

**Trabajo original**
**Combined effect of gene-gene interaction on the development of nodular goiter with autoimmune thyroiditis and thyroid adenoma in the inhabitants of Northern Bukovyna**


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(NGAIT) Nodular goiter with autoimmune thyroiditis

(AIT) Autoimmune thyroiditis

(TG) Thyroid gland

(TA) Thyroid adenoma

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Bocio nodular con tiroiditis autoinmune

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**A B S T R A C T**

This article presents the results of a comprehensive analysis of the combined influence of genetic polymorphisms associated with various links of apoptosis regulation (BCL-2, CTLA-4 and APO-1/Fas) on the development of nodular goiter with autoimmune thyroiditis and thyroid adenoma in the studied population. The analysis was performed using the Multifactor Dimensionality Reduction (MDR) method by calculating the prediction potential. Graphic models of gene-gene interaction with the highest cross-validation consistency created by the MDR method showed complex "synergistic or independent" impact of polymorphic loci of the CTLA-4 (+49G/A), Fas (-1377G/A) and BCL-2 (63291411 A>G) genes on the onset of thyroid pathology in general, or its individual types (nodular goiter with autoimmune thyroiditis and thyroid adenoma) in the population of Northern Bukovyna.

**Efecto combinado de la interacción genética sobre el desarrollo del bocio nodular con tiroiditis autoinmune y adenoma tiroideo en los habitantes de Bucovina del Norte**

**R E S U M E N**

Este artículo presenta los resultados de un análisis exhaustivo de la influencia combinada de polimorfismos genéticos asociados a diversos enlaces en la regulación de la apoptosis (BCL-2, CTLA-4 y APO-1/FAS) sobre el desarrollo de bocio nodular con tiroiditis autoinmune y adenoma tiroideo en la población estudiada. Para ello, se utilizó el método de reducción de dimensionalidad multifactorial (MDR) mediante el cálculo de los potenciales de predicción.

Los modelos gráficos de interacción gen-gen con la mayor consistencia de validación cruzada creada por el método MDR mostraron un complejo impacto «sinérgico o

independiente» de los loci polimórficos de los genes CTLA-4 (+49G/A), FAS (-1377G/A) y BCL-2 (63291411A>G) en el inicio de la patología tiroidea en general, o sus tipos individuales (bocio nodular con tiroiditis autoinmune y adenoma tiroideo) en la población de Bucovina septentrional.

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## Introduction

Recent studies have shown that genetic mutations, especially those of regulatory genes, cause the development of thyropathies, including NGAIT and TA<sup>1,2,3</sup>. Polymorphisms of the T-lymphocytes inhibitory activator genes of CTLA-4 (cytotoxic T lymphocyte-associated molecule-4) and PTPN22 (Protein tyrosine phosphatase, non-receptor type 22) play an important role in the development of AIT<sup>4,5,6</sup>. The genetic predisposition to the development of AIT is based on its association with certain antigens of the human leukocyte antigen (HLA) system, but these antigens are markers of a number of autoimmune diseases; therefore, they cannot be considered as specific "disease genes"<sup>7-10</sup>.

There are multiple mutations in different genes that can affect the lymphoid tissue function of the thyroid gland: an apoptotic regulator gene BCL-2 (B-cell lymphoma 2) (rs17759659), APO-1/FAS (apoptosis antigen 1/cluster of differentiation 95 (CD95)) (rs2234767) associated with AIT, located in chromosomes 2 (2q33), 6 (6p21), 8 (8q24), 12 (12q22) and 13 (13q32)<sup>7,8,9</sup>.

Dysregulation of apoptosis plays a key role in carcinogenesis and autoimmune processes in the thyroid gland<sup>10-13</sup>. In the study of the association of apoptosis-related genes Caspase 8 (-652 G N ins/del), Caspase 9 (-1263 A>G) and Bcl-2 (-938 C>A) polymorphisms and papillary thyroid carcinoma risk in Han Chinese population, Y.X. Wang et al.<sup>13</sup> analyzed the distribution of genotype frequency, as well as the association of genotype with clinic-pathological characteristics. Overall, no statistically significant association was observed in Caspase 8 (-652 G N ins/del). Nevertheless, the Caspase 9 -1263 GG genotype was associated with an increased risk of papillary thyroid carcinoma ( $p = 0.045$ ; odds ratio (OR) = 1.12). GG genotype thyroid cancers were significantly more common in older patients than AA or AG genotypes PTC and in cases of advanced pathological stages. However, BCL-2 -938 AA genotype in this study demonstrated a protective effect ( $p = 0.004$ ; OR = 0.35). Polymorphism in Caspase 9 (-1263 A>G) was observed to be associated with susceptibility of the tumor. The authors stated the need for further investigation to support their results.

M. Erdogan et al.<sup>14</sup> indicated that similarly to other types of malignancies, genetic factors in the pathogenesis of thyroid malignancies may also show changes in different populations. Fas/FasL-dependent apoptosis mechanism is in balance under normal conditions in patients with PTC.

Despite numerous studies, there is no clear understanding of the relationship between genetic background, expression of genes in thyroid tissue, activity of BCL-2 protein, FAS (APO-1), or inhibition of cytotoxic T-lymphocytes (CTLA-4) and the development of AIT and apoptosis activity in thyroid tissue<sup>15,16,17</sup>. Moreover, the possible interference of different genetic influences remains uncovered. Based

on the existing scientific database, we hypothesize the presence of associations of NGAIT and TA with BCL-2, CTLA-4, and APO-1/FAS single nucleotide polymorphisms.

The aim of the study is to analyze the combined influence of polymorphisms of apoptosis-regulatory genes (BCL-2, CTLA-4, and APO-1/FAS) on the development of NGAIT and TA in the population of Northern Bukovyna.

## Material and methods

The study was conducted at the Chernivtsi Regional Clinical Hospital (Northern Bukovyna, Ukraine) during 2013-2016 in 95 women with nodular goiter secondary to autoimmune thyroiditis (NGAIT). The age of the patients ranged from 23 to 72 years. Diagnosis was established based on clinical symptoms, laboratory findings (thyroid peroxidase antibodies (TPAB) – 60-250 U/ml, thyroglobulin antibodies (TGAB) – 60-500 U/ml; thyroid-stimulating hormone (TSH) – 4.10 mU/L) ultrasound examination and confirmed histologically after surgery. Among all patients, we selected a group of 30 women who, based on ultrasonography, fine needle aspiration biopsy (FNAB), intraoperative express biopsy and histological examination after surgery, were diagnosed with thyroid adenoma. Such distinction was made because this pathology is one of the most common among nodular goiters<sup>11</sup>. These patients' unaffected parenchyma of the morphologically unaltered contralateral lobe of the thyroid gland was also examined. Obtained indicators served as controls.

Clinical, hormonal, and genetic studies were performed in 25 practically healthy donors. The control and main groups were comparable in age ( $34.2 \pm 10.33$  and  $38.0 \pm 10.62$  years, respectively,  $p = 0.12$ ) and anthropometric data (body mass index – BMI  $23.5 \pm 2.71$  and  $24.3 \pm 4.88$  kg/m<sup>2</sup>, respectively,  $p = 0.43$ ). Indicators of the thyroid spectrum are presented in table 1. The study was approved by the Bioethics Committee of the Bukovinian State Medical University, Chernivtsi, Ukraine (Protocol #8, 14.06.2017).

**Table 1 - Thyroid parameters. The distribution of the indicators of the thyroid spectrum in the patients with thyroid pathology**

Indicators	NGAIT (n=95)	Thyroid adenoma (n=30)	p
Free T <sub>3</sub> level, ng/L	4.4 ± 0.93	4.4 ± 0.91	p = 0.93
Free T <sub>4</sub> level, ng/L	12.9 ± 3.42	16.6 ± 2.02	p < 0.0001
TSH, mUn/L	1.9 ± 0.76	4.9 ± 3.51	p < 0.0001
AT-TPO, mUn/L	255.7 ± 24.1	11.92 ± 3.19	p = 0.0009
AT-TG, mUn/L	340.58 ± 35.2	13.92 ± 4.08	p = 0.0009

In general, the differences between the groups were regular and confirmed an autoimmune process and a tendency towards decreasing function against the

background of AIT. The diagnosis was pathomorphologically confirmed after surgery.

All patients underwent surgical intervention based on generally accepted indications: large size of the goiter with compression and displacement of neck organs (compression syndrome), airway obstruction or suspected malignant neoplasm of the thyroid gland (III, IV, V group according to the Bethesda system for reporting thyroid cytopathology classification, FNAB findings). The volume of surgery varied from hemithyroidectomy to thyroidectomy.

### Genetic research

The DNA was isolated using the Thermo Scientific GeneJET Genomic DNA Purification kit (#K0721, Thermo Fisher Scientific), according to the manual, with proteinase K overnight incubation to complete cell lysis. The purified DNA was diluted in elution buffer and evaluated with Nanodrop2000C spectrophotometer. Only samples with concentration not lower than 15 ng/ml and values of A ratio (260/280) between 1.7 and 2.0 were used for genotyping. The obtained extracts were divided into aliquots: one was stored in the refrigerator at 4°C until assayed and the others were frozen at -20°C.

For genotyping the selected point polymorphism, TaqMan technology was used. Polymorphisms marked with the reference number SNP ID according to the dbSNP database have been studied. TaqMan® SN Genotyping Assays (40×) (4,351,379, Thermo Fisher Scientific) were used to test each of the polymorphisms (table 2).

**Table 2 - The nucleotide sequence of the region including the analyzed polymorphism**

Reference number SNP ID	Test number (Assay ID*)	Fragment of the region including the analyzed polymorphism
rs231775 (CTLA4)	C_2415786_20	GCACAAGGCTCAGCTGAACCTGGCT[A/G]CC AGGACCTGGCCCTGCACCTCTCCT
rs17759659 (BCL2)	C_33628167_10	TCTTCTTACCAAAGATTACAAATAC[A/G]GTG TTGATGGGAACGTGACCTAGTT
rs2234767 (FAS)	C_12123966_10	CAGAGTGTGTGCACAAGGCTGGCAC[A/G]CC CAGGGTCTTCTCATGGCACTAA

Note: according to www.thermofisher.com.

The volume of the reaction mixture was 5 µL and consisted of 2.5 µL Taq Man Genotyping Master Mix (20×) reagent (4,371,355, Thermo Fisher Scientific), 0.25 µL of probe solution and 2.25 µL of DNA solution. Genotyping was performed with the Quant Studio 6 instrument (Applied Biosystems, Thermo Fisher Scientific), 384-well block (table 3). Quant Studio™ Real-Time PCR (v.1.3) software was used for data collection and processing.

**Table 3 - Amplification was performed under the following conditions**

Activation	10 min	95°C	40*/60** cycles
Denaturation	15 sec	92°C	
Annealing/elongation	1 min	60°C	

The main part of the statistical analysis was carried out using the "Statistica 7.0" (SPSS) software. Nominal data are expressed as quantitative values and percentages. The Hardy-Weinberg equilibrium of the genotype distribution was checked using Online Encyclopedia for Genetic Epidemiology Studies (<http://www.oege.org/software/hwemr-calc.shtml>). Distribution of genotypes in the study and control groups was compared using Pearson's chi-squared test. The reliability of differences in averages in groups

with different genotypes was determined by the univariate analysis of variance (ANOVA) method. 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  $\chi^2$  (df = 1) criterion. The gene-gene interaction was studied with the MDR 3.0.2 method by calculating prediction potentials. The difference was considered reliable at  $p < 0.05$ .

## Results and discussion

The models of gene-gene interaction, including those which take into account the type of thyroid pathology, TG function and the degree of TG enlargement with the highest rates of cross-validation consistency, are shown in table 4.

**Table 4 - Models of gene-gene interactions among the surveyed individuals in general and considering thyroid gland pathology, the TG function and the degree of its enlargement**

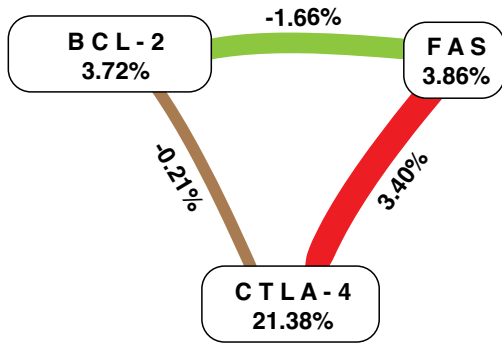
Groups	Combinations of genes in prognostic models	Model reproducibility	Testing cross-validation consistency	Model accuracy, %	OR; p
total	CTLA-4	10/10	15.68	74.29	OR = 35.0; $p < 0.001$
	CTLA-4, Fas	10/10	20.55	70.32	OR = 7.23; $p = 0.003$
	BCL-2, CTLA-4, Fas	10/10	25.45	75.73	OR = 26.0; $p = 0.014$
TA	CTLA-4	10/10	6.62	76.02	OR = 13.75; $p = 0.008$
	CTLA-4, Fas	10/10	13.56	69.39	OR = 5.71; $p = 0.052$
	BCL-2, CTLA-4, Fas	10/10	13.56	60.0	OR = 0.71; $p > 0.05$
NGAIT	CTLA-4	6/10	10.25	70.49	OR = 25.50; $p < 0.001$
	CTLA-4, Fas	9/10	13.64	68.12	OR = 14.85; $p < 0.001$
	BCL-2, CTLA-4, Fas	10/10	18.24	72.86	OR = 3.75; $p > 0.05$

Note. TA: thyroid adenoma; NGAIT: nodular goiter with autoimmune thyroiditis; TG: thyroid gland; OR: Odds Ratio.

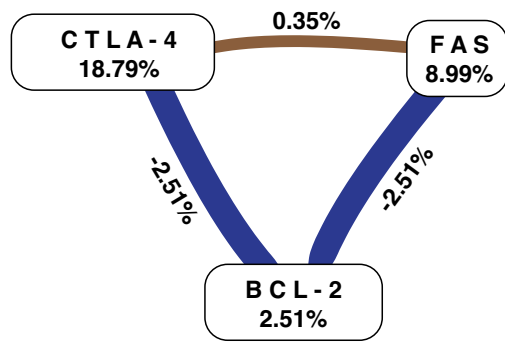
The MDR method showed reliability and a high reproducibility of a single-factor model with CTLA-4 gene involvement, both in the whole cohort of patients with thyroid pathology (100% reproducibility, model accuracy 74.29%, OR = 35.0;  $p < 0.001$ ) and in those with TA and NGAIT in particular (100% and 60% reproducibility; OR = 13.75,  $p = 0.008$  and OR = 25.50,  $p < 0.001$ , respectively).

Modeling of high-order gene-gene interactions showed a high risk of thyroid pathology in the population in general in the two-component model (CTLA-4 and FAS) with 100% reproducibility and high accuracy of risk prediction – 70.32% (OR = 7.23,  $p = 0.003$ ). A permutation test confirmed this risk for NGAIT (OR = 14.85,  $p < 0.001$ ) with boundary reliability for TA (OR = 5.71,  $p = 0.052$ ), despite 100% reproducibility of the model (10/10). In addition, the MDR method confirmed, by permutation test, the effectiveness of the three-locus model involving polymorphisms of the BCL-2, CTLA-4 and FAS genes with 100% reproducibility (10/10) and high accuracy of thyroid pathology risk prediction in general in the population of Northern Bukovyna (OR = 26.0,  $p = 0.014$ ), as well as with the highest Cross-validation Testing T-statistics – CV-TT.

The presence of CTLA-4 gene G-allele alone in the patient's genotype as well as the combination of minor G-allele of the CTLA-4 gene with GG-, AG- (in particular) genotypes of the FAS (0.717 and 0.467) gene, or the combination of minor



**Figure 1** - Cluster analysis of modeling gene-gene interaction of three genes CTLA-4, FAS and BCL-2 in general in patients with NGAIT and TA (Fruchterman-Rheingold circular dendrogram). The synergistic interaction is marked in red, the independent effect is in brown and green.



**Figure 2** - Cluster analysis of modeling gene-gene interaction of three genes CTLA-4, FAS and BCL-2 in patients with TA (Fruchterman-Rheingold circular dendrogram). The independent effect of genes on the onset of the pathology is marked in brown and blue.

G-allele of the CTLA-4 and BCL-2, FAS genes with GG-, or (stronger) AG-genotypes of the FAS (0.767 and 0.467) gene is associated with a high risk of NGAIT and TA in the studied population. In addition, the combination of homozygous wild allele of the CTLA-4 and FAS genes, as well as of CTLA-4, FAS, regardless of the BCL-2 gene genotypes (stronger with favorable GG-genotype) is associated with a lower risk of thyroid pathology (from -1.533 to -0.492).

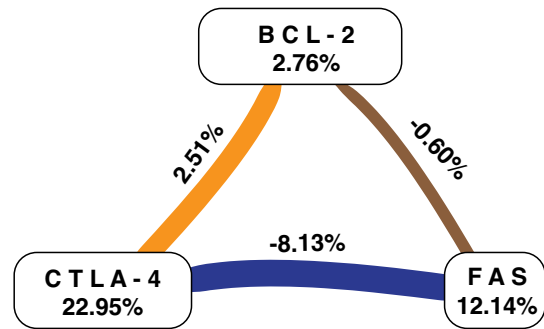
A cluster analysis chart of gene-gene interaction modeling results using the MDR method in thyroid pathology is shown in fig. 1. A close relationship (synergistic interaction) was found for CTLA-4 and FAS genes, with an entropy of 21.38% and 3.86%, respectively; increasing by 3.40% with their combined effect. The high rate of the CTLA-4 gene entropy confirms its importance and significant impact on the onset and course of thyroid pathology in the studied population. The inter-locus interaction between the FAS-BCL-2 and BCL-2-CTLA-4 genes manifested itself as "independent effects" of the impact (-1.66% and -0.21%, respectively).

It was established that the risk of TA is increased in carriers of the AG- genotype of the CTLA-4 gene (0.377,  $p = 0.008$ ); the risk is 2.56 times higher in the combination of heterozygotes (AG) of the CTLA-4 and FAS (0.967) genes and almost 3 times higher in the combination of heterozygotes (AG) of the CTLA-4, BCL-2 and FAS (1.167) genes. However, a corrective permutation test demonstrated a statistical irrelevance of the latter model.

The nature of inter-locus interaction confirms the high contribution of the CTLA-4 gene to the development of TA (18.79%), polymorphic locus of the FAS gene is twice (8.99%) and BCL-2 gene 7.5 times (2.51%) less influential. Analysis of gene-gene interaction modeling indicates an independent effect of each gene in the development of the disease (fig. 2).

The MDR method in NGAIT showed the probability of a one- and two-component model involving the CTLA-4 gene (+49G/A) in the G-allele carriers and CTLA-4 and FAS (-1377G/A) genes in the combination of G-allele (CTLA-4) and AG-genotype of the FAS gene (-1377G/A). The three-component model in the interaction of the three genes CTLA-4 (+49G/A), FAS (-1377G/A) and BCL-2 (63291411A>G) increases the risk of NGAIT, but only with the combination of minor G-allele of the CTLA-4 gene, G-allele of the BCL-2 gene and regardless of the genotypes of the FAS gene (stronger in AG-genotype), from 0.392 to 1.392.

The share of entropy of inter-locus interaction of three genes resulting from cluster analysis in patients with NGAIT is 22.92% (the highest) for CTLA-4 gene, 12.14% for FAS gene and 2.76% for BCL-2 gene (fig. 3). Synergistic interaction of CTLA-4 and BCL-2 genes in NGAIT increased the entropy by 2.51%. For other genes, the bond was set as an "independent



**Figure 3** - Cluster analysis of modeling gene-gene interaction of three genes CTLA-4, FAS and BCL-2 in patients with NGAIT. The synergistic interaction is marked in orange, the independent impact is in brown and blue.

impact" on development of NGAIT.

Graphic models of gene-gene interaction with the highest cross-validation consistency created by the MDR 3.0.2 method showed a complex "synergistic or independent" impact of the CTLA-4 (+49G/A), FAS (-1377G/A) and BCL-2 (63291411A>G) genes polymorphic loci on the onset of thyroid pathology in general or its individual types (NGAIT, TA) in the population of residents of Northern Bukovina.

The classification ability of the models despite the high reproducibility (60-100%) confirmed the likelihood of risks for a single-factor model involving the minor G-allele of the CTLA-4 gene (especially of AG-genotype) as well as for a two-locus model in the combination of the CTLA-4 and FAS genes AG-genotypes: onset of thyroid pathology in the general population (OR=35.0,  $p < 0.001$  and OR = 7.23,  $p = 0.003$ ), TA (OR = 13.75,  $p = 0.008$  and OR = 5.71,  $p = 0.052$ ), NGAIT (OR = 25.50,  $p < 0.001$  and OR = 14.85,  $p < 0.001$ ), condition of euthyroidism (exclusively with the participation of CTLA-4 gene OR = 16.0,  $p = 0.015$ ), subclinical hypothyroidism (OR = 31.0,  $p < 0.001$  and OR = 16.33,  $p < 0.001$ , respectively), clinical hypothyroidism (exclusively with the participation of CTLA-4 gene OR = 9.17,  $p = 0.043$ ), II degree thyroid hyperplasia (OR = 6.37,  $p = 0.022$  and OR = 4.36,  $p = 0.049$ ) with the accuracy varying from 68.12% to 97.41% and testing cross-validation consistency from 4.26 to 20.55, respectively. The two-locus combination of AG-genotype of the CTLA-4 and BCL-2 (1.038, OR = 12.0,  $p = 0.032$ ) proved its effectiveness in the III degree thyroid hyperplasia prognosis.

The incidence of the minor G-allele ( $P_G$  - G-allele conditional frequency) of the BCL-2 gene in this study did not differ<sup>18</sup> significantly from other European populations

of the Caucasian race ( $P_G = 0.50-0.56$ ,  $p > 0.05$ ). Instead, the frequency of wild A-alleles ( $P_A$  – A-allele conditional frequency) in our study is significantly lower compared to the Equatorial race and several populations of the Asian race ( $P_A = 0.52-0.54$  against  $P_G = 0.65-1.0$ ,  $p < 0.05$ ).

The frequency of the major A-allele of the CTLA-4 gene in residents of Bukovyna ( $P_A = 0.72-0.78$ ), as well as the minor G-allele ( $P_G = 0.22-0.28$ ) are similar to other European populations ( $P_A = 0.61-0.88$  and  $P_G = 0.12-0.39$ ,  $p > 0.05$ ), indicating the relative homogeneity of the gene's polymorphic locus. At the same time, there are significant differences in comparison with the individual populations of the Equatorial race, where the frequency of occurrence of alleles and genotypes has a wider spread and discrepancy, indicating higher heterogeneity ( $P_A = 0.54-0.67$  and  $P_G = 0.33-0.46$ ,  $p < 0.05$ ). Comparatively, lesser heterogeneity has been established for Asian race populations where the frequency of A-allele, according to the NCBI dbSNP genetic Database<sup>19</sup>, is reliably lower than in our study, and the G-allele, on the contrary, exceeds that of Bukovyna inhabitants ( $P_A = 0.29-0.37$  and  $P_G = 0.63-0.71$ ,  $p < 0.05$ ).

The alleles frequencies of the APO-1/FAS (rs2234767) gene in the studied individuals do not differ [20] from those in the Caucasian race ( $P_A = 0.09-0.12$ ,  $P_G = 0.88-0.91$  vs.  $P_A = 0-0.12$  and  $P_G = 0.88-1.0$ , respectively,  $p > 0.05$ ). The frequency of the minor A-allele in this study is lower, and that of the G-allele higher compared to the Asian populations ( $P_A = 0.21-0.42$ ,  $P_G = 0.58-0.79$ ) and Equatorial race ( $P_A = 0.54-0.67$ ,  $P_G = 0.33-0.46$ ).

This study significantly complements the existing understanding of the genetic background of NGAIT and TA in Caucasian population. Data obtained may be used for both screening and development of treatment approaches based on genetic susceptibility emphasizing apoptosis in thyroid tissue. However, further studies are needed to transform these results into treatment options.

## Conclusions

1. The three-genes-component (CTLA-4, FAS, BCL-2) model is associated with the risk of thyroid pathology in the Northern Bukovina population (OR = 26.0;  $p = 0.014$ ): AG-genotypes of three genes are associated with the risk of TA (1.167); AG-genotypes of the FAS and BCL-2 genes and the G-allele of the CTLA-4 gene are associated with the risk of NGAIT (1.392).

2. Interlocus interaction in thyroid pathology is characterized by a pronounced synergistic gene-gene relationship (CTLA-4, FAS, CTLA-4, and BCL-2 from 1.47% to 7.43%) or by an independent neutralizing effect (from -13.05% to 0.35%) with the highest entropy rate typical for CTLA-4 gene (18.79% in TA; 22.95% in NGAIT), which indicates its high contribution to the development of thyroid pathology (21.38%).

3. The entropy rate share emphasizing the FAS gene (3.86%): 8.99% in TA, 12.14% in NGAIT, is less significant (1.5-5.5 times) in the development of thyroid pathology.

## Conflictos de intereses

Los autores declaran no poseer conflictos de intereses.

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