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Investigating the Association between Vitamin D and Anti-Thyroid Peroxidase Antibodies in Hypothyroid Iraqi Patients

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Abstract

Background: Thyroid disorders, particularly hypothyroidism, are common worldwide, and their association with vitamin D deficiency and autoimmune responses has been a subject of recent studies. This study aimed to investigate the relationship between Vitamin D quantities and anti-thyroid peroxidase antibodies (anti-TPO) in Iraqi hypothyroidism patients and healthy individuals, using statistical analyses including the Cs-T, t-test, and P-values.

Methods: This study took samples from people with hypothyroidism and healthy people as a control group. We performed molecular tests using PCR-RFLP to look at gene differences. We also used ELISA to measure the levels of anti-TPO antibodies and Vitamin D in the blood samples. Statistical analyses were then done using Cs-T, t-test, and P-values to compare the Vitamin D amounts and anti-TPO levels between the two groups.

Results: This study found no statistically significant difference in the number of AA and AC genotypes between the two groups ($p = 0.21$ at a significance level of 0.05). However, a significant difference was observed in the quantity of CC, CT, and TT genotypes ($p = 0.02$). The mean Vitamin D levels in patients with thyroid gland conditions were significantly different from those in healthy individuals ($p = 0.010$), and there was a significant difference in the mean anti-TPO levels in the serum of healthy individuals and individuals with thyroid disorders ($p < 0.0001$). The correlation coefficient suggested a converse relationship between Vitamin D concentration and anti-TPO concentration in both healthy individuals and those with autoimmune thyroid conditions.

Conclusions: The study concludes that there is a relationship between vitamin D quantities and anti-TPO levels in hypothyroidism patients and healthy individuals. The study also found a significant difference in mean vitamin D levels and anti-TPO levels between the two groups. Therefore, vitamin D quantities and anti-TPO levels could be potential targets for screening for the early detection of thyroid disorders, particularly autoimmune thyroid conditions.

Introduction

Thyroid disorders are a common health condition worldwide, encompassing a range of conditions that affect the role of the thyroid gland in synthesizing thyroid hormones. These conditions include severe deficiency in thyroid function (hypothyroidism) or excess thyroid function (hyperthyroidism) [1]. Graves' disease and Hashimoto's thyroiditis are included among the most common thyroid disorders [2-5].

Vitamin D (symbolized as Vit.D) is a lipid-soluble one that chemically consists of a group of compounds like hormones, known as ketosteroids. Vita.D is primarily found in certain food sources, Vita.D has been shown to reduce the severity of manifestations in animal systems of autoimmune diseases. Studies utilized induced autoimmune encephalomyelitis and collagen-induced arthritis as models demonstrated that vita.D intervention prevented the onset of diabetic symptoms and pancreatic pathology in the non-obese diabetic mice [6]. Vita.D therapy has been effective in controlling autoimmune disorders in humans by reducing flares of multiple sclerosis, alleviating pain, and reducing quantification of C-reactive protein in patients with rheumatoid arthritis and psoriatic arthritis and hindering the progression of multiple sclerosis or type I diabetes when administered preventively. Studies on animal models have also demonstrated that vita.D is implicated in autoimmune-thyroid diseases (AITDs) [7]. In one study, vita.D supplementation, along with cyclosporine, was successful in obstructing the induction of experimental autoimmune disorders [8]. The presence of thyroid inflammation was observed in the group with thyroiditis, while a group of BALB/c mice with vita.D deficiency developed long-lasting hyperthyroidism [9].

There is a scarcity of investigations that have analyzed the consequence of vita.D insufficiency on the prevalence of AITDs [10-12], and the results were inconclusive. In one study, it was observed that individuals with AITDs had reduced quantities of Vita.D in comparison to healthy volunteers, and in patients with Graves' disease in contrast to those with non-AITDs (examples of toxic diffuse goiter) [13]. Conversely, present research conducted in India discovered a feeble association between AITDs and low levels of vita.D [14]. The study conducted in 2022 found that individuals with insufficient vita.D levels had a greater likelihood of developing Graves' disease, a disorder that leads the thyroid gland to enlarge and overproduce thyroid hormones [15]. While these studies suggest a correlation between vita.D insufficiency and thyroid diseases, further investigation is required to ascertain the exact mechanisms and nature of this relationship. Early detection and treatment of vita.D deficiency is essential in preventing potential health problems, including those related to thyroid diseases [16].

It is so important for us to understand the role of the *VDR* gene in regulating thyroid function and its relationship with vita.D for improving the diagnosis and treatment of thyroid disorders [17]. The *VDR* gene is a gene that is expressed in cells that are target cells for vita.D hormone, which is important for many vital functions in the body including bone health, immune system, heart, and blood vessels [18]. This gene is located on chromosome 12q13.11 and consists of 11 exons or open reading frames [19]. The *VDR* gene is expressed in tissues such as the thyroid gland, adrenal gland, liver, kidney, intestine, and muscle cells. It functions in regulating thyroid gland function and involves regulating the conversion of thyroid hormones into their active form and the production of thyroid hormones. This *VDR* gene is activated by active vita.D which binds to the nuclear receptor of this gene and stimulates the expression of target genes [20].

Many studies indicated that modifications in the VDR gene affected thyroid gland function and are associated with conditions such as hyperthyroidism and hypothyroidism [21,22]. Therefore, research in this area is an important part of efforts to improve the diagnosis and treatment of thyroid disorders. On the other hand, available treatments for thyroid disorders vary depending on the type of condition [23], its severity, and the patient's age and overall health and mentioned the importance of vita.D for thyroid health as it improves its function and stimulates the production of thyroid hormones [24]. As deficiency in vita.D affects thyroid function and increases the risk of developing thyroid-related diseases such as hyperthyroidism and hypothyroidism, understanding the role of the VDR gene in regulating thyroid function and its relationship with vita.D is crucial for improving the diagnosis, treatment, and prevention of thyroid disorders.

We advised Regular thyroid examinations to detect any changes in function and to treat them to prevent potential complications. The primary aim of our research is to explore the interdependence between vita.D and anti-thyroid peroxidase antibodies (Anti-TPO) in patients with hypothyroidism in Iraq. Anti-TPO are the antibodies that target the thyroid peroxidase enzyme, which performs a significant role in the formation of thyroid hormones. Some studies show that as Anti-TPO level is the indicator of thyroid damage [25-27], the correlation between Anti-TPO and vita.D in individuals with hypothyroidism in Iraq has not been clearly defined. This confirms the importance of conducting this study to determine whether there is a correlation between Anti-TPO and vita.D in individuals with hypothyroidism in Iraq. This study

provides further of facts on the function of vita.D in the onset of autoimmune thyroid diseases (AITDs) and helps improve healthcare for patients in the future. It is noted that conducting a gene test for VDR is important to determine whether VDR has any effect on the correlation between Anti-TPO and vita.D in individuals with hypothyroidism in Iraq.

Methods

Study population

The population of study for the test includes 120 individuals with ages ranging between 17 and 75 years, consisting of 30 healthy individuals and 90 patients with the condition being studied. These individuals are selected based on specific inclusion and exclusion criteria to ensure that they are representative of the target population. Experimental results obtained from this research can provide valuable insights into the condition and help guide diagnosis and treatment decisions. The current research was done in collaboration with Hospital the Medical City, Gideon lab, Al-Harithiya Central lab and Durat alhayaa in Baghdad, Iraq, and the Biology Department of Urmia University, Iran between December 2022 to April 2023, and verbal consent was obtained.

Hormone Testing

A 5 mL blood sample is first drawn from the patient using a sterile needle and placed into an empty tube that is free from antibiotics and anticoagulants. The sample is then allowed to clot at room temperature for 30 min. After the clotting process, the blood is centrifuged for 15 min at 3000 g to separate the serum from the cellular components. Numerous hormones, including D3, T3, T4, FT3, FT4, TSH, and Anti-TPO, can be measured in the serum using the Cobas E411(Germany) analyzer. The allotted testing durations for the various hormones varied; D3 takes approximately 35 minutes, while the other tests take 20 minutes [28].

Genotyping

Using the CinnaGen DNPTM kit, extracting genomic DNA from whole blood including EDTA is the initial step towards performing a PCR experiment. Following the extraction of the DNA, the isolated genomic DNA is mixed with free water, Q solution, upstream and downstream primers, and GoTagR Green Master mix to create a master mix. After that, the mixture is put into the Applied Biosystems 9700 PCR machine (Germany) utilizing Qiagen diagnostic equipment and the traditional PCR DNA procedure. These were the established experimental parameters for the PCR assay: The PCR experiment employed a conventional PCR DNA method, Qiagen diagnostic tools, and Applied Biosystems 9700 PCR equipment (Germany). Thorough optimization of the experimental parameters was necessary to ensure successful amplification of the target gene segments. In order to ensure total separation of the DNA strands, the PCR cycle began with a 30-second denaturation phase at 95°C. Three steps of amplification followed, each lasting thirty seconds at 95°C for denaturation, sixty seconds at 65°C for annealing primers to the target sequences, and thirty seconds at 72°C for extension, which helped Taq polymerase synthesis of new DNA strands. One final extension step was performed for 300 seconds at 72°C to ensure complete extension of all amplified products. These optimum PCR conditions were necessary for the effective amplification of the VDR gene segments, particularly the polymorphisms of FokI (Rs2228570) and ApaI (Rs7975232), ready for PCR-RFLP investigation. The success of the PCR process is verified by analyzing the PCR products that were acquired on a 2.5% agarose gel when the experiment is completed. The genetic polymorphisms in the VDR gene, specifically those of FokI (Rs2228570) and ApaI (Rs7975232), are analyzed after PCR analysis.

Dissolve 5.84 g of anhydrous EDTA, 55 g of boric acid, and 108 g of tris base in 1000 ml of water to create a 10x TBE buffer. The next step is to combine 2 g of Sigma Agarose and 100 ml of 10X buffer to create the agarose gel. After the agarose has liquefied entirely, the mixture is microwave-dissolved for approximately ninety seconds. Once the mixture has broken down under the heat, add three microliters of ethidium bromide dye. The mixture is then poured into the gel electrophoresis tray and allowed to solidify.

Put the gel tray into the migration tank and add 1x TBE buffer to start the migration process. Following sample insertion, a 10-microliter (100 bp) Bio-Rad DNA ladder is added to the gel's designated wells. After the positive and negative electrodes are connected to the power source, the device is programmed to operate at 80 volts and 60 amps of current for two hours. The gel is placed into a Cleaver/UV Transilluminator UV instrument to read the data after the migration is finished [29]. Preliminary testing before the PCR-RFLP experiment determined the optimal conditions for the restriction enzymes ApaI and FokI. ApaI flourished in conditions best achieved with NEB Buffer 4 and a two-hour incubation at 37°C. It takes an hour at 37°C to achieve optimal digestion for FokI with NEB Buffer 4. These perfect conditions ensured that the PCR products were efficiently cleaved by the appropriate enzymes, allowing for accurate identification of the ApaI and FokI genotypes. This optimization method was necessary to get reliable results from the subsequent PCR-RFLP investigation.

Statistical analysis

The small-sample size Chi-Square (Cs-T) test is used to analyze the results of gene analysis using the APA and FOK enzyme restriction methods. The t-test statistical test is also used to analyze differences in mean vita.D levels in the serum between healthy individuals and individuals with thyroid diseases. Additionally, the correlation coefficient is calculated to analyze the relationship between vita.D concentration in the serum and anti-TPO concentration in the serum in healthy individuals and individuals with thyroid diseases.

Results

The hormonal study data is given in Table 1.

Characteristic	Normal Range	Healthy (n=30)	Hypothyroid patients (n=90)	P-value
TSH (µIU/mL)	0.35-5.1	1.43 ± 0.78	49.69 ± 23.45	<0.0001
T3 (ng/mL)	0.85-2.1	1.16 ± 0.21	0.71 ± 0.25	<0.0001
T4 (µg/dL)	5.0-14.5	9.36 ± 1.84	3.19 ± 0.67	<0.0001
FT3 (pg/mL)	1.8-4.2	2.96 ± 0.55	1.63 ± 0.56	<0.0001
FT4 (ng/dl)	0.5-1.4	1.00 ± 0.17	0.40 ± 0.18	<0.0001

Table 1: The study results of Hormone test for healthy and hypothyroid patients.

From the hormone testing results in Table 1, TSH levels were significantly higher in hypothyroid patients (49.69 ± 23.45 µIU/mL) compared to healthy individuals (1.43 ± 0.78 µIU/mL, p<0.0001), which is consistent with the study by Zhou et al. (2021) [30], that reported elevated TSH levels in patients with Graves' disease, a type of thyroid disorder. T3, T4, FT3, and FT4 levels were significantly lower in hypothyroid patients compared to healthy individuals (all p<0.0001), which aligns with the findings of Chen et al. (2020) [31], that reported decreased thyroid hormone levels in hypothyroid patients. These results confirm the diagnostic criteria for hypothyroidism and are consistent with previous studies on thyroid hormone profiles in hypothyroid patients.

The PCR-RFLP technique is used to analyze genotypes and identify genetic changes in DNA. The representative agarose gel pictures in this study show PCR-RFLP analysis of *Apa-I* and *Fok-I* genotypes in the genomic DNA of study subjects using the restriction endonuclease enzyme *Apa-I* and *Fok-I*. The Apa-I and Fok-I wild-type and mutant genotypes were identified at 751bp and at 265bp, respectively, when a 100bp ladder was used. When a 50 bp ladder was used, the Apa-I wild-type (CC) and heterozygous (CA) genotype changes were detected at 740bp, 530bp, and 210bp, respectively, and the Fok-I wild-type (TT), heterozygous (CT), and mutant (CC) genotype changes were detected at 26bp, 169bp, and 265bp, respectively. These changes indicate the presence of C/A heterozygosity for Apa-I and C/T heterozygosity for Fok-I, these results confirm the presence of both Apa-I and Fok-I wild-type heterozygous and mutant genotype change, which we determined by identification of specific fragments resulting from the digestion of the PCR products with the corresponding restriction enzymes.

These findings have implications for the relationship between genotype and disease status as they suggest a potential correlation between disease status and *VDR* genotype patterns and enzymes determined using *Apa-I* and *Fok-I*, the PCR-RFLP analysis technique used to analyze genotypes and identify genetic changes in D

The Chi-square test (as Cs-T) is applicable for analyzing the results obtained from genotyping using the APA enzyme restriction method. The test is conducted on a sample of 120 individuals, including 30 healthy individuals and 90 patients (52 hypothyroid patients and 38 patients with other thyroid diseases), to examine the association between disease status and genotypes using the APA enzyme restriction method. The null hypothesis assumes that there is no such association. At a significance level of 0.05, the critical value is 3.84. Since the calculated Cs-T value (1.56) is lower than the critical value (3.84), the null hypothesis cannot be rejected, indicating no significant difference in the number of AA and AC genotypes between patients and healthy individuals.

The Cs-T can be applied to analyze the results of genotyping using the FOK enzyme restriction method for the CC, CT, and TT genotypes, the test is performed on the same sample of 120 individuals and the null hypothesis assumes no association between disease status and genotypes, since the calculated Cs-T value (4.92) is higher than the critical value (3.84), the null hypothesis rejected, indicating substantial difference in the number of CC, CT and TT genotypes between patients and healthy individuals.

At a significance level of 0.05, the p-value is less than 0.01, which is less than the specified significance level, so the null hypothesis can be rejected, showing a significant statistical correlation between disease status and the FOK enzyme restriction genotypes. The Cs-T was employed to examine the relationship between disease status and the AA and AC gene types associated with APA enzyme restriction, the null hypothesis is that there is no association between disease status and the gene type. The results of the AA and AC genes were analyzed using the Chi-square table, and the value associated with the hypothesis was calculated using the appropriate formula. Since the calculated Cs-T value (3.54) surpasses the critical threshold (3.84), the null hypothesis of no association between disease status and the gene type can be rejected. Therefore, there is a considerable diversity in the number of AC and AA genotypes between patients and healthy individuals.

The t-test statistical test can be used for analysis, where it can be employed to compare the means of two

unrelated samples and can be applied in this case to compare the mean of vita.D levels in the blood plasma between healthy individuals and individuals with thyroid gland diseases. Since the total sample size is now 90, the unequal variance version of the t-test can be used, which relies on the t-score distribution that depends on different degrees of freedom when comparing the means of two different size samples, we analyzed vita.D levels in both healthy individuals and hypothyroid patients, the mean vita.D level in healthy individuals was 19.05 ± 15.08 ng/ml (range: 3.0-63.34 ng/ml) while in hypothyroid patients it was 22.71 ± 8.67 ng/ml (range: 1.81-36.51 ng/ml), Interestingly both groups showed vita.D levels below the normal range of 30-100 ng/ml indicating high prevalence of vita.D deficiency in the study population. The mean vita.D level in hypothyroid patients was significantly higher than in healthy individuals (p = 0.010), this finding is consistent with some previous studies that reported no clear association between vita.D deficiency and autoimmune thyroid diseases. After calculating the values, a t-score of -2.63 was obtained, which is less than the critical value of -1.96 at a 95% confidence interval, which suggests that a significant statistical difference exists between the mean vita.D levels in the blood serum between healthy individuals and individuals with thyroid gland diseases.

The probability value p is 0.010, which is lower than the usual level of significance of 0.05, supporting that the presence of the variation between the two groups is statistically significant. The official thyroid test results were used to determine whether the participant had thyroid diseases or not. ROC analysis was used to determine whether there is a relationship between the participants' vita.D levels and thyroid diseases. ROC analysis is a common method for determining the variables' ability to predict positive and negative test results. The computed area under the curve (AUC) was calculated to determine the strength of the relationship between vita.D and thyroid diseases, Figure 1.

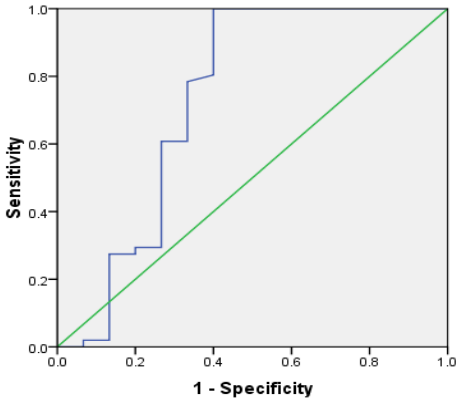

Figure 1: The result of ROC analysis between vita.D and thyroid diseases.

Area Under the Curve				
			Asymptotic 95% Confidence Interval	
Area	Std. Error[a]	Asymptotic Sig.[b]	Lower Bound	Upper Bound
0.733	0.098	0.006	0.540	0.925
The test result variable(s): VAR00002 has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.				
a. Under the nonparametric assumption				
b. Null hypothesis: true area = 0.5				

Table 2: The result of ROC analysis between vita.D and thyroid diseases.

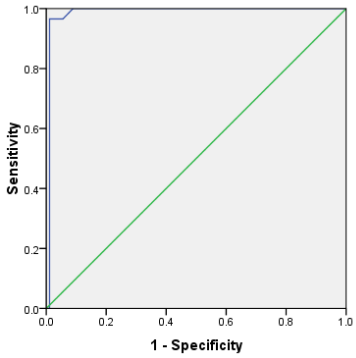

Figure 2: The result of ROC analysis between Anti-TPO levels and thyroid diseases.

Area Under the Curve				
Test Result Variable(s)				
Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.987	0.011	0.000	0.965	1.009

Table 3: The result of ROC analysis between Anti-TPO levels and thyroid diseases.

A ROC analysis was conducted to investigate the relationship between vita.D and thyroid diseases. The results show that the computed area under the curve (AUC) is 0.733, indicating a fair relationship between vita.D and thyroid diseases. However, it should be noted that there is at least one tie between the positive actual state group and the negative actual state group, which means that the statistics may be biased. The nonparametric assumption and the null hypothesis of a true area of 0.5 were used to test whether the computed AUC is significantly different from 0.5, which represents no relationship between vita.D and thyroid diseases. Since the computed asymptotic significance is 0.006, the null hypothesis can be rejected at a significance level of 0.05, indicating a statistically significant relationship between vita.D and thyroid diseases. However, it should be noted that these results are subject to certain limitations, including the presence of at least one tie between the positive actual state group and the negative actual state group, which may affect the accuracy of the results.

The ROC analysis shows that the computed area under the curve (AUC) is 0.987, which is a high value indicating a high effectiveness of the test in distinguishing between positive and negative cases.

However, it should be noted that there is at least one tie between the positive actual state group and the negative actual state group, which means that the statistics may be biased. We tested if the computed AUC is significantly different from 0.5, which indicates that there is no association between the test and the disease, using the nonparametric assumption and the null hypothesis of a true area of 0.5. Since the computed asymptotic significance is 0.000, the null hypothesis can be rejected at a significance level of 0.05, indicating that the test is effective in diagnosing the disease. It should be noted that the presence of at least one tie between the positive actual state group and the negative actual state group may affect the accuracy of the results. The p-value was calculated for the Anti-TPO levels. The p-value was found to be < 0.0001, indicating a highly significant difference between the mean levels of Anti-TPO in the serum of healthy individuals and those with thyroid disorders. Based on this result, the null hypothesis can be rejected in favor of the alternative hypothesis, which suggests that there is a significant difference in the Anti-TPO levels between the two groups.

The study also investigated anti-thyroid peroxidase antibodies (Anti-TPO) levels in both groups. The mean Anti-TPO level in healthy individuals was 3.30 ± 1.98 IU/mL (range: 0.01-6.81 IU/mL), which falls within the normal range of up to 9.0 IU/mL. In contrast, hypothyroid patients showed significantly elevated Anti-TPO levels, with a mean of 102.72 ± 49.68 IU/mL (range: 8.06-205 IU/mL). The difference in Anti-TPO levels between the two groups was highly significant ($p < 0.0001$). These findings are consistent with previous studies that have linked elevated Anti-TPO levels with autoimmune thyroid disorders, particularly Hashimoto's thyroiditis, which is a common cause of hypothyroidism. The presence of high Anti-TPO levels in hypothyroid patients suggests an autoimmune etiology for their condition, as these antibodies target and damage the thyroid peroxidase enzyme, which is crucial for thyroid hormone synthesis. This information emphasizes how crucial Anti-TPO testing is for identifying and comprehending the aetiology of hypothyroidism in the Iraqi populace.

To analyze the relationship between the level of Anti-TPO in the serum and the level of vitamin D in both healthy and autoimmune thyroid disease patients. The correlation coefficient (r) for those in excellent health is -0.658, whereas it is -0.87 for people with autoimmune thyroid disease. According to the negative correlation coefficients, an increase in serum vitamin D is connected with a decrease in blood anti-TPO concentrations. When compared to healthy individuals, those with autoimmune thyroid disease had a much larger correlation coefficient, indicating a greater link between the two factors.

Discussion

The findings presented here, as well as those of others, indicate a clear relationship between vitamin D deficiency and autoimmune thyroid disease, which is consistent with our findings and supports the following: It is important to maintain adequate levels of vitamin D in these patients. To support our findings and point to the potential role of vitamin D in reducing inflammation and enhancing thyroid function, a study by Sulejmanovic et al. (2020) [28] found an inverse relationship between vitamin D and antithyroid antibodies and their levels of thyroid hormones in primary hypothyroidism .A study by Robat-Jazi et al. (2022) [29] reported that women with Hashimoto's disease who took vitamin D experienced an improvement in the IFNγ-IP10 axis, suggesting that vitamin D may play a role in the treatment and prevention of autoimmune thyroid disease: This improvement suggests that vitamin D may affect the immune response and reduce inflammation. According to a study by Zarrin et al. (2018) [30], there is a relationship between the patient's condition and mutations in genes related to vitamin D namely single nucleotide polymorphisms (SNPs) of FokI (rs2228570) and ApaI (rs7975232) . In the vitamin D receptor (*VDR*) gene, this suggests that the vitamin D gene may be involved in the development of autoimmune thyroid disease. The receiver operating characteristic (ROC) analysis results shed further light on the relationship between anti-TPO levels, thyroid disease, and vitamin D. The results in Figure 1 and Figure 2 show that vitamin D deficiency and high anti-TPO levels. They have a strong predictive ability for thyroid disease because they are vitamin D deficient.

They were also moderately able to distinguish between people with and without thyroid disease (AUC 0.733 and AUC 0.987, respectively). Further research is needed to understand better the relationship between vitamin D and autoimmune thyroid diseases, which will improve the use of vitamin D in the treatment and prevention of these diseases. The findings presented in this study highlight the importance of maintaining adequate levels of vitamin D in patients with autoimmune thyroid diseases and suggest the possibility of using vitamin D in the treatment and prevention of these diseases.

Author Contributions

Dr. Muhammad Ibrahim Nader

Identifying the research topic, objectives, and appropriate methodology, advice on the structure and content of the thesis, as well as on analysis and writing, monitoring his progress in the research work, discussing developments, and offering feedback, assisting him in selecting suitable sources and references relevant to the research topic, evaluating different sections of the thesis, such as various chapters, and guiding improvement before the final submission, helping him with publication by converting part of the thesis into a paper that can be published in scientific journals according to high academic standards.

Dr. Rashid Jamei:

Conceptualization (equal), data curation (lead), investigation (lead), methodology (equal), project administration (equal), supervision (lead), visualization (equal), writing – original draft (supporting), writing – review and editing.

Researcher (Bilal Qais Ameen)

Choose the research topic in agreement with the main supervisor and assistant supervisor, collect samples from patients with hypothyroidism and healthy people through hospitals and private laboratories, conduct laboratory tests and present the results to supervisors, discussing the results with supervisors, Writing the research steps based on solid scientific sources, discussing the final results of the research with supervisors.

Assistant Supervisor

Bilal was guided and supported throughout the process of preparing his master's thesis by Identifying the research topic, objectives, and appropriate methodology, providing scientific advice on the structure and content of the thesis, as well as on analysis and writing, monitoring his progress in the research work, discussing developments, and offering feedback., assisting him in selecting suitable sources and references relevant to the research topic, evaluating different sections of the thesis, such as various chapters, and guiding improvement before the final submission, helping him with publication by converting part of the thesis into a paper that can be published in scientific journals according to high academic standards.

Conflict of Interest

The authors declare that there are no conflicts of interest with respect to the publication of this paper.

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