

Evaluation and epigenetic impact of B12, vitamin D, folic acid and anemia in Hashimoto's thyroiditis: a clinical and molecular docking study

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Cite this article as: Aslan ES, Gür S. Evaluation and epigenetic impact of B12, vitamin D, folic acid and anemia in Hashimoto's thyroiditis: a clinical and molecular docking study. *J Health Sci Med.* 2023;6(4):705-712.

Received: 28.01.2023

Accepted: 28.05.2023

Published: 30.07.2023

ABSTRACT

Aims: Our approach in this study is to investigate the collective effect of various parameters, including vitamin B12 (B12), vitamin D (Vit-D), folic acid, and iron deficiency, on Hashimoto's thyroiditis (HT) disease. This differs from existing literature that has examined these parameters individually.

Methods: The study evaluated age, gender, thyroid stimulating hormone (TSH), free-T4 (FT4), free-T3 (FT3), Vit-D levels, as well as autoantibodies against thyroid peroxidase (anti-TPO) and anti-thyroglobulin iron (anti-TG) levels, ferritin, B12, and folic acid parameters in a total of 30 HT patients and 37 non-HT patients. These parameters were assessed by analyzing the patients' routine blood test results using automated analysis methods.

Results: A negative correlation was found between the blood, Vit-D level and anti-TG (as the vit-D value increases, the anti-TG decreases) ($r=-0.417$; $p=0.001$; $p<0.01$). There was a statistically significant and weak correlation between blood Vit-D measurements and anti-TPO measurements ($r=-0.341$; $p=0.005$; $p<0.01$).

Conclusion: The study findings demonstrated that there was no statistically significant difference in the measurements and correlation between hemoglobin, FT3, FT4, TSH, ferritin, vitamin B12, and folic acid levels in patients with and without a diagnosis of HT ($p>0.05$). However, the study emphasized the critical role of vitamin D in the pathophysiology and treatment of HT. Furthermore, molecular docking simulations indicated that folic acid could potentially act as a potent inhibitor of human extracellular signal-regulated kinase (ERK2), which has been reported to play a key role in HT.

Keywords: Hashimoto's thyroiditis, otoimmun disease, vitamin D, anti-TPO, ferritin, vitamin B12

INTRODUCTION

Hashimoto's thyroiditis (HT) is the most common autoimmune thyroid disease. Approximately 20-20% of the cases are attributed to genetic predisposition, while remaining causes are linked to environmental conditions and epigenetic factors. In studies focusing on autoimmune thyroid diseases, it has been observed that HT leads to the overexpression of certain genes involved in immune function or the activation of immune cells. Furthermore, research has shown that epigenetic mechanisms, such as DNA methylation, histone modification, and miRNA, play a role in regulating specific genes, which may contribute to the autoimmune attack against thyroid tissues and leads to appearance of the disease.

The histological features of the autoimmune thyroiditis include follicular destruction, granular atrophy, and

fibrosis due to lymphocytic infiltration of T cells.¹ HT is characterized by varying levels of clinical hypothyroidism and the presence of autoantibodies against thyroid peroxidase (anti-TPO) and thyroglobulin (anti-thyroglobulin). This disease is 4-10 times more common in women than men, particularly in the age range of 30-50 years.² The predominant antibody in autoimmune hypothyroidism, anti-TPO, is an essential biomarker, as it is present in over 90% of patients.³

Thyroid disorders may occur due to deficiencies in iodine, selenium, iron, zinc, minerals and vitamins A, C, B6, B5, and D, all of which are necessary for thyroid hormone synthesis and metabolism.^{4,5} Environmental factors contribute to the pathogenesis of autoimmune thyroid disease at a rate of 20-30%, while genetic factors account for 70%.⁶ Polymorphisms in the vitamin D receptor

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(VDR) gene predispose individuals to conditions such as Addison's disease, type 1 diabetes, and autoimmune diseases, including Hashimoto's, under the influence of environmental conditions and epigenetic factors. Studies on cytokine genes, such as interferon-gamma (IFN- γ), interleukin-4 (IL-4), and transforming growth factor beta (TGF- β), which regulate the balance between T-helper 1 (Th1) and T-helper 2 (Th2) mechanisms, have shown a correlation with the development and severity of Hashimoto's.⁷

Although epigenetic mechanisms, such as DNA methylation, miRNA and histone modifications, have been demonstrated in some genes associated with autoimmune thyroid diseases, the precise molecular mechanisms underlying these epigenetic changes are still not fully understood.

This study aimed to evaluate the frequency of B12, vitamin D, folic acid, and iron deficiency, which play a role in the pathogenesis and epigenetic effects on HT, compared to individuals without thyroid disease.

METHODS

The study was carried out with the permission of Biruni University Clinical Researches Ethics Committee (Date: 09.04.2021, Decision No: 2021/50-40). All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki.

Patients who applied to the outpatient clinic between January and December 2021 and 67 patients between the ages of 21 and 72 who were diagnosed with HT were included in the study.

Patients with no known chronic disease, no history of drug use, insulin resistance and autoimmune HT were included in this study retrospectively. Anti-TPO and/or anti-TG antibody positivity was considered as the main condition in terms of autoimmune thyroiditis.

Patients who had undergone thyroidectomy, radioactive iodine (RAI) treatment, had malabsorption syndromes, gastrectomy, ileal resection, followed a vegetarian diet, were pregnancy, or had received B12, Folic acid, iron, and Vit-D supplementation in the last six months were excluded from the study.

Patients with 30 Hashimoto's and 37 non-Hashimoto's control groups were compared in terms of gender, age, TSH, FT4, FT3, Vit-D, anti-TPO, anti-TG and iron levels. Then, patients were divided into three groups according to their Vit-D levels: <10ng/mL (severe deficiency), 10-19 ng/mL (mild to moderate deficiency), and 20-50 ng/mL (optimum levels). The relationship between Vit-D and anti-TPO levels of the group was checked to understand the correlation between them. B12 values were also

evaluated as 180-914 ng/L and folate deficiency found as <4.0 mcg/L between groups.⁸

G-Power Analysis

According to the literature, autoimmune Graves' disease has been reported to occur in approximately 77% of women.⁹ When the expected rate for the variable was considered to be 60%, a power analysis using R (software/programming-version 3.6.2 – CRAN) determined that a minimum of 65 patients in total should be included to achieve a 90% power.

Statistical Analysis

The statistical analysis was conducted using the NCSS (Number Cruncher Statistical System) 2007 program from Kaysville, Utah, USA. Descriptive statistical methods, including mean, standard deviation, median, frequency, percentage, minimum, and maximum, were employed to evaluate the study data. The Shapiro-Wilk test and visual examinations were used to assess the normal distribution of quantitative data. The independent groups' t-test was utilized to compare two groups with normally distributed quantitative variables, while the Mann-Whitney U test was employed for non-normally distributed quantitative variables. For comparing qualitative data, the Pearson chi-square test and Fisher-Freeman-Halton exact test were utilized. Diagnostic screening tests, such as sensitivity, specificity, positive predictive value, and negative predictive value, along with receiver operating characteristic (ROC) analysis, were performed to determine the cut-off value for the parameters. Statistical significance was accepted as $p < 0.05$.

- **Sensitivity:** The test can identify patients among actual patients.
- **Specificity:** The test can identify the healthy people among the real healthy people.
- **Positive predictive value (PKD):** The probability that the patient/subject has the disease/condition when restricted to those patients/subjects who test positive.
- **Negative predictive value (NKD):** The probability that the patient will not have the disease/condition when restricted to all patients/subjects who test negative.

Molecular Docking

All calculations were carried out using Schrödinger suite (Schrodinger, In. Version 2022-2, LLC). A high-resolution Human extracellular signal-regulated kinase 2 (ERK2) crystal structure (PDB ID: 5NHH, 1.94 Å) was downloaded from protein data bank and prepared. The preparation protocol includes adding missing hydrogen bonds, correction of bond orders, optimization and finally, minimization of whole

protein to avoid steric clashes. Prior to optimization step, water molecules were removed but the co-crystallized ligand (native ligand) were kept which were used as reference for binding site generation using Grid module. Folic acid and native ligand were also prepared using LigPrep module, see **Figure 1**. All possible 3D conformations, tautomers, ionization states at physiological conditions were generated. Glide XP method was used in molecular docking.

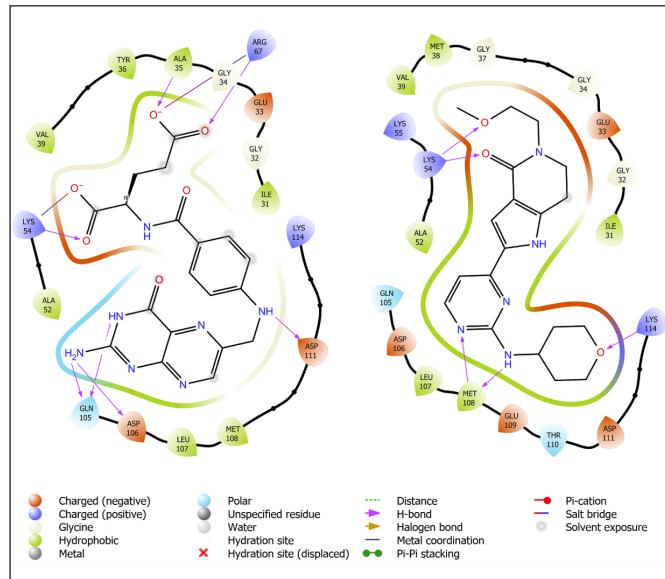


Figure 1. Ligand interaction diagram of folic acid (left) and co-crystallized ligand with ERK2.

RESULTS

The study was conducted with a total of 67 cases, 74.6% (n=50) female and 25.4% (n=17) male, who applied to the outpatient clinic between January 2021 and December 2021 (**Figure 2**).

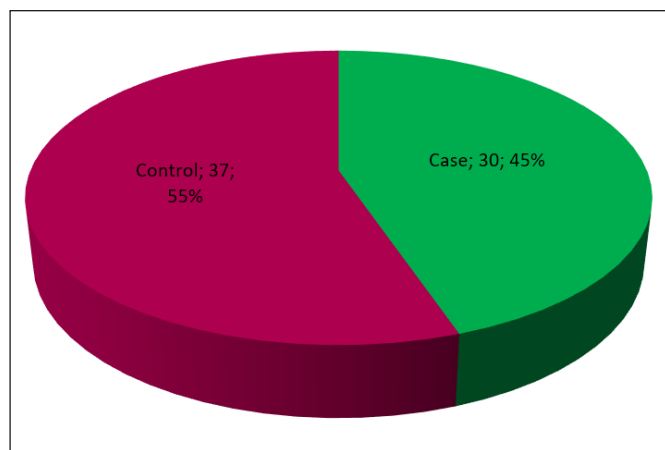


Figure 2. Distribution of groups

The ages of the cases ranged from 21 to 75, with a mean age of 43.61±13.19 (**Table 1, 2**).

Table 1. Evaluation of descriptive characteristics by groups

	Total	Grup	
		Patients (n=30)	Control (n=37)
Sex			^a 0.140
Female	50 (74.6)	25 (83.3)	25 (67.6)
Male	17 (25.4)	5 (16.7)	12 (32.4)
Age			^b 0.374
Avr±Ss	43.76±13.19	45.37±13.38	42.46±13.06
Median (Min-Max)	42 (21-75)	44.5 (28-75)	42 (21-68)

^aPearson Chi-Square Test, ^bStudent T Test

The ages and genders of the cases did not show a statistically significant difference according to the groups (p>0.05).

Anti-TPO measurements in the patient group were statistically significantly higher than those in the control group (p=0.001; p<0.01).

Anti-thyroglobulin measurements in the patient group were statistically significantly higher than those in the control group (p=0.001; p<0.01).

Vit-D measurements (**Figure 3**) in the patient group were statistically significantly lower than those in the control group (p=0.004; p<0.01).

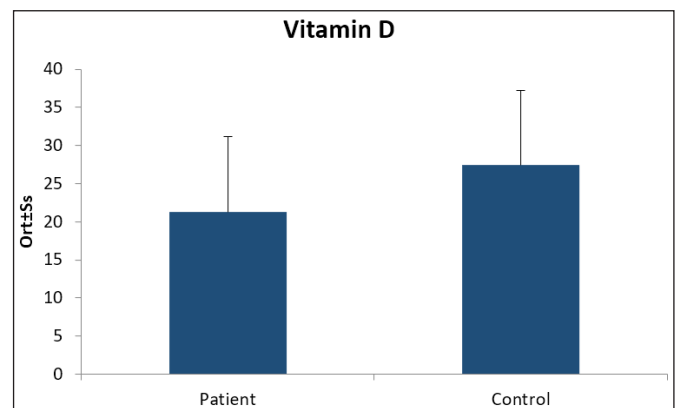


Figure 3. Distribution of vitamin-D measurement by groups

Haemoglobin, FT3, FT4, TSH, Ferritin, Vitamin B12, and Folic acid measurements of the cases did not show a statistically significant difference according to the groups (p>0.05).

For the 20.1 cut-off value of the anti-thyroglobulin level, sensitivity is 92.86%; specificity is 83.78%; positive predictive value is 81.20%, and negative predictive value is 93.30% (**Table 3**). In the obtained ROC curve (**Figure 4**), the standard error of 92.5% was determined as 3.6% for the underlying area. In addition, a statistically significant correlation was found with the 20.1 cut-off value of the anti-thyroglobulin level in predicting the disease (p=0.001; p<0.01).

Table 2. Evaluation of measurements by groups

	Total	Groups		P
		Case	Control	
Haemoglobin (gr/dl)				^b 0.155
Mean±SD	13.57±1.51	13.27±1.36	13.80±1.59	
Median (Min-Max)	13.5 (10.3-16.9)	13.2 (10.3-15.8)	13.6 (10.4-16.9)	
FT3 (pg/ml)				^c 0.434
Mean±SD	3.18±0.94	3.29±1.39	3.12±0.50	
Mean±SD	3.1 (2.1-9.5)	3 (2.1-9.5)	3.2 (2.1-4)	
FT4 (ng/dl)				^c 0.645
Median (Min-Max)	1.26±0.31	1.30±0.41	1.23±0.22	
Mean±SD	1.2 (0.9-2.9)	1.2 (0.9-2.9)	1.2 (0.9-2)	
TSH (uIU/ml)				^c 0.265
Median (Min-Max)	2.69±1.75	2.89±1.92	2.54±1.63	
Mean±SD	2.2 (0-8.1)	2.9 (0-7.6)	2.1 (0.5-8.1)	
Anti-TPO (Anti-M) (IU/ml)				^c 0.001**
Mean±SD	87.90±128.9	185.87±140.97	8.47±3.75	
Median (Min-Max)	13.5 (4.5-540)	150.5 (13.6-540)	8 (4.5-21.9)	
Anti-Thyroglobulin(IU/ml)				^c 0.001**
Mean±SD	163.69±495.24	353.26±718.12	20.23±12.30	
Median (Min-Max)	19.8 (9.5-3800)	152 (14.6-3800)	17.4 (9.5-75.3)	
Ferritin (ng/ml)				^c 0.587
Mean±SD	77.00±86.25	77.81±80.78	76.41±91.13	
Median (Min-Max)	53 (6-435)	52 (8-400)	54 (6-435)	
Vitamin B12 (pg/ml)				^b 0.919
Mean±SD	426.36±155.52	424.20±164,21	428.11±150.39	
Median (Min-Max)	409 (161-828)	388 (161-828)	448 (181-755)	
Folic Asid (ng/ml)				^b 0.666
Mean±SD	8.29±4.26	8.54±4.21	8.08±4.35	
Median (Min-Max)	7.7 (1.7-20)	8.2 (2.6-18.7)	7.7 (1.7-20)	
<4 mcg/L	12 (18.2)	5 (17.2)	7 (18.9)	
≥4 mcg/L	54 (81.8)	24 (82.8)	30 (81.1)	
Vitamin D - (25 Hydroxy) (ng/ml)				^c 0.004**
Mean±SD	24.69±10.23	21.30±9.92	27.43±9.75	
Median (Min-Max)	22 (6-62)	20.5 (6-47)	25 (14-62)	
<10 ng/mL	2 (3.0)	2 (6.7)	0 (0)	
10-19 ng/mL	19 (28.4)	12 (40.0)	7 (18.9)	
20-62 ng/mL	46 (68.7)	16 (53.3)	30 (81.1)	

aPearson Chi-Square Test, bStudent T Test, cMann Whitney U Test, dFisher Freeman Halton Test, **p<0.01, *p<0.05

Table 3. Diagnostic screening tests and ROC curve results for anti-thyroglobulin, anti-TPO, and vitamin-D

	Diagnostic Scan					ROC Curve		P
	Cut off	Sensitivite	Spesifisite	Positive predictive value	Negative predictive value	Area	95% confidence interval	
Anti-thyroglobulin	≥20.1	92.86	83.78	81.20	93.90	0.925	0.855-0.996	0.001**
Anti-TPO	≥22.1	96.67	100	100	97.40	0.997	0.989-1.000	0.001**
Vitamin-D	≤22	70.00	64.86	61.80	72.70	0.706	0.578-0,835	0.004**

**p<0.01, r: Spearman Correlation Coefficient, **p<0,01

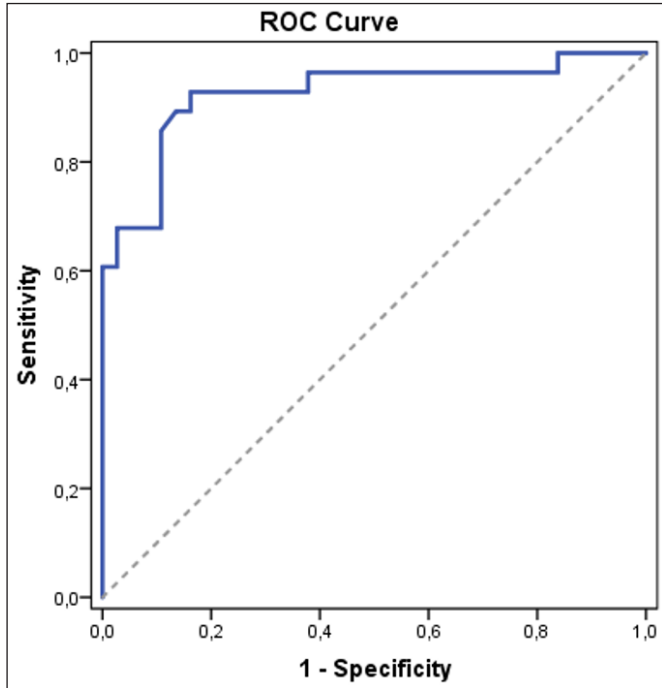


Figure 4. ROC curve for anti-thyroglobulin in predicting disease

For the 22.1 cut-off value of the anti-TPO level, sensitivity is 96.67%; specificity is 100%; positive predictive value is 100%, and negative predictive value is 97.4%. In the obtained ROC curve (Figure 5), the area under it was determined as 99.7%, with a standard error of 0.3%.

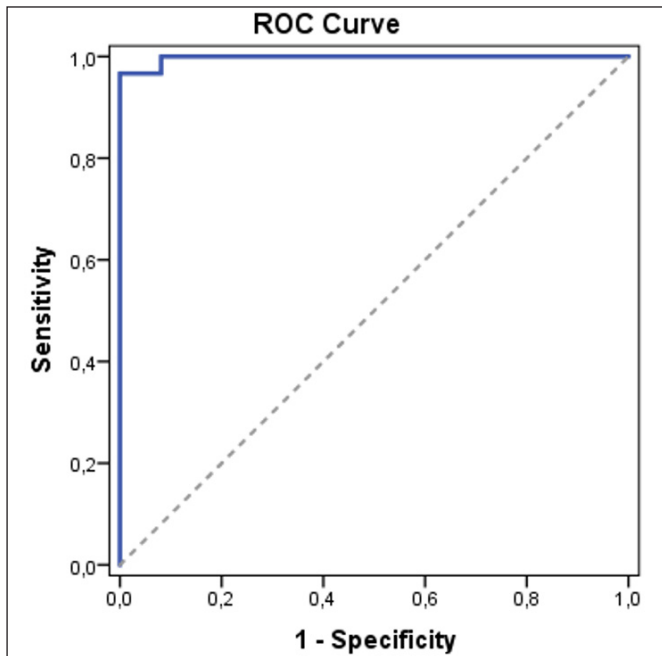


Figure 5. ROC curve for anti-TPO in predicting disease

A statistically significant correlation was found with the 22.1 cut-off value of the Anti-TPO value in predicting the disease ($p=0.001$; $p<0.01$).

For the 22 cut-off values of the Vit-D level, sensitivity is 70%; specificity 64.86%; positive predictive value is 61.80%, and negative predictive value is 72.70%. The area under the ROC curve (Figure 6) was determined

as 70.6%, with a standard error of 6.5%. A statistically significant correlation was found with the 22 cut-off values of the Anti-Thyroglobulin level in predicting the disease ($p=0.004$; $p<0.01$).

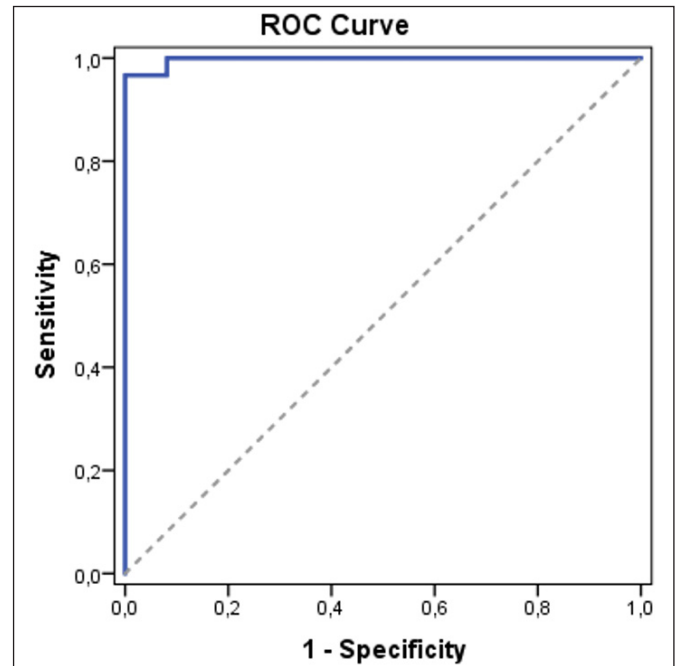


Figure 6. ROC curve for anti-TPO in predicting disease

A statistically significant weak correlation (Table 4) was found between the Vit-D measurements of the subjects and the Anti-TPO measurements (Figure 7), with a negative direction (as the Vit-D value increases, the Anti-TPO value decreases) ($r=-0.341$; $p=0.005$). ; $p<0.01$).

	Vitamin-D	
	r	P
Anti-TPO	-0.341	0.005**
Anti-thyroglobulin	-0.417	0.001**

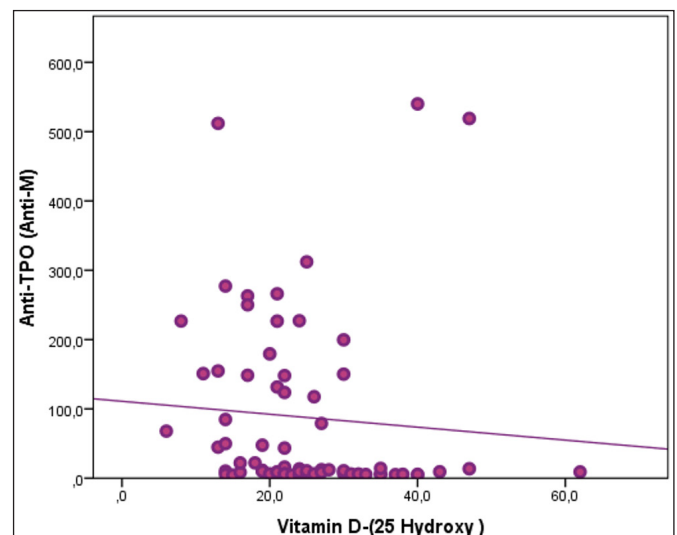


Figure 7. Relationship graph of vitamin D and anti-TPO

A statistically significant moderate correlation was found between the Vit-D measurements of the cases and the Anti-Thyroglobulin measurements (Figure 8), in the negative direction (as the Vit-D value increases, the Anti-Thyroglobulin value decreases) ($r=-0.417$; $p=0.001$; $p<0.01$).

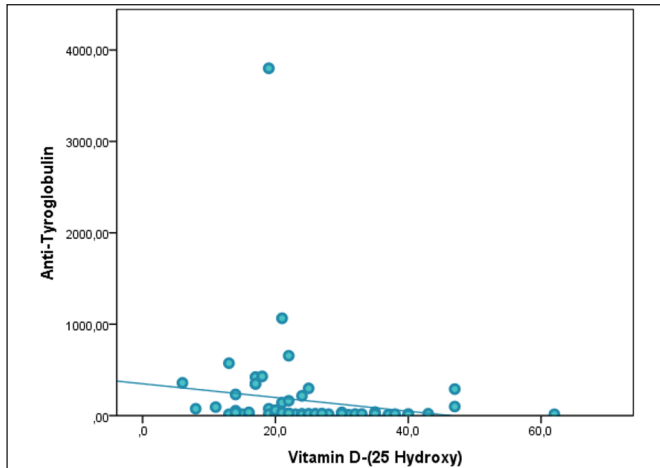


Figure 8. Relationship graph of Vitamin D and Anti-Thyroglobulin

DISCUSSION

As accepted in the literature, HT is recognized as the most common autoimmune disease worldwide. The susceptibility to HT is influenced by various factors, including genetic predispositions, environmental exposures, epigenetic factors, and immune system dysregulation.^{4,5} Literature suggests that high iodine intake, iodine deficiency, selenium deficiency, as well as deficiencies in vitamin B12, iron, and vitamin D, can increase the risk of HT.^{10,11} In this study, we aimed to investigate the relationship between HT severity and important factors in its pathogenesis, between vitamin D, iron, and vitamin B12.

Numerous studies have demonstrated the association between hypothyroidism and vitamin D deficiency in patients with HT. One study reported vitamin D deficiency in 96% of HT patients, with severe deficiency (serum levels <10 ng/mL) observed in 56% of these individuals. Furthermore, this study revealed a negative correlation between anti-thyroid peroxidase (anti-TPO) levels and vitamin D, suggesting that vitamin D deficiency may play a crucial role in autoimmune hypothyroidism.⁸

In another study investigating the effects of vitamin D supplementation in HT patients, 34 female individuals diagnosed with HT and being treated with levothyroxine for at least six months were included. These participants had normal vitamin D levels (serum 25-hydroxyvitamin D levels above 30 ng/mL). The study compared vitamin D levels after six months in two groups: one group received vitamin D supplements ($n=18$) and the other group did not receive vitamin D supplements ($n=12$). Results showed that serum vitamin D levels and titers of thyroid antibodies (anti-thyroglobulin and anti-

TPO) decreased in the group that received vitamin D supplements. This indicates an inverse relationship between serum vitamin D levels and thyroid antibody titers.¹⁰

Although it is believed that there is an association between HT risk and vitamin D, viral infections involved in the pathogenesis of HT can lead to damage to vitamin D receptors (VDR) and reduce the utilization of vitamin D.¹¹ Similar findings were observed in our study. When comparing vitamin D levels between 30 patients with Hashimoto's and 37 patients without a Hashimoto's diagnosis, a significant difference in vitamin D levels was found. Our study also revealed results consistent with other studies, showing a negative correlation between vitamin D levels and anti-thyroglobulin (as vitamin D levels increase, anti-thyroglobulin levels decrease) ($r=-0.417$; $p=0.001$; $p<0.01$).

Furthermore, a statistically significant weak correlation was found between vitamin D and anti-thyroid peroxidase (anti-TPO) measurements ($r=-0.341$; $p=0.005$; $p<0.01$) (8). Considering the relationship between iron deficiency and HT, it is known that iron deficiency can decrease the levels of T3 and T4 in circulation and reduce the peripheral conversion of T4 to T3 due to the role of iron metabolism in the synthesis and metabolism of thyroid hormones.¹²

In a study examining the frequency of iron deficiency in patients with positive thyroid-specific antibodies, significant decreases were observed in hemoglobin, hematocrit, mean corpuscular volume (MCV), ferritin, iron, and transferrin saturation in patients positive for anti-TPO and anti-thyroglobulin compared to the control group. Additionally, a significant correlation was found between anti-TPO levels and serum iron, transferrin saturation, and ferritin values.¹³

However, in our study, no significant differences were found in hemogram and ferritin measurements between patients with and without a diagnosis of HT ($p>0.05$). Furthermore, since the HT patients in our study were under treatment and their thyroid function (FT3, FT4, and TSH measurements) was being monitored, no statistically significant differences were observed in these parameters ($p>0.05$).

Despite the availability of various diagnostic methods, the cytomorphological features observed in fine needle aspiration cytology (FNAC) smears remain the gold standard for HT diagnosis. HT is a common cause of hypothyroidism in women.⁸

An accurate cytological diagnosis can prevent the need for surgical intervention. However, a multidisciplinary approach that includes clinical, radiological, biochemical and cytological parameters should be used to detect subclinical hypothyroidism and guide treatment.^{8,14}

The study showed that the incidence of Vit-B12 deficiency is 46% in patients with autoimmune hypothyroidism, and there is a negative correlation between Anti-TPO and Vit-B12 levels. Therefore, pernicious anaemia accompanied by autoimmune diseases or atrophic gastritis may be likely.

In a study, a relationship was found between Vitamin D deficiency, defined as 25(OH)D <10 ng/mL (~25 nmol/L), and the presence of thyroid antibodies, indicating a higher frequency of autoimmune thyroid disease, particularly HT.¹⁶ Another study reported lower 25(OH)D levels in individuals with autoimmune thyroid disease.¹⁷ These consistent findings once again highlight the importance of treating Vitamin D deficiency alongside HT in the management of patients.¹⁸⁻²⁰

The significance of epigenetic mechanisms in autoimmune diseases is increasingly understood in current research. Addressing the negative impact of epigenetic changes in HT, which arises from an imbalance in immune response, necessitates restoring the balance of B12 and folic acid and achieving optimal Vitamin D levels, which play crucial roles in epigenetic mechanisms. The effect of epigenetic mechanisms and changes should not be disregarded, considering individual variations in the methylation balance of each patient. The objective of this study is to underscore the comprehensive evaluation of these vitamin and mineral deficiencies in order to reverse the adverse effects of epigenetic mechanisms and enhance the patient's clinical presentation.

In most autoimmune thyroid studies and publications, the effects of values such as Vitamin D, B12, folic acid, and anemia have been examined separately. However, in this investigation, we believe that epigenetic factors should be evaluated as a whole mechanism, and all these values should be considered together. We emphasize the importance of examining them collectively to demonstrate the comprehensive factors involved.

Molecular docking simulations can yield promising results when a high-resolution X-ray crystal structure of the target macromolecule is available.²¹⁻²³ Therefore, in the present study, molecular docking was also conducted. Previous evidence has linked HT with mitogen-activated protein kinase 1 (MAPK).²⁴ A recent study using network pharmacology demonstrated that MAPK is one of the key targets in treating HT.²⁵ This study also reported strong interactions between compounds for HT disease and MAPK1 proteins through molecular docking. Consequently, in our study, extracellular signal-regulated kinase 2 (ERK2), which belongs to the MAPK family, was selected as the primary target. To our knowledge, no literature has reported the interaction of folic acid with ERK2.

As an internal validation, the native ligand was initially docked into the binding region of ERK2. The resulting binding pose was compared with the X-ray crystal conformation, yielding a root-mean-square deviation (RMSD) value of 0.35, indicating the successful performance of the selected docking method. Docking of folic acid into the binding pocket of ERK2 produced a high score very close to that of the native ligand (-9.16 vs. -10.18 kcal/mol). As shown in **Figure 1**, folic acid interacts with ERK2 through multiple hydrogen bonds and salt bridges. LYS54 appears to be the common amino acid residue that both folic acid and the native ligand interact with. Although the bulky size of folic acid might result in steric clashes with amino acid residues, the favorable interactions in the folic acid-ERK2 complex outweigh the native ligand. These overall interactions may account for the high docking score. The observed critical interactions can provide new insights for future studies aiming to design and optimize novel therapeutic agents against HT.

While many studies have evaluated epigenetic modifications in autoimmune thyroid patients, few have investigated epigenetic modifications associated with early disease diagnosis, treatment outcomes, and the risk of recurrence during follow-up. A better understanding of these epigenetic changes can contribute to the correct diagnosis of autoimmune thyroiditis, guide appropriate treatment approaches, and yield accurate results.

Low Vitamin D levels are closely related to activated autoimmune diseases such as HT and autoimmune hepatitis. Furthermore, Vitamin D exerts an important effect on immunoregulatory cells, and deficiency is strongly associated with impaired suppressor activity of immunoregulatory cells, leading to enhanced inflammation and increased autoimmunity in autoimmune diseases.²⁶

CONCLUSION

We aimed to highlight the significance of addressing vitamin D deficiency in the treatment of patients with HT and its importance in the overall management of HT.

Based on molecular docking simulations, folic acid showed strong affinity towards ERK2, which is known to play a key role in HT. This finding suggests the potential for designing novel therapeutic agents targeting ERK2 in the treatment of HT.

One limitation of our study is the relatively small number of patients, and further analysis with larger sample sizes across multiple centers is necessary to confirm the correlation and validate the docking studies. Therefore, the initial findings presented here provide a basis for conducting future clinical studies with a larger number of patients.

Furthermore, additional research in this field could contribute to a better understanding of the pathogenesis of the disease, potentially leading to the development of diagnostic and prognostic tools. Moreover, investigating the environmental and epigenetic factors involved in HT can shed light on their interplay with the disease and provide valuable insights for patient management.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was carried out with the permission of Biruni University Clinical Researches Ethics Committee (Date: 09.04.2021, Decision No: 2021/50-40).

Informed Consent: Written informed consent was obtained from all participants who participated in this study.

Referee Evaluation Process: Externally peer reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

Author Contributions: All the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

Acknowledgments: I can acknowledge the all authors. The support of Dr. Abdulilah Ece is gratefully acknowledged in computational part.

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