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مستويات إنترفيرون غاما (IFN–γ) وإنترلوكين–٢١ (IL–21) في المصل وخلايا NKT الثابتة الدورية كمؤشرات مناعية في مرض التهاب الغدة الدرقية لهاشيموتو غير المعالج

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المستخلص:

يُعدّ التهاب الغدة الدرقية لهاشيموتو مرضًا مناعيًا ذاتيًا نوعيًا للعضو، إذ قد تعكس السيتوكينات الالتهابية ومجموعات الخلايا اللمفاوية ذات الخصائص الشبيهة بالمناعة الفطرية حالةً من اضطراب التنظيم المناعي النشط.

الهدف: تهدف هذه المقالة إلى تقييم مستويات الإنترفيرون–غاما في المصل (IFN–γ)، والإنترلوكين–٢١ (IL–21)، والخلايا القاتلة الطبيعية التائية الثابتة الدورانية (iNKT; CD3+6B11+) لدى مرضى التهاب الغدة الدرقية لهاشيموتو المشخّصين حديثًا وغير المعالجين، اعتمادًا حصريًا على البيانات المستمدة من الأطروحة. **الطرائق:** أُجريت دراسة حالة–شاهد شملت ٥٠ مريضًا غير معالج من مرضى التهاب الغدة الدرقية لهاشيموتو و٥٠ فردًا سليمًا كمجموعة ضابطة. تم قياس IFN–γ و IL–21 باستخدام تقنية الامتزاز المناعي المرتبط بالإنزيم (ELISA)، في حين كُشفت خلايا iNKT بواسطة قياس التدفق الخلوي. كما قُيّمت الدقة التشخيصية باستخدام تحليل منحنى خصائص التشغيل للمستقبِل (ROC). **النتائج:** أظهرت النتائج ارتفاعًا ملحوظًا في مستوى IFN–γ لدى مرضى هاشيموتو مقارنةً بمجموعة السيطرة (٢٧,٨٤±٨,٤٠ مقابل ٦,٢٤±١,٨١ بيكوغرام/مل؛ P=0.001). كما ارتفع مستوى IL–21 كذلك (٤٦,٤٧±١٠,٤٢ مقابل ٢٠,٢١±٣,٥٣ بيكوغرام/مل؛ P=0.001). وازدادت خلايا iNKT بصورة معنوية (٠,٥٤±٠,٢٩%

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مقابل ٠,٣١±٠,١٣%؛ P=0.001). وأظهر تحليل ROC قدرة تمييزية ممتازة لكل من IFN−γ (AUC=0.949) و IL−21 (AUC=0.930)، في حين أظهرت خلايا iNKT أداءً تشخيصيًا متوسطًا (AUC=0.751). **الاستنتاج:** يمثل كل من IFN−γ و IL−21 يمثلان مؤشرات حيوية تكميلية محتملة تتطلب تحققاً خارجياً، بينما توفر خلايا iNKT معلومات داعمة ذات دلالة إحصائية، إلا أنها أضعف من حيث القوة التشخيصية.

الكلمات المفتاحية: التهاب الغدة الدرقية لهاشيموتو؛ IFN−γ؛ IL−21؛ خلايا iNKT؛ ELISA؛ قياس التدفق الخلوي.

Serum IFN-γ, IL-21 and Circulating iNKT Cells as Immunological Markers in Untreated Hashimoto's Thyroiditis

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Abstract

Hashimoto's thyroiditis (HT) is an organ-specific autoimmune disease in which inflammatory cytokines and innate-like lymphocyte subsets may reflect active immune dysregulation. Objective: This article evaluates serum interferon-gamma (IFN-γ), interleukin-21 (IL-21), and circulating invariant natural killer T (iNKT; CD3+6B11+) cells in untreated newly diagnosed HT patients. Methods: A case-control study included 50 untreated HT patients and 50 healthy controls. IFN-γ and IL-21 were measured by ELISA, and iNKT cells were detected by flow cytometry. Diagnostic accuracy was assessed by receiver-operating-characteristic analysis. Results: IFN-γ was markedly higher in HT patients than controls (27.84±8.40 vs. 6.24±1.81

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pg/mL; P=0.001). IL-21 was also elevated (46.47±10.42 vs. 20.21±3.53 pg/mL; P=0.001). iNKT cells increased significantly (0.54±0.29% vs. 0.31±0.13%; P=0.001). Receiver Operating Characteristic (ROC) analysis showed potential adjunct biomarkers requiring external validation for IFN-γ (AUC=0.949) and IL-21 (AUC=0.930), while iNKT cells showed moderate diagnostic performance (AUC=0.751). Conclusion: IFN-γ and IL-21 represent potential adjunct biomarkers requiring external validation, whereas iNKT cells provide significant but weaker supportive information.

Keywords: *Hashimoto's thyroiditis; IFN-γ; IL-21; iNKT cells; ELISA; Flow cytometry.*

1- Introduction

Hashimoto's thyroiditis (HT) is a chronic organ-specific autoimmune thyroid disease and one of the leading causes of acquired hypothyroidism in iodine-sufficient populations. The disease was classically defined by lymphocytic infiltration of the thyroid gland, progressive destruction of thyroid follicles, and the presence of thyroid-specific autoantibodies, particularly anti-thyroid peroxidase and anti-thyroglobulin antibodies. Although routine diagnosis depends mainly on TSH, FT3, FT4, anti-TPO, anti-TG, and ultrasound findings, these conventional measures do not fully explain the underlying immune activity that drives tissue injury and disease progression. Therefore, a focused immunological assessment was useful, especially in untreated newly diagnosed patients, because treatment can modify both thyroid function and systemic inflammatory status (Caturegli *et al*., 2014; Ralli *et al*., 2020).

HT pathogenesis is not a simple endocrine abnormality. It begins with loss of immune tolerance to thyroid antigens and develops through interaction among genetic susceptibility, environmental triggers, epigenetic regulation, and persistent immune activation. In susceptible individuals, thyroid antigens are presented to autoreactive T cells, which then support both cellular thyroid injury and B-cell-mediated autoantibody production. This

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process produces a self-amplifying cycle: follicular-cell damage releases additional thyroid antigens, antigen-presenting cells enhance local immune activation, and inflammatory cytokines increase the recruitment and persistence of pathogenic lymphocytes within thyroid tissue (Ajjan and Weetman, 2015; Weetman, 2021).

Among the inflammatory pathways involved in HT, the Th1 axis remains particularly important. IFN-γ was produced mainly by activated T cells, cytotoxic lymphocytes, and natural killer related populations. Biologically, IFN-γ can increase major histocompatibility complex expression on thyroid follicular cells, enhance macrophage activation, stimulate chemokine production, and intensify autoreactive recognition of thyroid antigens. For this reason, increased serum IFN-γ was not merely a secondary laboratory change; it was compatible with active cellular immune injury and chronic inflammatory amplification in untreated HT. Previous clinical work has also supported the relevance of IFN-γ-related inflammation in patients with Hashimoto's thyroiditis (Özışık *et al*., 2023; Wrońska and Szczuko, 2024).

IL-21 represents a different but complementary immunological axis. It was produced mainly by T follicular helper cells, T peripheral helper cells, and Th17-related cells. Functionally, IL-21 promotes B-cell proliferation, plasma-cell differentiation, immunoglobulin production, and persistence of Th17-type responses. In HT, this gives IL-21 a strong mechanistic link with humoral autoimmunity because thyroid autoantibodies are not isolated diagnostic signs; they reflect B-cell help and long-standing immune activation against thyroid antigens. Thus, measuring IL-21 may help interpret the bridge between cellular immune dysregulation and autoantibody-dominant disease expression (Qi et al., 2023; Adamska-Fita *et al*., 2024).

iNKT cells add a further layer to this immune model. These CD1d-restricted innate-like T cells express both T-cell and natural killer features and can rapidly secrete cytokines after activation. Their role in autoimmune thyroid disease was complex because they may support inflammatory circuits through IFN-γ and other cytokines, while in different microenvironments they may also participate in regulatory or balancing

immune responses. Consequently, a change in circulating CD3+6B11+ iNKT cells should not be interpreted automatically as purely pathogenic or purely protective. A more rigorous interpretation is that iNKT-cell frequency reflects participation of innate-like immune mechanisms, whereas its diagnostic strength depends on whether the frequency change was large and specific enough to discriminate patients from controls (Roman-Gonzalez et al., 2009; Sharma et al., 2011; Adamska-Fita *et al*., 2024).

This immunological perspective matters because HT can remain clinically heterogeneous for a long time. Some patients present with clear overt hypothyroidism, whereas others show subclinical disease, positive antibodies, or nonspecific symptoms before pronounced thyroid-hormone failure becomes evident. In such cases, immune markers may clarify whether the patient was only biochemically abnormal or was also undergoing active inflammatory amplification. The point was not to overclaim cytokines as independent diagnostic tools; that would be scientifically weak without external validation. Instead, cytokine and lymphocyte measurements can serve as adjunctive evidence explaining why two patients with similar TSH or antibody status may differ in inflammatory burden, tissue damage, and progression risk (Wang *et al*., 2026).

The present focus on untreated patients was also methodologically important. Levothyroxine can correct hormone deficiency, and clinical follow-up may reduce inflammatory stress by stabilizing thyroid function, but treatment does not directly measure the baseline immune disturbance present at first diagnosis. Studying untreated cases therefore provides a cleaner window into the natural immune profile of HT before therapy, disease duration, and treatment adherence obscure the initial relationship between thyroid autoimmunity and systemic immune markers. For this reason, IFN-γ, IL-21, and circulating iNKT cells were selected as biologically coherent markers: they represent inflammatory T-cell activation, B-cell-supportive cytokine signaling, and innate-like lymphocyte participation, respectively. This layered design gives the article a stronger rationale than simply reporting statistically significant laboratory differences.

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A further reason for broadening the immunological introduction was that thyroid autoantibodies, despite their diagnostic value, do not fully describe the current activity of the immune response. Anti-TPO and anti-TG antibodies confirm the autoimmune nature of the disease. Still, antibody positivity may persist for long periods and may not change in a linear manner with symptoms, thyroid-hormone concentration, or the degree of inflammatory-cell activation. Likewise, TSH was a sensitive functional marker of thyroid status, but it was a pituitary response to circulating hormone availability rather than a direct measure of immune-mediated thyroid injury. This creates a clear analytical gap: endocrine tests define thyroid function, antibody tests define autoimmune identity, but cytokine and cell-subset markers may more directly describe the active immune environment. In untreated HT, this distinction was essential because the earliest immune disturbance can precede or exceed the visible hormonal abnormality (Caturegli *et al*., 2014; Weetman, 2021; Wrońska *et al*., 2024).

The three selected markers also differ in their expected clinical meaning. IFN-γ was most suitable for reflecting inflammatory pressure and antigen-presentation intensity; IL-21 was most suitable for reflecting B-cell help and the maintenance of autoantibody-producing responses; and iNKT cells were most suitable for indicating the participation of rapid innate-like immune mechanisms. If all three markers rose together, the result suggests that untreated HT wasn't restricted to one immune pathway. If the cytokines show stronger diagnostic performance than iNKT frequency, the interpretation should be strict: serum cytokine levels may capture active functional signaling more directly than a simple circulating cell percentage. This distinction prevents overstatement and strengthens the manuscript, because it allows the results to be ranked biologically and diagnostically rather than reported as equally important laboratory abnormalities.

Receiver-operating-characteristic analysis was therefore used in this article as a supportive diagnostic evaluation, not as a claim of immediate clinical substitution. A high Area Under the Curve (AUC) in a case-control dataset indicates strong separation between the selected patients and controls, but it did not automatically prove that the marker will perform

equally well in larger, mixed, or borderline clinical populations. The proper interpretation was that ROC results identify markers worthy of further validation. This was especially relevant for IFN-γ and IL-21 because very strong separation in untreated patients could be biologically meaningful, yet still requires replication in multi-center cohorts that include euthyroid antibody-positive subjects, subclinical hypothyroidism, treated patients, and other autoimmune or inflammatory conditions (Caturegli *et al*., 2014; Ralli *et al*., 2020).

On this basis, the expanded introduction frames the article around a defensible question: do IFN-γ, IL-21, and circulating iNKT cells provide meaningful adjunct information in untreated Hashimoto's thyroiditis beyond routine endocrine and antibody testing? This wording was deliberately careful. It avoids the weak claim that these markers replace established diagnostic tools, and it avoids the vague claim that every statistically significant immune marker was clinically useful. Instead, it places the markers into a mechanistic hierarchy and asks whether the measured differences are large enough to support diagnostic discrimination and biological interpretation in the thesis cohort.

Against this background, the present article evaluates three immunological markers representing connected but distinct compartments of HT immunopathogenesis: serum IFN-γ as an indicator of Th1-dominant inflammatory pressure, serum IL-21 as an indicator of Tfh/Th17-supported B-cell activation, and circulating CD3+6B11+ iNKT cells as an indicator of innate-like lymphocyte involvement. The strength of the present work lies in its restriction to untreated newly diagnosed patients, which reduces the confounding influence of thyroid-hormone replacement and allows a clearer view of baseline immune disturbance. The central aim was therefore not to replace established endocrine and serological tests, but to determine whether these immune markers can serve as adjunct biomarkers that improve biological interpretation and support diagnostic discrimination in untreated HT (Weetman, 2021; Wang *et al*., 2026).

2- Methods

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2-1 Study design and participants

A case-control study was conducted from November 1, 2024, to April 1, 2026, at Al-Karama Teaching Hospital in Wasit Governorate. This study included 50 patients with untreated, newly diagnosed HT (7 males, 43 females) aged 20–64 years and 50 healthy controls (8 males, 42 females) aged 21–59 years. Physicians diagnosed the HT patients clinically. Patients were interviewed directly by using an anonymous questionnaire form that covered age, sex, weight, and family history of HT. This study evaluated FT3, FT4, and TSH by mini VIDAS; anti-TPO, anti-Tg, IL-21, and IFN-γ by ELISA; and iNKT cells (CD3+6B11+) by flow cytometry. Ultrasound imaging was not used for diagnosis in this study. Exclusion criteria included previous thyroidectomy, current thyroid hormone therapy, parathyroid disorders, other autoimmune diseases, liver, kidney, or pancreatic disease, malignancy, smoking, alcohol use, and recent blood transfusion within the preceding six months.

2-2 Ethical Approval

This study was approved by the ethical committee of the Faculty of Medicine, University of AL-Qadisiyah. and a permission from AL-Karama Teaching Hospital, Wasit. All the patients were informed about the research and given permission.

2-3 Blood sampling

Eight milliliters of blood were obtained from each patient and control via venous puncture using a single-use syringe under sterile technique. The blood sample was then divided equally into 3 milliliters in a gelatin tube, 2 milliliters in an EDTA tube, and 3 milliliters in a sodium heparin tube. The 3-milliliter blood sample in the gel tube was isolated after being allowed to coagulate at room temperature and centrifuged at 2500 rpm for 10 minutes. It was then transferred to an Eppendorf tube and stored frozen at -20°C until used. The study is based on 100 blood samples, 50 from untreated HT patients and 50 from healthy individuals, and did not employ a duplicated sampling method.

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2-4 Immunological assays

Serum IFN-γ and IL-21 were determined using Human IFN-γ ELISA Kit and Human IL-21 ELISA Kit (Elabscience, USA, catalog number E-EL-H2450), according to the manufacturer's procedure. Thyroid autoantibodies were measured using anti-thyroperoxidase and anti-thyroglobulin autoantibody kits (Sun Long, China), according to the manufacturer's procedure. Thyroid hormones were assessed by Mini VIDAS kits for TSH, FT3, and FT4 (Biomerieux, France), according to the manufacturer's procedure. No procedural details beyond the company protocols are expanded here in order to keep the Materials and Methods section concise and consistent with the journal format.

2-4-1 Principle of the Mini VIDAS assay of TSH, FT3, and FT4

The assay principle combines a one-step enzyme immunoassay sandwich method had been combined with a final fluorescent detection (ELFA). The Solid Phase Receptacle (SPR) serves as the solid phase and the pipetting device for the assay. Reagents for the assay are ready-to-use and predispensed in the sealed reagent strips. All of the assay steps are performed automatically by the instrument. The reaction medium is cycled in and out of the SPR several times. The sample (serum or plasma) is transferred into the well containing anti- TSH antibody labeled with alkaline phosphatase (conjugate). The sample/conjugate mixture is cycled in and out of the SPR. The antigen binds to antibodies coated on the SPR and to the conjugate forming a "sandwich". Unbound components are eliminated during the washing steps. During the final detection step, the substrate (4Methylumbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of this substrate into a fluorescent product (4-Methyl-umbelliferone) the fluorescence of which is measured at 450 nm. The intensity of the fluorescence is proportional to the concentration of antigen present in the sample. At the end of the assay, the results are automatically calculated by the instrument in relation to the calibration curve stored in memory, and then printed out.

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2-4-2 Principle of Anti-TPO and Anti-TG

The Sandwich-ELISA method was employed by ELISA kit. This kit comes with a pre-coated micro-Elisa strip plate that is specific to TPO-Ab and Anti-TG. Each well of a Micro Elisa strip plate was used for a different antigen, and standards or samples were added to each one. Following this, a TPO-specific antigen that is in each well of the Micro Elisa strip plate, horseradish peroxidase (HRP) was added. and left to incubate. Dissolved components were removed by washing TMB substrate solution goes to all wells. Only Ab and HRP-conjugated TPO, TG antigen wells would turn from blue to yellow when the stop solution was applied. Spectrophotometry measures 450 nm optical density (OD). The OD value rose with TPO-Ab and Anti-TG concentration. The optical density (OD) of samples can be compared to the standard curve to assess TPO-Ab and Anti-TG content.

2-4-3 Principle of the IL-21 and IFN-γ ELISA test

In this study, ELISA kits were used based on sandwich-coated antibody-specific IL-10, IL-17, IL-21, and IFN-γ. Samples or standards were then added to the appropriate enzyme-linked immunosorbent assay (ELISA). The ELISA microplate provided in this kit, along with the plates and wells, were combined with the selected antibody. Biotin detection antibody-specific IL-10, IL-17, IL-21, and IFN-γ and avidin-horseradish peroxidase (HRP) conjugate were added to each well and incubated. Free components were then washed off. After the substrate solution was added to each well, only those wells containing IL-10, IL-17, IL-21, and IFN-γ, the biotin detection antibody, and the avidin-HRP conjugate appeared blue. The enzyme substrate reaction was terminated by adding a stop solution (sulfuric acid), resulting in a yellow color change. Optical density (OD) was immediately measured using a spectrophotometer at a wavelength of 450 ± 2 nm. The optical density (OD) value is directly proportional to the concentration of serum indicators (IL-10, IL-17, IL-21, and IFN-γ), and their concentration in the samples was determined by comparing the optical density (OD) of the sample to the standard curve.

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2-5 Flow cytometry

Circulating iNKT cells were identified as CD3+6B11+ cells. Monoclonal antibody conjugated FITC dye for CD3 and monoclonal antibody conjugated PE dye for 6B11 were supplied by EXBIO (Czech Republic, catalog number for CD31F-202-T100\100 test, and for 6B11 1P-927-T100\ 100 test), and lysis/fixation reagents were supplied by Unique-Lyse (USA). Data acquisition was performed using a BriCyte E6 flow cytometry machine (India), according to the manufacturer's procedure.

2-5-1 Steps of INKT Cells Flow cytometry

The steps of Flowcytometry assay was according to instructions of Exbio company.

1. One hundred micron of EDTA anticoagulated tube [whole blood] was added into test tube.
2. Antibodies, 5 µl of CD3 and 6B11 monoclonal Ab, were added to the test tube (polystyrene tube) according to the instructions provided by the manufacturer's product insert. The resulting mixture should be subjected to vortex and incubated in a dark environment at room temperature for the designated duration.
3. Following thorough mixing with a vortex, 100 µl of the Reagent A was added to each sample, and it was incubated for 10 minutes at temperature of room in the dark.
4. One ml (1 milliliter) of reagent B was added to every sample, thoroughly vortex, and incubate for about 20 minutes at room temperature in the dark.
5. Centrifuged for approximately five minutes at 300–600 xg, and the supernatant was disposed of. Wash the cells with 2 milliliters of cold PBS/BSA (4°C), centrifuge them at 300–400 xg for 5 minutes at 4°C, and then throw away the supernatant. If necessary, suspend cells in 200 µl of 0.5% paraformaldehyde in PBS or 200 µl of cold (4°C) PBS.
6. Acquire data by flow cytometry (BriCyte E6/ India, analyze fixed cells within 24 hr.
7. Phenotyping of lymphocytes is achieved by gating in accordance with the side scatter (SSC) histogram, which is dependent on the granularity of cells,

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and the forward scatter (FSC) histogram, which is dependent on the size of cells.

8. Flowcytometry result was expressed as mean levels concentration of positive cells of CD3 and 6B11 immune markers.

2-6 Statistical analysis

Statistical analyses were performed using SPSS version 26. Data normality was assessed using the Kolmogorov-Smirnov test. Results were expressed as medians with 25th and 75th interquartile ranges (IQR) or as means with standard deviations (SD), as appropriate. Comparisons between two independent groups were conducted using the Mann-Whitney U test or Student's t-test. Correlations were examined by Spearman's correlation test. Statistical significance was set at P <= 0.05.

3- Results

The demographic chapter demonstrated that age and sex were comparable between patients and controls, while BMI was significantly higher in untreated HT patients (the formula for calculating BMI was $(\frac{Kg}{m^2})$. Therefore, the immune markers were interpreted within a cohort that was broadly comparable by age and sex but had an important BMI-related difference. Serum IFN-γ showed the clearest inflammatory separation between study groups. Untreated HT patients had a mean value of 27.84±8.40 pg/mL compared with 6.24±1.81 pg/mL in healthy controls, with a highly significant difference. Serum IL-21 also showed marked elevation in HT patients, with 46.47±10.42 pg/mL compared with 20.21±3.53 pg/mL in controls. Circulating iNKT cells were significantly higher in untreated HT patients, but the absolute separation was smaller than for the cytokines.

Table 1: Demographic characteristics among study groups.

Variables	Untreated HT	Healthy control	*P*-value
Age (years)			
Mean ±SD	41.01 ± 11.12	38.97 ± 10.27	0.343 † NS
Range	20–64 years	21–59 years	

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< 30, *n* (%)	10 (20.0%)	8 (16.0%)	0.784 ¥ NS
30-39, *n* (%)	17 (34.0%)	20 (40.0%)	
≥ 40, *n* (%)	23 (46.0%)	22 (44.0%)	
Gender			
Male, *n* (%)	7 (14.0%)	8 (16.0%)	0.779 ¥ NS
Female, *n* (%)	43 (86.0%)	42 (84.0%)	
Body mass index (BMI) kg/m2			
Mean ± SD	27.5 ± 7.8	24.1 ± 5.0	0.01 † S
Range	19.20–45.22	19.75– 39.67	
Normal Weight, *n* (%)	13 (26.0%)	28 (56.0%)	0.001 ‡ HS
Overweight, *n* (%)	27 (54.0%)	18 (36.0%)	
Obesity, *n* (%)	10 (20.0%)	4 (8.0%)	

n: number of cases; SD: standard deviation; †: independent samples t-test; ¥: Chi-square test; NS: not significant at P > 0.05.

Table 2: Mean levels of thyroid hormones among study groups

Groups		TSH level (μIU/L)	FT3 level (pmol/l)	FT4 level (pmol/l)
Patients	Mean ± SD	7.95± 0.98	2.46± 0.21	8.25±0.92
Control	Mean ± SD	2.26± 0.73	5.65± 0.80	14.35± 0.55
p-value		0.001**	0.001**	0.001**

SD: standard deviation; **: high significant at $P < 0.001$

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Table 3: Mean levels of thyroid autoantibodies among study groups

Groups		Anti-TPO pg/mL	Anti-TG pg/mL
Patients	**Mean ± SD**	198.15 ± 25.66	99.35 ± 11.67
Control	**Mean ± SD**	11.02 ± 7.33	18.21 ± 3.39
p-value		0.001**	0.001**

SD: standard deviation; **: high significant at P < 0.001

Table 4: Mean levels of IFN-γ, IL-21, and iNKT cells among study groups.

Marker	Unit	Untreated HT Mean ± SD	Control Mean ± SD	P value
IFN−γ	pg/mL	27.84 ± 8.40	6.24 ± 1.81	0.001
IL−21	pg/mL	46.47 ± 10.42	20.21 ± 3.53	0.001
iNKT (CD3+6B11+)	%	0.54 ± 0.29	0.31 ± 0.13	0.001

SD: standard deviation; HT: Hashimoto's thyroiditis; iNKT: invariant natural killer T cells. NS: not significant at P > 0.05.

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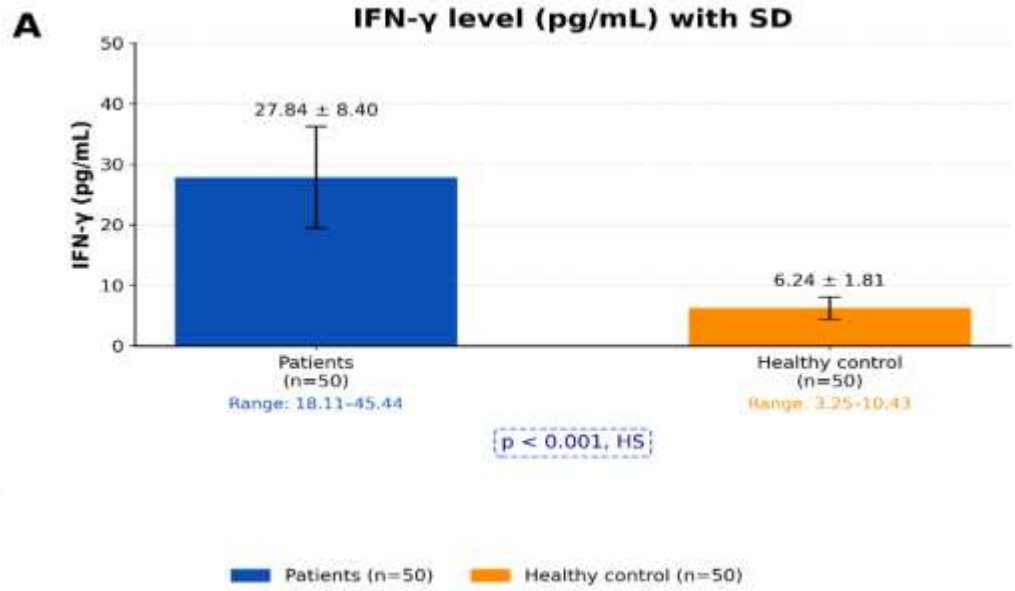

Figure 1. Mean levels of IFN-γ among untreated HT patients and healthy controls.

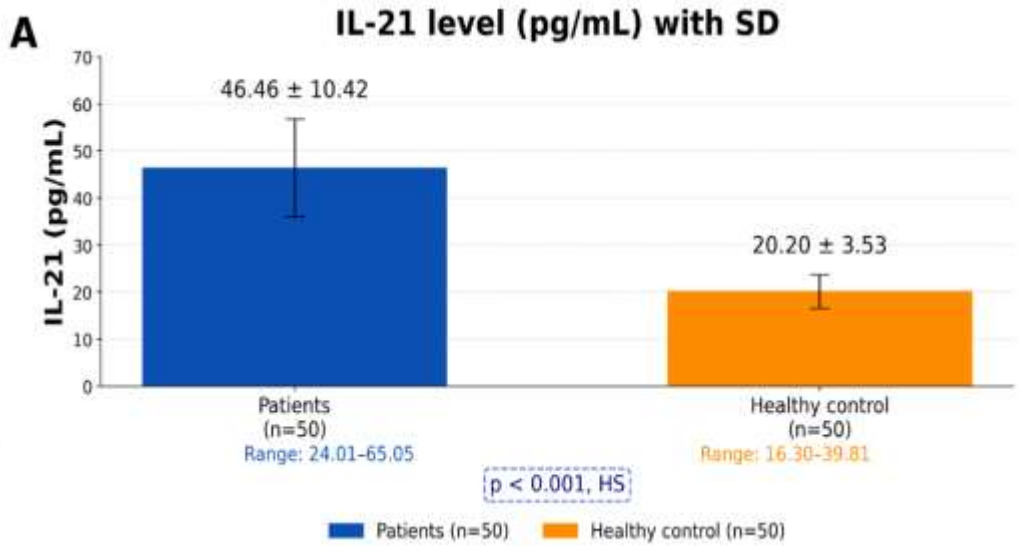

Figure 2. Mean levels of IL-21 among untreated HT patients and healthy controls.

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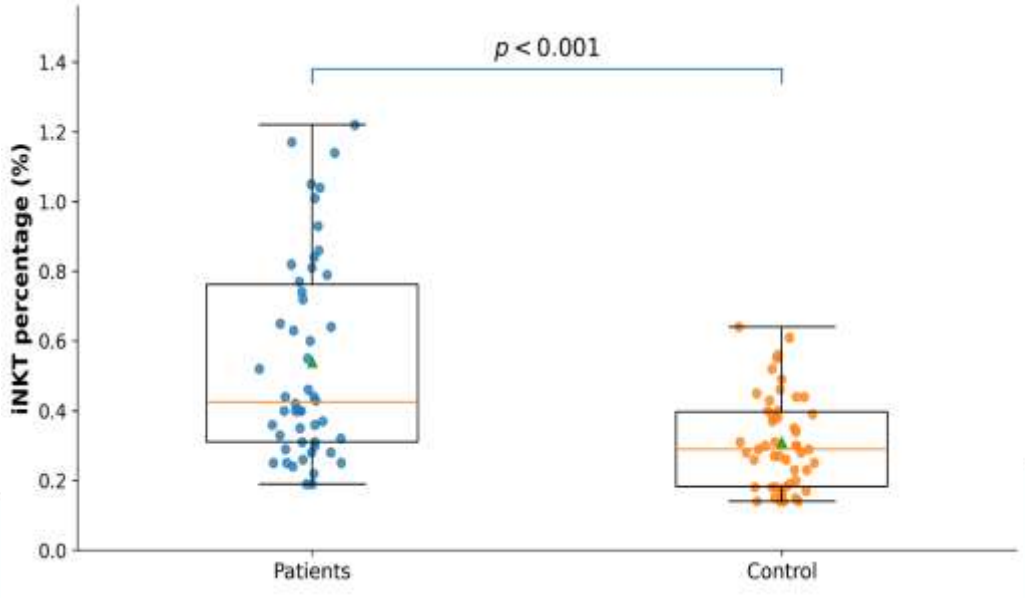

Figure 3. iNKT cell percentages among untreated HT patients and healthy controls.

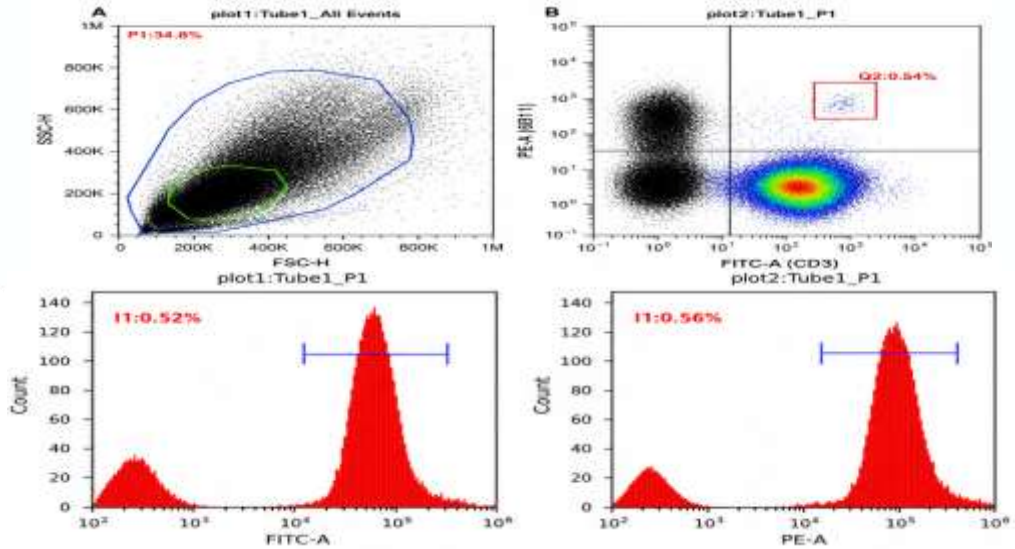

Figure 4: Plot and all events results of INKT markers among HT patients

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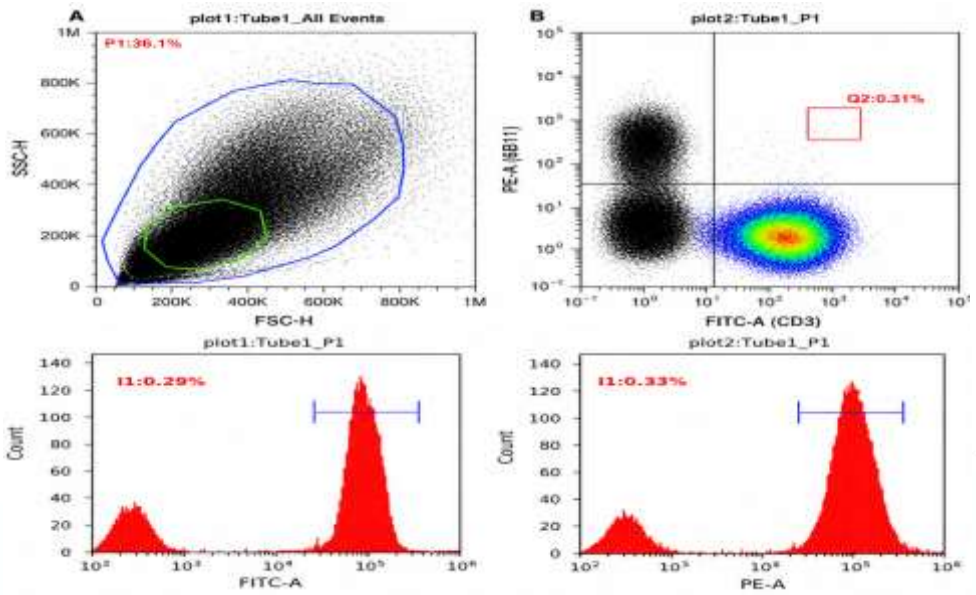

Figure 5: Plot and all events results of INKT markers among controls groups

3.1 ROC analysis strengthened this pattern.

IFN-γ showed perfect discrimination in the thesis dataset at a cutoff ≥18.1 pg/mL, while IL-21 showed near-perfect discrimination at a cutoff ≥24 pg/mL. In contrast, iNKT cells had a lower AUC and less balanced sensitivity and specificity, indicating that iNKT was statistically significant but diagnostically weaker than the two cytokines.

Table 5: ROC performance of IFN−γ, IL−21, and iNKT cells.

Marker	Cutoff	P value	Sensitivity %	Specificity %	PPV %	NPV %	AUC (95% CI)
IFN−γ	≥18.1	<0.001	88.3	90.0	89.9	88.5	0.949 (0.887–0.990)
IL−21	≥24	<0.001	94.5	95.0	95.7	93.2	0.930 (0.891–0.995)
iNKT	≥0.32	<0.001	72	62	65.5	68.9	0.751 (0.655–0.847)

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ROC: receiver-operating-characteristic; AUC: area under the curve; CI: confidence interval; PPV: positive predictive value; NPV: negative predictive value.

Association analysis further refined the interpretation. IFN-γ and IL-21 were strongly and positively correlated with BMI, while iNKT cells were not significantly correlated with BMI. Similarly, IFN-γ and IL-21 were significantly higher in patients with positive family history, whereas iNKT cells did not show a significant family-history difference. This suggests that IFN-γ and IL-21 may reflect broader inflammatory-metabolic and familial-autoimmune burden, while iNKT cell elevation may represent a more general immune dysregulation signal.

Table 6: Association of immune markers with BMI

Viarable	Association with BMI		
	Spearman ρ (rho)	p-value	q-value (FDR)
IFN-γ	0.890	<0.001	<0.001
IL-21	0.895	<0.001	<0.001
iNKT	0.089	0.540	0.535

Spearman correlation. BMI comparisons were tested using Mann-Whitney U test. q-values are FDR-adjusted p-values. . NS: not significant at P > 0.05.

4- Discussion

This article shows that untreated HT was accompanied by a strong inflammatory cytokine signature and a measurable increase in circulating iNKT cells. The most important result was the marked elevation of IFN-γ. This finding was biologically coherent because IFN-γ was central to Th1-mediated autoimmunity. In thyroid tissue, IFN-γ can increase antigen presentation, enhance macrophage activation, promote inflammatory chemokine production, and intensify cytotoxic immune responses against follicular cells. Therefore, the elevation of IFN-γ in untreated patients was not incidental; it fits the expected immunopathology of active autoimmune thyroid injury (Weetman, 2021; Özışık *et al.*, 2023). IFN-γ also showed strong diagnostic performance in the thesis dataset, with an AUC of 0.949. This value suggests potential adjunct biomarkers that require external

validation in the present case-control sample, but it should be interpreted cautiously. Case-control designs often compare clearly diseased cases with healthy controls, which can inflate diagnostic estimates. Thus, IFN-γ should be described as an adjunct marker in this cohort, not as a stand-alone clinical replacement for TSH, FT3, FT4, anti-TPO, or anti-TG. This distinction matters; any manuscript that presents IFN-γ as a definitive diagnostic test without external validation would be scientifically weak. IL-21 showed an equally important but mechanistically different pattern. Its elevation supports an active B-cell-help axis in HT. Because IL-21 promotes B-cell differentiation and plasma-cell activity, it plausibly connects cellular immune dysregulation with humoral autoimmunity and thyroid autoantibody production (Qi *et al*., 2023). The near-perfect ROC performance of IL-21 indicates that it may be especially useful as a supportive marker of active autoimmune status. In addition, the strong association with BMI and family history suggests that IL-21 may be linked to both systemic inflammatory burden and familial-autoimmune susceptibility in the current cohort.

The iNKT finding needs a stricter interpretation. Although CD3+6B11+ iNKT cells were significantly elevated, their ROC performance was clearly weaker than IFN-γ and IL-21. This means that iNKT cells are biologically relevant but diagnostically limited. Their moderate AUC, lower sensitivity, and lower specificity indicate that they cannot be promoted as a strong diagnostic discriminator. A fair interpretation was that iNKT cells reflect activation of innate-like immune circuits in untreated HT, possibly contributing to cytokine amplification or immune regulation depending on the functional phenotype. This interpretation also explains why iNKT cells did not correlate strongly with BMI or family-history status. Unlike IFN-γ and IL-21, iNKT frequency alone may not capture functional polarization, cytokine secretion capacity, tissue trafficking, or local thyroid involvement. Peripheral blood iNKT percentages may therefore underestimate or misrepresent their real biological role. This aligns with the broader literature, in which iNKT cells show context-dependent behavior and variable results across autoimmune thyroid studies (Adamska-Fita *et al*., 2024).

Taken together, the thesis results support a layered immunological model: IFN-γ indicates Th1 inflammatory pressure; IL-21 indicates B-cell and Tfh/Th17 support; and iNKT cells indicate innate-like immune participation, but with weaker diagnostic utility. The strongest manuscript claim should therefore be that IFN-γ and IL-21 are high-performing adjunct biomarkers in untreated HT, while iNKT cells are a significant but secondary

marker. This claim was defensible, precise, and not overstated. The study has limitations. The sample size was moderate, the design was case-control, and the data are derived from a single clinical setting. The analysis was based on circulating blood markers rather than thyroid-tissue immunophenotyping. The study also lacks longitudinal follow-up after treatment, so it cannot determine whether IFN-γ, IL-21, or iNKT cells normalize with therapy or predict progression. Future work should use larger multi-center cohorts, include treated and untreated patients, and combine serum cytokines with functional flow-cytometry assays.

5- Conclusion

Untreated newly diagnosed HT patients showed significantly higher serum IFN-γ, serum IL-21, and circulating CD3+6B11+ iNKT cells compared with healthy controls. IFN-γ and IL-21 demonstrated potential adjunct biomarkers requiring external validation, not replacements for routine thyroid function tests or thyroid autoantibodies. iNKT cells were significantly elevated but showed only moderate diagnostic performance, making them a supportive marker rather than a primary discriminator. The most scientifically defensible conclusion was that IFN-γ and IL-21 reflect dominant inflammatory and B-cell-supportive pathways in untreated HT, while iNKT cells indicate additional innate-like immune dysregulation.

6– References

1. Adamska-Fita, E., Śliwka, P. W., Karbownik-Lewińska, M., Lewiński, A., & Stasiak, M. (2024). The absence of thyroid-stimulating hormone receptor expression on natural killer T cells: Implications for the immune-endocrine interaction. *International Journal of Molecular Sciences, 25*(21), Article 11434. https://doi.org/10.3390/ijms252111434
2. Ajjan, R. A., & Weetman, A. P. (2015). The pathogenesis of Hashimoto's thyroiditis: Further developments in our understanding. *Hormone and Metabolic Research, 47*(10), 702–710. https://doi.org/10.1055/s-0035-1548832
3. Caturegli, P., De Remigis, A., & Rose, N. R. (2014). Hashimoto thyroiditis: Clinical and diagnostic criteria. *Autoimmunity Reviews, 13*(4–5), 391–397. https://doi.org/10.1016/j.autrev.2014.01.007
4. Özışık, H., Çekin, A., Suner, A., Durmaz, B., Özel, B., Gunel, N. S., Özgen, G., & Erdoğan, M. (2023). Evaluation of IL-10, MCP-1, IFN

gamma, and protectin D1 levels in patients with Hashimoto's thyroiditis. *Irish Journal of Medical Science*, *192*(1), 177–184. https://doi.org/10.1007/s11845-022-03231-3

5. Qi, J., Liu, C., Bai, Z., Li, X., & Yao, G. (2023). T follicular helper cells and T follicular regulatory cells in autoimmune diseases. *Frontiers in Immunology*, *14*, Article 1178792. https://doi.org/10.3389/fimmu.2023.1178792
6. Ralli, M., Angeletti, D., Fiore, M., D'Aguanno, V., Lambiase, A., Artico, M., de Vincentiis, M., & Greco, A. (2020). Hashimoto's thyroiditis: An update on pathogenic mechanisms, diagnostic protocols, therapeutic strategies, and potential malignant transformation. *Autoimmunity Reviews*, *19*(10), Article 102649. https://doi.org/10.1016/j.autrev.2020.102649
7. Roman-Gonzalez, A., Moreno, M. E., Alfaro, J. M., Uribe, F., Latorre-Sierra, G., Rugeles, M. T., & Montoya, C. J. (2009). Frequency and function of circulating invariant NKT cells in autoimmune diabetes mellitus and thyroid diseases in Colombian patients. *Human Immunology*, *70*(4), 262–268. https://doi.org/10.1016/j.humimm.2009.01.012
8. Sharma, R. B., Fan, X., Caturegli, P., Rose, N. R., & Burek, C. L. (2011). Invariant NKT cell lines derived from the NOD.H2h4 mouse enhance autoimmune thyroiditis. *Journal of Thyroid Research, 2011*, Article 895923. https://doi.org/10.4061/2011/895923
9. Wang, L., Zhu, X., Xu, S., Zhou, B., Wu, Y., Li, Z., Zhao, Y., Li, S., Cheng, F., & Zhu, L. (2026). Hashimoto's thyroiditis: From pathogenesis to clinical management. *Frontiers in Endocrinology, 17*, Article 1729316. https://doi.org/10.3389/fendo.2026.1729316
10. Weetman, A. P. (2021). An update on the pathogenesis of Hashimoto's thyroiditis. *Journal of Endocrinological Investigation, 44*(5), 883–890. https://doi.org/10.1007/s40618-020-01477-1
11. Wrońska, K., Hałasa, M., & Szczuko, M. (2024). The role of the immune system in the course of Hashimoto's thyroiditis: The current state of knowledge. *International Journal of Molecular Sciences, 25*(13), Article 6883. https://doi.org/10.3390/ijms25136883.

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