ORIGINAL ARTICLE

Expression profile of interferon-gamma (IFN- γ) mRNA as diagnostic molecular signatures of Hashimoto's Thyroiditis

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ABSTRACT

Background: *Hashimoto's thyroiditis (HT) is a T cell-mediated autoimmune disease that primarily affects females. IFN- γ is a critical cytokine that has been related to the pathogenesis of HT.* ***Objectives****: We aimed to evaluate serum and expression levels of interferon-gamma (IFN- γ) in Egyptian women with HT and to assess the association between serum and expression levels of IFN- γ with clinical and laboratory characteristics of HT.* ***Methodology****: This case-control study included 120 women with HT and 70 controls. IFN- γ mRNA expression was analyzed using real-time polymerase chain reaction. Serum IFN- γ was measured using enzyme-linked immunosorbent assay.* ***Results:*** *Serum IFN- γ level and the level of IFN- γ mRNA are both sensitive and specific to be used as diagnostic markers for HT with cut off values of 28.57 pg/ml and 3.55 respectively. Both showed a significant positive correlation with TPO-Ab and Tg-Ab, obesity indices, dyslipidemia, and TSH, while they have a negative correlation with FT3, FT4.* ***Conclusions****: Serum IFN- γ level and the level of IFN- γ mRNA are both sensitive and specific to be used as diagnostic markers for HT, significantly correlated with thyroid autoantibodies and thyroid function tests.*

INTRODUCTION

Hashimoto'sthyroiditis (HT), is a chronic thyroid inflammation that affects women predominantly. [1,2] It is regarded as the most common organ-specific autoimmune disease. [3,4,5] It is characterized by follicular lymphocytic infiltration in the thyroid gland with germinal centres' formation, atrophy of the follicular epithelial cells, Hurthle cell change, and gradual fibrous replacement of the thyroid parenchyma. [6] Disturbed balance of pro-and anti-inflammatory cytokines play an essential role in autoimmune thyroid diseases' pathogenesis. Correlation between the high level of Interferon (IFN- γ) and autoimmune thyroiditis has been widely established. [7]

Interferon (IFN)-γ- released from the infiltrating lymphocytes during the autoimmune process induces the expression of human leukocyte antigen (HLA) class II on thyroid follicular cells, increases the production of intra-thyroidal cytokines, augments the interactions between chemokines and their receptors, and the interactions between co-stimulatory molecules. Moreover, it inhibits apoptosis and thus shares in the propagation of the thyroid inflammatory autoimmunity process. Consequently, activated CD4+ and CD8+ T cells, macrophages, B cells, and plasma cells accumulate in the thyroid. Destruction of thyroid cells in HT is also associated with antibody-mediated immune processes, including thyroid autoantibodies against thyroid peroxidase (TPO) and thyroglobulin (Tg).[8]

This study aims to make use of the serum level of IFN- γ and the level of IFN-γ gene expression as diagnostic markers of HT. Additionally, we aimed to find the association between the level of them and other markers used in HT diagnosis as diagnostic anti-thyroid antibodies and thyroid hormones.

METHODOLOGY

This case-control study included 120 women with HT and 70 healthy women matched to the case group regarding age and ethnic origin.

Inclusion Criteria:

- The control group included healthy individuals.
- The case group included adult females suffer from HT. We recruited patients from outpatient clinics of the Endocrinology Unit of Internal Medicine Department, Faculty of Medicine, Zagazig University, Egypt. Diagnosis of HT was obtained based on clinical findings and serum anti-thyroid antibodies; anti-TPO-Ab and/or anti-Tg-Ab.

Exclusion criteria:

We excluded women with a history of hyperandrogenic states (such as Cushing's syndrome, non-classical congenital adrenal hyperplasia, 21-hydroxylase deficiency, and androgen-secreting tumours), liver or kidney diseases, hypertension, angina,

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myocardial infarction, and stroke. We also excluded pregnant patients.

All participants underwent complete history taking, thorough clinical examination, full clinical assessment and anthropometric measures. Thyroid function tests assessed thyroid dysfunction.

Blood sampling and testing

We collected 5 ml of blood samples between days 3 and 6 of the menstrual cycle Samples were divided into two portions: 1 ml of whole blood was collected into EDTA tubes, for RNA extraction. The remaining were collected into plain evacuated tubes for sera separation. We stored sera at-20°C until analysis.

- **Thyroid function test and anti-thyroid autoantibody levels**

To assess thyroid function, the serum concentration of thyrotrophin (TSH), free T3 (FT3), and free T4 (FT4) by was measured by Electrochmeiluminescence on Cobas 6000 analyzer (ROCHE DIAGNOSTICS). Anti-thyroid autoantibody levels including anti-TPO and anti-TG were measured using Accu-Bind ELISA kit (Monobind Inc., Lake Forest, CA 92630, USA), Accu-Bind ELISA kit (Monobind Inc., Lake Forest, CA 92630, USA) respectively.

- **Serum IFN- γ level assay**

Serum IFN- γ level was assayed using Accu-Bind ELISA kit (Monobind Inc., Lake Forest, CA 92630, USA).

- **IFN- γ gene expression**

RNA isolation, cDNA preparation

According to the manufacturer's protocol, RNA extraction was done using total RNA Purification kit (Jena Bioscience, Germany). The SCRIPT Reverse Transcriptase kit (Jena Bioscience, Germany) was used to produce the first-strand cDNA, M-MLV RT enzyme and Oligo (dT) primer were used. In ice, we mixed 10 µl RNA template, and 1 µl Oligo-(dT) primer together, then 1 µl dNTP Mix, 4 µl SCRIPT RT buffer, 1 µl RNase inhibitor, 1 µl Dithiothreitol stock solution, 0.5 µl SCRIPT reverse transcriptase and1.5 µl RNase-free water were added then incubated at 30°C for 10 min and 50°C for 60 min. The cDNA was stored at -20°C for analysis.

Quantitative real-time polymerase chain reaction (RT-PCR)

Stratagene Mx3005P qPCR System (Agilent Technologies, Germany) was used for quantitative real-time RT-PCR for IFN- γ gene using the qPCR Green Master (Jena Bioscience, Germany).

PCR reaction with 20 µl final volume was prepared by adding 10 µl qPCR Green Master, 0.5 µl forward primer (10 µM) with a sequence of 5'-GCATCCAAAAGAGTGTGGAG-3', 0.5 µl reverse primer (10 µM) with a sequence of 5'-GACAGTTCAGCCATCACTTGG-3', 5 µl template cDNA and 4 µl PCR grade water were all added into real-time PCR wells. The cycling program was prepared according to 95º C for 10 min, then 40 cycles each is composed of (95º C for 15 secs, 58º C for 1 min. β-actin gene (forward primer 5′-TTG CCG ACA GGA TGC AGA A-3′ and reverse primer 5′-GCC GAT CCA CAC GGAGTA CT-3′) was used as the reference gene. We normalized the quantity of the target gene w by subtracting the cycle threshold (CT) for β -actin from that of the target gene (ΔCT sample). The same calculation was done with values of controls (ΔCT control). Subsequently, ΔΔCT was estimated as the difference of (ΔΔCT = ΔCT sample – ΔCT control). The relative expression was considered as fold change by a 2−ΔΔCT relative to that of the control.

Statistical Analysis

We statistically analyze data using the Statistical Package for the Social Sciences for Windows (version 21.0; SPSS, Chicago, USA).

RESULTS

Clinical and laboratory characteristics of the studied groups.

Patients with HT had significantly higher BMI values, waist/hip ratio, and controls. As expected, patients with HT had significantly lower levels of FT3, and FT4 compared with controls. On the contrary, TSH, anti-TPO and anti-TG were significantly higher in HT patients than in control. Regarding serum IFN- γ, IFN- γ mRNA, they were significantly higher in HT group cases than controls (Table 1, P< 0.05).

Table 1: Anthropometric and biochemical characteristics in HT patients

Parameter	Controls group (*n*=70)	HT group, (*n*=120)	*P* value
Age (years)	25.04± 9.63	27.6±9.35	0.113
Systolic blood pressure (mmHg)	124.34±6.25	125.52±7.07	0.473
diastolic blood pressure (mmHg)	76.4±6.95	77.68±6.2	0.718
Waist/hip ratio	0.98±0.14	1.18±0.23	<0.001*
BMI (kg/m²)	21.93±1.98.	26.74±4.56	<0.001*
TG (mg/dl)	142.4±8.85	149.64±7.56	<0.001*
FT3(pg/ml)	2.57±0.51	2.16±0.14	<0.001*
FT4(ng/dl)	1.68±0.14	1.13±0.51	<0.001*
TSH (µIU/ml)	2.87±0.14	8.16±1.3	<0.001*
Anti TPO(IU/ml)	49.01±2.4	272.8±68.6	<0.001*
Anti TG (IU/ml)	0.58±0.14	4.16±1.19	<0.001*
IFN- γ (pg/mL)	1.44±0.040	27.02±7.02	<0.001*
IFN- γ mRNA	1.3±0.05	3.03±0.69	<0.001*

The Accuracy of circulating serum IFN- γ (pg/mL), for the diagnosis of HT by ROC analysis

The power of serum IFN- γ to diagnose HT among the studied group was evaluated using ROC analysis. The AUC was 0.944 (95% CI = 0.896–0.992) with sensitivity = 90.5%, specificity = 99.3%, and the cutoff values (28.57), (Figure

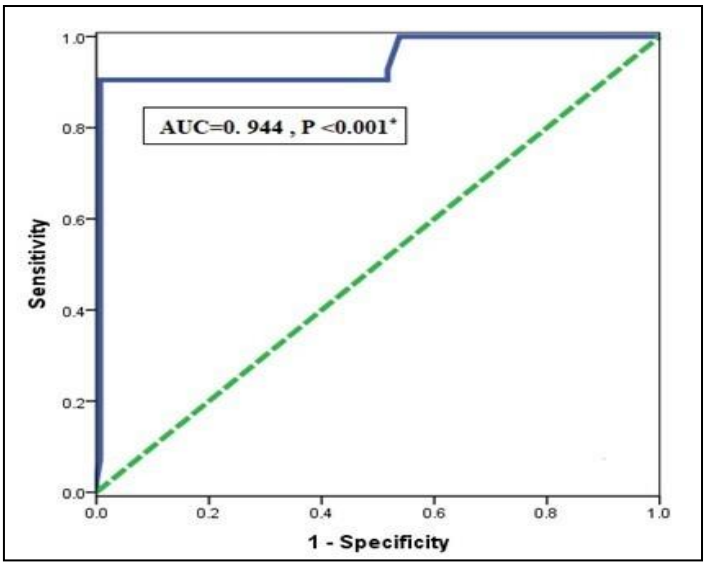

Fig. 1: The Accuracy of circulating serum IFN- γ (pg/mL), for the diagnosis of HT by ROC analysis

The Accuracy of circulating IFN- γ gene expression for diagnosis of HT and UEI by ROC analysis

The power of circulating IFN- γ gene expression to diagnose HT among the studied group was evaluated using ROC analysis. The AUC was 0.970 (95% CI = 0.944–0.995) with sensitivity = 92.9%, specificity = 99.6%, and the cutoff values (3.55), (Figure 2).

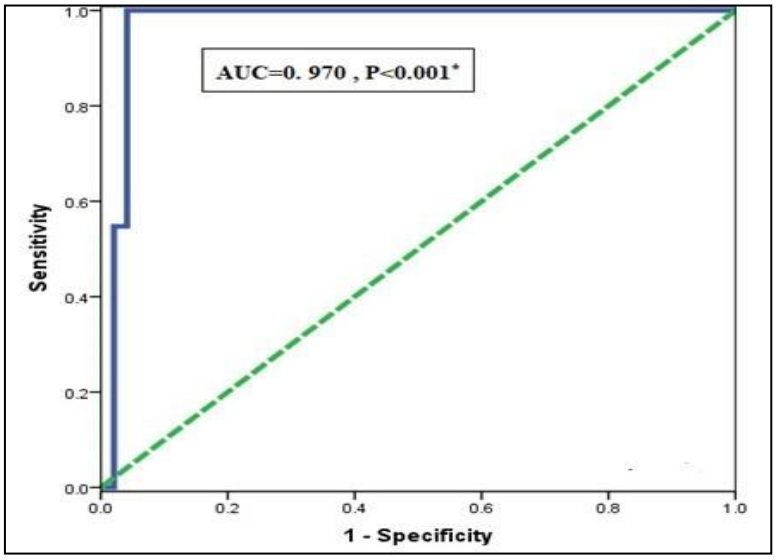

Fig. 2: The Accuracy of circulating IFN- γ gene expression for the diagnosis of HT and UEI by ROC analysis

The Accuracy of the combination of circulating serum IFN- γ and IFN- γ gene expression for diagnosis of HT and UEI by ROC analysis

Regarding the combination of circulating serum IFN- γ and IFN- γ gene expression for diagnosing HT among the studied group. The AUC was 0.964 (95% CI = 0.931–0.997) with sensitivity = 92.9%, specificity = 99.6%, Figure 3).

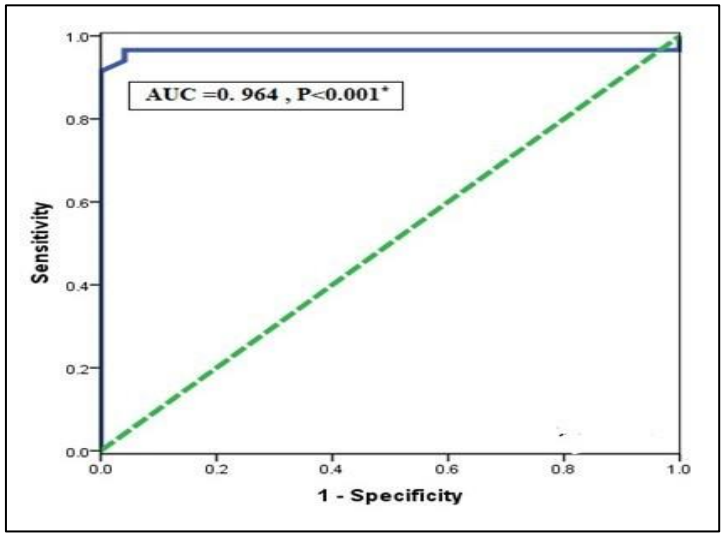

Fig. 3: The Accuracy of the combination of circulating serum IFN- γ and IFN- γ gene expression for the diagnosis of HT and UEI by ROC analysis

Correlation between serum IFN- γ and IFN- γ gene expression with clinical and laboratory characteristics among women with HT

There was significant positive correlation with serum IFN- γ with systolic blood pressure, BMI, waist/hip ratio, TSH, LH, anti-TPO, and anti-TG. On the other hand, there was significant negative correlation with FT3 and FT4 (Table 2, <0.001*).

Regarding IFN-γ gene expression, there was significant positive correlation with diastolic blood pressure, BM I, TC, TSH, LH, AFC, anti-TPO, and anti-TG on the other hand, there was a significant negative correlation with FT3, and FT4 (Table 2), P<0.001*.

Table 2: Correlation between IFN- γ serum and gene expression levels with clinical and laboratory characteristic among HT groups.

Variable	Serum IFN- γ		IFN- γ gene expression	
	r	p	r	p
SBP (mmHg)	0.394	<0.001*	0.129	0.286
DBP (mmHg)	0.157	0.231	0.497	<0.001*
Waist/hip ratio	0.271	<0.001*	0.015	0.894
BMI (kg/m^2)	0.386	<0.001*	0.344	<0.001*
TG (mg/dl)	0.221	0.090	0.016	0.895
FT3(pg/ml)	-0.859	<0.001*	-0.942	<0.001*
FT4(ng/dl)	-0.608	<0.001*	-0.729	<0.001*
TSH (μIU/ml)	0.704	<0.001*	0.780	<0.001*
Anti TPO(IU/ml)	0.612	<0.001*	0.682	<0.001*
Anti TG (IU/ml)	0.692	<0.001*	0.815	<0.001*

TSH: thyroid-stimulating hormone, FT3: free triiodothyronine, FT4: free thyroxine, Anti TG: anti-thyroglobulin antibodies, anti-TPO: anti-thyroid peroxidase antibodies; HT: Hashimoto thyroiditis.* P < 0.05

Linear regression analyses in HT patients to test the influences of the main independent variables against serum IFN- γ (dependent variable).

Linear regression analysis test showed that TSH and FT3 were the only variables independently correlated with serum IFN- γ (P< 0.001).

Linear regression analyses in HT patients to test the influences of the main independent variables against circulating IFN- γ gene expression (dependent variable).

Linear regression analysis test showed that FT3 was the only variable independently correlated with circulating IFN- γ gene expression (P< 0.001).

DISCUSSION

Gathering studies have reported that increased expression of IFN-γ results in cell-mediated immune destruction of many organs, leading to activation of humoral immune response and increased cytokine production, leading to initiation or progression of autoimmune disease. IFN-γ is a potent macrophage activator and promotes inflammation and effector $CD8^+$ T cell cytotoxicity. Moreover, IFN-γ contributes to the amplification of the immune response by inducing MHC class II molecules' expression on thyroid cells.[9]

To our knowledge, this is the first study proposes to use circulating serum and expression levels of IFN- γ as diagnostic markers for HT and find out their possible associations with clinical and laboratory characteristics of thyroid disease.

As expected, in the present study, women with HT had significantly higher values of obesity indices and TSH. On the other hand, they had significantly lower levels of FT3, FT4, and compared with controls.

Serum IFN- γ level and the level of IFN- γ mRNA expression were significantly higher in HT group cases than controls. Similar results were obtained by Qin et al. how found a statistically significant increase in the expression of IFN- γ mRNA compared to the control. [10,11] Similar results were obtained from other research groups who also relate the morphological and functional alteration that occur in HT to Th1 cytokines predominantly IFN- γ. [12,13,14,15,16] The crucial role of IFN- γ in the pathogenesis of HT has been illustrated by founding that aggravation of the disease can occur through increase the concentration of IFN- γ. This can be achieved by systemic injection of IFN- γ, [17] or blocking CTLA molecules which subsequently activate T effector lymphocytes, [18] while a systemic infusion of a mAb neutralizing IFN- γ abrogated EAT. [19]

On the other hand, other studies suggest that IFN- γ was shown to suppress EAT because a more severe form of thyroiditis occurred when IFN- γ was blocked by systemic injection of a neutralizing Ab. [20] Also, mice lacking the IFN- receptor developed EAT equally well, [21] this immune-suppressive mechanism of INF can be explained by the ability of the IFN to induce inhibitory molecules or induce apoptosis of the activated T cell. [22] However, all these experimental studies were conducted on transgenic mice in which each bread has its specific immunological profile, and thus their results cannot be easily generalized.

We investigated our results by ROC test to assess the power of serum IFN- γ and IFN- γ gene expression in differentiating patients with HT from the control group. According to our results, the ability of serum IFN- γ to diagnose HT among the studied group was both sensitive and specific (sensitivity = 90.5%, specificity = 99.3%) with a cutoff value of 28.57 pg/ml. Moreover, both sensitivity and specificity increase when IFN- γ gene expression was used (sensitivity = 92.9%, specificity = 99.6%), with cutoff values (3.55). When we made a combination of circulating serum IFN- γ and IFN- γ gene expression for diagnosing HT among the studied group, we found that the sensitivity and specificity remain IFN- γ gene expression.

To evaluate the associations between serum IFN- γ and IFN- γ gene expression with clinical and laboratory characteristic among women with HT, there was a significant positive correlation with thyroid autoantibodies (TPO-Ab and Tg- ab) as studies potentiate these associations by other researchers. Karanikas et al. found that the level of IFN-γ expression is positively related to the disease and anti-TPO titre activity.[9] This can be explained by the fact that after initiating the immune response thyroglobulin, T lymphocytes specific to thyroid tissue migrate to the thyroid. IFN-γ production induces increases expression of thyrocyte MHC class-II molecules. This leads to the expansion of autoreactive T cells, the inflammatory response, and accumulation of activated macrophages CD4+ and CD8+ T cells, B cells, and plasma cells in the thyroid tissue. [23]

Serum IFN-γ and IFN-γ gene significantly correlated with obesity indices, dyslipidemia, and TSH while there was a negative correlation with FT3, FT4. A research work supported these findings by Kimura et al., who found a positive correlation between IFN- γ, low level of total T4 and higher levels of TSH.[24] This can be explained by the fact that cell-mediated immunity stimulates the induction of autoantibodies and self-reactive T cells against Tg, and thyroid peroxidase. Thyroid infiltration of lymphocytes and other immune cells, thyroid enlargement and fibrosis, and the progressive destruction of thyrocytes eventually result in hypothyroidism. [25]

In this study, TSH and FT3 were the only variables independently associated with serum IFN- γ while FT3 was only variables independently associated with IFN- γ gene expression by linear regression analysis. These findings need further studies to be elucidated.

CONCLUSION

We conclude that Serum IFN- γ level and the level of IFN- γ mRNA are both sensitive and specific to be used as diagnostic markers for HT with cut off values of 28.57 pg/ml and 3.55 respectively. They showed a significant positive correlation between TPO-Ab and Tg- ab, obesity indices, dyslipidemia, and TSH, while they negatively correlated with FT3, FT4. TSH and FT3 were the only variables independently associated with serum IFN- γ while FT3 was only independently associated with IFN- γ gene expression. We recommend other studies to test the potential use of serum IFN- γ and IFN- γ gene expression as HT's diagnostic markers.

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

Ethics approval and consent to participate

Written informed consent was taken from all of the participants. The ethical committee of Faculties of Medicine, of Zagazig, University approved this study. The reference number is not applicable.

REFERENCES

1. Zhu C, Ma J, Liu Y, et al. Increased frequency of follicular helper T cells in patients with autoimmune thyroid disease. J Clin Endocrinol Metab. 2012; 97: 943–950.
2. Vanderpump MP. The epidemiology of thyroid disease. Br Med Bull. 2011;99: 39–51.
3. Jacobson DL, Gange SJ, Rose NR, et al. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. Clin Immunol Immunopathol. 1997;84: 223–243.
4. Caturegli P, De Remigis A, Rose NR. Hashimoto thyroiditis: clinical and diagnostic criteria. Autoimmun Rev. 2014;13: 391–397.
5. McLeod DS, Cooper DS. The incidence and prevalence of thyroid autoimmunity. Endocrine. 2012; 42: 252–265.
6. Tomas T, Sreedharan S, Khadilkar UN, D Deviprasad et al. Clinical, biochemical & cytomorphologic study on hashimoto's thyroiditis. Indian Journal of Medical Research. 2014;140: 729–735.
7. Bossowski A , Harasymczuk J, Moniuszko A, et al. Cytometric evaluation of intracellular IFN-g andIL-4 levels in thyroid follicular cells from patients with autoimmune thyroid diseases. Thyroid Research. 2011;4:13.
8. Mansoor A, Magtooph M. The Role of Interferon (IFN- γ) in thyroid autoimmunity. Journal of Education For Pure Science-University Of Thi-Qar 2019;9(1):221-226.
9. Karanikas G, Schuetz M, Wahl K, et al. Relation of anti-TPO autoantibody titre and T-lymphocyte cytokine production patterns in Hashimoto's thyroiditis. Clin Endocrinol 2005;63(2):191-196.
10. Qin Q, Liu P, Liu L, et al. The increased but non-predominant expression of Th17- and Th1-specific cytokines in Hashimoto's thyroiditis but not in Graves' disease Braz J Med Biol Res 2012; 45(12);1202-1208.
11. Lu Q, Luo X, Mao C, et al. Caveolin-1 regulates autophagy activity in thyroid follicular cells and is involved in Hashimoto's thyroiditis disease. Endocr J 2018;65(9):893-901.
12. Rasmussen AK, Kayser L, Feldt-Rasmussen U, et al. Influence of tumour necrosis factor-alpha, tumour necrosis factor-beta and interferon-gamma, separately and added together with interleukin-1 beta, on the function of cultured human thyroid cells. J Endocrinol 1994;143:359–365.
13. Ajjan RA, Watson PF, McIntosh RS, et al.Intrathyroidal cytokine gene expression in Hashimoto's thyroiditis. Clin Exp Immunol 1996; 105: 523–528.
14. Paschke R, Schuppert F, Taton M, et al. Intra-thyroidal cytokine gene expression profiles in autoimmune thyroiditis. J Endocrinol 1994;141: 309–315.
15. Weetman AP. Cellular immune responses in autoimmune thyroid disease. Clin Endocrinol 2004;61: 405– 413.
16. Rekha PL, Ishaq M, Valluri V. A differential association of interferon-gamma high-producing allele T and low-producing allele A (+874 A/T) with Hashimoto's thyroiditis and Graves' disease. Scand J Immunol. 2006;64(4):438-443.
17. Kawakami Y, Kuzuya N, Watanabe T, et al. Induction of experimental thyroiditis in mice by recombinant interferon administration. Acta Endocrinol. 1990;122:41.
18. Sharma R, Di Dalmazi G, Caturegli P. Exacerbation of Autoimmune Thyroiditis by CTLA-4 Blockade: A Role for IFNγ-Induced Indoleamine 2, 3-Dioxygenase. Thyroid 2016;26(8):1117-1124.
19. Tang H, Mignon-Godefroy K, Meroni PL, et al. The effects of a monoclonal antibody to interferon-on experimental autoimmune thyroiditis (EAT): prevention of disease and decrease of EAT-specific T cells. Eur. J. Immunol.1993;23:275.
20. Stull SJ, Sharp GC, Kyriakos M, et al. induction of granulomatous experimental autoimmune thyroiditis in mice within vitro activated effector T cells and anti-IFN- antibody. J. Immunol. 1992;149:221
21. Alimi, E, Huang S, Brazillet MP, et al. Experimental autoimmune thyroiditis (EAT) in mice lacking the IFN- receptor gene. Eur. J. Immunol. 1998;28:201.

22. Jobert GB, Afanasyeva M, Talor MV, et al., Thyroid-Specific Expression of IFN-g Limits Experimental Autoimmune Thyroiditis by Suppressing Lymphocyte Activation in Cervical Lymph Nodes J Immunol. 2003;170:5523-5529.
23. Ganesh BB, Bhattacharya P, Gopisetty A, et al. Role of Cytokines in the Pathogenesis and Suppression of Thyroid Autoimmunity. Journal of Interferon & Cytokine Research 2011;31:10721-10731.
24. Kimura H, Kimura M, Westra WH, et al. Increased thyroidal fat and goitrous hypothyroidism induced by interferon-gamma. Int J Exp Pathol 2005;86 (2):97-106.
25. Weetman AP. Autoimmune thyroid disease: propagation and progression. Eur J Endocrinol. 2003;148(1):1–9.