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Evaluation of interleukins 37 and 38 and vitamin D status in the serum of women with Graves' disease

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Abstract

Background: Graves' disease (GD) is an autoimmune thyroid disorder and recent studies have proposed a role for interleukin (IL)-37, IL-38, and vitamin D (VitD) in the pathophysiology of disease. Therefore, this study investigated the expression of IL-37, IL-38, and VitD in the serum of GD patients and correlations of their levels with some demographic and clinical characteristics.

Methods: Serum IL-37, IL-38, and VitD levels were evaluated in 90 women with GD and 93 control women using enzyme-linked immunosorbent assay kits. Depending on therapy, six patients were newly diagnosed (ND; untreated), and 50 patients were receiving only carbimazole (CMZ), while 34 patients were also on CMZ but also received one (31 patients), two (one patient), or three (two patients) doses of radioactive iodine (RAI).

Results: IL-37 levels were significantly higher in GD patients than in controls, while IL-38 and VitD levels were significantly decreased. As indicated by the area under the curve (AUC), receiver operating characteristic curve analysis demonstrated the potential of IL-37, IL-38, and VitD as biomarkers to distinguish GD patients from controls (AUC = 0.953, 0.959, and 0.793, respectively). Multinomial logistic regression analysis showed that altered levels of IL-37, IL-38, and VitD were most likely associated with the pathogenesis of GD. IL-37 was negatively correlated with IL-38 and VitD, while IL-38 and VitD were positively correlated.

Conclusion: Serum Il-37 levels were upregulated in women with GD, while IL-38 and VitD levels showed downregulated levels. The latter two were positively correlated while they showed a negative correlation with IL-37.

KEYWORDS
Graves' disease, IL-37, IL-38, therapy, vitamin D

1 | INTRODUCTION

Graves' disease (GD), an autoimmune disorder specific to the thyroid, is the leading cause of hyperthyroidism due to an enlarged and hyperactive thyroid gland, associated with extra-thyroidal manifestations, such as orbitopathy (ocular abnormalities) and pretibial myxedema (localized dermopathy).[1] It is more common in women than in men (7:1), and the peak incidence is between 35 and 40 years.[2] Although the exact etiology of GD has not been well elucidated, the interaction between the genetic predisposition of the host and environmental triggers is proposed to contribute to a dysregulated immune response, a hallmark of the disease's immunopathogenesis.[3] Anti-thyroid antibodies have been determined in the serum of GD patients and the presence of autoreactive B and T cells infiltrating the thyroid has been documented. These observations indicate a breakdown of immune tolerance against thyroid antigens, particularly against thyroid-stimulating hormone receptor (TSHR), and illustrate the autoimmune pathway involved in GD.[4] Autoimmunity in GD is also associated with dysregulated systemic levels of cytokines, which have been shown to influence both the induction phase and the effector phase of the inflammatory immune response, and play a major role in the immunopathogenesis of GD.[5]

Cytokines, which are immunomodulators with proinflammatory and anti-inflammatory functions, are produced by many immune and non-immune cells to regulate inflammatory reactions during the innate and adaptive immune response.[6] Proinflammatory cytokines in GD have been the focus of an increasing number of studies, which have demonstrated upregulated levels of these cytokines in serum of patients and their association with disease pathogenesis, including those belonging to the interleukin (IL)-1, IL-6, IL-17, and tumor necrosis factor (TNF) cytokine families.[7,8] Besides, anti-inflammatory cytokines have been an additional focus of studies in GD, and the evidence collected supports their involvement in the pathogenesis. In this regard, two newly discovered cytokines of the IL-1 family have attracted attention in GD, IL-37, and IL-38, but studies are limited. IL-37 (originally known as IL-1 F7) is constitutively expressed in various human cells, particularly monocytes, macrophages, dendritic cells, tonsil B cells, and plasma cells, with the ability to maintain immune homeostasis.[9] This cytokine showed significantly upregulated expression in serum and blood mononuclear cells (PBMCs) of GD patients compared to controls.[10] IL-38 (originally known as IL-1HY2) is secreted by various immune and non-immune cells, including PBMCs, B cells, keratinocytes, and fibroblast-like synoviocytes, with ability to regulate the production and function of proinflammatory cytokines through binding to several receptors, such as IL-36R and IL-1 receptor 1 (IL-1R1).[11] Regarding GD, a recent study demonstrated that IL-38 levels were significantly lower in patients than in controls and suggested the potential of IL-38 as a new diagnostic biomarker for the disease.[12]

This study also focused on vitamin D (vitamin D), a fat-soluble vitamin primarily involved in bone metabolism. However, studies have also highlighted additional extra-skeletal effects of VitD, such as anti-inflammatory and immunomodulatory functions, and indicated the link between low levels of VitD and the risk of multiple diseases, including metabolic syndromes, autoimmune diseases, and cardiovascular disease.[13] In autoimmune thyroid diseases, such as GD and Hashimoto's thyroiditis, low levels of VitD have also been demonstrated and associated with the progression of these diseases.[14,15] It is interesting to note that recent suggestive evidence indicates that VitD deficiency is associated with upregulated levels of IL-37, and that VitD may have an immunomodulatory effect on the expression of this cytokine.[16,17]

Although current diagnostic criteria are sufficient to diagnose GD, they do not provide information about the immunopathological mechanisms involved. The aforementioned studies indicate that IL-37, IL-38, and VitD may be considered important biomarkers that aid in understanding this topic and may be relevant to disease pathogenesis. Therefore, the current study aimed to investigate serum IL-37 and IL-38 levels in women with GD and correlate these levels with VitD status and some demographic and clinical characteristics. Deciphering these correlations may be necessary to understand the inflammatory mechanism in GD. To the investigators' knowledge, this issue has not been addressed in GD.

2 | MATERIALS AND METHODS

2.1 | Patients and controls

The Ethics Committee at the Iraqi Ministry of Health and Environment approved the study (Reference No. 4923 on January 31, 2022), and all participants provided written consent. The study included 90 women with GD and 93 healthy women (control group; CTRL). Patients were referred to the outpatient clinic at Baghdad Center for Radiotherapy and Nuclear Medicine (Medical City Complex, Baghdad) during the period from January to August 2022. Patients were registered, diagnosed and treated at the center, which offers free diagnosis and treatment service and through regular pre-scheduled visits by consultants. Included patients were only those who met the diagnostic criteria for GD,[18] and were 18 years of age or older. Diagnostic criteria included ultrasonography examination (diffuse goiter), thyroid function tests (high serum levels of total triiodothyronine [TT3] and thyroxine [T4] and low serum levels of thyroid-stimulating hormone [TSH]), positive TSH receptor antibody test (TRAb), and radioiodine uptake and thyroid scan (diffuse uptake). Pregnant women, male patients and those with Hashimoto's thyroiditis were excluded. Depending on therapy, six patients were newly diagnosed (ND; untreated), and 50 patients were receiving only carbimazole (CMZ), while 34 patients were also on CMZ but also received one (31 patients), two (one patient), or three (two patients) doses of radioactive iodine (RAI). The CTRL group included blood donors and health service workers who were not suffering from infectious or autoimmune diseases. Age, weight, height, and body mass index (BMI) were recorded for all participants. In addition, the following laboratory tests were performed as follows: hemoglobin (Hb), platelet count,

white blood cell (WBC) count, granulocyte-to-lymphocyte ratio (GLR), TT3, T4, TSH, TRAb, IL-37, IL-38, and VitD. Patients were also characterized by age at onset, disease duration, and type of therapy. Extra-thyroidal manifestations, such as orbitopathy and dermopathy, were not recorded.

2.2 | Blood sample collection

Five milliliters blood were collected from each participant in a clot activator and gel tube. The tube was left to stand for 30 minutes at room temperature (20–25°C), and then centrifuged for 15 min at 4°C to separate serum. The serum was divided into aliquots and kept frozen at −20°C until assessment of the required tests. For patients, blood was collected during their outpatient visit and that was 3–4 h after the last dose of CMZ for those undergoing treatment.

2.3 | Thyroid tests

Serum TT3, T4, and TSH levels were determined using commercially available kits following the manufacturer's instructions (Catalogue No. 0025282, 0025258, and 0025294, respectively; Tosoh Bioscience, Japan). A fully automated test (Elecsys Anti-TSHR, Roche Diagnostics, Germany) was used to determine TRAb.

2.4 | IL-37, IL-38, and VitD immunoassay

Serum levels of IL-37, IL-38, and VitD (1,25-dihydroxyvitamin D) were measured using enzyme-linked immunosorbent assay (ELISA) assay kits following the manufacturer's instructions (Catalogue No. MBS165041, MBS269990 and MBS580159, respectively; MyBioSource Inc.). The standard curve range of the kits was 0–240 ng/L, 0–1000 pg/ml and 0–150 ng/ml, respectively.

2.5 | Statistical analysis

Significance was determined using one-way ANOVA for parametric variables, Mann–Whitney U-test and Kruskal–Wallis test for non-parametric variables, and Pearson's Chi-squared test for categorical variables. Receiver operating characteristic (ROC) curve analysis was performed to estimate the area under the curve (AUC), 95% confidence interval (CI), cutoff point, sensitivity, and specificity. The cut-off point was optimized using Youden index (YI). Age- and BMI-adjusted multinomial logistic regression analysis was performed to calculate odds ratio (OR) and 95% CI after classifying subjects into low and high-production groups based on median level in CTRL (≤ and > median), respectively. Spearman's rank correlation analysis as applied to determine correlation coefficient (r_s). The significance was set at a probability (p) value ≤0.05. Statistical analysis was performed using GraphPad Prism version 8.0.0 (San Diego, CA, USA) and IBM

SPSS Statistics 25.0 (Armonk, NY: IBM Corp.). G*power software (version 3.1.9.7) was used to calculate power of sample size.[19]

3 | RESULTS

3.1 | Power of sample size

The sample size of GD patients ($n = 90$) and CTRL ($n = 93$) was tested using the G*power software. The power of the sample size was estimated under 0.05 two-tailed alpha error probability and 0.5 d effect size, and was 0.92. The statistically acceptable power of the sample size is 0.80,[20] and this may validate the current sample size of patients and CTRL.

3.2 | Baseline characteristics

Mean age did not show a significant difference between GD patients and CTRL (41.3 ± 11.7 vs. 42.8 ± 13.7 years; $p = 0.429$). Patients and CTRL were classified into two age groups, ≤40 and >40 years, and there was no significant difference in the distribution of these age groups ($p = 0.723$). Most patients and CTRL were either overweight (35.6% and 32.3%, respectively) or obese (40.0% and 46.2%, respectively), and there were no significant differences in this context ($p = 0.694$). The age at onset was 37.4 ± 12.0 years, but 54.4% of GD patients were classified in the age range 18–40 years. Disease duration was 3.8 ± 4.1 years, which was distributed as <1 (18.9%), 1–5 (57.8%) and >5 (23.3%) years. Six patients were ND (6.7), 50 patients were receiving CMZ (55.5%) and 34 patients received CMZ + RAI (37.8%) (Table 1).

3.3 | Baseline laboratory results

Hb, platelet count, WBC count and granulocyte count did not show significant differences between GD patients (ND, CMZ and CMZ + RAI) and CTRL, while lymphocyte count ($p < 0.001$) and GLR ($p = 0.033$) showed significant differences between these groups. The levels of TT3 ($p = 0.002$), T4 ($p < 0.001$) and TSH ($p = 0.002$) were significantly different between the ND, CMZ, CMZ + RAI, and CTRL groups. The main contributor to this significance was increased levels of TT3 and T4 and decreased levels of TSH in the ND group compared to the other groups (Table 2).

3.4 | IL-37, IL-38, and VitD levels

IL-37 levels were significantly higher in GD patients than in CTRL (84.8 [IQR: 74.5–104.0] vs. 61.9 [IQR: 57.4–65.6] ng/L; $p < 0.001$). On the contrary, IL-38 levels were significantly decreased in GD patients compared to CTRL (43.0 [IQR: 41.0–46.5] vs. 70.0 [IQR: 62.0–86.0] pg/ml; $p < 0.001$). VitD levels were also significantly lower in

TABLE 1 Baseline characteristics of Graves' disease patients and controls.

Characteristic; mean ± SD or n (%)	GD; n = 90	CTRL; n = 93	p-Value
Age; years	41.3 ± 11.7	42.8 ± 13.7	0.429
Age group; years			
≤40	43 (47.8)	42 (45.2)	0.723
>40	47 (52.2)	51 (54.8)	
Weight; kg	74 ± 14	76 ± 12	0.403
Height; cm	162 ± 4	162 ± 5	0.557
BMI; kg/m²	28.0 ± 4.7	28.8 ± 4.2	0.248
BMI group			
Healthy weight	22 (24.4)	20 (21.5)	0.694
Overweight	32 (35.6)	30 (32.3)	
Obese	36 (40.0)	43 (46.2)	
Age at onset; years	37.4 ± 12.0	NA	NA
Age at onset group; years			
<18	6 (6.7)	NA	NA
18–40	49 (54.4)	NA	NA
>40	35 (38.9)	NA	NA
Disease duration; years	3.8 ± 4.1	NA	NA
Disease duration group; years			
<1	17 (18.9)	NA	NA
1–5	52 (57.8)	NA	NA
>5	21 (23.3)	NA	NA
Therapy			
ND	6 (6.7)	NA	NA
CMZ	50 (55.5)	NA	NA
CMZ + RAI	34 (37.8)	NA	NA

Abbreviations: BMI, body mass index; CMZ, Carbimazole; CTRL, Controls; GD, Graves' disease; NA, Not applicable; NDC, Newly diagnosed (untreated); p, Two-tailed probability (significance was determined using one-way ANOVA [parametric variables] or Pearson's Chi-square test [categorical variable]); RAI, Radioactive iodine; SD, Standard deviation.

GD patients than in CTRL (12.8 [IQR: 8.9–25.9] vs. 33.6 [21.9–45.0] ng/ml; $p < 0.001$) (Figure 1).

When IL-37, IL-38, and VitD levels were stratified by groups characteristic of GD patients (age, age at onset, disease duration, BMI and therapy), only two significant differences were observed. First, IL-38 levels were significantly different between the age at onset groups (<18, 18–40, and >40 years). This difference was due to higher IL-38 levels in the <18 year group compared to the 18–40 and >40 year groups (50.6 [IQR: 44.7–65.3] vs. 43.3 [IQR: 41.2–46.5] and 43.3 [IQR: 41.1–54.3] pg/ml, respectively; $p = 0.034$). Second, vitamin D levels were inversely parallel to the BMI groups, healthy weight patients showed the highest level, while obese patients showed the lowest level (healthy weight: 23.3 [IQR: 12.2–33.6]; overweight: 11.3 [IQR: 9.1–19.8]; Obese: 9.5 [IQR: 8.0–24.6] ng/ml; $p = 0.017$). In addition, IL-37 tended to show higher levels in

ND patients compared to CMZ and CMZ + RAI groups (99.9 [IQR: 85.3–108.7] vs. 82.2 [IQR: 72.3–99.8] and 87.4 [IQR: 77.3–108.7] ng/L, respectively; $p = 0.145$). Besides, the lowest levels of VitD were found in ND patients (9.2 [IQR: 8.6–16.1] vs. 12.6 [IQR: 9.3–30.0] and 18.4 [IQR: 8.3–29.3] ng/ml, respectively; $p = 0.143$). IL-38 levels did not show these differences (Table 3).

3.5 | ROC curve analysis

ROC curve demonstrated the potential of IL-37 (AUC = 0.953; 95% CI = 0.923–0.982; $p < 0.001$; cut-off point = 67.9 ng/L; Youden index [YI] = 0.75; sensitivity = 87.1%; specificity = 87.8%), IL-38 (AUC = 0.959; 95% CI = 0.928–0.990; $p < 0.001$; cut-off point = 57.5 pg/ml; YI = 0.84; sensitivity = 91.4%; specificity = 92.2%) and VitD (AUC = 0.793; 95% CI = 0.730–0.857; $p < 0.001$; cut-off point => 24.0 ng/ml; YI = 0.44; sensitivity = 72.0%; specificity = 72.2%) as biomarkers to distinguish GD patients from CTRL (Figure 2).

3.6 | Multinomial logistic regression analysis

Age- and BMI-adjusted multinomial logistic regression analysis was conducted to assess the significance of IL-37, IL-38, and VitD in the risk of GD. To perform the analysis, patients and CTRL were classified as low- and high-production groups (LPG and HPG, respectively) based on the median level of each variable in CTRL (≤ and > median, respectively). It was found that IL-37 HPG was significantly associated with an increased risk of GD (OR = 34.77; 95% CI = 9.94–121.64; $p < 0.001$). On the contrary, IL-38 (OR = 0.03; 95% CI = 0.01–0.12; $p < 0.001$) and VitD (OR = 0.22; 95% CI = 0.11–0.43; $p < 0.001$) HPGs were associated with a lower risk of GD (Table 4).

3.7 | Spearman's correlation analysis

Spearman's correlation analysis demonstrated that IL-37 was negatively correlated with IL-38 ($r_s = -0.617$; $p < 0.001$) and vitamin D ($r_s = -0.424$; $p < 0.001$), while IL-38 and VitD were positively correlated ($r_s = 0.367$; $p < 0.001$) (Figure 3). When the analysis was performed to include IL-37, IL-38, and VitD in relation to some variables of GD patients, only VitD showed significant negative correlations with BMI ($r_s = -0.306$; $p = 0.003$) and T4 ($r_s = -0.207$; $p = 0.05$) (Table 5).

4 | DISCUSSION

The current study disclosed that serum IL-37 levels were significantly elevated in GD patients compared to age- and BMI-matched CTRL, while IL-38 and VitD levels were significantly lower in

TABLE 2 Baseline laboratory results of Graves' disease patients (classified by therapy) and controls.

Characteristic; mean ± SD or median (IQR)	GD; n = 90			CTRL; n = 93	p-Value
	ND; n = 6	CMZ; n = 50	CMZ + RAI; n = 34		
Hb; mg/dl	12.6 ± 1.1	12.2 ± 1.2	12.4 ± 1.0	12.5 ± 0.7	0.21
Platelets; ×10⁹/L	264.3 ± 47.3	252.9 ± 73.6	266.3 ± 66.0	273.5 ± 64.4	0.381
WBC; ×10⁹/L	7.5 ± 1.1	7.4 ± 1.9	8.1 ± 1.7	7.1 ± 1.8	0.061
Granulocyte; ×10⁹/L	2.6 ± 1.2	3.0 ± 1.6	3.1 ± 1.5	3.0 ± 1.0	0.907
Lymphocyte; ×10⁹/L	2.4 ± 0.7	2.7 ± 1.7	3.7 ± 2.4	2.2 ± 0.6	**<0.001**
GLR	1.1 ± 0.5	1.6 ± 1.3	1.0 ± 0.6	1.5 ± 0.7	**0.033**
TT3; ng/ml	2.18 (1.98–2.69)	1.11 (1.00–1.24)	1.12 (0.93–1.22)	1.05 (0.85–1.36)	**0.002**
T4; µg/dl	13.75 (12.30–24.00)	6.65 (5.40–8.40)	7.00 (5.80–8.70)	7.60 (6.10–9.90)	**<0.001**
TSH; µIU/ml	0.01 (0.01–0.30)	2.41 (0.52–9.63)	2.67 (1.10–8.40)	2.20 (1.05–3.48)	**0.002**

Note: Significance was determined using one-way ANOVA (parametric variables) or Kruskal-Wallis test (non-parametric variable).

Abbreviations: CMZ, Carbimazole; CTRL, Controls; GD, Graves' disease; IQR, Interquartile range; NDC, Newly diagnosed (untreated); p, two-tailed probability (significant p-value is indicated in bold); RAI, Radioactive iodine; SD, Standard deviation; T4, Thyroxine; TSH, Thyroid-stimulating hormone; TT3, Total triiodothyronine.
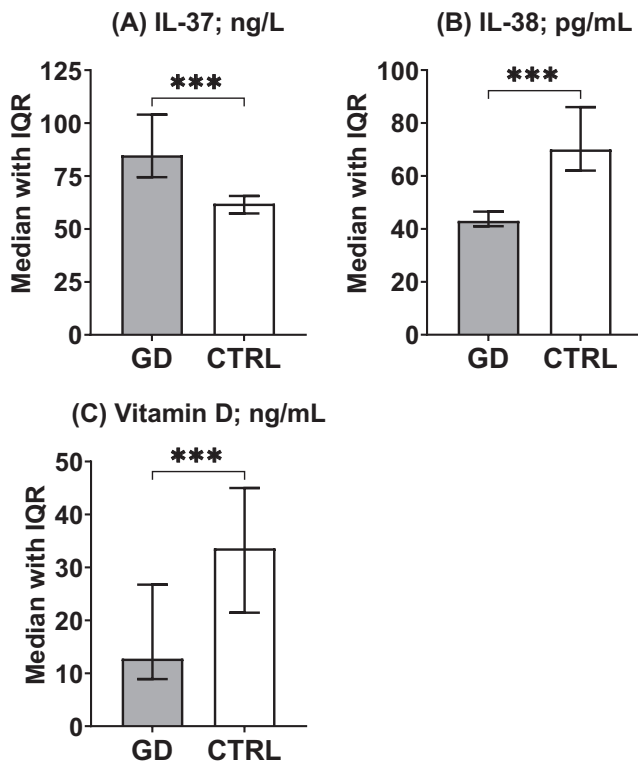

FIGURE 1 Column bar graph of IL-37 (A), IL-38 (B) and vitamin D (C) levels in the serum of Graves' disease (GD) patients and controls (CTRL). Column represents median. Bar represents interquartile range (IQR). Significant differences between medians were assessed using Mann–Whitney U test (***p < 0.001). IL-37 levels were significantly higher in GD patients than in CTRL (84.8 [IQR: 74.5–104.0] vs. 61.9 [IQR: 57.4–65.6] ng/L; p < 0.001). On the contrary, IL-38 levels were significantly decreased in GD patients compared to CTRL (43.0 [IQR: 41.0–46.5] vs. 70.0 [IQR: 62.0–86.0] pg/ml; p < 0.001). Vitamin D levels were also significantly lower in GD patients than in CTRL (12.8 [IQR: 8.9–25.9] vs. 33.6 [21.9–45.0] ng/ml; p < 0.001).

patients. Besides, IL-38 and VitD were positively correlated, while both showed a negative correlation with IL-37. Increased levels of IL-37 and decreased levels of VitD were more pronounced in ND patients, who also showed elevated levels of TT3 and T4 and decreased levels of TSH.

IL-37 is a unique member of the IL-1 family with the ability to suppress inflammation associated with upregulated innate and adaptive immune responses through inhibiting proinflammatory cytokine production to reduce the pathological effects of inflammation.[9] Production of IL-37 is induced in response to proinflammatory stimuli to control inflammation and limit excessive tissue damage. Dysregulated levels of IL-37 have been reported in the serum of patients with various inflammatory and autoimmune diseases. This observation sparked interest in understanding the biological properties of this cytokine and its potential as a therapeutic target.[21] In vivo and ex vivo studies demonstrated that IL-37 can significantly reduce the activity of proinflammatory signaling mediators through interaction with two IL-1 receptors (IL-1R8 and IL-18Rα) on the cell surface, and silencing of both receptors was associated with impairment of IL-37 anti-inflammatory activity.[22] Constitutive or induced production of several key proinflammatory cytokines, including IL-1, IL-6, and TNF, has been shown to be inhibited by the upregulated expression of IL-37 to reduce inflammatory insults.[23]

In the current study, upregulated serum IL-37 levels in GD patients were associated with a 34.77-fold increased risk of disease and showed excellent ability to discriminate between patients and CTRL (AUC = 0.953). Consistent with these findings, a previous study demonstrated that serum IL-37 levels and mRNA expression of the IL37 gene in PBMCs were significantly higher in GD patients compared with CTRL. Besides, the study showed that IL-37 levels were positively correlated with the anti-inflammatory cytokines IL-6, IL-17, and TNF-α, and in vitro evidence was provided that IL-37 suppresses

TABLE 3 Serum IL-37, IL-38, and vitamin D levels stratified by the characteristic groups of Graves' disease patients.

Group	IL-37; ng/L Median (IQR)	p-Value	IL-38; pg/ml Median (IQR)	p-Value	VitD; ng/ml Median (IQR)	p-Value
Age; years						
≤40	84.6 (74.4–100.9)	0.71	43.4 (41.346.7)	0.477	16.2 (9.2–33.5)	0.135
>40	85.3 (74.5–108.70		43.7 (41.2–54.3)		10.7 (8.4–22.2)	
Age at onset; years						
<18	87.3 (62.9–106.2)	0.944	50.6 (44.7–65.3)	**0.034**	33.6 (25.9–39.1)	0.156
18–40	84.6 (77.1–100.8)		43.3 (41.2–46.5)		11.1 (8.9–19.5)	
>40	85.3 (74.5–111.2)		43.3 (41.1–54.3)		16.9 (8.8–30.0)	
Disease duration; years						
<1	85.3 (80.4–108.7)	0.359	42.4 (41.5–43.8)	0.28	11.8 (8.6–19.1)	0.094
1–5	86.4 (73.8–107.5)		44.2 (41.9–50.6)		18.7 (9.4–32.4)	
>5	83.2 (74.5–90.2)		43.4 (40.0–46.3)		9.9 (8.0–20.8)	
BMI						
Healthy weight	80.6 (70.6–103.3)	0.499	44.1 (42.1–49.3)	0.217	23.3 (12.2–33.6)	**0.017**
Overweight	88.5 (76.6–101.0)		42.6 (40.8–45.4)		11.3 (9.1–19.8)	
Obese	83.8 (77.2–108.0)		44.0 (41.2–49.3)		9.5 (8.0–24.6)	
Therapy						
ND	99.9 (85.3–108.7)	0.145	43.6 (42.8–43.9)	0.79	9.2 (8.6–16.1)	0.143
CMZ	82.2 (72.3–99.8)		43.5 (41.3–47.0)		12.6 (9.3–30.0)	
CMZ+RAI	87.4 (77.3–108.7)		43.6 (41.2–52.3)		18.4 (8.3–29.3)	

Note: Significance was determined using Mann–Whitney *U* test (comparing two groups) or Kruskal–Wallis test (comparing more than two groups). Abbreviations: BMI, body mass index; CMZ, Carbimazole; IQR, interquartile range; ND, newly diagnosed (untreated); *p*, two-tailed probability (significant *p*-value is indicated by bold); RAI, Radioactive iodine; VitD, Vitamin D.

the production of these cytokines in PBMCs from GD patients.[10] No other relevant study has been performed in GD, but other inflammatory and autoimmune diseases, such as Behçet's disease (BD), rheumatoid arthritis (RA), ankylosing spondylitis and systemic lupus erythematosus (SLE), have also been associated with abnormal expression of IL-37.[21] Higher levels of IL-37 were found in ND patients than in patients under therapy (CMZ or CMZ+RAI) but the difference was not significant. This may indicate the involvement of IL-37 in the early episodes of GD and the therapy may have a negative effect on the level of this cytokine. The non-significant difference could be attributed to the small number of ND patients included (six patients only). Although there is no direct evidence to support this finding, serum IL-37 levels were significantly decreased in patients with sepsis after 7 days of treatment with anti-infective and other symptomatic treatment.[24]

In contrast to IL-37, serum IL-38 levels were significantly lower in GD patients than in CTRL, and these levels were not affected by therapy. Individuals with low production of IL-38 were more likely to develop GD versus individuals with higher production (OR = 28.93). In addition, lower levels of IL-38 were significant predictors of GD (AUC = 0.959). IL-38 is a recently identified cytokine of the IL-1 family that has anti-inflammatory effects. These effects are mediated by interaction with several receptors, including

IL-36R (interleukin-36 receptor), IL-1RAPL1 (IL-1 receptor accessory protein-like 1) and IL-1R1 (IL-1 receptor 1) to prevent binding to other proinflammatory cytokines, such as IL-1, IL-17, and TNF, and to suppress subsequent signaling pathways.[11] With regard to inflammatory and autoimmune diseases, the expression, regulation and biological functions of IL-38 have been the focus of recent studies but inconsistent results have been reported. Autoimmune diseases such as RA and SLE have been shown to be associated with elevated serum levels of IL-38,[25,26] while, BD patients and diabetic patients with latent tuberculosis showed low serum levels of IL-38.[27,28] In GD, as in the current study, serum IL-38 levels were significantly lower in patients than in CTRL, and the authors suggested that IL-38 levels could be considered as a potential novel prognostic biomarker for GD.[12] Taken together, these findings suggest that IL-38 may play roles in immune cell homeostasis with cytokine regulation as a mechanism involved in autoimmune diseases. Indeed, IL-38 has been shown to be involved in regulating the differentiation and function of many immune cells, including T cells, PBMCs, macrophages, and dendritic cells, and thus may influence the development of autoimmune diseases.[29]

VitD levels decreased markedly in GD patients and in fact 77.8% of them showed levels below 30 ng/mL (insufficient) and 65.6% were classified as deficient (below 20 ng/ml). This observation may link VitD

to the development and/or pathogenesis of GD. VitD is an important component of the endocrine system that contributes to the interaction between bones, parathyroid hormone, kidneys and intestines to maintain calcium homeostasis, thus preserving vital physiological process and skeletal integrity.[30] In addition, as immune cells have been
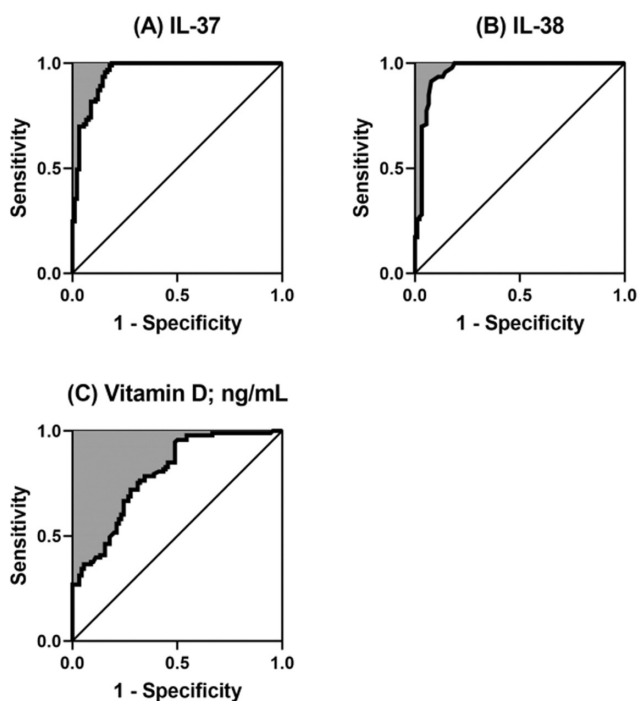

FIGURE 2 Receiver operating characteristic (ROC) curve analysis of IL-37 (A), IL-38 (B) and vitamin D (C) levels in Graves' disease (GD) patients versus controls (CTRL). The analysis demonstrated the potential of IL-37 (area under the curve [AUC] = 0.953; 95% confidence interval [CI] = 0.923–0.982; $p < 0.001$; cut-off point = 67.9 ng/L; Youden index [YI] = 0.75; sensitivity = 87.1%; specificity = 87.8%), IL-38 (AUC = 0.959; 95% CI = 0.928–0.990; $p < 0.001$; cut-off point = 57.5 pg/ml; YI = 0.84; sensitivity = 91.4%; specificity = 92.2%) and vitamin D (AUC = 0.793; 95% CI = 0.730–0.857; $p < 0.001$; cut-off point => 24.0 ng/ml; YI = 0.44; sensitivity = 72.0%; specificity = 72.2%) as biomarkers to distinguish GD from CTRL.

shown to express the VitD receptor (VDR) and the activation enzyme, 1-α-hydroxylase, studies have indicated that VitD can perform various immunomodulatory and anti-inflammatory functions, and thus may have a role in the pathophysiology of autoimmune diseases.[13] Indeed, a meta-analysis of more than 130 studies has shown that low levels of vitamin D are associated with an increased risk of several autoimmune diseases, such as type 1 diabetes, SLE, multiple sclerosis, RA and inflammatory bowel disease.[31] In GD, as in the current study, a meta-analysis study demonstrated that most GD patients are deficient in VitD or have low levels.[32] However, in a recent meta-analysis study, low VitD levels were significant only among GD patients ≥40 years old.[33] This study also showed that the lowest levels of VitD were observed in obese patients and found a significant negative correlation between VitD and BMI. Obesity is a complex chronic disease associated with an increased risk of inflammation, and several lines of evidence indicates that overweight and obese individuals display altered levels of inflammatory markers such as IL-6, IL-18, TNF- and C-reactive protein.[34] Therefore, low VitD levels and obesity may create an ideal environment for the development of autoimmunity, especially if we consider that obesity is associated with decreased regulatory B and T cell response and enhanced Th1 and Th17 cell response.[35] Both responses are dysregulated in autoimmune diseases including GD,[36] and this may link VitD and overweight/obesity to the pathophysiology of GD.

Correlation analysis demonstrated that IL-37 was negatively correlated with IL-38 and VitD, while both the latter two were positively correlated. There is no direct evidence to support these findings, but in juvenile idiopathic arthritis, increased serum IL-37 levels were associated with decreased levels of IL-38 compared to CTRL.[37] Regarding IL-37 and VitD, studies have shown that VitD deficiency and low levels are associated with increased expression of IL-37, an observation that supports the negative correlation between IL-37 and VitD and highlights the role of both markers in exacerbating inflammation.[16,17] As for the positive correlation between IL-38 and VitD, this topic has not been addressed and further studies are warranted.

The low number of GD patients, particularly ND cases, is an important limitation of the study. Besides, Extra-thyroidal manifestations,

TABLE 4 Age- and body mass index-adjusted multinomial logistic regression analysis of IL-37, IL-38, and vitamin D in Graves' disease patients versus controls.

Variable	Production group	GD; $n = 90$		CTRL; $n = 93$		OR (95% CI)	Reciprocal OR (95% CI)	p-Value
		n	%	n	%			
IL-37	Low (≤median)	3	3.3	47	50.5	Reference (1.0)	0.03 (0.01–0.10)	**<0.001**
	High (>median)	87	96.7	46	49.5	34.77 (9.94–121.64)	Reference (1.0)	
IL-38	Low (≤median)	87	96.7	47	50.5	Reference (1.0)	28.93 (8.50–98.48)	**<0.001**
	High (>median)	3	3.3	46	49.5	0.03 (0.01–0.12)	Reference (1.0)	
VitD	Low (≤median)	74	82.2	47	50.5	Reference (1.0)	4.56 (2.30–9.04)	**<0.001**
	High (>median)	16	17.8	46	49.5	0.22 (0.11–0.43)	Reference (1.0)	

Abbreviations: CI, confidence interval; CTRL, Controls; GD, Graves' disease; OR, Odds ratio; p, two-tailed probability (significant p-value is indicated in bold); VitD, Vitamin D.
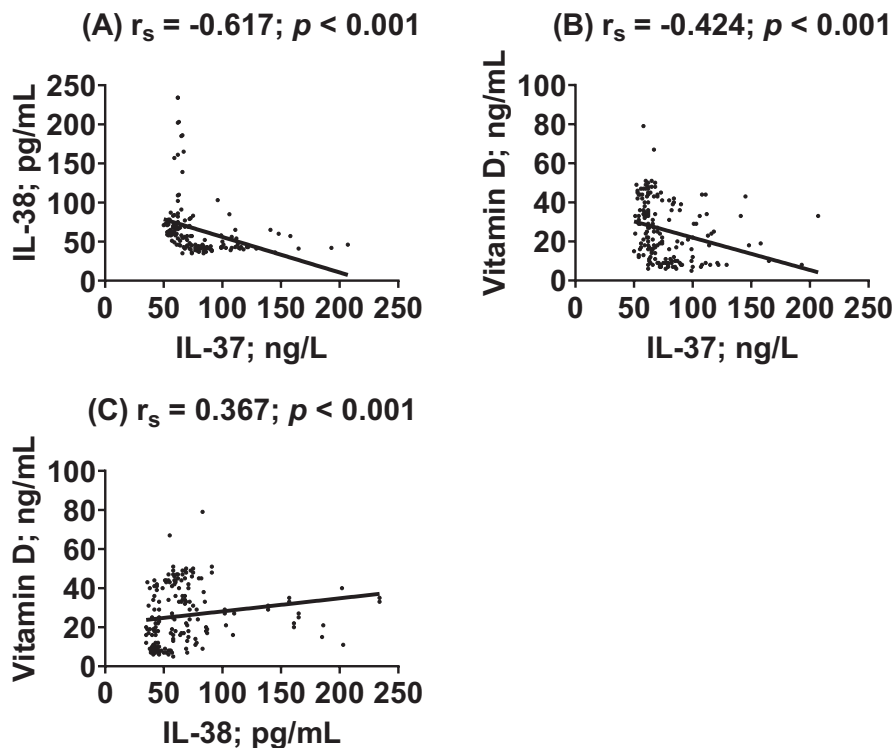
FIGURE 3 Spearman's rank correlation analysis between IL-37, IL-38, and vitamin D levels among all participants (Graves' disease and controls) showing correlation coefficient (r_s). IL-37 was negatively correlated with IL-38 ($r_s = -0.617$; $p < 0.001$) and vitamin D ($r_s = -0.424$; $p < 0.001$), while IL-38 and vitamin D were positively correlated ($r_s = 0.367$; $p < 0.001$).

TABLE 5 Spearman's rank correlation analysis of IL-37, IL-38, and vitamin D levels in relation to some variables of Graves' disease patients.

Variable	Statistics	IL-37	IL-38	VitD
Age	r_s	0.051	−0.031	−0.083
	p-Value	0.634	0.773	0.439
Age at onset	r_s	0.059	0.011	−0.047
	p-Value	0.579	0.919	0.661
Disease duration	r_s	−0.078	0.044	−0.090
	p-Value	0.466	0.677	0.401
BMI	r_s	0.100	0.031	−0.306
	p-Value	0.347	0.773	**0.003**
TT3	r_s	0.196	−0.110	−0.144
	p-Value	0.064	0.301	0.175
T4	r_s	0.120	0.051	−0.207
	p-Value	0.262	0.631	**0.05**
TSH	r_s	0.009	0.035	0.141
	p-Value	0.931	0.746	0.184
WBC	r_s	0.042	0.176	−0.122
	p-Value	0.694	0.098	0.25
Granulocytes	r_s	0.074	0.067	−0.049
	p-Value	0.490	0.533	0.646
Lymphocytes	r_s	0.184	0.022	−0.134
	p-Value	0.083	0.837	0.207
GLR	r_s	−0.084	−0.004	0.057
	p-Value	0.433	0.973	0.591

Abbreviations: BMI, body mass index; GLR, Granulocyte-to-lymphocyte ratio; p, two-tailed probability (significant p-value is indicated in bold); r_s, Spearman's correlation coefficient; T4, Thyroxine; TSH, Thyroid-stimulating hormone; TT3, Total triiodothyronine; VitD, Vitamin D; WBC, White blood cell.

including orbitopathy and dermopathy, have not been identified, and IL-37, IL-38, and VitD may also have a role in these manifestations.

5 | CONCLUSION

Serum IL-37 levels were upregulated in women with GD, while IL-38 and VitD levels showed downregulated levels. The latter two were positively correlated while they showed a negative correlation with IL-37. The study proposed that altered serum levels of IL-37, IL-38, and VitD may be associated with the pathogenesis of GD.

CONFLICT OF INTEREST
The authors declare that they have no potential financial or non-financial conflicts of interest.

DATA AVAILABILITY STATEMENT
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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