correlation with anti-TPO antibodies.[31] The activation of the NF-κB pathway is considered a critical step in the inflammatory cascade of thyroid autoimmunity.[20,32]

The observation that Btk levels in non-HT patients were significantly lower than in HT patients, yet slightly higher than in healthy controls, may reflect a generalized, non-specific inflammatory state associated with hypothyroidism itself, independent of autoimmunity. This is supported by previous research indicating that hypothyroidism can induce a state of low-grade systemic inflammation.[33] However, because Btk is an intracellular kinase, measuring its concentration in serum does not directly reflect its intracellular enzymatic activity; rather, elevated serum levels likely represent increased immune cell turnover, apoptosis, or active secretion during inflammatory responses.

A noteworthy finding is the positive correlation between Btk and NF-κB levels (ρ=0.45, p=0.013) within HT patients. This provides clinical evidence consistent with the mechanistic link established in molecular studies, where Btk acts as a critical upstream kinase in the BCR signaling pathway that activates NF-κB.[24,34] Upon BCR engagement by an autoantigen, Btk is activated and subsequently phosphorylates downstream targets, including the IκB kinase complex, which leads to the degradation of the IκB inhibitor and the translocation of NF-κB to the nucleus.[24,35] This activation cascade promotes B-cell proliferation, differentiation into plasma cells, and autoantibody production.[29,36] Our results are consistent with this model, as we observed significant positive correlations between both Btk and NF-κB and the levels of anti-TPO and anti-Tg antibodies. This suggests that the Btk/NF-κB axis may be associated with the humoral autoimmune response that characterizes Hashimoto's thyroiditis.

It is important to note, however, that NF-κB is a highly non-specific marker of inflammation, activated in a wide array of physiological and pathological conditions; thus, its elevation in HT, while significant, is not unique to this specific autoimmune disease.[19,22] This non-specificity should be considered when interpreting the diagnostic performance of NF-κB reported in this study.

The preliminary ROC analysis suggested that NF-κB may have diagnostic value in discriminating HT patients from both non-HT hypothyroid patients (AUC=0.95) and healthy controls (AUC=0.94). Importantly, a combined biomarker model incorporating both Btk and NF-κB yielded the highest diagnostic accuracy (AUC=0.94 for HT vs. non-HT), suggesting that a multi-marker approach may provide superior discriminatory power. However, these results should be interpreted with caution given the relatively small sample size (n=30 per patient group), the absence of an independent validation cohort, and the potential for overfitting in small datasets. The use of a case-control design with clinically distinct groups often overestimates di-

agnostic accuracy compared to a prospective cohort of consecutive patients with diagnostic uncertainty. Therefore, the reported AUC values and diagnostic thresholds (cutoff points) should be considered exploratory and hypothesis-generating rather than definitive clinical recommendations.

To establish the true clinical utility of these biomarkers, future studies must validate these cutoff points in independent, external cohorts. Such validation should ideally employ a prospective design in a diverse population presenting with undifferentiated thyroid dysfunction, allowing for the calculation of positive and negative predictive values in a real-world clinical setting.

Our demographic and clinical findings are largely consistent with the established epidemiology of Hashimoto's thyroiditis, both globally and within the Iraqi context. The significant female predominance (83.33%) in our HT cohort mirrors the findings of numerous Iraqi studies, including those by Al-Zamali et al. and Mansour et al., which reported approximately 83% female prevalence in hypothyroidism cases.[37-39] This strong gender bias is a well-documented hallmark of autoimmune thyroid disease worldwide.[40] Furthermore, our finding that a significant proportion of HT patients (66.67%) had a positive family history of hypothyroidism aligns with the known strong genetic component of the disease, with heritability estimates as high as 75%.[41]

This study confirms the well-established association between Hashimoto's thyroiditis and increased body mass index. Our HT patients had a significantly higher mean BMI compared to healthy controls, and 80% were classified as overweight or obese. This is consistent with several Iraqi studies that have linked hypothyroidism and HT with higher BMI.[33,42,43] The relationship between obesity and thyroid autoimmunity is considered bidirectional: chronic low-grade inflammation associated with obesity, driven by adipokines and pro-inflammatory cytokines, can trigger or exacerbate autoimmune responses, while the metabolic slowdown in hypothyroidism can contribute to weight gain.[44-46]

The inverse correlation with age ($\rho=-0.59$ for Btk and $\rho=-0.89$ for NF-κB) is an interesting finding that warrants further investigation, as it may suggest a more aggressive inflammatory phenotype in younger HT patients, consistent with some epidemiological observations.[47]

Study limitations

This study has several important limitations that must be acknowledged. First, the cross-sectional design precludes any definitive conclusions regarding causality; we can only report associations between biomarker levels and disease status. Second, the sample size is relatively small (30 patients per group), which limits the statistical power, particularly for subgroup analyses, and increases the risk of overfitting in the ROC models. Third, participants were recruited from three centers within a single governorate (Karbala, Iraq), which may not be representative of the broader population. Fourth, no independent external validation cohort was used to confirm the ROC-derived diagnostic thresholds; therefore, the reported performance metrics may be overestimated. Fifth, important potential confounding variables, including smoking status and ongoing levothyroxine therapy, were not adjusted for in the primary analyses, and their influence on biomarker levels cannot be excluded. No multivariable adjustment was performed for BMI, smoking, and levothyroxine treatment, and this may have influenced the observed biomarker differences. Sixth, data on certain environmental factors, such as iodine status and vitamin D levels, as well as detailed clinical severity indices, were not collected. Seventh, while we demonstrated a correlation between Btk and NF-κB, our study did not involve direct molecular techniques to confirm the phosphorylation cascade in patient cells. Eighth, the reliance on commercial ELISA kits without extensive in-house validation of intra/inter-assay variability and matrix effects for these specific novel analytes introduces potential measurement error. Finally, the exploratory nature of this biomarker study means that the findings should be regarded as preliminary.

Conclusion

This exploratory study provides preliminary evidence that serum levels of Bruton's tyrosine kinase (Btk) and nuclear factor kappa B (NF-κB) are significantly elevated in patients with Hashimoto's thyroiditis compared to both non-autoimmune hypothyroid patients and healthy individuals. NF-κB, alone and in combination with Btk, showed promising initial diagnostic performance in ROC analysis, although these findings require validation in larger, independent cohorts before any conclusions regarding clinical applicability can be drawn. The significant positive correlations observed between Btk/NF-κB levels and thyroid autoantibodies, coupled with their association with a more severe biochemical hypothyroid profile, suggest their involvement in the autoimmune and inflammatory processes central to HT pathogenesis. These results warrant further investigation in adequately powered, prospective studies to confirm the diagnostic value and to explore the potential of the Btk/NF-κB axis as a therapeutic target in Hashimoto's thyroiditis.

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Declarations

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Author contributions

Conceptualization, H.M.A-R.M. and S.N.A-H.; Methodology, H.M.A-R.M. and S.N.A-H.; Formal Analysis, H.M.A-R.M.; Investigation, H.M.A-R.M.; Resources, S.N.A-H.; Data Curation, H.M.A-R.M.; Writing – Original Draft Preparation, H.M.A-R.M.; Writing – Review & Editing, S.N.A-H.; Visualization, H.M.A-R.M.; Supervision, S.N.A-H.; Project Administration, S.N.A-H..

Conflicts of interest

The authors declare no competing interests.

Data availability

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

Ethics approval

The protocol was approved by the Research Ethics Committee at the College of Medicine, University of Kufa, Najaf, Iraq (Approval No.: 4540/2025).

Use of AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used AI-assisted technologies solely for language refinement and grammar checking. The authors reviewed and edited the content as needed and take full responsibility for the content of this publication.

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