Ibrahim *et al.*
Egyptian Journal of Medical Human Genetics (2023) 24:76
https://doi.org/10.1186/s43042-023-00455-5

Egyptian Journal of Medical Human Genetics

RESEARCH Open Access

B-lymphocyte-activating factor is a potential biomarker associated with susceptibility to Graves' disease in Iraqi women

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Abstract

Background B-lymphocyte-activating factor (BAFF) is a cytokine involved in regulating the development and maturation of B lymphocyte and has been shown to be up-regulated in patients with Graves' disease (GD). However, the association of *TNFSF13B* variants (the gene that encodes BAFF) with the risk of GD has not been well explored. In this case–control study, the aim was to evaluate the role of BAFF, in terms of serum level and polymorphism, in the etio-pathogenesis of GD. Therefore, serum BAFF concentrations were analyzed in Iraqi women with GD and age-matched control women (n = 90 and 93, respectively) using an ELISA kit. In addition, two promoter variants of the *TNFSF13B* gene, rs9514827 (T > C) and rs9514828 (C > T), were genotyped using a PCR–RFLP-based assay.

Results Median BAFF concentrations (interquartile range) were significantly elevated in GD patients compared to controls (1525 [1327–1840] vs. 689 [585–807] pg/mL; probability [*p*] < 0.001). Elevated BAFF concentrations were a reliable predictor of GD as indicated by the area under the curve of 0.971. BAFF was positively correlated with triiodothyronine (correlation coefficient [r_s] = 0.216; $p = 0.041$) and thyroxine ($r_s = 0.269$; $p = 0.01$) in GD patients. Mutant alleles, rs9514827 *C* (odds ratio [OR] = 2.00; $p = 0.008$; corrected *p* [*pc*] = 0.048) and rs9514828 *T* (OR = 2.15; $p = 0.002$; $pc = 0.012$), as well as genotypes, rs9514827 CC (OR = 4.29; $p = 0.032$; $pc = 0.192$) and rs9514828 TT (OR = 4.57; $p = 0.003$; $pc = 0.018$), were associated with a greater risk of GD. Besides, the C-T haplotype (rs9514827-rs9514828) was also linked to an elevated risk of GD among Iraqi women (OR = 2.71; $p = 0.006$; $pc = 0.024$).

Conclusions BAFF showed up-regulated levels in the serum of women with GD. In light of this, BAFF has been proposed as a reliable prognostic biomarker for GD. Regarding its relationship to thyroid hormones, BAFF showed a positive correlation with triiodothyronine and thyroxine. Both variants (rs9514827 and rs9514828) of the *TNFSF13B* gene showed an association with susceptibility to GD, and rs9514828 may have up-regulatory effects on BAFF levels.

Keywords Graves' disease, B-lymphocyte-activating factor, Vitamin D, rs9514827, rs9514828

Background

Graves' disease (GD) is an autoimmune and inflammatory disorder characterized by the production of autoantibodies directed towards thyroid antigens due to failure of immunological tolerance. As a consequence, an alteration in the expression of thyroid hormones occurs, including an up-regulation of triiodothyronine (T3) and thyroxine (T4) and a down-regulation of thyroid stimulating hormone (TSH) [1]. GD is among the most common causes of hyperthyroidism with an estimated annual incidence of 20 cases/100,000 individuals worldwide.

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Besides, it is more common in women than in men, especially in the age range 30–60 years [2]. There are no relevant data for Iraq, but a recent retrospective observational study reported hyperthyroidism in 6.1% of 17,878 patients (67.7% women) from a specialized center for diabetes, endocrinology and metabolism in southern Iraq during the period 2008–2019 [3].

Although B and T lymphocytes are well-known to play a role in mediating the pathogenesis of GD, B lymphocytes are the source of anti-thyroid antibodies and thus a cornerstone of thyroid-directed humoral autoimmunity [4]. B lymphocytes play an essential role in regulating immune system functions and directly contribute to inflammatory and autoimmune reactions through many pathways including antibody production, antigen presentation, activation of $CD4^{+}$T lymphocytes, expression of co-stimulatory molecules, and promotion of pro-inflammatory cytokines [5]. Disruption of these tightly regulated immune pathways may be associated with an increased risk of autoimmune responses. Although the exact mechanisms involved are not well defined, loss of B lymphocyte tolerance is a prominent feature of autoimmunity in many autoimmune diseases including GD [6]. In addition, it has been indicated that rescue of transitional B lymphocytes at checkpoints of tolerance and promotion of their maturation requires activation of B-lymphocyte-activating factor (BAFF), which is also involved in providing these cells with essential survival signals and prevents their deletion [7].

BAFF, also referred as BLyS (B lymphocyte stimulator), is a cytokine classified under a superfamily of cytokines called tumor necrosis factor (TNF). BAFF can be produced by a variety of cells including neutrophils, monocytes/macrophages, dendritic cells, B lymphocytes, activated T lymphocytes, epithelial cells, and stromal cells [8]. Observational and experimental studies in humans and transgenic animals indicate that up-regulated expression of BAFF is associated with an increased number of B lymphocytes, enhanced immunoglobulin production, and the consequent development of autoimmune manifestations. BAFF-neutralizing agents have been shown to be effective in counteracting these effects and suppressing autoimmune and inflammatory responses [9]. Indeed, elevated BAFF levels have been found in a number of autoimmune diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematous (SLE), and are associated with the pathological events in these disorders [7, 10]. In GD patients, it has been demonstrated that plasma concentrations and gene expression of BAFF are up-regulated, while immunomodulators such as steroids, have been associated with declined serum concentrations of BAFF [11–13]. Convincing evidence also suggests that GD is associated with low levels of vitamin D (VitD), a steroid molecule responsible for regulating bone metabolism and calcium homeostasis. In addition, VitD has been shown to have a role in controlling the expression of factors involved in regulating immune function [14, 15]. However, the nature of the functional relationship between BAFF and VitD has yet to be explored, and an understanding of this relationship is essential because both factors have immunomodulatory effects and are proposed to have a role in the pathophysiology of autoimmune disorders including GD [7, 13, 16].

BAFF is encoded by *TNFSF13B* (HGNC: 11929), a seven-exon gene mapped to human chromosome 13 at position 13q33.3 [17]. Several genetic variants of this gene (single-nucleotide polymorphisms; SNPs) have been described and some have been indicated to influence susceptibility to various autoimmune disorders, including rs9514828 with SLE [18], rs2893321 with myasthenia gravis [19], and rs9514828 with RA [20]. With regard to GD, Lin and colleagues were the first to analyze two *TNFSF13B* SNPs, rs2893321 and rs1041569, and reported that rs2893321 was associated with susceptibility to disease in Mexicans [21]. Another study looked at two additional SNPs, rs9514828 and rs4000607, in British patients with GD, and a significant association was found with rs4000607, while rs9514828 showed no association [22]. Recently, three *TNFSF13B* SNPs, rs9514827, rs1041569 and rs9514828, were explored in 62 patients with GD and the authors concluded that the rs9514828 *T* allele may be a variant of susceptibility to GD in Tunisians [23].

This study aimed to analyze BAFF in the serum of Iraqi women with GD in order to understand its pathological role. The aim was expanded to assess the effects of treatment, body mass index (BMI), and VitD status on BAFF production, as the data available in this regard are not overwhelming. Furthermore, a molecular study was conducted to investigate the role of two *TNFSF13B* SNPs (rs9514827 and rs9514828) in susceptibility to GD and to evaluate the effect of their genotypes on BAFF synthesis. To the best of the researchers' knowledge, these genetic variants have not been explored in Iraqis.

Materials and methods

Populations studied

During January-August 2022, a case–control study was conducted on 90 women with GD (mean age = 41.3 years; standard deviation [SD] = 11.7 years) and 93 age-matched healthy control women (CTRL group; mean age = 42.8 years; SD = 13.7 years). Patients were registered and diagnosed at a radiotherapy and nuclear medicine center. They visited the center according to regular appointments for the purpose of treatment. Fifty patients were on carbimazole (CMZ) therapy only, while 34 patients

received one, two, or three doses of radioiodine (RAI) plus CMZ (31, 1, and 2 patients, respectively). In addition, six newly diagnosed (ND) patients who received no treatment were included. Only patients ≥ 18 years of age who met the criteria for the diagnosis of GD were included [24]. These criteria included diffuse goiter, presence of TSH receptor antibodies (TRAb), and diffuse uptake of RAI. Patients with other thyroid diseases and pregnant women were excluded. Age, age at onset, and disease duration data were collected from the patients' hospital registry. Besides, height and weight were measured in meter (m) and kilogram (kg), respectively, and used to calculate body mass index (BMI). In view of this, patients with GD were classified as having a normal weight (NOR) or overweight/obese (O/O) [25]. VitD was also determined and patients' status was classified as insufficient (INSFT; < 30 ng/mL) or sufficient (SFT; 30–50 ng/mL) [26]. The CTRL group included blood donors and health service personnel, who were apparently healthy individuals without autoimmune disorders or infectious diseases.

Blood sample collection

A morning blood sample (5 mL) was collected from each participant and for patients who were receiving CMZ treatment, the collection was 3–4 h after the last dose. Blood was distributed into a plain tube (3 mL) and an EDTA tubes (2 mL, respectively). Plain tube blood was processed to collect serum by centrifugation (1200×*g* for 10 min at 4 °C). Serum was frozen at − 20 °C until quantification of TT3, T4, TSH, TRAb, BAFF and VitD (1,25-dihydroxyvitamin D) concentrations. EDTA blood was used for DNA isolation using the ReliaPrep Blood gDNA Miniprep System Kit (Promega, USA) according to the manufacturer's protocol. Isolated DNA was frozen at − 20 °C until genotyping of *TNFSF13B* SNPs.

Immunoassay of thyroid hormones, BAFF and VitD

Commercially available enzyme-linked immunosorbent assay kits were used to quantify TT3, T4, TSH, TRAb, BAFF, and VitD concentrations following procedures recommended by the manufacturer (Catalogue No.: 0025282, 0025258, 0025294 [Tosoh Bioscience, Japan], MBS175962 and MBS580159 [MyBioSource Inc., USA], respectively).

Selection and detection of SNPs rs9514827 and rs9514828

Following previous studies, two *TNFSF13B* SNPs (rs9514827 and rs9514828) were selected to evaluate their association with susceptibility to GD [21–23]. Tagged SNPs, rs9514827 (T > C) and rs9514828 (C > T), were genotyped according to the principles of polymerase chain reaction (PCR)-restriction fragment length polymorphisms (RFLPs) assay as described previously [18]. For rs9514827, the DNA was amplified with the forward primer 5′-ATTCCCTGTCAGAATTTTCTCT-3′ and the reverse primer 5′-CCTATAACTCCCACA ATAAGGTGAC-3′. The corresponding primers for rs9514828 were 5′-TTGTACACCGACCTGTTAGG-3′ and 5'-TGGAAGTAAGTCCACTGGGAAT-3′, respectively. The PCR amplification was performed in 25 µL reaction mix that was made-up of 12.5 µL of GoTaq Green Master Mix (Promega, USA), 1 µL of forward primer, 1 µL of reverse primer, 5 µL of DNA (50–60 ng/mL), and 5.5 µL of nuclease-free water. A BioRad thermal cycler (USA) was used to amplify target DNA under optimized conditions that included an initial cycle of denaturation (94 °C; 3 min), followed by 35 cycles of denaturation (30 s at 94 °C), annealing 20 s at 57 °C), and extension (30 s at 72 °C). A final cycle of extension was also conducted at 72 °C for 1 min. Amplified products of rs9514827 (468 bp) were digested with 3 units of *Aci-I* restriction enzyme (Sibenzyme, Russia) for 2 h at 37 °C. The molecular size of the digested fragments as detected by electrophoresis on 2% agarose was 468 bp for TT genotype, 468 bp, 308 bp and 160 bp for TC genotype, and 308 bp and 160 bp for CC genotype. Amplified products of rs9514828 (398 bp) were also digested with 3 units of *Aci-I* restriction enzyme but for 6 h at 37 °C. The molecular size of the digested fragments as detected by electrophoresis on 2% agarose was 261 bp and 137 bp for CC genotype, 398 bp, 261 bp and137 bp for CT genotype, and 398 bp for TT genotype [18].

Statistical analysis

Parametric variables were presented by mean and standard deviation (SD) and significance was determined with one-way analysis of variance (ANOVA) test. Non-parametric variables were presented by median and interquartile range (IQR: 25–75%) and significance was determined with Mann–Whitney *U* test. Absolute number and frequency (percentage) were used to describe allele and genotype frequencies, and significance was determined with two-tailed Fisher exact test. Pearson Chi-square goodness of fit test was used to analyze Hardy–Weinberg equilibrium (HWE). Receiver operating characteristic curve (ROC) analysis was used to assess the prognostic performance of serum BAFF concentrations through estimating area under the curve (AUC) and its 95% confidence interval (CI), cut-off value optimized with Youden index (YI), sensitivity and specificity. Odds ratio (OR) and its 95% CI were calculated using multinomial logistic regression (MLR) analysis, which was conducted under allele, recessive, dominant, over-dominant, and co-dominant genetic models. Correlations between variables were analyzed with Spearman's rank-order

correlation test and presented as a correlation coefficient (r_s). A significant level was set at a two-tailed probability (p) value of 0.05. Bonferroni correction was used to correct p-value (pc). IBM SPSS Statistics 25.0 (IBM Corp., Armonk, NY) was used to perform the statistical analysis, while GraphPad Prism version 9.4.1 (San Diego, CA, USA) was used to generate the plots. A two-locus haplotype of SNPs rs9514827 and rs9514828 was estimated using the SHEsis software [27]. Power of sample size was calculated using G*power software (version 3.1.9.7) [28].

Results

Sample power

Sample size power was calculated using G*power software with the following entries: two-tailed α error p of 0.05, effect size d of 0.5, 90 GD patients, and 93 CTRL. The calculated sample power (1 − β error p) was 0.92, which was statistically sufficient to validate the sample size. The statistically acceptable sample power is 0.8 [28].

Baseline data

The mean age was identical in GD patients and CTRL and no significant difference was found (41.3 [SD: 11.7] vs. 42.8 [SD: 13.7] years; $p = 0.429$). The age at onset of patients was 37.4 (SD: 12.0) years and the disease duration was 3.8 (SD: 4.1) years. GD patients and CTRL shared the same mean BMI but it was above the NOR range (18–24.9 kg/m^2) and subjects in both groups were classified as O/O (≥25 kg/m^2). Median concentrations of TT3 (1.13 [IQR: 1.00–1.30] vs. 1.05 [IQR: 0.85–1.36] ng/mL; $p = 0.11$) and TSH (2.23 [IQR: 0.50–8.40] vs. 2.20 [IQR: 1.05–3.48] μIU/mL; $p = 0.514$) showed no significant differences between GD patients and CTRL, while T4 concentrations were significantly decreased in patients (6.90 [IQR: 5.60–8.70] vs. 7.60 [IQR: 6.10–9.90] μg/dL; $p = 0.026$). Serum VitD concentrations were significantly lower in GD patients than in CTRL (12.8 [IQR: 8.9–25.9] vs. 33.6 [IQR: 21.9–45.0] ng/mL; $p < 0.001$) (Table 1).

BAFF concentrations

Serum BAFF concentrations were significantly elevated in GD patients compared with CTRL (1525 [IQR: 1327–1840] vs. 689 [IQR: 585–807] pg/mL; $p < 0.001$). Stratification of BAFF concentrations by therapy, age at onset, disease duration, BMI or VitD status revealed no significant difference with respect to each stratum (Fig. 1).

ROC curve analysis

The prognostic performance of serum BAFF concentrations was assessed using the ROC curve analysis, which demonstrated the significance of BAFF as a biomarker for differentiating patients with GD from CTRL. BAFF occupied an AUC of 0.971 and the 95% CI was 0.939–1.0 ($p < 0.001$). At a cut-off point of 902 pg/mL (YI = 0.89), the calculated sensitivity for BAFF was 94.6% and the specificity was 94.4% (Fig. 2).

Table 1 Baseline data describing the characteristics of patients with Graves' disease and controls

Characteristic; mean ± SD or median (IQR)	GD; n = 90	CTRL; n = 93	*p* value
Age; year	41.3 ± 11.7	42.8 ± 13.7	0.429
Onset age; year	37.4 ± 12.0	NA	NA
Disease duration; year	3.8 ± 4.1	NA	NA
BMI; kg/m^2	28.0 ± 4.7	28.8 ± 4.2	0.248
TT3; ng/mL	1.13 (1.00–1.30)	1.05 (0.85–1.36)	0.11
T4; μg/dL	6.90 (5.60–8.70)	7.60 (6.10–9.90)	**0.026**
TSH; μIU/mL	2.23 (0.50–8.40)	2.20 (1.05–3.48)	0.514
VitD; ng/mL	12.8 (8.9–25. 9)	33.6 (21.9–45.0)	**< 0.001**

SD standard deviation, *IQR* interquartile range, *GD* Graves' disease, *CTRL* controls, *BMI* body mass index, *TT3* total triiodothyronine, *T4* total thyroxine, *TSH* thyroid stimulating hormone, *VitD* Vitamin D, *NA* not applicable, *p* Two-tailed probability (significant *p* value is indicated in bold). Significance was determined using one-way ANOVA test to compare means and Mann–Whitney U test to compare medians

Spearman's rank-order correlation analysis

A bivariate analysis was performed between BAFF and BMI, TT3, T4, TSH, and VitD among GD patients and CTRL. BAFF was positively correlated with TT3 ($r_s = 0.216$; $p = 0.041$) and T4 ($r_s = 0.269$; $p = 0.01$) among women with GD, whereas no significant correlation was found among the CTRL group ($r_s = -0.454$; $p < 0.001$) (Table 2).

MLR analysis of SNPs rs9514827 and rs9514828

The SNPs rs9514827 and rs9514828 were genotyped in 90 GD patients and 70 CTRL. Genotype frequencies of rs9514827 (TT, TC, and CC) and rs9514828 (CC, CT, and TT) showed no significant deviation from HWE in the CTRL sample ($p = 0.881$ and 0.61, respectively). To examine the association of the two SNPs with susceptibility to GD, the MLR analysis was performed under five genetic models; allele, recessive, dominant, overdominant, and co-dominant. Besides, a two-locus haplotype analysis (rs9514827 and rs9514828) was also conducted. The *C* allele frequency of rs9514827 (mutant allele) was significantly greater in GD patients than in the CTRL group (33.3 vs. 20.0%; OR = 2.00; 95 CI 1.19–3.35; $p = 0.008$; $pc = 0.048$). The frequencies of TC + CC genotypes (dominant model: 53.3 vs. 35.7%; OR = 2.06; 95% CI 1.09–3.89; $p = 0.037$) and CC genotype (co-dominant model: 13.3 vs. 4.3%; OR = 4.29; 95 CI 1.13–16.26; $p = 0.032$) were also significantly greater in GD patients

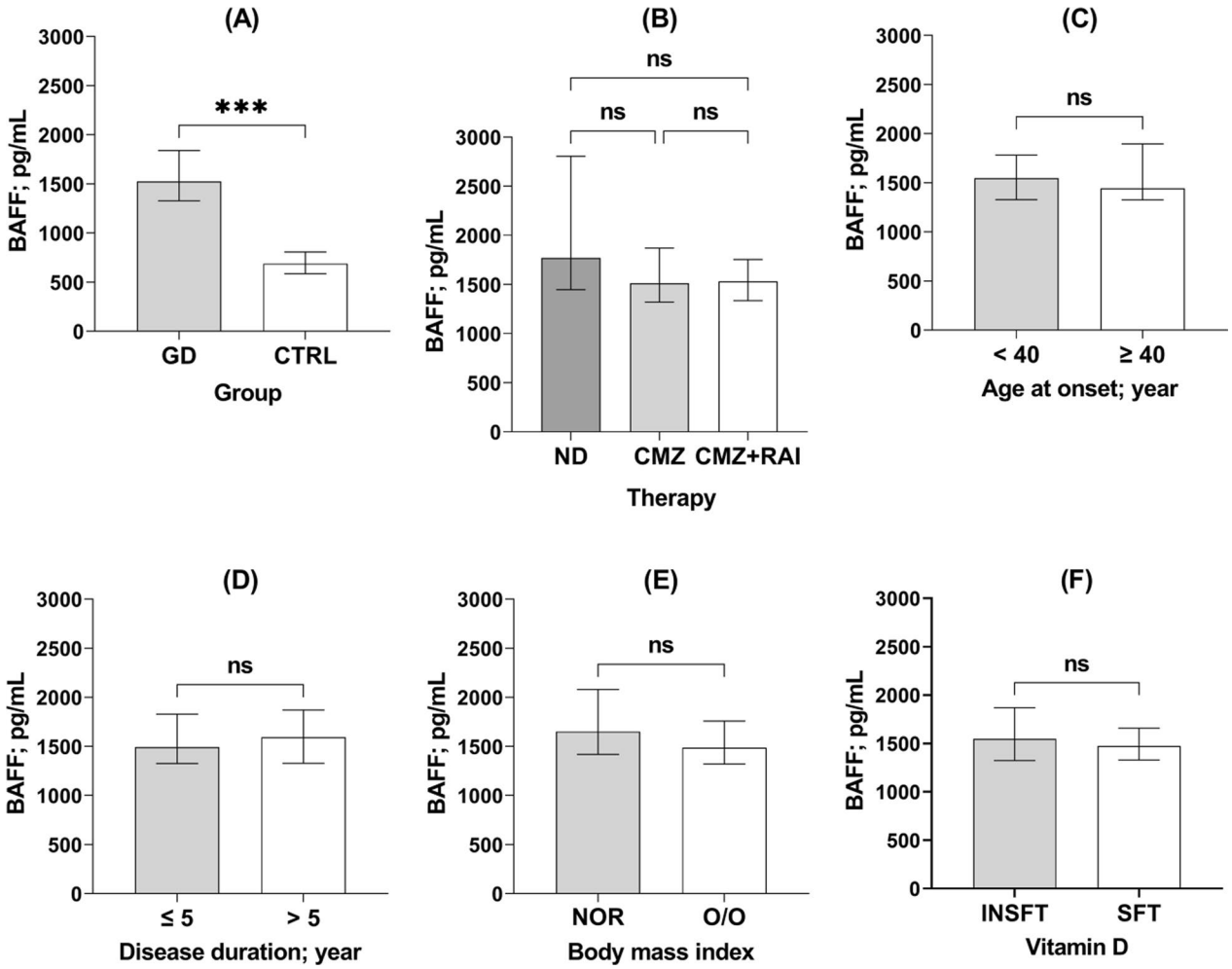

Fig. 1 Column-bar plots of serum B-lymphocyte-activating factor (BAFF) levels: **A** Graves' disease (GD) patients and controls (CTRL), **B** therapy groups (*ND* newly diagnosed, *CMZ* carbimazole, *RAI* radioactive iodine), **C** age at onset groups; **D** disease duration groups, **E** body mass index group (*NOR* normal-weight, *O/O* overweight/obese); and **F** vitamin D groups (*INSFT* insufficient, *SFT* sufficient). Columns indicates median, while bars represent interquartile range (IQR). Significance was assessed using Mann–Whitney *U* test (***$p < 0.001$; *ns* not significant). BAFF levels were significantly elevated in GD patients compared to CTRL (Plot **A**: 1525 [IQR: 1327–1840] vs. 689 [IQR: 585–807] pg/mL; $p < 0.001$). When BAFF levels were stratified by some characteristics of GD patients (Plots **B, C, D, E** and **F**), there was no significant difference in each stratum

but the Bonferroni-corrected *p*-value did not attend a significant level ($pc = 0.222$ and 0.192, respectively). Analysis of rs9514827 under recessive and over-dominant genetic models revealed no significant association with susceptibility to GD ($p = 0.059$ and 0.32, respectively). For rs9514828, the mutant *T* allele (44.4 vs. 27.1%; OR = 2.15; 95 CI 1.34–3.45; $p = 0.002$; $pc = 0.012$), CT + TT genotypes (recessive model: 28.9 vs. 8.6%; OR = 4.33; 95% CI 1.68–11.16; $p = 0.001$; $pc = 0.006$), and TT genotype (co-dominant model: 28.9 *vs.* 8.6%; OR = 4.57; 95 CI 1.69–12.41; $p = 0.003$; $pc = 0.018$) exhibited significantly greater frequencies in GD patients compared to CTRL. Analysis of rs9514828 under dominant and over-dominant revealed that this variant was not significantly associated with susceptibility to GD ($p = 0.081$ and 0.501, respectively). Haplotype analysis demonstrated that C-T haplotype (in the order: rs9514827-rs9514828) was also linked to an elevated risk of GD, while a decreased risk was associated with the TC haplotype (40.2 vs. 60.3%; OR = 0.44; 95% CI 0.28–0.69; $p < 0.001$; $pc = 0.001$) (Table 3).

Impact of rs9514827 and rs9514828 genotypes on BAFF concentrations

Serum concentrations of BAFF were analyzed in all participating subjects (GD patients plus CTRL; n = 160) after stratification by genotypes of the rs9514827 and rs9514828 SNPs. The CC genotype of rs9514827 tended to show higher BAFF concentrations compared to the

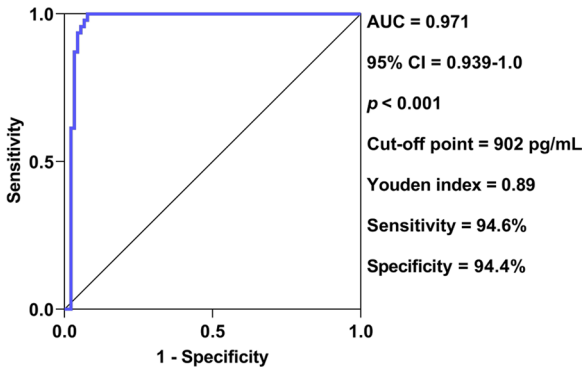

Fig. 2 Receiver operating characteristic curve analysis of B-lymphocyte-activating factor (BAFF) in Graves' disease (GD) patients versus CTRL. The area under the curve (AUC), 95% confidence interval (CI), probability (*p*), cut-off point, sensitivity, and specificity are shown. Youden index was used to adjust the cut-off point. As evidenced by an AUC of 0.971, BAFF made an excellent distinction between patients with GD and CTRL

Table 2 Spearman's rank-order correlation analysis between B-lymphocyte-activating factor and body mass index, total triiodothyronine, total thyroxine, thyroid stimulating hormone, and vitamin D (VitD) among women with Graves' disease and control women

Variable	BAFF; pg/mL			
	GD; n = 90		CTRL; n = 93	
	r_s	*p* value	r_s	*p* value
BMI; kg/m^2	−0.108	0.31	−0.118	0.259
TT3; ng/mL	0.216	**0.041**	0.116	0.27
T4; µg/dL	0.269	**0.01**	−0.106	0.311
TSH; µIU/mL	−0.182	0.087	−0.006	0.956
VitD; ng/mL	−0.06	0.575	−0.127	0.226

BAFF B-lymphocyte-activating factor, *GD* Graves' disease, *CTRL* controls, r_s correlation coefficient, *p* Two-tailed probability (significant *p* value is indicated in bold), *BMI* body mass index, *TT3* total triiodothyronine, *T4* total thyroxine, *TSH* thyroid stimulating hormone, *VitD* vitamin D

TT genotype but the difference did not attend a significant level ($p = 0.09$). For rs9514828, the TT genotype was associated with significantly elevated serum concentrations of BAFF compared to the CC and CT genotypes (1516 [IQR: 884–1943] *vs.* 799 [IQR: 619–1517] and 871 [IQR: 649–1454] pg/mL; $p = 0.005$ and 0.02, respectively), while there was no significant variation between CC and CT genotypes in this context ($p = 0.64$) (Fig. 3).

Discussion

In this study serum BAFF concentrations were analyzed in a group of women with GD and compared these concentrations with those of age- and BMI-matched CTRL. BAFF showed markedly elevated concentrations in GD patients and ROC curve analysis demonstrated the biomarker significance of this cytokine in disease prediction (AUC = 0.971). In addition, BAFF was positively correlated with TT3 and T4. It was also found that two promoter SNPs of the *TNFSF13B* gene, rs9514827 and rs9514828, were associated with susceptibility to GD, particularly at the haplotype level. Besides, the results indicated that the TT genotype of rs9514828 was associated with greater serum concentrations of BAFF. Together, these findings suggest a role for BAFF in the etio-pathogenesis of GD.

GD is an organ-specific autoimmune disorder affecting the thyroid gland, and as demonstrated by observational and experimental studies, B cells play a vital role in the pathophysiology of the disease because these cells are the source of pathogenic autoantibodies (i.e. TRAb) against the TSH receptor. Thus, binding of TRAb to the TSH receptor can activate target cells and may lead to higher levels of T3 and T4 (thyroid hormones) [29]. Studies have demonstrated that the essential survival signals for B cells are provided by BAFF, and therefore up-regulated functional activity of these cells can be associated with elevated concentrations of BAFF [30]. In fact, massive expansion of activated autoreactive B cells and autoantibody production was evident in mice in association with over-expression of BAFF [31]. Consistent with our findings, previous studies also reported elevated plasma concentrations and up-regulated gene expression of BAFF in patients with GD [11–13]. Furthermore, blockade of BAFF in a mouse model of GD was associated with a significant decrease in hyperthyroidism [32]. Therefore, blocking the BAFF interaction has been proposed as one of the novel therapeutic options in GD [33]. Together, these data indicate the pathological significance of elevated BAFF concentrations in the serum of patients with GD particularly in terms of prognosis (AUC = 0.971; sensitivity = 94.6%; specificity = 94.4%).

The action of BAFF on B cells is mediated through interaction with three surface receptors, namely BAFF receptor 3 (BR3), transmembrane activator and calcium-modulating and cyclophilin ligand interactor (TACI), and B cell maturation antigen [30]. BR3 is essential for the development of immature B cells into transitional B cells, as this receptor is involved in mediating pro-survival signals to rescue B cells from premature death [34]. In contrast, TACI is associated with B-cell apoptosis and mediates IL-10 production by regulatory B-lymphocytes to exert an immunosuppressive function [35]. Altered expression of BR3 and TACI has recently been demonstrated in peripheral B cells of GD patients, with the former being over-expressed and the latter showing lower expression. Accordingly, the authors suggested

Table 3 Association analysis of *BAFF* single nucleotide polymorphisms (rs9514827 and rs9514828) with Graves' disease

***BAFF* SNP/genetic model**	**Allele/genotype/haplotype**	**GD; n = 90**		**CTRL; n = 70**		**OR (95% CI)**	***p* value (*pc*)**
		n	**%**	**n**	**%**		
rs9514827 T > C							
Allele	*T*	120	66.7	112	80.0	Reference; 1.0	
	C	60	33.3	28	20.0	2.00 (1.19–3.35)	**0.008 (0.048)**
Recessive	TT + TC	78	86.7	67	95.7	Reference; 1.0	
	CC	12	13.3	3	4.3	3.44 (0.94–12.58)	0.059 (0.354)
Dominant	TT	42	46.7	45	64.3	Reference; 1.0	
	TC + CC	48	53.3	25	35.7	2.06 (1.09–3.89)	**0.037** (0.222)
Over-dominant	TT + CC	54	60.0	48	68.6	Reference; 1.0	
	TC	36	40.0	22	31.4	1.45 (0.76–2.80)	0.32 (1.0)
Co-dominant	TT	42	46.7	45	64.3	Reference; 1.0	
	TC	36	40.0	22	31.4	1.75 (0.89–3.45)	0.104 (0.624)
	CC	12	13.3	3	4.3	4.29 (1.13–16.26)	**0.032** (0.192)
p-HWE			0.881				
rs9514828 C > T							
Allele	*C*	100	55.6	102	72.9	Reference; 1.0	
	T	80	44.4	38	27.1	2.15 (1.34–3.45)	**0.002 (0.012)**
Recessive	CC + CT	64	71.1	64	91.4	Reference; 1.0	
	TT	26	28.9	6	8.6	4.33 (1.68–11.16)	**0.001 (0.006)**
Dominant	CC	36	40.0	38	54.3	Reference; 1.0	
	CT + TT	54	60.0	32	45.7	1.78 (0.95–3.34)	0.081 (0.486)
Over-dominant	CC + TT	62	68.9	44	62.9	Reference; 1.0	
	CT	28	31.1	26	37.1	0.76 (0.40–1.47)	0.501 (1.0)
Co-dominant	CC	36	40.0	38	54.3	Reference; 1.0	
	CT	28	31.1	26	37.1	1.14 (0.56–2.29)	0.72 (1.0)
	TT	26	28.9	6	8.6	4.57 (1.69–12.41)	**0.003 (0.018)**
p-HWE				0.61			
Two-locus haplotype (rs9514827-rs9514828)	C–C	27.7	15.4	17.5	12.5	1.27 (0.67–2.42)	0.465 (1.0)
	C-T	32.3	17.9	10.5	7.5	2.71 (1.30–5.65)	**0.006 (0.024)**
	T-C	72.3	40.2	84.5	60.3	0.44 (0.28–0.69)	**< 0.001 (0.001)**
	T-T	47.7	26.5	27.5	19.7	1.47 (0.87–2.51)	0.153 (0.612)

BAFF B-lymphocyte-activating factor, *SNP* single nucleotide polymorphism, *HWE* Hardy–Weinberg equilibrium, *GD* Graves' disease, *CTRL* controls, Odds ratio, *CI* Confidence interval, *p* two-tailed probability, *pc*: Bonferroni corrected probability (significant *p* value is indicated in bold)

that autoimmunity in GD could be attributed to altered expressions of BAFF and its receptors through the enhancement and inhibition of the activities of the BR3 and TACI signaling pathways, respectively [13]. Consistent with this suggestion, blocking the interaction between BAFF and BR3 was associated with decreased B cell proliferation, decreased B cell survival, and decreased autoantibody production [36]. Therefore, targeting such an interaction between BAFF and its receptors may have therapeutic potential in GD.

With respect to thyroid hormones, serum TT3 and TSH concentrations showed no significant differences between GD patients and CTRL, while T4 concentrations were significantly lower in patients. T3 and T4 are secreted by the thyroid gland after stimulation with TSH, a hormone produced by the anterior pituitary gland. Irregular production of these hormones can lead to hypothyroidism or hyperthyroidism. Hyperthyroidism is the hallmark of GD as T3 and T4 show up-regulated concentrations in patients' serum, while TSH is down-regulated [1]. This pattern of TT3, T4, and TSH concentrations in current GD patients was not followed as described above. However, the median concentration of the three hormones was within the reference range (TT3: 1.13 vs. 0.8–2.0 ng/mL; T4: 6.90 vs. 5.5–12.2 μg/dL; TSH: 2.23 vs. 0.3–4.5 μIU/mL) [37]. The normalization of TT3, T4 and TSH concentrations may be due to therapy effects as most patients (84 of 90; 93.3%) were receiving

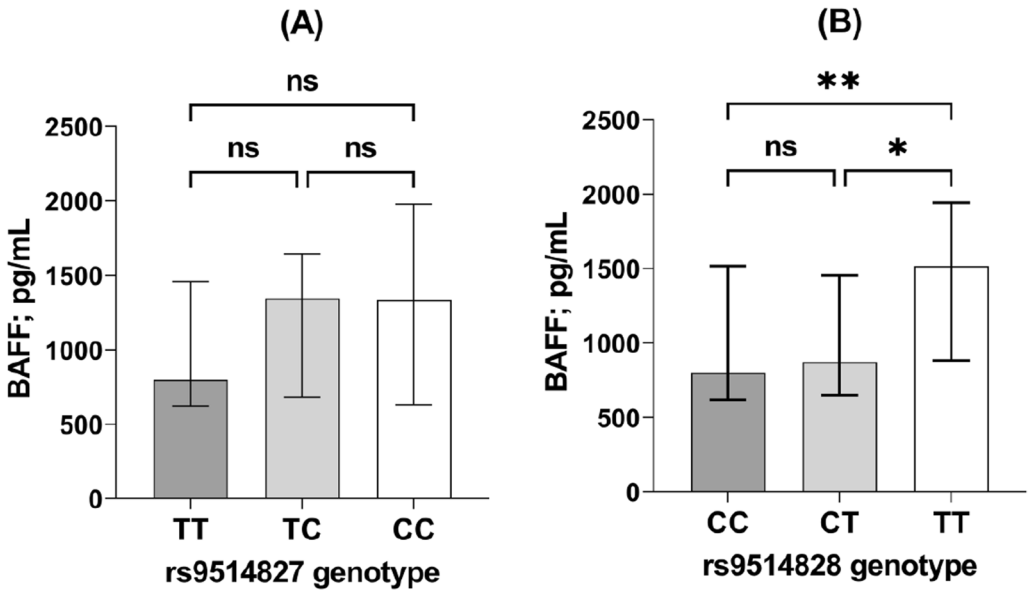

Fig. 3 Column-bar plots of serum B-lymphocyte-activating factor (BAFF) levels stratified by genotypes of *BAFF* single nucleotide polymorphisms (Plot **A**: rs9514827; Plot **B**: rs9514828) in Graves' disease patients plus controls. Columns indicates median, while bars represent interquartile range (IQR). Significance was assessed using Mann–Whitney *U* test (*$p < 0.05$; **$p < 0.01$; ns: not significant). The CC genotype of rs9514827 tended to show higher BAFF levels compared to the TT genotype but the differences were not significant ($p = 0.16$). For rs9514828, the TT genotype was associated with significantly increased serum levels of BAFF compared to the CC and CT genotypes (1516 [IQR: 884–1943] vs. 799 [IQR: 619–1517] and 871 [IQR: 649–1454] pg/mL; $p = 0.005$ and 0.02, respectively), while there was no significant difference between CC and CT genotypes ($p = 0.64$)

treatment with CMZ or CMZ + RAI. This is evidenced by the fact that CMZ is a well-known anti-thyroidal drug that targets the synthesis of thyroid hormones and causes a decrease in their concentrations [38].

In Spearman's correlation analysis, BAFF was found to be positively correlated with TT3 and T4 in GD patients but not in CTRL. In light of this, a functional relationship can be proposed between BAFF and thyroid hormones T3 and T4 in GD patients. This topic has recently been addressed and the results showed that high systemic concentrations of TT3 and T4 are associated with overexpression of BAFF and may have effects on B-cell differentiation [39]. In addition, it has been found that elevated concentrations of TT3 can induce polarization of M1 macrophages and this may lead to increased expression of BAFF [40].

With regard to VitD, although there was no correlation with BAFF, current GD patients were predominantly characterized by VitD insufficiency/deficiency (< 30 ng/mL). Therefore, low concentrations of VitD could be promoted to become a potential risk factor associated with the etio-pathogenesis of GD as recently suggested by our group [16]. Besides being an important component of the endocrine system, studies have indicated that VitD can perform various immune functions and thus may have an essential role in the pathophysiology of autoimmune diseases [41]. However, the relationship between VitD and BAFF in GD has not been investigated, but in patients with Sjögren's syndrome (an autoimmune disorder), BAFF concentrations increased while VitD concentrations decreased, a negative relationship was also found between them but it was not significant [42]. Accordingly, simultaneous evaluation of BAFF and its receptors and VitD may represent a cornerstone in understanding the pathophysiology of GD and further studies are warranted.

Family and twin studies have revealed the role of genetic factors in conferring an individual's susceptibility to GD. In this context, several genetic loci have been described as harboring alleles and genotypes with potential for predisposition to GD [2]. It has been suggested that *TNFSF13B*, the gene encoding BAFF protein, is among such loci that may have a role in determining susceptibility to GD [22]. This proposal motivated us to expand our understanding of the role of BAFF in the pathogenesis of GD by analyzing two promoter SNPs of the *TNFSF13B* gene (rs9514827 and rs9514828) in a cohort of 90 GD patients and 70 CTRL. The two SNPs show moderate linkage disequilibrium and are located in the 5' untranslated region within 5kb of the first exon of the gene [20]. The results were interesting and mutant alleles (rs9514827 *C* and rs9514828 *T*) and genotypes (rs9514827 CC and rs9514828 TT) were associated with an increased risk

of GD. The OR for the corresponding alleles was 2.00 and 2.15, respectively (allele model), and for the corresponding genotypes it was 4.29 and 4.57, respectively (co-dominant model). Furthermore, haplotype analysis revealed that the C-T haplotype (rs9514827-rs9514828) was associated with a 2.71-fold increased risk of GD, whereas the TC haplotype was associated with a reduced risk of the disease (OR = 0.44). In addition, the mutant TT genotype of rs9514828 was associated with elevated serum BAFF concentrations compared to CC and CT genotypes. To date, one previous study has explored the association between the rs9514827 variant and GD in Tunisians, and no significant association with susceptibility to the disease was found. In addition, the rs9514827 genotypes were not associated with serum BAFF concentrations [23]. Other autoimmune diseases, such as SLE and RA, also showed no association with the rs9514827 variant [18, 20]. The present study may be the first to report a significant association between this variant and the risk of GD in Iraqi women. Ethnic difference may be responsible for this discrepancy in results and further studies are warranted. Regarding the rs9514828 SNP, only two studies have investigated the association between this variant and susceptibility to GD. In the first, no association with GD was found in a cohort from the United Kingdom [22], while in the second, the rs9514828 *T* allele was associated with an increased risk of GD in Tunisians [23]. The rs9514828 *T* allele was also found to be more prevalent in RA patients and the rs9514828 variant was considered a risk factor for the disease in a Mexican population. Moreover, the rs9514828 TT genotype was associated with elevated expression of *TNFSF13B* gene [20]. Taken together, these data indicate that the rs9514827 and rs9514828 SNPs may be associated with susceptibility to GD in Iraqi women, in addition to being involved in controlling BAFF production. In this context, rs9514828 polymorphism has been shown to be present in potential transcription factor-binding sites, and thus may affect the regulation of *TNFSF13B* gene expression [43].

The study encountered an important limitation, namely, the low sample size of GD patients (particularly ND patients of whom there were only six) and CTRL. In addition, the effects of BAFF on extrathyroidal manifestations, such as orbitopathy and dermopathy, were not analyzed. Importantly, bioinformatic analysis of the rs9514827 and rs9514828 SNPs with respect to some of the transcription factor-binding sites in the *TNFSF13B* gene was not performed. Such an issue requires extensive investigation and may contribute to understanding the effects of SNP genotypes on BAFF synthesis.

Conclusions

BAFF showed up-regulated levels in the serum of women with GD. In light of this, BAFF has been proposed as a reliable prognostic biomarker for GD. Regarding its relationship to thyroid hormones, BAFF showed a positive correlation with triiodothyronine and thyroxine. Both variants (rs9514827 and rs9514828) of the *TNFSF13B* gene showed an association with susceptibility to GD, and rs9514828 may have up-regulatory effects on BAFF levels.

Abbreviations

ANOVA	One-way analysis of variance
AUC	Area under curve
BAFF	B-lymphocyte-activating factor
BMI	Body mass index
BR3	BAFF receptor 3
CI	Confidence interval
CMZ	Carbimazole
CTRL	Controls
GD	Graves' disease
HWE	Hardy–Weinberg equilibrium
INSFT	Insufficient
IQR	Interquartile range
MLR	Multinomial logistic regression
ND	Newly diagnosed
NOR	Normal weight
O/O	Overweight/obese
p	Probability
pc	Corrected probability
PCR	Polymerase chain reaction
RA	Rheumatoid arthritis
RAI	Radioiodine
RFLP	Restriction fragment length polymorphism
ROC	Receiver-operating characteristic
r_s	Correlation coefficient
SD	Standard deviation
SFT	Sufficient
SLE	Systemic lupus erythematous
SNP	Single nucleotide polymorphism
T4	Thyroxine
TACI	Transmembrane activator and calcium-modulating and cyclophilin ligand interactor
TRAb	TSH receptor antibodies
TSH	Thyroid stimulating hormone
TT3	Total triiodothyronine
VitD	Vitamin D
YI	Youden index

Acknowledgements

We are grateful to the medical staff at the Baghdad Center for Radiotherapy and Nuclear Medicine (Baghdad Medical Complex) for their kind cooperation during the completion of this study.

Author contributions

HYI, GMS and MSA contributed to laboratory work, data handling, writing and revising the manuscript. AHA managed data, carried out statistical analyses and wrote the manuscript. All authors read and approved the final manuscript.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and materials

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

Declarations

Ethics approval and consent to participate

The participants provided their written informed consent to be included in the study. The study protocol was approved by the Ethics Committee at the Baghdad Medical Complex (Iraqi Ministry of Health and Environment; Reference No. 4923, January 31, 2022).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 12 August 2023 Accepted: 13 November 2023
Published online: 17 November 2023

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