

Association of the blood serum cytokines' rate and lymphocytes' apoptosis with polymorphic variants of the *BCL-2* (rs17759659), *CTLA-4* (rs231775) and *APO-1/FAS* (rs2234767) genes in patients with nodular goiters in autoimmune thyroiditis and thyroid adenoma

MICHAEL IVANOVICH SHEREMET¹⁾, LARYSA PETRIVNA SYDORCHUK²⁾,
 VIKTOR OLEKSANDROVYCH SHIDLOVSKYI³⁾, RUSLAN IGOROVYCH SYDORCHUK¹⁾,
 OLEKSANDR VIKTOROVYCH SHIDLOVSKYI³⁾, VITALIY VASILYOVICH MAKSYMUK¹⁾,
 VOLODYMYR VOLODYMYROVYCH BEZRUK⁴⁾, NINA PETRIVNA TKACHUK¹⁾,
 VIKTOR MARKIYANOVICH BATIG⁵⁾, OLEKSANDR VIKTOROVYCH MYTCHENOK⁵⁾,
 TETYANA OLEKSANDRIVNA BEZRUK⁶⁾, KRISTINA ANDREYVNA CHYMPOI⁶⁾

¹⁾Department of Surgery No. 1, Bukovinian State Medical University, Chernivtsi, Ukraine

²⁾Department of Family Medicine, Bukovinian State Medical University, Chernivtsi, Ukraine

³⁾Department of Surgery with Urology No. 1, "I. Horbachevsky" Ternopil State Medical University, Ternopil, Ukraine

⁴⁾Department of Pediatrics, Neonatology and Perinatology of Medicine, Bukovinian State Medical University, Chernivtsi, Ukraine

⁵⁾Department of Therapeutic Stomatology, Bukovinian State Medical University, Chernivtsi, Ukraine

⁶⁾Department of Internal Medicine and Infectious Diseases, Bukovinian State Medical University, Chernivtsi, Ukraine

Abstract

The paper analyses results of serum cytokines and lymphocyte apoptosis study in patients with nodular goiter against the background of autoimmune thyroiditis (NGAIT) and thyroid adenoma (TA) based on the cell preparedness to apoptosis (content of lymphocytes carrying apoptosis marker – CD95⁺-receptor), the number of apoptotic lymphocytes (annexin V⁺-lymphocytes) and the content of proapoptotic tumor necrosis factor-alpha (TNF- α), interleukin (IL)-1 β and IL-6, as well as anti-inflammatory IL-4 cytokine in serum, considering the polymorphism of *BCL-2* (rs17759659), *CTLA-4* (rs231775) and *APO-1/FAS* (rs2234767) genes. The results show that under the damaging action of peroxidation products in the thyroid structures, activation of *Fas*- and caspase-dependent mechanisms of influence on pro- and anti-apoptotic targets, the induced hyperproduction and release of TNF- α from thyroid-stimulated lymphocytes stimulate an additional synthesis of other pro-inflammatory cytokines IL-1 β and IL-6, as well as compensatory anti-inflammatory proteins including IL-4. There is a synchronized increase in secretion of the soluble form of TNF- α receptor (sTNFR), which prevents binding the corresponding cytokine to a specific membranous shedding of a number of receptors and separates the apoptotic signals. The above-mentioned changes associate with the polymorphic variants of *BCL-2* (rs17759659), *CTLA-4* (rs231775) genes and only for some figures, they are almost three times weaker with *FAS* (rs2234767).

Keywords: nodular goiter, autoimmune thyroiditis, cytokines, genetics, apoptosis, thyroid gland.

Introduction

The incidence of autoimmune thyroiditis (AIT) is growing every year and in the future, an upward trend is expected to remain, following introduction of new diagnostic methods and techniques [1–5]. Mechanisms underlying the deregulation of the immune system with autoimmune pathologies, including AIT are relevant and require further studying. In addition, recent studies have shown that genetic mutations, especially those of regulatory genes cause the development of thyropathies, including nodular goiter against the background of autoimmune thyroiditis (NGAIT) [6–7].

A promising area of this research is immunodependent

apoptosis of the thyroid cells [8–10]. The mechanisms of inhibition and immune activation are associated with apoptosis modulation of immune cells including that through the effector molecules of cytotoxic T-lymphocytes (CD95⁺-lymphocytes), CD95 antigen (also known as *Fas/Apo-1*) and *Fas* ligand, cascade of cytokine reactions, etc. The key mediators of inflammation onset, including in AIT are pre-immune and immune cytokines: tumor necrosis factor-alpha (TNF- α), interleukin (IL)-1 β and IL-6, IL-4, IL-8 and others, respectively [11]. However, the same cytokine may lead to multidirectional effect (stimulate or limit the proliferation, differentiation, migration and effector function of immune cells), depending on its concentration, such as a specific receptor on

the cell and the state of its activation [12]. TNF- α is known to be produced by macrophages and by activated thyroid T-lymphocytes and T-cells that are at rest. TNF- α increases the cytotoxic activity of lymphocytes that infiltrate the thyroid gland and is involved in the process of apoptosis-mediated death, that is, to some extent, it is a component of not only damaging, but defensive reaction of the microorganism that controls the “strength” of autoimmune process [13, 14]. Feedback mechanisms controlling inflammation reaction manifestations are associated with production of anti-inflammatory interleukins (IL-4, IL-10, IL-13) and soluble inhibitors of pro-inflammatory cytokines, which have immunosuppressive effects.

The aim of the study is to evaluate the cytokines in peripheral blood and lymphocyte apoptosis in patients with NGAIT and thyroid adenoma (TA) by determining the cell preparedness to apoptosis (content of the lymphocytes carrying apoptosis marker – CD95⁺-receptor), the number of apoptotic lymphocytes (annexin V⁺-lymphocytes), and the content of proapoptotic factors – TNF- α , IL-1 β and IL-6 and anti-inflammatory IL-4 cytokine in the serum, considering the polymorphism of *BCL-2* (*rs17759659*), *CTLA-4* (*rs231775*) and *APO-1/FAS* (*rs2234767*) genes.

Patients, Materials and Methods

One hundred and twenty-five women with NGAIT were examined in 2013–2016 in Chernivtsi Regional Clinical Hospital (Ukraine). The age of patients ranged from 23 to 72 years. The diagnosis was established clinically and laboratory [thyroid peroxidase antibodies (TPABs) 60–250 U/mL; thyroglobulin antibodies (TGABs) 60–500 U/mL; thyroid-stimulating hormone (TSH) 4.1 mU/L], ultrasonographically and histologically after surgery.

We singled out a group of 30 women who had been diagnosed with thyroid adenoma after the surgery by ultrasound, fine-needle aspiration biopsy (FNAB) and histological conclusion. The group was identified because this pathology is one of the most common forms of nodular goiter. Twenty-five practically healthy donors were ex-

amined as well. Ten mL of the peripheral blood drawn from the ulnar vein in the morning on an empty stomach served as the material for the study.

Lymphocytes were isolated by sedimentation of blood in ficoll-verografin gradient 1.077 h/cm³. Determination of lymphocytes carrying an apoptosis marker CD95⁺-receptor was conducted in extracorporeal conditions using monoclonal antibodies CD95 (Caltag, Austria) in a lymphocytotoxic test. The level of apoptosis in the population of peripheral blood lymphocytes was determined by placing the ex-depression on the external monolayer of plasma membrane of phosphatidylserine molecules by fluorescence microscopy using fluorescein isothiocyanate (FITC)-labeled annexin V⁺ through a set of annexin V⁺-FITC (Beckman Coulter, France).

To study the concentrations of TNF- α , flow cytometry and simplex sets made by the Bender Medsystems GmbH (Austria) were applied using Multiplex technology, which makes it possible to determine the necessary amount of cytokine in one sample.

The DNA was isolated by means of a set of Thermo Scientific Gene JET Genomic DNA Purification kit (# K0721, Thermo Fisher Scientific) reagents, according to the manual, with incubation with proteinase K overnight to complete cell lysis. The purified DNA was diluted in Elution Buffer and evaluated with Nanodrop 2000C spectrophotometer. Only samples with concentration of not lower than 15 ng/mL and the values of the ratio of *A* (260/280) between 1.7 and 2.0 were used for genotyping. The obtained extracts were divided into aliquots, one of which was placed in a refrigerator at 4°C until its use, while others were frozen at -20°C.

To normalize the amount of DNA, all samples were brought to a concentration of 2 ng/ μ L using nuclease-free water. The TaqMan technology was used to genotype the selected point polymorphism. Polymorphisms marked with the reference number SNP (single-nucleotide polymorphism) ID, according to the dbSNP database. To test each of the polymorphisms, the TaqMan SN Genotyping Assays (40 \times) (4,351,379, Thermo Fisher Scientific) were used (Table 1).

Table 1 – The nucleotide sequence of the region including the analyzed polymorphism

Reference No. SNP ID	Test No. (Assay ID*)	Fragment of the region including the analyzed polymorphism
<i>rs231775</i> (<i>CTLA-4</i>)	C__2415786_20	GCACAAGGCTCAGCTGAACCTGGCT[A/G]CCAGGACCTGGCCCTGCACTCTCCT
<i>rs17759659</i> (<i>BCL-2</i>)	C__33628167_10	TCTTCTTACCAAAGATTCACAATAC[A/G]GTGTTGATGGGAACGTGACCTAGTT
<i>rs2234767</i> (<i>FAS</i>)	C__12123966_10	CAGAGTGTGTGCACAAGGCTGGCAC[A/G]CCCAGGGTCTTCTCATGGCACTAA

SNP: Single-nucleotide polymorphism. *According to the www.thermofisher.com website.

The volume of the reaction mixture was 5 μ L and consisted of: 2.5 μ L Taq Man Genotyping Master Mix (20 \times) (4,371,355, Thermo Fisher Scientific) reagent, 0.25 μ L of probe solution and 2.25 μ L of DNA solution. Genotyping was performed with the Quant Studio 6 (Applied Biosystems, Thermo Fisher Scientific) instrument, 384-well block.

Amplification was performed under the conditions presented at Table 2.

To collect and process the data the *Quant Studio*TM *Real-Time PCR* (v.1.3) software was used.

Table 2 – The conditions used for amplification

Activation	10 minutes	95°C	40*/60** cycles
Denaturation	15 seconds	92°C	
Annealing / elongation	1 minute	60°C	

*To amplify the polymorphisms associated with *CTLA-4* and *FAS* genes; **To amplify the polymorphisms associated with *BCL-2* gene.

The main part of the statistical analysis was carried out using the Statistica 7.0 (SPSS – Statistical Package for the Social Sciences) software. Nominal data is presented in the form of quantitative values and percentages. The

Hardy–Weinberg equilibrium of the genotype distribution was checked using the *Online Encyclopedia for Genetic Epidemiology Studies* (<http://www.oege.org/software/hwe-mr-calc.shtml>). To compare the distribution of genotypes in the experimental and control groups, the Pearson's (χ^2) *chi*-squared test was used. The reliability of differences of mean values in groups with different genotypes was determined by the method of univariate analysis of variance (ANOVA). The impact of factors on the development of thyroid pathology was assessed using a binary logistic regression model for the relative risk (RelR), risk ratio (RR) and odds ratio (OR) with 95% confidence interval (95% CI), taking into account the χ^2 criterion ($df=1$). The difference was considered reliable at $p<0.05$.

Results

It was established that the rate of lymphocytes representing CD95⁺-receptor (Table 3), marker of apoptosis, prevailed reliably in homozygous carriers of minor allele *G-BCL-2* gene (*rs17759659*) over those in people with *AA*- and *AG*-genotypes by 27.09% ($p_{AA}=0.034$) and 14% ($p_{AG}=0.002$). The average apoptotic activity of lymphocytes by the number of annexin V⁺-presenting cells among them in patients with thyroid pathology, by contrast, was much lower than in the control group by 35.06–44.46% ($r\leq 0.045$) without reliable dependence on the genotypes of *BCL-2* (*rs17759659*) gene. The latter, amid the high content of CD95⁺, indicates a disorder in the implementation of receptor-dependent apoptosis of lymphocytes. This

condition might be due to an enhanced synthesis of soluble forms of *Fas*-receptor (soluble *Fas* – *sFas*), which can accumulate in the microenvironment of lymphocytes and compete with membrane-localized receptor in ligand binding. There is evidence [11] that *sFas* inhibits receptor-mediated apoptosis and elimination of activated lymphocytes and promotes the development of autoaggressive cell clones and progression of an autoimmune process. Under these conditions the content of TNF- α and IL-1 β , as the apoptogenic cytokines and proinflammatory IL-6 in our patients significantly exceeded the indices of the control group by 2.89–4.37 times ($p\leq 0.006$) 3.31–4.91 times ($p\leq 0.008$) and 1.79–2.17 times ($p<0.001$), respectively. We have not found clear dependence of the above-mentioned changes on polymorphism of the *BCL-2* (*rs17759659*) gene. Instead, the content of anti-inflammatory IL-4, on the contrary, was slightly lower in patients than in the individuals from the control group, but reliably only in carriers of *GG*-genotype of the *BCL-2* gene by 15.14% ($p=0.038$).

Univariate analysis of variance confirmed the association of polymorphic site of the *BCL-2* (*rs17759659*) gene with indicators of “readiness” to apoptosis of lymphocytes – the number of CD95⁺-receptors ($F=28.36$, $p<0.001$), somewhat less with apoptotic activity of lymphocytes by the number of annexin V⁺-lymphocytes ($F=4.17$, $p=0.018$), and with cytokines: TNF- α ($F=47.47$, $p<0.001$), IL-1 β ($F=16.13$, $p<0.001$), IL-6 ($F=23.62$, $p<0.001$) and IL-4 ($F=9.19$, $p<0.001$) (Table 3).

Table 3 – The rates of lymphocyte apoptosis and serum cytokines considering the polymorphic variants of the *BCL-2* (*rs17759659*) gene

Rates	Control, n=25	Genotypes of the <i>BCL-2</i> gene in patients		
		AA, n=10	AG, n=110	GG, n=5
CD95 ⁺ -lymphocytes [%]	11.39±0.65	15.06±0.91; $p=0.011$	16.79±1.02; $p=0.004$	19.14±0.77; $p<0.001$ $p_{AA}=0.034$; $p_{AG}=0.002$
Annexin V ⁺ -lymphocytes [s.u.]	17.77±0.38	11.54±1.06; $p=0.045$	10.64±1.15; $p=0.02$	9.87±1.06; $p=0.013$
TNF- α [pg/mL]	1.95±0.13	8.52±1.0; $p=0.001$	5.64±0.98; $p=0.006$ $p_{AA}=0.028$	7.71±1.08; $p=0.002$
IL-1 β [pg/mL]	1.93±0.13	7.48±1.21; $p=0.003$	6.38±1.30; $p=0.008$	9.47±1.39; $p=0.008$
IL-4 [pg/mL]	1.85±0.08	1.83±0.13	1.73±0.11	1.57±0.10; $p=0.038$
IL-6 [pg/mL]	2.79±0.08	5.68±0.41; $p<0.001$	5.0±0.44; $p<0.001$	6.06±0.45; $p<0.001$

TNF- α : Tumor necrosis factor-alpha; IL: Interleukin; p : Reliability of difference of values compared to those in the control group; p_{AA} : Reliability of difference of values compared to those in AA-genotype carriers; p_{AG} : Reliability of difference of values compared to those in AG-genotype carriers.

Among patients with thyroid pathology prevailed those with a high level (>50th percentile) of CD95⁺-lymphocytes and TNF- α by 16.8% ($p=0.008$) against a moderately ($\leq 50^{\text{th}}$ percentile) increased content of IL-1 β in the serum by 58.4% ($p<0.001$) and IL-6 by 56.8% ($p<0.001$) and a slight increase ($\leq 25^{\text{th}}$ percentile) of IL-4 by 10.4% ($p>0.05$), with low concentration of annexin V⁺-lymphocytes (>50th percentile) – by 16.8% ($p=0.008$), primarily in patients with *G*-allele of the *BCL-2* (*rs17759659*) gene (people with *AG*- and *GG*-genotypes): by 18.23% ($p=0.006$), 61.74% ($p<0.001$) and 60% ($p<0.001$), respectively, in IL-4 – by 11.3% ($p=0.057$) and annexin V⁺-lymphocytes – by 18.23% ($p=0.006$) (Table 4).

The content of lymphocytes with CD95⁺-receptors, TNF- α and IL-1 β in serum prevailed in the carriers of the main *A*-allele of the *CTLA-4* (*rs231775*) gene (Table 5)

over those in patients with *GG*-genotype and individuals from the control group: in CD95⁺-lymphocytes – by 36.08% ($p_{AA}=0.01$) and 37.14% ($p_{AG}=0.011$), in TNF- α – by 2.14 ($p_{AA}=0.015$) and 2.07 times ($p_{AG}=0.012$), in IL-1 β – by 2.2 ($p_{AA}=0.017$) and 2.07 times ($p_{AG}=0.018$), respectively. The apoptotic activity of lymphocytes by the number of annexin V⁺-presenting cells on the contrary was significantly lower in carriers of the wild-*A*-allele of the *CTLA-4* (*rs231775*) gene, than in patients with the *GG*-genotype and the control group by 32.40% ($p_{AA}=0.017$) and 36.56% ($p_{AG}=0.018$), respectively. The level of IL-6 prevailed in the serum of patients with thyroid pathology, but without reliable dependences, taking into account the polymorphic variants of the *CTLA-4* (*rs231775*) gene (Table 5).

Univariate analysis of variance confirmed the

association of polymorphic site of the *CTLA-4* (*rs231775*) gene with the rates of “readiness” to apoptosis of lymphocytes – the number of cells with CD95⁺-receptors ($F=24.28$, $p<0.001$), somewhat stronger

with apoptotic activity of lymphocytes by the number of annexin V⁺-lymphocytes ($F=51.24$, $p<0.001$), and with proinflammatory cytokines: TNF- α ($F=21.29$, $p<0.001$), IL-1 β ($F=15.28$, $p<0.001$) and IL-6 ($F=22.44$, $p<0.001$).

Table 4 – The rates of lymphocyte apoptosis in the peripheral blood and serum cytokines in the patients with thyroid pathology considering the polymorphic variants of the *BCL-2* (*rs17759659*) gene

Rates	Changes in the rates, <i>n</i>	Genotypes of the <i>BCL-2</i> gene, <i>n</i> =125 (percent)		
		AA, <i>n</i> =10	AG, <i>n</i> =110	GG, <i>n</i> =5
CD95 ⁺ -lymphocytes [%]	Moderate increase ($\leq 50^{\text{th}}$ percentile), <i>n</i> =52	5 (50%)	46 (41.82%)	1 (20%)
	Significant increase ($> 50^{\text{th}}$ percentile), <i>n</i> =73	5 (50%)	64 (58.18%)	4 (80%)
	χ^2 ; <i>p</i>	$p>0.05$	$\chi^2=5.89$; $p=0.015$	$p>0.05$
Annexin V ⁺ -lymphocytes [s.u.]	Moderate decrease ($\leq 50^{\text{th}}$ percentile), <i>n</i> =52	5 (50%)	46 (41.82%)	1 (20%)
	Significant decrease ($> 50^{\text{th}}$ percentile), <i>n</i> =73	5 (50%)	64 (58.18%)	4 (80%)
	χ^2 ; <i>p</i>	$p>0.05$	$\chi^2=5.89$; $p=0.015$	$p>0.05$
TNF- α [pg/mL]	Moderate decrease ($\leq 50^{\text{th}}$ percentile), <i>n</i> =32	1 (10%)	31 (28.18%)	0
	Moderate increase ($\leq 50^{\text{th}}$ percentile), <i>n</i> =20	4 (40%)	15 (13.64%)	1 (20%)
	Significant increase ($> 50^{\text{th}}$ percentile), <i>n</i> =73	5 (50%)	64 (58.18%)	4 (80%)
	χ^2 ; <i>p</i>	$\chi^2=3.90$; $p>0.05$	$\chi^2=51.08$; $p<0.001$	$p>0.05$
IL-1 β [pg/mL]	Moderate increase ($\leq 50^{\text{th}}$ percentile), <i>n</i> =99	6 (60%)	90 (81.82%)	3 (60%)
	Significant increase ($> 50^{\text{th}}$ percentile), <i>n</i> =26	4 (40%)	20 (18.18%)	2 (40%)
	χ^2 ; <i>p</i>	$\chi^2<1.0$; $p>0.05$	$\chi^2=89.09$; $p<0.001$	$p>0.05$
IL-4 [pg/mL]	An increase $\leq 25^{\text{th}}$ percentile, <i>n</i> =69	5 (50%)	62 (56.36%)	2 (40%)
	Normal, <i>n</i> =31	1 (10%)	29 (26.36%)	1 (20%)
	Significant decrease ($> 50^{\text{th}}$ percentile), <i>n</i> =25	4 (40%)	19 (17.27%)	2 (40%)
	χ^2 ; <i>p</i>	$\chi^2=3.90$; $p>0.05$	$\chi^2=41.23$; $p<0.001$	$\chi^2<1.0$; $p>0.05$
IL-6 [pg/mL]	Moderate increase ($\leq 50^{\text{th}}$ percentile), <i>n</i> =98	6 (60%)	89 (80.91%)	3 (60%)
	Significant increase ($> 50^{\text{th}}$ percentile), <i>n</i> =27	4 (40%)	21 (19.09%)	2 (40%)
	χ^2 ; <i>p</i>	$\chi^2<1.0$; $p>0.05$	$\chi^2=84.07$; $p<0.001$	–

TNF- α : Tumor necrosis factor-alpha; IL: Interleukin.

Table 5 – The rates of lymphocyte apoptosis and serum cytokines considering the polymorphic variants of the *CTLA-4* (*rs231775*) gene

Rates	Control, <i>n</i> =25	Genotypes of the <i>CTLA-4</i> gene in patients		
		AA, <i>n</i> =59	AG, <i>n</i> =62	GG, <i>n</i> =4
CD95 ⁺ -lymphocytes [%]	11.39 \pm 0.65	16.82 \pm 1.28; $p=0.006$	16.95 \pm 1.31; $p=0.006$	12.36 \pm 0.55 $p_{AA}=0.01$; $p_{AG}=0.011$
Annexin V ⁺ -lymphocytes [s.u.]	17.77 \pm 0.38	10.89 \pm 1.21; $p=0.002$	10.22 \pm 1.05; $p<0.001$	16.11 \pm 1.49 $p_{AA}=0.009$; $p_{AG}=0.01$
TNF- α [pg/mL]	1.95 \pm 0.13	5.98 \pm 1.02; $p=0.005$	5.78 \pm 0.88; $p=0.004$	2.79 \pm 0.74 $p_{AA}=0.015$; $p_{AG}=0.012$
IL-1 β [pg/mL]	1.93 \pm 0.13	6.83 \pm 1.40; $p=0.008$	6.44 \pm 1.25; $p=0.007$	3.11 \pm 0.55; $p=0.046$ $p_{AA}=0.017$; $p_{AG}=0.018$
IL-4 [pg/mL]	1.85 \pm 0.08	1.71 \pm 0.12	1.72 \pm 0.11	1.80 \pm 0.10
IL-6 [pg/mL]	2.79 \pm 0.08	5.12 \pm 0.48; $p=0.003$	5.07 \pm 0.43; $p=0.002$	3.56 \pm 0.34; $p=0.037$

TNF- α : Tumor necrosis factor-alpha; IL: Interleukin; *p*: Reliability of difference of values compared to those in the control group; p_{AA} : Reliability of difference of values compared to those in AA-genotype carriers; p_{AG} : Reliability of difference of values compared to those in AG-genotype carriers.

Qualitative analysis of the above-mentioned changes in rates considering the genotypes of the *CTLA-4* (*rs231775*) gene showed (Table 6) that among patients with thyroid pathology prevailed those with a high level ($> 50^{\text{th}}$ percentile) of CD95⁺-lymphocytes and TNF- α by 15.7% ($\chi^2=5.97$, $p=0.014$), moderately ($\leq 50^{\text{th}}$ percentile) increased content of serum IL-1 β – by 60.32% ($p<0.001$) and IL-6 – by 58.68% ($p<0.001$) and a slight increase ($\leq 25^{\text{th}}$ percentile) of IL-4 – by 10.74% ($p>0.05$) with low concentration of annexin V⁺-lymphocytes ($> 50^{\text{th}}$ percentile) – by 15.7% ($\chi^2=5.97$, $p=0.014$), primarily in patients with major A-allele of the *CTLA-4* (*rs231775*) gene.

The analyzed rates of apoptosis and serum cytokine profile in the patients did not depend on polymorphic variants of the *FAS* (*rs2234767*) gene and corresponded to the general trend of the surveyed population (Table 7). In patients the content of CD95⁺-lymphocytes, TNF- α , IL-1 β and IL-6 prevailed over those of the control group by 44.16% ($p=0.006$) and 47.94% ($p=0.004$); for TNF- α – by 2.85 ($p=0.005$) and 3.07 times ($p=0.004$); for IL-1 β – by 3.54 and 3.08 times ($p=0.008$); for IL-6 by 1.75 ($p=0.003$) and 1.85 ($p=0.002$) times, respectively. The number of annexin V⁺-lymphocytes on the contrary was significantly lower than in the control group – by 37.08% ($p=0.001$) and 40.52% ($p<0.001$), respectively.

Univariate analysis confirmed the association of polymorphic site of the *FAS* (*rs2234767*) gene only with the apoptotic activity of lymphocytes by the number of annexin V⁺-lymphocytes ($F=8.79$, $p=0.004$), and by proinflammatory cytokines: TNF- α ($F=4.12$, $p=0.045$), IL-1 β ($F=10.48$, $p=0.002$) and IL-6 ($F=7.17$, $p=0.008$).

Table 6 – The rates of lymphocyte apoptosis in the peripheral blood and serum cytokines in the patients with thyroid pathology considering the polymorphic variants of the CTLA-4 (*rs231775*) gene

Rates	Changes in the rates, <i>n</i>	Genotypes of the CTLA-4 gene, <i>n</i> =125 (percent)		
		AA, <i>n</i> =59	AG, <i>n</i> =62	GG, <i>n</i> =4
CD95 ⁺ -lymphocytes [%]	Moderate increase ($\leq 50^{\text{th}}$ percentile), <i>n</i> =52	23 (38.98%)	28 (45.16%)	1 (25%)
	Significant increase ($> 50^{\text{th}}$ percentile), <i>n</i> =73	36 (61.02%)	34 (54.84%)	3 (75%)
	χ^2 ; <i>p</i>	$\chi^2=5.73$; $p=0.017$	$\chi^2=1.16$; $p>0.05$	$p>0.05$
Annexin V ⁺ -lymphocytes [s.u.]	Moderate decrease ($\leq 50^{\text{th}}$ percentile), <i>n</i> =52	23 (38.98%)	28 (45.16%)	1 (25%)
	Significant decrease ($> 50^{\text{th}}$ percentile), <i>n</i> =73	36 (61.02%)	34 (54.84%)	3 (75%)
	χ^2 ; <i>p</i>	$\chi^2=5.73$; $p=0.017$	$\chi^2=1.16$; $p>0.05$	$p>0.05$
TNF- α [pg/mL]	Moderate decrease ($\leq 50^{\text{th}}$ percentile), <i>n</i> =32	14 (23.73%)	18 (29.03%)	0
	Moderate increase ($\leq 50^{\text{th}}$ percentile), <i>n</i> =20	9 (15.25%)	10 (16.13%)	1 (25%)
	Significant increase ($> 50^{\text{th}}$ percentile), <i>n</i> =73	36 (61.02%)	34 (54.84%)	3 (75%)
	χ^2 ; <i>p</i>	$\chi^2=31.45$; $p<0.001$	$\chi^2=21.68$; $p<0.001$	$p>0.05$
IL-1 β [pg/mL]	Moderate increase ($\leq 50^{\text{th}}$ percentile), <i>n</i> =99	45 (76.27%)	52 (83.87%)	2 (50%)
	Significant increase ($> 50^{\text{th}}$ percentile), <i>n</i> =26	14 (23.73%)	10 (16.13%)	2 (50%)
	χ^2 ; <i>p</i>	$\chi^2=32.58$; $p<0.001$	$\chi^2=56.90$; $p<0.001$	$p>0.05$
IL-4 [pg/mL]	An increase $\leq 25^{\text{th}}$ percentile, <i>n</i> =69	37 (62.71%)	30 (48.39%)	2 (50%)
	Normal, <i>n</i> =30	8 (13.56%)	22 (35.48%)	0
	Significant decrease ($> 50^{\text{th}}$ percentile), <i>n</i> =26	14 (23.73%)	10 (16.13%)	2 (50%)
	χ^2 ; <i>p</i>	$\chi^2=35.75$; $p<0.001$	$\chi^2=14.71$; $p<0.001$	$p>0.05$
IL-6 [pg/mL]	Moderate increase ($\leq 50^{\text{th}}$ percentile), <i>n</i> =98	45 (76.27%)	51 (82.26%)	2 (50%)
	Significant increase ($> 50^{\text{th}}$ percentile), <i>n</i> =27	14 (23.73%)	11 (17.74%)	2 (50%)
	χ^2 ; <i>p</i>	$\chi^2=32.58$; $p<0.001$	$\chi^2=51.61$; $p<0.001$	$p>0.05$

TNF- α : Tumor necrosis factor-alpha; IL: Interleukin.

Table 7 – The rates of lymphocyte apoptosis and serum cytokines considering the polymorphic variants of the APO-1/*FAS* (*rs2234767*) gene

Rates	Control, <i>n</i> =25	Genotypes of the <i>FAS</i> gene in patients	
		AG, <i>n</i> =23	GG, <i>n</i> =102
CD95 ⁺ -lymphocytes [%]	11.39 \pm 0.65	16.42 \pm 1.11; $p=0.006$	16.85 \pm 1.07; $p=0.004$
Annexin V ⁺ -lymphocytes [s.u.]	17.77 \pm 0.38	11.18 \pm 1.06; $p=0.001$	10.57 \pm 0.85; $p<0.001$
TNF- α [pg/mL]	1.95 \pm 0.13	5.55 \pm 0.89; $p=0.005$	5.99 \pm 0.95; $p=0.004$
IL-1 β [pg/mL]	1.93 \pm 0.13	6.84 \pm 1.39; $p=0.008$	5.94 \pm 1.16; $p=0.008$
IL-4 [pg/mL]	1.85 \pm 0.08	1.71 \pm 0.09	1.74 \pm 0.08
IL-6 [pg/mL]	2.79 \pm 0.08	4.87 \pm 0.42; $p=0.003$	5.15 \pm 0.46; $p=0.002$

TNF- α : Tumor necrosis factor-alpha; IL: Interleukin;

Among the homozygous carriers of the wild-*G*-allele of the *FAS* (*rs2234767*) gene, there were reliably more individuals (Table 8) with a significant content ($> 50^{\text{th}}$ percentile) of CD95⁺-lymphocytes, low rates of annexin V⁺-lymphocytes ($> 50^{\text{th}}$ percentile) and a slight increase in the serum ($\leq 25^{\text{th}}$ percentile) IL-4 by 15.68% ($p<0.05$). Also, there were more individuals with a significant rate of TNF- α (by 39.13%; $p=0.003$ and 15.69%; $p<0.001$), increase of IL-1 β and IL-6 (by 65.22%, 56.86% and 54.90%; $p<0.001$, respectively), which did not depend on polymorphic variants of the *FAS* (*rs2234767*) gene.

The risk of NGAIT and TA in the observed population increases with the significant rise of CD95⁺-lymphocytes and TNF- α ($> 50^{\text{th}}$ percentile) in serum and decrease of annexin V⁺-lymphocytes ($> 50^{\text{th}}$ percentile) by 1.45 times (OR: 2.09; 95% CI OR: 1.24–2.52; $p=0.006$), and with

an increase of IL-1 β in the serum – by 4.23 times (OR: 17.87; 95% CI OR: 9.26–34.48; $p<0.001$), but only in the carriers of the minor *G*-allele of the *BCL-2* (*rs17759659*) gene (Table 9). On the other hand, the likelihood of thyroid pathology decreases with the low content of anti-inflammatory IL-4 cytokine (OR: 0.05; 95% CI OR: 0.03–0.10; $p<0.001$) and high levels of IL-6 ($> 50^{\text{th}}$ percentile) (OR: 0.06; 95% CI OR: 0.03–0.12; $p<0.001$).

Increasing CD95⁺-lymphocytes and TNF- α ($> 50^{\text{th}}$ percentile) and decreasing annexin V⁺-lymphocytes ($> 50^{\text{th}}$ percentile) in serum increase the risk of AIT and TA in the observed population 1.56 fold (OR: 2.45; 95% CI OR: 1.17–5.13; $p=0.017$), but only in homozygous carriers of the main A-allele (*AA*-genotype) of the *CTLA-4* (*rs231775*) gene (Table 10). A moderate serum IL-1 β

increase also increases this risk by 6.35 times (OR: 23.57; 95% CI OR: 6.13–90.67; $p < 0.001$), while a slight elevation of IL-4 ($\leq 25^{\text{th}}$ percentile) does it by 5.23 times (OR: 12.33; 95% CI OR: 3.31–46.01; $p < 0.001$), but again, only in carriers of AA-genotype of the *CTLA-4* (*rs231775*) gene. The lowest probability of thyroid pathology in the observed population is in the owners of G-allele of the above-mentioned gene with the low content of anti-inflammatory IL-4 cytokine (OR: 0.03; 95% CI OR: 0.01–0.12; $p < 0.001$) and high IL-6 levels

($> 50^{\text{th}}$ percentile) (OR: 0.03; 95% CI OR: 0.01–0.13; $p < 0.001$).

The risk of AIT and TA in the examined population increases with a significant increase of CD95⁺-lymphocytes and TNF- α ($> 50^{\text{th}}$ percentile) and decrease of annexin V⁺-lymphocytes ($> 50^{\text{th}}$ percentile) in the serum – by 1.37 times (OR: 1.88; 95% CI OR: 1.08–3.28; $p = 0.018$), but only in homozygous carriers of wild G-allele (GG-genotype) of the *FAS* (*rs2234767*) gene (Table 11).

Table 8 – The rates of lymphocyte apoptosis in the peripheral blood and serum cytokines in the patients with thyroid pathology considering the polymorphic variants of the APO-1/*FAS* (*rs2234767*) gene

Rates	Changes in the rates, <i>n</i>	Genotypes of the <i>FAS</i> gene, <i>n</i> =125 (percent)	
		AG, <i>n</i> =23	GG, <i>n</i> =102
CD95 ⁺ -lymphocytes [%]	Moderate increase ($\leq 50^{\text{th}}$ percentile), <i>n</i> =52	9 (39.13%)	43 (42.16%)
	Significant increase ($> 50^{\text{th}}$ percentile), <i>n</i> =73	14 (60.87%)	59 (57.84%)
	χ^2 ; <i>p</i>	$\chi^2=2.17$; $p > 0.05$	$\chi^2=5.02$; $p = 0.025$
Annexin V ⁺ -lymphocytes [s.u.]	Moderate decrease ($\leq 50^{\text{th}}$ percentile), <i>n</i> =52	9 (39.13%)	43 (42.16%)
	Significant decrease ($> 50^{\text{th}}$ percentile), <i>n</i> =73	14 (60.87%)	59 (57.84%)
	χ^2 ; <i>p</i>	$\chi^2=2.17$; $p > 0.05$	$\chi^2=5.02$; $p = 0.025$
TNF- α [pg/mL]	Moderate decrease ($\leq 50^{\text{th}}$ percentile), <i>n</i> =32	5 (21.74%)	27 (26.47%)
	Moderate increase ($\leq 50^{\text{th}}$ percentile), <i>n</i> =20	4 (17.39%)	16 (15.69%)
	Significant increase ($> 50^{\text{th}}$ percentile), <i>n</i> =73	14 (60.87%)	59 (57.84%)
	χ^2 ; <i>p</i>	$\chi^2=11.88$; $p = 0.003$	$\chi^2=44.02$; $p < 0.001$
IL-1 β [pg/mL]	Moderate increase ($\leq 50^{\text{th}}$ percentile), <i>n</i> =99	19 (82.61%)	80 (78.43%)
	Significant increase ($> 50^{\text{th}}$ percentile), <i>n</i> =26	4 (17.39%)	22 (21.57%)
	χ^2 ; <i>p</i>	$\chi^2=19.57$; $p < 0.001$	$\chi^2=65.96$; $p < 0.001$
IL-4 [pg/mL]	An increase $\leq 25^{\text{th}}$ percentile, <i>n</i> =69	10 (43.48%)	59 (57.84%)
	Normal, <i>n</i> =30	9 (39.13%)	21 (20.59%)
	Significant decrease ($> 50^{\text{th}}$ percentile), <i>n</i> =26	4 (17.39%)	22 (21.57%)
	χ^2 ; <i>p</i>	$\chi^2=4.04$; $p > 0.05$	$\chi^2=41.38$; $p < 0.001$
IL-6 [pg/mL]	Moderate increase ($\leq 50^{\text{th}}$ percentile), <i>n</i> =98	19 (82.61%)	79 (77.45%)
	Significant increase ($> 50^{\text{th}}$ percentile), <i>n</i> =27	4 (17.39%)	23 (22.55%)
	χ^2 ; <i>p</i>	$\chi^2=19.57$; $p < 0.001$	$\chi^2=61.49$; $p < 0.001$

TNF- α : Tumor necrosis factor-alpha; IL: Interleukin.

Table 9 – Polymorphic variants of the BCL-2 (*rs17759659*) gene as risk factors of thyroid pathology considering the rates of apoptosis and non-specific inflammation

Genotypes of the <i>BCL-2</i> gene		RelR	OR	95% CI RR	95% CI OR	<i>p</i>
Increase in the number of CD95 ⁺ -lymphocytes ($> 50^{\text{th}}$ percentile)	AA	0.71	0.69	0.22–2.34	0.19–2.52	> 0.05
	AG, GG	1.45	2.09	1.11–1.89	1.24–3.54	0.006
Decrease in the number of annexin V ⁺ -lymphocytes ($> 50^{\text{th}}$ percentile)	AA	0.71	0.69	0.22–2.34	0.19–2.52	> 0.05
	AG, GG	1.45	2.09	1.11–1.89	1.24–3.54	0.006
Increase in TNF- α ($> 50^{\text{th}}$ percentile)	AA	0.71	0.69	0.22–2.34	0.19–2.52	> 0.05
	AG, GG	1.45	2.09	1.11–1.89	1.24–3.54	0.006
Significant increase in IL-1 β ($> 50^{\text{th}}$ percentile)	AA	0.67	0.44	0.27–1.66	0.07–2.66	> 0.05
	AG, GG	0.24	0.06	0.16–0.35	0.03–0.11	< 0.001
Moderate increase in IL-1 β ($\leq 50^{\text{th}}$ percentile)	AA	1.50	2.25	0.60–3.73	0.38–13.47	> 0.05
	AG, GG	4.23	17.87	2.87–6.22	9.26–34.48	< 0.001
Decrease in IL-4 ($> 50^{\text{th}}$ percentile)	AA	0.67	0.44	0.27–1.66	0.07–2.66	> 0.05
	AG, GG	0.22	0.05	0.15–0.33	0.03–0.10	< 0.001
Increase in IL-4 ($\leq 25^{\text{th}}$ percentile)	AA	0.71	0.69	0.22–2.34	0.19–2.52	> 0.05
	AG, GG	1.25	1.57	0.97–1.63	0.94–2.65	0.057
Increase in IL-6 ($> 50^{\text{th}}$ percentile)	AA	0.67	0.44	0.27–1.66	0.07–2.66	> 0.05
	AG, GG	0.25	0.06	0.17–0.36	0.03–0.12	< 0.001

RelR: Relative risk; OR: Odds ratio; RR: Risk ratio; CI: Confidence interval; TNF- α : Tumor necrosis factor-alpha; IL: Interleukin.

Table 10 – Polymorphic variants of the CTLA-4 (rs231775) gene as risk factors of thyroid pathology considering the rates of apoptosis and non-specific inflammation

Genotypes of the CTLA-4 gene		ReIR	OR	95% CI RR	95% CI OR	p
Increase in the number of CD95 ⁺ -lymphocytes (>50 th percentile)	AA	1.56	2.45	1.07–2.29	1.17–5.13	0.017
	AG, GG	1.28	1.63	0.90–1.80	0.82–3.24	>0.05
Decrease in the number of annexin V ⁺ -lymphocytes (>50 th percentile)	AA	1.56	2.45	1.07–2.29	1.17–5.13	0.017
	AG, GG	1.28	1.63	0.90–1.80	0.82–3.24	>0.05
Increase in TNF- α (>50 th percentile)	AA	1.56	2.45	1.07–2.29	1.17–5.13	0.017
	AG, GG	1.28	1.63	0.90–1.80	0.82–3.24	>0.05
Significant increase in IL-1 β (>50 th percentile)	AA	1.98	2.28	0.62–6.28	0.59–8.78	>0.05
	AG, GG	0.21	0.03	0.12–0.35	0.01–0.12	<0.001
Moderate increase in IL-1 β (\leq 50 th percentile)	AA	6.35	23.57	2.18–18.55	6.13–90.67	<0.001
	AG, GG	0.93	0.61	0.77–1.12	0.16–2.39	>0.05
Decrease in IL-4 (>50 th percentile)	AA	1.98	2.28	0.62–6.28	0.59–8.78	>0.05
	AG, GG	0.21	0.03	0.12–0.35	0.01–0.12	<0.001
Increase in IL-4 (\leq 25 th percentile)	AA	5.23	12.33	1.77–15.38	3.31–46.01	<0.001
	AG, GG	0.55	0.13	0.41–0.73	0.03–0.47	<0.001
Increase in IL-6 (>50 th percentile)	AA	1.98	2.28	0.62–6.28	0.59–8.78	>0.05
	AG, GG	0.22	0.03	0.13–0.37	0.01–0.13	<0.001

ReIR: Relative risk; OR: Odds ratio; RR: Risk ratio; CI: Confidence interval; TNF- α : Tumor necrosis factor-alpha; IL: Interleukin.

Table 11 – Polymorphic variants of the APO-1/FAS (rs2234767) gene as risk factors of thyroid pathology considering the rates of apoptosis and non-specific inflammation

Genotypes of the APO-1/FAS gene		ReIR	OR	95% CI RR	95% CI OR	p
Increase in the number of CD95 ⁺ -lymphocytes (>50 th percentile)	AG	1.05	1.13	0.73–1.52	0.45–2.86	>0.05
	GG	1.37	1.88	1.04–1.88	1.08–3.28	0.018
Decrease in the number of Annexin V ⁺ -lymphocytes (>50 th percentile)	AG	1.05	1.13	0.73–1.52	0.45–2.86	>0.05
	GG	1.37	1.88	1.04–1.88	1.08–3.28	0.018
Increase in TNF- α (>50 th percentile)	AG	1.05	1.13	0.73–1.52	0.45–2.86	>0.05
	GG	1.37	1.88	1.04–1.88	1.08–3.28	0.018
Significant increase in IL-1 β (>50 th percentile)	AG	0.81	0.77	0.31–2.11	0.24–2.48	>0.05
	GG	1.24	1.31	0.47–3.25	0.40–4.24	>0.05
Moderate increase in IL-1 β (\leq 50 th percentile)	AG	1.05	1.31	0.85–1.30	0.40–4.24	>0.05
	GG	0.95	0.77	0.77–1.15	0.24–2.48	>0.05
Decrease in IL-4 (>50 th percentile)	AG	0.81	0.77	0.31–2.11	0.24–2.48	>0.05
	GG	1.24	1.31	0.47–3.25	0.40–4.24	>0.05
Increase in IL-4 (\leq 25 th percentile)	AG	0.75	0.56	0.46–1.23	0.22–1.40	>0.05
	GG	1.33	1.78	0.81–2.18	0.72–4.45	>0.05
Increase in IL-6 (>50 th percentile)	AG	0.81	0.77	0.31–2.11	0.24–2.48	>0.05
	GG	1.30	1.38	0.50–3.39	0.43–4.47	>0.05

ReIR: Relative risk; OR: Odds ratio; RR: Risk ratio; CI: Confidence interval; TNF- α : Tumor necrosis factor-alpha; IL: Interleukin.

Discussion

Disorders in implementation of receptor-dependent apoptosis of lymphocytes in AIT and TA is characterized by an increase in the content of lymphocytes, which represent the apoptosis marker – CD95⁺-receptor, and indicate the readiness to the cell killing as well as of proinflammatory cytokines TNF- α , IL-1 β and IL-6, while reducing the apoptotic activity of lymphocytes by the number of annexin V⁺-presenting cells in peripheral blood and of inflammatory IL-4. The above-mentioned changes associate the most comprehensively with polymorphic sites of the *BCL-2* (rs17759659) ($F=28.36$, $p<0.001$) and *CTLA-4* (rs231775) ($F=24.28$, $p<0.001$) genes and only for certain indices, almost three times weaker with *FAS* (rs2234767) ($F=8.79$, $p=0.004$).

The content of lymphocytes with CD95⁺-receptors prevailed in homozygous carriers of the minor *G*-allele of the *BCL-2* (rs17759659) gene and those with the main *A*-allele of the *CTLA-4* (rs231775) gene: by 14.0–27.09% ($p\leq 0.034$) and 36.08–37.14% ($p\leq 0.011$), respectively. The rates of the most apoptotic cytokines TNF- α and IL-1 β , as well as IL-6 are in general higher in serum of the patients with thyroid pathology (by 2.89–4.37 times ($p\leq 0.006$), 3.31–4.91 times ($p\leq 0.008$) and 1.75–2.17 times ($p<0.001$), respectively, but without reliable unidirectional dependencies on polymorphism of the *BCL-2* (rs17759659) and *FAS* (rs2234767) genes. The carriers of the wild-*A*-allele of the *CTLA-4* (rs231775) gene have the higher concentration of pro-inflammatory TNF- α and IL-1 β 2.07–2.14 times ($r\leq 0.015$) and 2.07–2.2 times ($p\leq 0.018$),

respectively, and apoptotic activity by the number of annexin V⁺-lymphocytes, on the contrary, is lower by 32.4% ($p_{AA}=0.017$) and 36.56% ($p_{AG}=0.018$), respectively. The polymorphic site of the *CTLA-4* (*rs231775*) gene, according to univariate analysis of variance, confirmed the association of the highest apoptotic activity of the lymphocytes ($F=51.24$, $p<0.001$).

The risk of AIT and TA in the examined population grow with an increase of CD95⁺-lymphocytes and TNF- α (>50th percentile) and decrease of annexin V⁺-lymphocytes (>50th percentile): in carriers of the minor *G*-allele of the *BCL-2* (*rs17759659*) gene – by 1.45 times (OR: 2.09; 95% CI OR: 1.24–2.52; $p=0.006$); in the homozygous carriers of the main *A*-allele (*AA*-genotype) of the *CTLA-4* (*rs231775*) gene – by 1.56 times (OR: 2.45; 95% CI OR: 1.17–5.13; $p=0.017$); in the homozygous carriers of the wild *G*-allele (*GG*-genotype) of the *FAS* (*rs2234767*) gene – by 1.37 times (OR: 1.88; 95% CI OR: 1.08–3.28; $p=0.018$).

Thus, the results show that in terms of damaging action of peroxidation products in the thyroid structures, activation of *Fas*- and caspase-dependent mechanisms of influence on pro- and antiapoptotic targets the induced hyperproduction and the release of TNF- α from thyroid-stimulating lymphocytes promotes an additional synthesis of other proinflammatory cytokines IL-1 β and IL-6, as well as compensatory contra-inflammatory proteins including IL-4. The secretion of the soluble form of TNF- α receptor (sTNFR) increases synchronically, which prevents binding the corresponding cytokine to a specific membrane shedding receptor and separates the apoptotic signals.

Conclusions

A moderate increase in IL-1 β in serum also increases the risk of AIT and TA in residents of Northern Bukovina (Ukraine) by 6.35 times, and slight elevation of IL-4 – by 5.23 times, but only in carriers of *AA*-genotype of the *CTLA-4* (*rs231775*) gene. The likelihood of thyroid pathology reduces with the low content of IL-4 anti-inflammatory cytokine and high levels of IL-6 in carriers of mutation *G*-allele of the *BCL-2* gene and *G*-allele of the *CTLA-4* (*rs231775*) gene and does not depend on the polymorphic variants of the *FAS* (*rs2234767*) gene.

The results show that under the damaging action of peroxidation products in the thyroid structures, activation of *Fas*- and caspase-dependent mechanisms of influence on pro- and antiapoptotic targets, the induced hyperproduction and release of TNF- α from thyroid-stimulated lymphocytes stimulate an additional synthesis of other pro-inflammatory cytokines IL-1 β and IL-6, as well as compensatory anti-inflammatory proteins, including IL-4. There is a synchronized increase in the sTNFR secretion, which prevents binding the corresponding cytokine to a specific membranous shedding of a number of receptors and separates the apoptotic signals. The above-mentioned changes associate with the polymorphic variants of *BCL-2* (*rs17759659*) and *CTLA-4* (*rs231775*) genes and only for some figures, they are almost three times weaker with *FAS* (*rs2234767*) gene.

Prospects for further research

Finding specific pathogenic factors and mechanisms of deregulation of apoptosis in immunocompetent blood cells (lymphocytes, monocytes, neutrophils) and thyrocytes and the association of these changes with polymorphic variants of *BCL-2* (*rs17759659*), *CTLA-4* (*rs231775*) and *APO-1/FAS* (*rs2234767*) genes, in patients with nodular forms of goiter, against the background of autoimmune thyroiditis and from thyroid adenoma, will allow to establish the causes of their onset and thus, to formulate pathogenically-justified methodological approaches to the correction of immunopathological changes that form the basis of their development.

Conflict of interests

The authors declare no conflict of interests concerning this article.

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Corresponding author

Michael Ivanovich Sheremet, Assistant Professor, MD, PhD, Department of Surgery No. 1, Bukovinian State Medical University, 191 Holovna Street, UA-58018, Chernivtsi, Ukraine; Phone +380–956064607, e-mail: mihayl71@gmail.com

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