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Vitamin D alters the transcriptional profile of blood cells in patients with primary hypothyroidism

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Our work aimed to study the influence of cholecalciferol on neurogenesis, neurotrophins and their receptors pathway-focused genes expression in patients with autoimmune thyroiditis and hypothyroidism. The pathway-specific PCR array (Neurotrophins and Receptors RT2 Profiler PCR Array, "QIAGEN", Germany) was used to identify and validate the neurogenesis regulatory genes expression in patients with thyroid pathology and control group. The results showed that in patients with autoimmune thyroiditis-caused primary hypothyroidism, the expression of BDNF, FAS, FGF2, HSPB1, IL10, NGF, NGFR, NRG1, NTF3, NTF4, TGFB1, and TP53 were increased after treatment with cholecalciferol and L-thyroxine. The mRNA levels of BAX, GDNF, CD40, FOS, GDNF, GFRA1, IL1B, and STAT3 were significantly decreased in patients with autoimmune thyroiditis-caused primary hypothyroidism after treatment with cholecalciferol and L-thyroxine compared to the patients who received only L-thyroxine. Therefore, the addition of vitamin D to standard therapy alters the transcriptional profile of blood cells in patients with primary hypothyroidism.
Key words: vitamin D; neurogenesis; mRNA; autoimmune thyroiditis; hypothyroidism.

INTRODUCTION

Thyroid disease has been reported to be the most widespread endocrine abnormality, and autoimmune thyroiditis (AIT) may be one of the most common autoimmune diseases [1]. Existing research data on the occurrence of vitamin D deficiency in relation to thyroid disease mostly demonstrates the association of vitamin D deficiency with a higher frequency of autoimmune thyroiditis [2]. The substantiation for finding a role of vitamin D in aforementioned diseases is that this hormone can affect and alter various brain processes, including the efficiency of communication in some neural circuits implicated in cognition, memory, and emotional behavior [3].

Vitamin D deficiency, primarily in the elderly, may lead to impaired learning, memory, and cognitive processes [4]. The role of vitamin D in cognition and related processes and behavior is not unexpected, since vitamin D regulates protein expression and neurotrophic factors involved in synaptic plasticity and physiological health of neurons [5].

Adult neurogenesis is strongly associated with high sensitivity to hormones, neurotransmitters, and growth factors in the neurogenic niche. It has been demonstrated that the thyroid hormone profoundly affects neurogenesis [6]. This can lead to the onset of neurological complications occurring from thyroid pathology [7-10]. The modern search for effective targeted therapy is based on existing transcriptome and proteome data [11].

Our work aimed to study the influence of cholecalciferol on neurotrophins and their receptors pathway-focused genes expression in patients with autoimmune thyroiditis and hypothyroidism living in the western regions of Ukraine.

METHODS

Our research was conducted in Bukovinian State Medical University, Chernivtsi Regional Endocrinology Center, and I. Horbachevsky Ternopil National Medical University, Ukraine. The study

included 56 patients with hypothyroidism (HT) caused by autoimmune thyroiditis (AIT). These patients were divided into two groups. Patients in the test group (group 1, n = 28) received cholecalciferol at a dose of 4000 IU/day (28,000 IU/week) and L-thyroxine (88.39 ± 12.70 μg/day). Patients in the control group (n = 28) were prescribed only L-thyroxine (87.50 ± 12.73 μg/day). Examinations were performed at the beginning and end of the 12-week treatment.

Ethical approval. The study fully ensured standards described in the 1975 Helsinki Declaration of Human Rights (amended in 2008). The participants completed and signed written informed consent before voluntarily enrolling in the research. The Ethics Committee of the HSEEU "Bukovinian State Medical University", I. Horbachevsky Ternopil National Medical University, and Chernivtsi Regional Endocrinology Center, Ukraine have approved this study.

To diagnose HT, we were guided by recommendations required by the American Association of Clinical Endocrinologists 2012. The corresponding clinical features were considered when verifying AIT, namely, the results of a sonogram of the thyroid gland (reduced echogenicity) and circulating antibodies to thyroid antigens, anti-TPO, and anti-TG were detected. Blood samples from the patients were taken in the morning (8 to 10 am) after a night fast. Using STAT FAX303/Plus analyzer ("Awareness Technology Inc", USA), we determined levels of thyroxine (fT4, normal range 6.0-13.0 pmol/l for males and 7.0-13.5 pmol/l for females), thyroid-stimulating hormone (TSH, normal range 0.3-4.0 mIU/ml), anti-thyroid peroxidase (anti-TPO, normal range 0-30 IU/ml) and anti-thyroglobulin (anti-TG, normal range 0-65 IU/ml) in each individual participated in the study.

The study exclusion criteria were the following: less than 18 years of age, malignancy, inflammation resulting from rheumatic diseases or acute/chronic infection, diabetes mellitus, vascular, chronic diseases of liver and kidneys, and pregnancy. Individuals administering drugs that could influence thyroid function were also ruled out from the study.

When determining 25-OH Vitamin D levels in the serum of patients, we applied the ELISA using the 25-OH Vitamin D Total (Vit D-Direct) Test System ELISA Kit (Monobind Inc.®, United States, Product Code: 9425-300) on E.I.A. Reader Sirio S ("Seac", Italy). We used a pathway-specific PCR array (Neurotrophins and Receptors RT^2 Profiler PCR Array, "QIAGEN", Germany) to identify and verify Neurogenesis pathway-focused genes expression in randomly selected 12 individuals from each group using real-time PCR due to the procedure described below.

Experimental procedures. RNA isolation. Total RNA was isolated from white blood cells using NucleoZOL ("Macherey-Nagel", Germany) according to the manufacturer's instructions. NucleoZOL is designed for the isolation of total RNA (small and large RNA) in single or separate fractions from a variety of sample materials, such as cells, tissue, and liquids of human or animal origin. White blood cells were lysed and homogenized in NucleoZOL reagent based on guanidinium thiocyanate and phenol.

cDNA synthesis. The RNA quality was determined and it was reverse transcribed. The concentration and quality of the isolated total RNA were determined on a NanoDrop spectrophotometer (Thermo Scientific™, USA). For the reverse transcription procedure was performed with a cDNA conversion RT² First Strand Kit ("QIAGEN", Germany, Cat. No. 330401), RNA samples with ratio A260/A280 within the range of 1.8-2.2 were selected. The RT2 HT First Strand Kit procedure comprises 2 steps: elimination of genomic DNA contamination and reverse transcription, this was to enable fast and easy handling of 96 RNA samples simultaneously. After genomic DNA elimination, the RNA samples underwent reverse transcription with an RT master mix, as well as random hexamers and oligo-dT prime reverse transcription to capture more difficult-to-detect genes.

PCR Array. The cDNA samples were then used with RTI Profiler PCR Array ("QIAGEN", Cat. No. PAHS-031Z) in combination with RTI SYBR® Green qPCR Mastermix ("QIAGEN", Cat. No. 330504), following the complete RT2 Profiler PCR Array procedure (www.qiagen.com). Each array contained 5 separate housekeeping genes - ACTB (Actin, beta), B2M (Beta-2-microglobulin), GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), HPRT1 (Hypoxanthine phosphoribosyltransferase 1), RPLP0 (Ribosomal protein, large, P0) that were used for normalization of the sample data. The CT values were normalized based on an automatic selection from the full panel of reference genes. Any CT value >35 was considered to be a negative result. The RT2 Profiler PCR Array data analysis software calculates the fold change based on the widely used and agreed upon the delta-delta CT method. The data analysis web portal calculates fold change/regulation using the delta-delta CT method, in which delta CT is calculated between the gene of interest (GOI) and an average of reference genes (HKG), followed by delta-delta CT calculations (delta CT (test group)-delta CT (control group)). Fold change is then calculated using the $2^{-\text{delta-delta CT}}$ formula. This data analysis report was exported from the "QIAGEN" web portal at GeneGlobe. This software allows to best defining reference genes for normalization.

Statistical analysis of PCR array data. The RT2 Profiler PCR Array Data Analysis software does not perform any statistical analysis beyond the calculation of P-values using Student's t-test (two-tail distribution and equal variances between two samples) based on the triplicate $2^{-\text{delta CT}}$ values for each gene in the experimental group, compared to the control group. Microarray quality control (MAQC) published results indicating that a ranked list of genes based on fold-change and associated p-value calculation was sufficient to demonstrate reproducible results across multiple microarrays and PCR Arrays, including the RT2 Profiler PCR Arrays.

RESULTS

Using the Pathway-Focused PCR Array Profiling (Neurotrophins and Receptors RT2 Profiler PCR Array) we examined the influence of cholecalciferol on transcription factors and regulators pathway-focused genes expression in patients with primary AIT-caused HT.

Table shows only those transcription factors and regulators pathway-focused genes whose expression had changed statistically significant.

We have detected changes in the expression of genes involved in the production of cytokines and their receptors in patients with AIT-caused primary HT after treatment with cholecalciferol and L-thyroxine. The analysis showed that the expression of IL10 increased 29.78-fold (Table; Figs. 1; 2), while the expression of IL1B decreased (6.85-fold; Fig. 2).

As shown in Table, the mRNA-level in the expression of transcription factors and regulators genes in AIT-caused HT patients after treatment with cholecalciferol and L-thyroxine also changed: the expressions of TP53 (Table; Figs. 1; 2) increased (16.83-fold) whereas the expressions of FOS (5.17-fold) and STAT3 (11.72-fold) decreased.

The results from neurotrophins & receptors pathway-focused genes expression analysis in the AIT-caused HT patients after treatment with cholecalciferol and L-thyroxine are presented in Table; Figs. 1; 2. Our analysis of genes expression showed that the expression of NGF and NGFR increased 7.57 and 9.47-fold, respectively. In contrast, the expressions of GFRA1 was decreased (4.43-fold). The mRNA levels of NTF3 (5.42-fold) and NTF4 (3.93-fold) significantly increased (Table; Figs. 1; 2). Also, we noted that the mRNA level of NRG1 was significantly increased (3.68-fold).

The results from neurogenesis pathway-focused genes expression analysis indicated that in the AIT-caused HT patients after treatment with cholecalciferol and L-thyroxine the expression of BDNF and FGF2 were increased by 24.02 and 11.39 times correspondingly,

whereas the expression of GDNF was 11.96-fold decreased (Table; Figs. 1; 2).

The results from apoptosis and cell cycle pathway-focused genes expression analysis indicated that in the AIT-caused HT patients after treatment with cholecalciferol and L-thyroxine, the expression of anti-apoptotic genes BCL2 and TGFA was increased by 3.2 and 4.5 times correspondingly, whereas HSPB1 was upregulated (16.91-fold) (Table). The expression of pro-apoptotic genes, such as BAX, was significantly decreased (35.85-fold). Whereas the expression of FAS was increased (12.78-fold) (Table; Figs. 1; 2). At the same time, the expression of promiscuous genes, regulators of apoptosis, have changed in different ways. The

Changes in the transcriptional activity of neurotrophins and receptors genes in the patients with AIT-caused HT + vitamin D vs the control group

Unigene	Refseq	Symbol	Description	Fold change	P
Hs.502182	NM_001709	BDNF	Brain-derived neurotrophic factor	24.02	0.016160
Hs.667309	NM_000043	FAS	Fas (TNF receptor superfamily, member 6)	12.78	0.011407
Hs.284244	NM_002006	FGF2	Fibroblast growth factor receptor substrate 2	11.39	0.000004
Hs.520973	NM_001540	HSPB1	Heat shock 27kDa protein 1	16.91	0.004734
Hs.193717	NM_000572	IL10	Interleukin 10	29.78	0.000032
Hs.2561	NM_002506	NGF	Nerve growth factor (beta polypeptide)	7.57	0.002776
Hs.415768	NM_002507	NGFR	Nerve growth factor receptor	9.47	0.001834
Hs.453951	NM_013957	NRG1	Neuregulin 1	3.68	0.008191
Hs.99171	NM_002527	NTF3	Neurotrophin 3	5.42	0.024310
Hs.266902	NM_006179	NTF4	Neurotrophin 4	3.93	0.039421
Hs.645227	NM_000660	TGFB1	Transforming growth factor, beta 1	37.96	0.000039
Hs.437460	NM_000546	TP53	Tumor protein p53	16.83	0.000011
Hs.624291	NM_004324	BAX	BCL2-associated X protein	-35.85	0.002270
Hs.472860	NM_001250	CD40	CD40 molecule, TNF receptor superfamily member 5	-9.34	0.021128
Hs.25647	NM_005252	FOS	FBJ murine osteosarcoma viral oncogene homolog	-5.17	0.015483
Hs.248114	NM_000514	GDNF	Glial cell derived neurotrophic factor	-11.96	0.000029
Hs.388347	NM_005264	GFRA1	GDNF family receptor alpha 1	-4.43	0.000297
Hs.126256	NM_000576	IL1B	Interleukin 1, beta	-6.85	0.006074
Hs.463059	NM_003150	STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	-11.72	0.003227

Fold-Change ($2^{-\text{delta-delta CT}}$) is the normalized gene expression ($2^{-\text{delta CT}}$) in the test group (AIT-caused HT + vitamin D) divided by the normalized gene expression ($2^{-\text{delta CT}}$) in the control group (standard therapy).

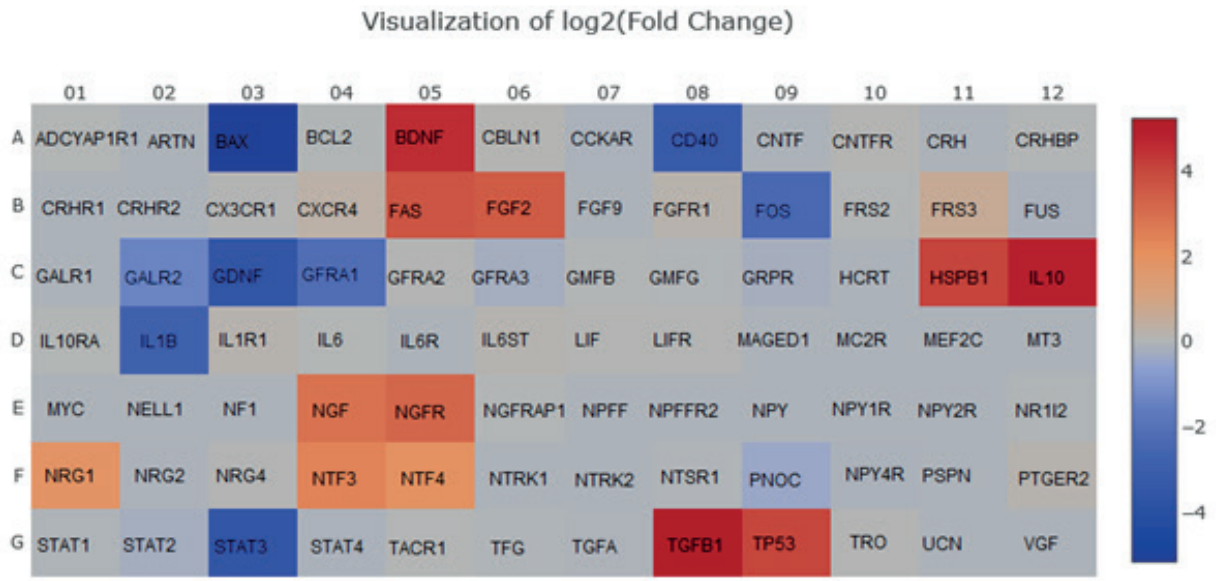

Fig. 1. Heat Map plot visualization of log2 (fold change) gene expression. The data are presented as the test group (AIT-caused HT + vitamin D) vs the control group (standard therapy)

expression of CD40 was markedly decreased (9.34-fold), while TGFB1 expression was increased (37.96-fold).

DISCUSSION

Vitamin D is an immunomodulator that plays a key role in the onset and progression of AIT. It binds to intracellular vitamin D receptors (VDR) and has a lot of biological effects [12]. The interaction between VDR and its ligand, as well as vitamin D responsive elements found in the promoter regions of hundreds of target genes, regulating the expression of over 500 genes. These genes are involved in various physiological functions, including brain development, neurological functions, inflammation, cell cycle control, immunomodulation, and apoptosis [13]. Furthermore, Vitamin D has been shown to regulate various neurotrophic factors, such as nerve growth factor (NGF), demonstrating its effect on neuronal proliferation, differentiation, survival, and growth. For example, 1-hydroxylase knockout mice, which cannot produce the active form of vitamin D (1,25(OH)2D), showed increased cell proliferation in the hippocampal dentate gyrus, decreased newborn neuron survival, and increased apoptosis in adult mice [14].

Vitamin D stimulates the production of NGF in the hippocampus, which promotes neurite outgrowth while inhibiting cellular proliferation [15]. In our study, the expression of NGF and NGFR was increased in the AIT-caused HT patients after treatment with cholecalciferol and L-thyroxine, compared to the patients who received only L-thyroxine ($P < 0.001$).

Neurotrophic chemicals are essential for nervous system development and maintenance. Researchers were able to determine the expression of neurotrophic factors by NSCs after culturing using 1,25(OH)2D3. They discovered that 1,25(OH)2D3 significantly increased NT-3, BDNF, CNTF, and GDNF expression in NSCs [16]. We found that in the AIT-caused HT patients the mRNA levels of NTF3 and NTF4 were significantly increased, whereas the expression of GDNF was decreased. It was shown that 1,25(OH)2D3 increased the expression of NT-3, NGF, and GDNF in neural cells [17]. In our study, the BDNF expression was upregulated in the AIT-caused HT patients with after

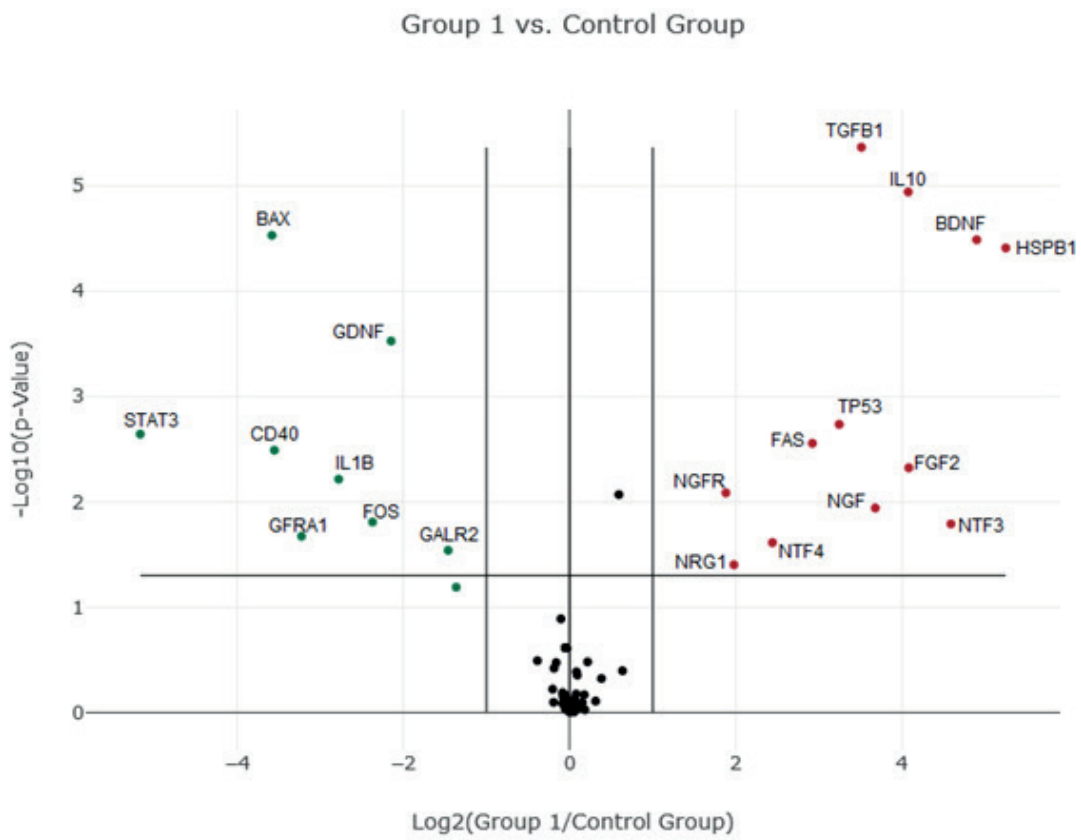

Fig. 2. This Volcano Plot identifies significant gene expression changes by plotting the log2 of fold changes in gene expression on the x-axis versus their statistical significance on the y-axis. The center vertical line indicates unchanged gene expression, while the two outer vertical lines indicate the selected fold regulation threshold. The horizontal line indicates the selected P-value threshold. Genes with data points in the far upper left (down-regulated) and far upper right (up-regulated) sections meet the selected fold regulation and P-value thresholds. By combining the fold change results with the p-value statistical test results, genes with both large and small statistically significant expression changes are easily visualized. The data are presented as Group 1 (AIT-caused HT + vitamin D) vs the control group (standard therapy)

treatment with cholecalciferol and L-thyroxine compared to the patients who received only L-thyroxine ($P < 0.001$).

Because vitamin D supplementation significantly increased mRNA expression, the researchers believe that BDNF, IGF-I, NGF, and their associated receptors (TrkA and TrkB) may play a vital role [16]. It was shown that vitamin D administration significantly increased NGF production in primary cultures of the hippocampus and cortical neurons [17]. Vitamin D intake increases expression of proteins and neurotrophic factors involved in synaptic plasticity and physiological neuronal health [5]. FGF2 is essential for the central nervous system maintenance and neurodevelopment [18, 19]. Thyroid hormone disruption is linked to affective disorders, in addition to increasing sensitivity to depressive symptoms in hypothyroid people and increasing anxiety in hyperthyroid patients [19]. In our study, the mRNA level of FGF2 was markedly increased in the AIT-caused HT patients after treatment with cholecalciferol and L-thyroxine, compared with the patients who received only L-thyroxine.

IL-10 is a critical Treg cytokine for maintaining and managing inflammation. IL-10 stimulates synthesis of antibodies, which are required for development of HT, particularly TPOAbs [20]. Furthermore, the network's intricate relationships between cytokines can cause them to switch from producing traditional anti-inflam-

matory properties to proinflammatory ones. This complex cytokine interplay may explain how IL-10 contributes to HT vulnerability by enhancing the pathogenic effect of B-cell autoantibody synthesis [21]. The expression analysis of genes involved in the production of cytokines and their receptors showed that the expression of IL10 increased, while the expression of IL1B decreased in the AIT-caused HT patients after treatment with cholecalciferol and L-thyroxine, compared to the patients who received only L-thyroxine. 1,25-(OH)2D3, an active form of vitamin D, inhibited the secretion of critical Th1 proinflammatory cytokines by antigen-presenting cells, preventing the development of cytotoxic Th1 lymphocytes and increasing the synthesis of the Th2 cytokine (IL-4) [22].

According to Christakos S. [23], vitamin D3 regulates several biological processes, including apoptotic cell death. Vitamin D anti-apoptotic action in experimental models of ischemic-reperfusion injury was demonstrated by decreased caspase expression [24]. Bcl-2 family proteins also control apoptosis. This class includes pro-apoptotic proteins, like Bax, and anti-apoptotic proteins [25]. In our study, the expression of anti-apoptotic genes such as BCL2 and TGFA was increased, whereas HSPB1 was upregulated. The expression of pro-apoptotic genes, such as Bax, was significantly decreased, whereas the expression of FAS was increased. The results from apoptosis and cell cycle pathway-focused genes expression analysis indicated that in the AIT-caused HT patients after treatment with cholecalciferol and L-thyroxine the expression of promiscuous genes, such as CD40, was markedly decreased, while TGFB1 expression was increased. Recent research has revealed that G1 cell cycle arrest regulates vitamin D anti-apoptotic and anti-proliferative effects. According to Chen et al. [26], vitamin D has an anti-proliferative effect on B cell development and the formation of autoantibodies.

Monitoring vitamin D levels is important because of the general appearance of vitamin D deficiency, especially in patients with AIT and hypothyroiditis. Given its neuroprotective properties, vitamin D supplementation is a simple, low-cost, and effective way to address a variety of health concerns.

CONCLUSIONS

1.The results showed that in the AIT-caused HT patients the expression of BDNF, FAS, FGF2, HSPB1, IL10, NGF, NGFR, NRG1, NTF3, NTF4, TGFB1, and TP53 were increased after treatment with cholecalciferol and L-thyroxine. The mRNA levels of BAX, GDNF, CD40, FOS, GDNF, GFRA1, IL1B, and STAT3 were significantly decreased in the AIT-caused HT patients after treatment with cholecalciferol and L-thyroxine, compared to the patients who received only L-thyroxine.

2. We expect more appropriate randomized double trials to be performed in the future blinded placebo-controlled studies with longer follow-up to confirm the effect of vitamin D supplementation in the treatment of AIT and hypothyroidism, especially in patients with vitamin D deficiency.

The authors of this study confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of co-authors of the article.

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ДОДАВАННЯ ВІТАМІНУ D ДО СТАНДАРТНОЇ ТЕРАПІЇ ЗМІНЮЄ ТРАНСКРИПЦІЙНИЙ ПРОФІЛЬ КЛІТИН КРОВІ У ПАЦІЄНТІВ З ПЕРВИННИМ ГІПОТИРЕОЗОМ

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Метою нашої роботи було вивчення впливу холекальциферолу на експресію генів, орієнтованих на шляхи

нейрогенезу, нейротрофінів та їх рецепторів у пацієнтів з аутоімунним тиреоїдитом (AIT) та гіпотиреозом. Ми застосували специфічний ПЛР-еррей (Neurotrophins and Receptors RT2 Profiler PCR Array, «QIAGEN», Німеччина) для визначення експресії генів-регуляторів нейрогенезу у пацієнтів із патологією щитоподібної залози. Результати показали, що у пацієнтів з первинним гіпотиреозом внаслідок AIT експресія генів BDNF, FAS, FGF2, HSPB1, IL10, NGF, NGFR, NRG1, NTF3, NTF4, TGFB1 і TP53 була підвищена після лікування холекальциферолом і L-тироксином. Концентрації мРНК BAX, GDNF, CD40, FOS, GDNF, GFRA1, IL1B і STAT3 був значно зниженим у пацієнтів з первинним гіпотиреозом внаслідок аутоімунного тиреоїдиту після лікування холекальциферолом і L-тироксином порівняно з пацієнтами, які отримували лише L-тироксин. Отже, додавання вітаміну D до стандартної терапії змінює транскрипційний профіль клітин крові у пацієнтів з первинним гіпотиреозом.

Ключові слова: вітамін D; нейрогенез; мРНК; аутоімунний тиреоїдит; гіпотиреоз.

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