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ROLE OF *BLASTOCYSTIS SP.* AND *TOXOCARA SPP.* AS PARASITIC ALLERGENS: A DIAGNOSTIC STUDY ON PATIENTS WITH ACUTE AND CHRONIC SPONTANEOUS URTICARIA

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The dissertation comprises 152 standard pages and is illustrated with 19 figures and 12 tables. The bibliography comprises 214 refferences, of which 9 are in Cyrillic and 205 are in Latin.

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The materials for the dissertation thesis defense are available on the website of Medical University - Pleven: www.mu-pleven.bg

The public defence of the dissertation thesis will be held on the 24th of October 2025 at 13:00 in Ambroas Pare Hall, in accordance with Order № 2089 of the Rector of MU - Pleven, in front of a scientific jury with the following composition:

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LIST OF ABBREVIATIONS:

AU - Acute urticaria

CNS - Central nervous system

CSU - Chronic spontaneous urticaria

DKC - Diagnostic-consultation center

DNA - Deoxyribonucleic acid

FECT - Formalin ether concentration technique

ECP - Eosinophil cationic protein

ELISA - Enzyme linked immunosorbent essay

IgE - Immunoglobulin E

IgG - Immunoglobulin G

IU/ml - International units per mililitre PCR - Polymerase chain reaction

SR - Sample Ratio

ST - Subtype

I. Introduction

Allergic diseases are among the most common in human pathology and in recent years there has been an increase in their incidence, especially in developed countries. The exact mechanism and role of the external environment for the development of allergic diseases is still unclear. Because of how often parasitoses present with urticaria different authors have studied a potential link between the two disease groups. The toxo-allergic mechanism is part of the complex pathological processes realized during parasite invasion. Different products released as a part of a parasite's activity in the host cause immunopathologic reactions which are part of the disease process and are responsible for the systemic allergization of the infected person. Vacuolar forms of *Blastocystis sp.* and antitoxocara IgG antibodies are often present in patients with urticaria and because of this we have studied the role of *Blastocystis* and *Toxacara* as allergens.

Blastocystis sp. is a common anaerobic protozoan that lives in the lumen of the large intestine in both humans and animals. Clinically the presentation of blastocystosis varies from asymptomatic carriership to gastro-intestinal and/or allergic symptoms with varying severity. The parasite is divided into 34 subtypes (ST) and it is considered that its genetic diversity is the reason for the varying clinical presentation of the disease. According to literature data, *Blastocystis sp.* is the most commonly isolated intestinal parasite from patients with clinical allergy and the development of allergic symptoms is associated with the presence of ST3 of the parasite. Cases of urticaria are rarer among patients infected with ST1 or ST4. It is considered, that there are allelic differences in each subtype of the parasite, which could explain the differences in clinical

presentation of the diseases among patients infected with the same subtype.

Toxocariasis is a helminthic zoonotic disease caused by migration of *Toxocara canis* and *Toxocara cati* larvae in the organism of the human host. The disease presents in several clinical forms: visceral, ocular, toxocariasis of the CNS, common and covert toxocariasis. During migration the larvae release excretory-secretory antigens with allergize the host. The different antigens stimulate the synthesis of immunoglobulin E, peripheral eosinophilia and local secretion of eosinophilic protein around the parasitic larvae. This process leads to the development of asthma-like symptoms, urticaria and other types of allergic reactions.

In our study we analyzed the rate of infection with *Blastocystis sp.* and *Toxocara spp.* among hospitalized patients with urticaria. We also studied the subtype diversity of *Blastocystis sp.* in patients with acute and chronic spontaneous urticaria. We analyzed the levels of total serum IgE and eosinophil cationic protein (ECP) and their relationship with the levels of antitoxocara IgG antibodies in patients with urticaria.

Our results will help medical specialists for the early diagnosis of „covert" parasitoses among patients with acute or chronic urticaria. This will also allow to assess the necessity of etiological treatment, which would allow the reversal of allergic symptoms.

II. GOAL AND TASKS

The goal of this dissertation is to study the role of *Blastocystis sp*. and *Toxocara spp.* as parasitic allergens in patients with acute and chronic spontaneous urticaria.

In order to achieve the goal we have formulated the following tasks:

1. To study and compare the rate of infection with *Blastocystis sp.* in patients with acute and chronic spontaneous urticaria and among healthy controls.

2. To determine the subtype of *Blastocystis* isolates in healthy carriers and in patients with acute and chronic spontaneous urticaria.

3. To study and compare the rate of antitoxocara IgG antibody carriership in patients with acute and chronic spontaneous urticaria and in healthy controls.

4. To compare the levels of total serum IgE in healthy individuals and in patients with acute and chronic urticaria that are carriers of antitoxocara IgG antibodies.

5. To study and compare the levels of ECP in patients with acute and chronic spontaneous urticaria infected with *Toxocara spp.* and in healthy individuals.

III. MATERIALS

1. Examined subjects:

The current study includes 1679 patients with clinical urticaria, hospitalized in the Allergology Clinic of University Hospital - Pleven or that were examined routinely in the Parasitology lab of DKC 2 - Pleven. For comparison we have also tested 1424 healthy individuals. Based on the research focus, subjects have been distributed as follows:

1.1. Patients with urticaria and healthy individuals examined for blastocystosis.

We tested 1197 patients with acute and chronic spontaneous urticaria and 1300 healthy individuals without prior history of allergic disease for infection with *Blastocystis sp*.

1.2. Patients with urticaria and people with allergic symptoms, infected with *Blastocystis sp*., isolated from which have been genotyped for their subtype specificity.

We genotyped blastocystis isolates from 45 patients with urticaria and 24 healthy parasite carriers without any allergic symptoms.

1.3. Patients with urticaria and healthy individuals tested for the presence of antitoxocara IgG antibodies.

We studied the frequency of antitoxocara IgG antibody carriership among 297 patients with acute and chronic urticaria and in 50 healthy individuals with no history of allergic symptoms.

1.4. Patients with urticaria and healthy individuals without allergic symptoms tested for their levels of total serum IgE.

We examined the levels of total serum IgE in 46 patients with urticaria that are carriers of antitoxocara IgG antibodies, 46 patients with urticaria without any antitoxocara IgG antibodies and in 50 healthy

individuals without prior history of allergic diseases and without antitoxocara IgG antibodies.

1.5. Patients with urticaria and healthy individuals without allergic symptoms tested for their levels of ECP.

We examined the levels of eosinophil cationic potein in 48 patients with urticaria that are carriers of antitoxocara IgG antibodies, 45 patients with urticaria without any antitoxocara IgG antibodies and in 50 healthy individuals without prior history of allergic diseases and without antitoxocara IgG antibodies.

2. Examined materials.

2.1. Fecal samples examined for *Blastocystis sp*.

We examined fresh fecal probes, delivered in chemically pure containers, under a microscope for *Blastocystis sp*.

2.2. Jones' growth medium.

Jones' growth medium was used to cultivate *Blastocystis sp*. from fecal samples obtained from patients with urticaria and healthy parasite carriers. We used a suspension from the medium to isolate DNA of the parasite for later genotyping.

2.3. Serum samples tested for antitoxocara IgG antibodies, total serum IgE and ECP.

We liberated serum samples from venous blood, obtained from the cubital vein early in the morning in a closed system without anticoagulant, following guidelines for proper collection of biologic material. Each subject's serum sample was preserved at -20^0C in sealed, chemically pure transport vials, that were marked with personal data of each patient. We defrosted the serums once at the day of the specific immunological test.

IV. METHODS

1. Parasitological methods:

1.1. Native methods.

1.1.1. Native fecal smear - used for the detection of intenstinal protozoans at 400x magnification. Prepared with physiological solution.

1.1.2. Fecal smear stained with Lugol's solution - stained native smear used for the detection of intenstinal protozoans at 400x magnification.

1.2. Concentration methods:

1.2.1. Formalin ether concentration technique (FECT) (Ritchie, 1948) - An enrichment method that contains a mixture of a fecal sample with 10% formalin solution and ether. Centrifuged for 3 minutes at 1500 rpm. The resulting sediment was examined either natively or as a smear stained with Lugol's solution.

1.3. Culture methods:

1.3.1. Culturing in Jones' growth medium (Jones', 1946). Jones' medium is used to cultivate *Blastocystis sp.* for the purposes of isolating DNA of the parasite. It is a xenic culture which contains $Na_2HPO_4.12H_2O$, KH_2PO_4; NaCl, yeast extract and has a pH of 7.2. *Blastocystis* isolates are cultured at 36-37^{0}C, and are recultured every 2 to 3 days.

2. Molecular - biological methods.

2.1. Isolation of *Blastocystis sp.* genome DNA. Genome DNA of *Blastocystis sp.*was isolated using the manual extraction kit NucleoSpin Tissue Kit (Macherey-Nagel, Germany), following the protocol provided by the manufacturer.

2.2. PCR typing with diagnostic STS primers. We used 7 pairs of STS primers for the genotyping of blastocystis isolates. The sequence of the primers is based on Yoshikawa et al. (2004).

3. Immunoenzyme methods:

3.1. Immunoenzyme method ELISA for the detection of specific antitoxocara IgG antibodies. Serum samples from patients with acute and chronic spontaneous urticaria and healthy individuals were tested for antitoxocara IgG antibodies with the kit RIDASCREEN ® Toxocara IgG ELISA (R-Biopharm AG - Germany), following the instructions of the manufacturer.

3.2. Detection of total serum IgE levels using the immunoenzyme method ELISA. Total serum IgE levels were detected in serum from patients with urticaria and healthy individuals with the diagnostic kit ELISA IgE (NovaTec Immunodiagnostica GmbH - Germany).

3.3. Detection of Eosinophil cationic protein (ECP) levels using the immunoenzyme method ELISA. ECP levels were detected with the diagnostic kit Human Eosionophil Cationic Protein (ECP) ELISA Kit (CUSABIO - China), following the instructions of the manufacturer.

4. Collection of information from the study subjects. Information was collected in personal subject files which contain personal data, patient history, clinical and lab data.

5. Statistical methods. Data was analyzed using Statistical Package for the Social Sciences v.25 (SPSS Inc., Chicago, IL, USA) and EXCEL. We used Pearson and Kruskal-Wallis tests to detect statistical differences between the groups at a significance level $P \leq 0.05$. The strength of the effect between the tested variables was evaluated using Cramer's V coefficient by taking into account the degrees of freedom (df).

V. OWN RESEARCH

1. Rate of infection with *Blastocystis sp.* in patients with acute and chronic spontaneous urticaria.

We tested 1197 patients with acute and chronic spontaneous urticaria and 1300 healthy individuals for the presence of intestinal protozoans and helminths. The urticaria group includes 479 subjects with acute urticaria and 718 with chronic spontaneous urticaria. The distribution of the examined individuals is shown on table №1.

Table №1 *Distribution of the tested individuals in the compared groups based on diagnosis, sex and age.*

Type of allergic reaction	**Acute urticaria**	**Chronic spontaneous urticaria**	**Healthy controls**
	n (%)	**n (%)**	**n. (%)**
Sex			
Male	154 (32.2%)	232 (32.3%)	427 (32.8%)
Female	325 (67.8%)	486 (67.7%)	873 (67.2%)
Total	*479 (100.0%)*	*718 (100.0%)*	*1300 (100.0%)*
Age Mdn (Min, Max)	*51 (3, 82)*	*50 (3, 86)*	*40 (1, 82)*

We isolated *Blastocystis sp.* from 115 of the tested individuals – rate of infection of 9,6%. In the control group out of 1300 healthy subjects, infection with *Blastocystis sp.* was found in 15 people (rate - 1.2%).

We found a statistically significant higher rate of infection with *Blastocystis sp.* among patients with urticaria compared to the control group (χ^2=92.793, df=2, *P*=0.001, V=0.193), though the strenght of the effect is small (V<0.21 at df=2).

Figure № 1 shows the frequency of *Blastocystis sp.* infection among patients with acute and chronic sponatenous urticaria and among the control group.

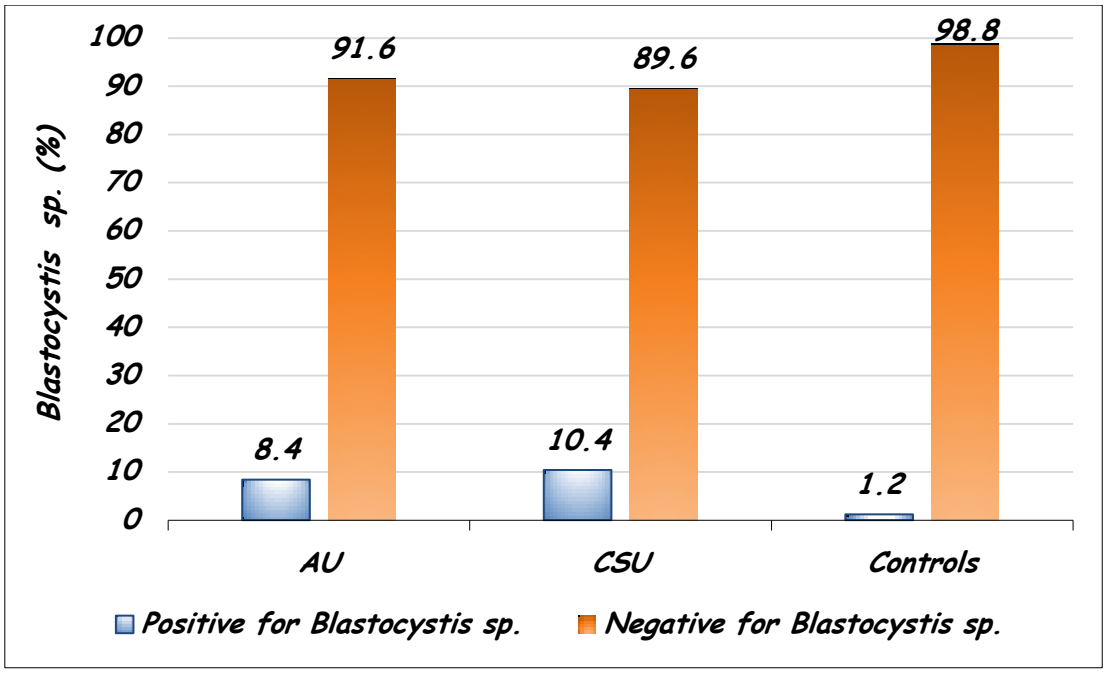

Fig. № 1 *Frequency of infection with Blastocystis sp. in ecah of the compared groups (%)*

The rate of blastocystosis in patients with acute urticaria is 8.4%, while the rate among patients with chronic spontaneos urticaria is 10.4%. We did not find any statistically significant difference in the rate of infection with *Blastocystis sp.* based on the type of urticaria (χ^2=1.452; df=1; p=0.228).

Among the patients positive for *Blastocystis sp.* the age groups 41-60 years (37.40% of the infected) and 61-86 years (31.30%) predominate. The relative share of patients with blastocystosis of the age group 1 - 20 years is 11.30%, and the share of infected patients of the age group 21-40 years is 20%.

Figure № 2 shows the frequency of blastocystosis among patients with urticaria and in the healthy controls based on age group.

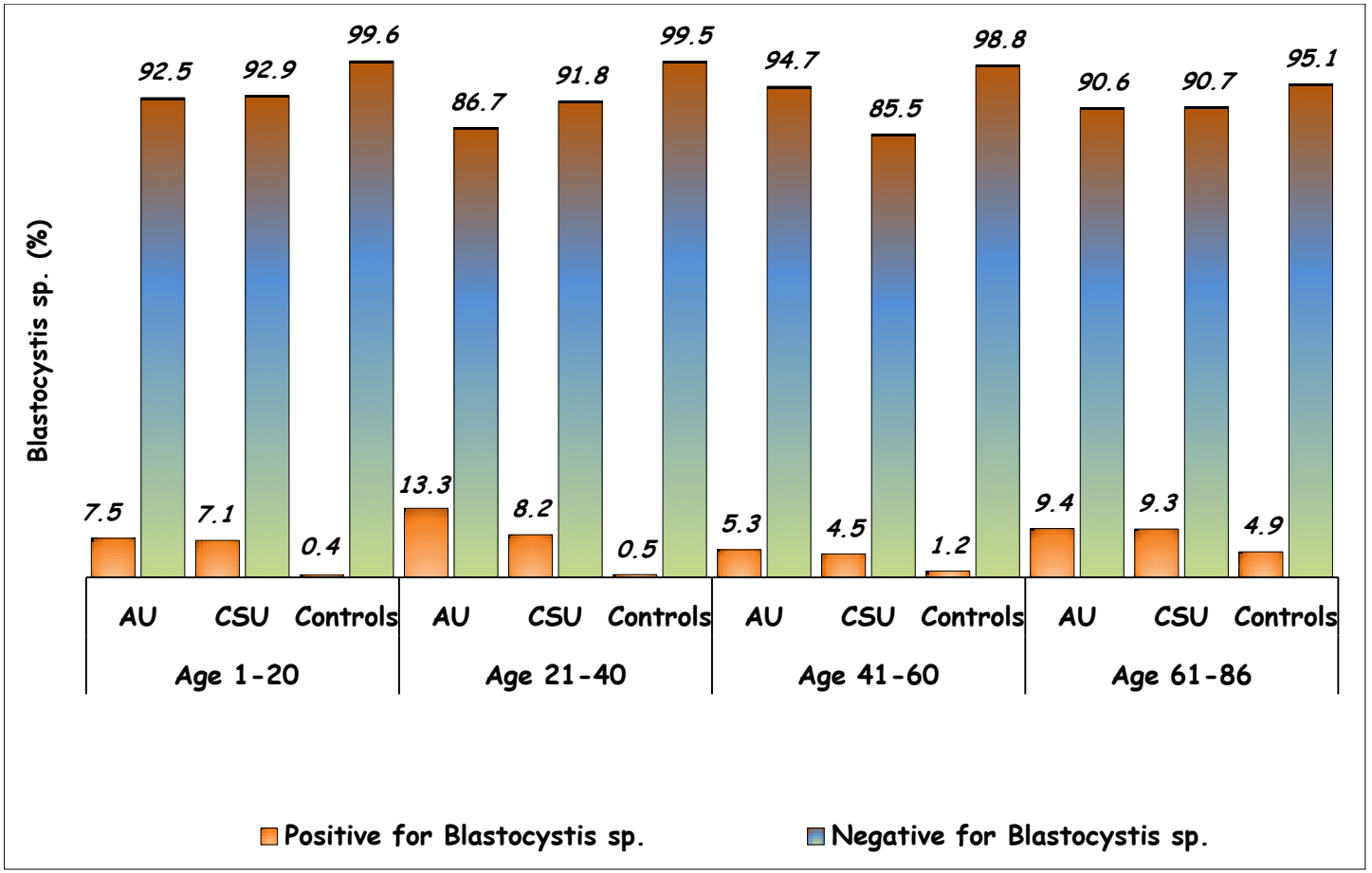

Fig. № 2 *Frequency of infection with Blastocystis sp. in the compared groups – age stratification (%)*

The age stratification shows that the connection between the two studied variables (age and frequency) is statistically significant in most age groups, with the exception of the 61-86 age group: Age - 1-20 (χ^2=16.463, df=2, P=0.001, V=0.193; Age - 21-40 χ^2=38.069, df=2, P=0.001, V=0.246; Age 41-60 χ^2=57.161, df=2, P=0.001, V=0.249). The change in the strength of the effect shows that age is a confounding factor.

The relative share of infected women is 66,1% of all patients with urticaria and blastocystosis, while men account for 33,9% (fig. №3).

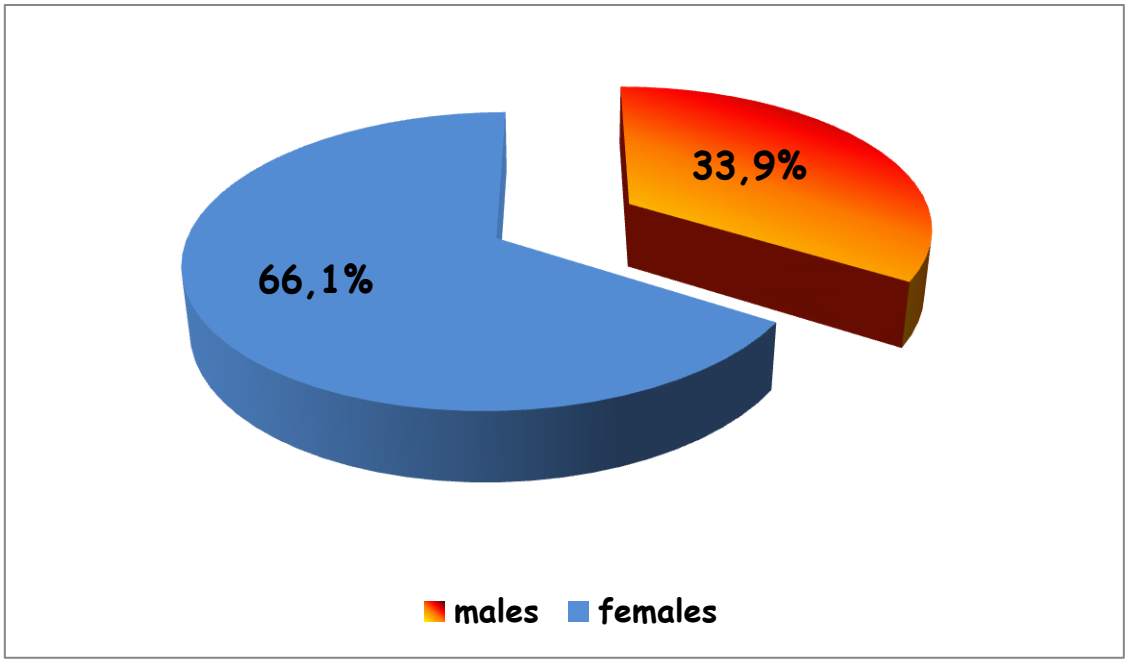

Fig. № 3 *Relative share of patients with acute and chronic spontaneous urticaria, positive for blastocystosis, based on sex (%)*

Despite the higher relative share of female patients positive for *Blastocystis sp.*, the rate of infection is higher in males with urticaria – 10.1% in males, compared to 9.4% in females.

The frequency of infection with *Blastocystis sp*. depending on the type of allergic reaction and sex is shown on figure № 4.

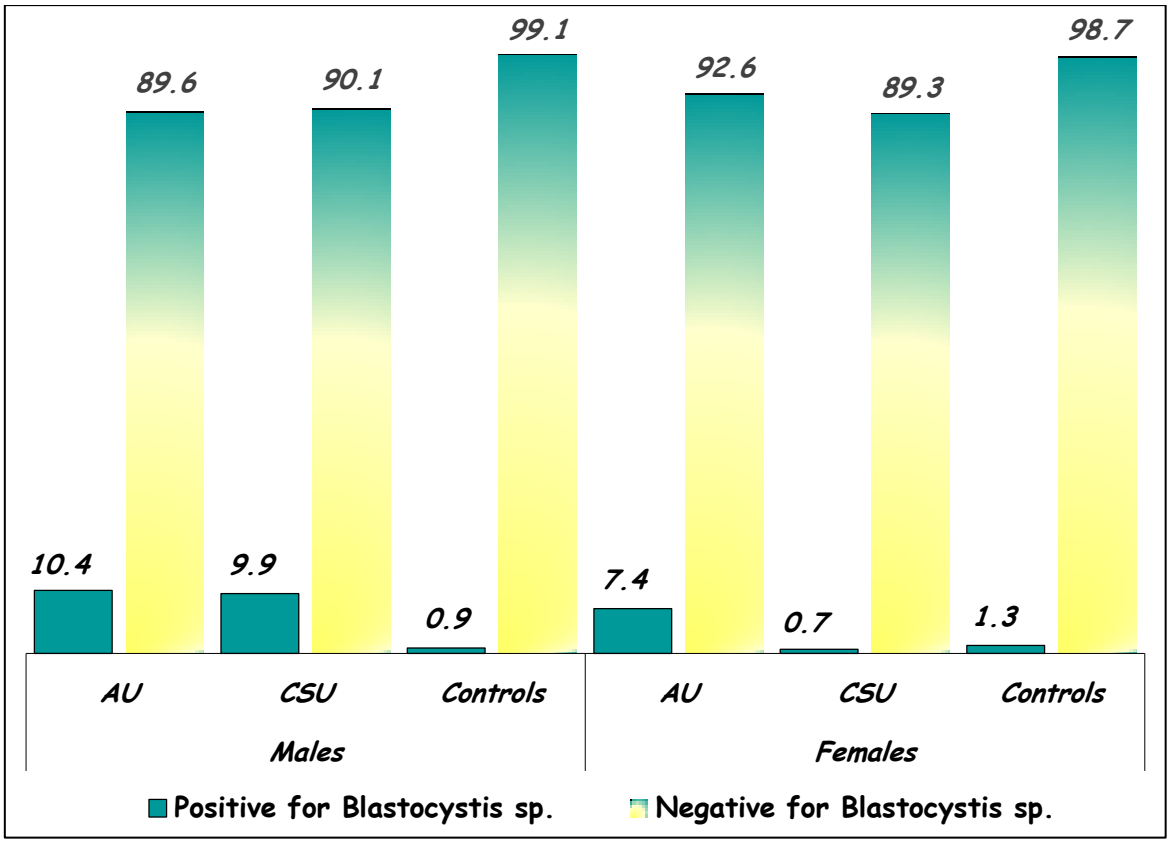

Fig. № 4 *Frequency of infection with Blastocystis sp. in the compared groups – stratification by sex (%)*

Among the tested patients with acute urticaria, males have a blastocystosis frequency of 10.4%, which relative higher that the rate in females – 7.4%. In patients with CSU, the frequency of infection was higher in females - 10.7%, compared to 9.9% in males.

When stratifying based on sex, the relation between the two tested variables is statistically significant: Males χ^2=34.050, df=2, P=0.001, V=0.205; Females χ^2=60.825, df=2, P=0.001, V=0.190. Due to the change in the strength of the effect, similarly to age, sex is a condounding factor.

Figure № 5 presents a logistic regression model (singular multinomial regression), which shows the independent role of *Blastocystis sp*. in the etiology of allergic reactions.

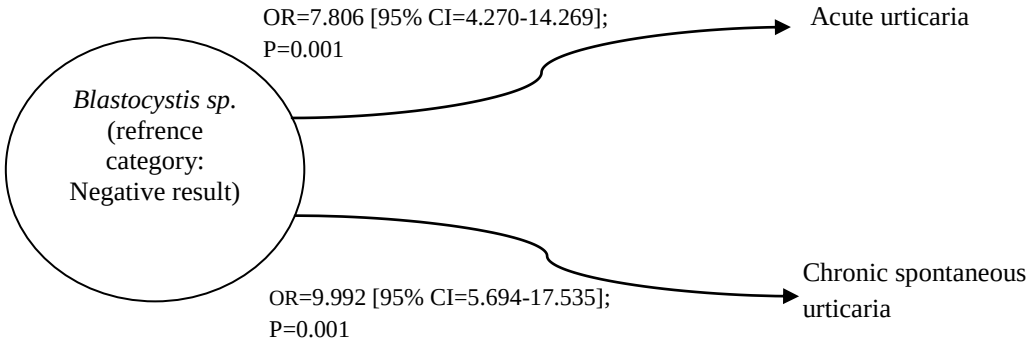

Fig. №5 *Results of the logistic regression analysis*

The odds of urticaria patients being infected with *Blastocystis sp.* compared to the individuals from the control group is roughly 8 times higher in patients with AU - OR=7.806 (95% CI=4.270-14.269; P=0.001) and roughly 10 times higher in patients with CSU - OR=9.992 (95% CI=5.694-17.535; P=0.001).

2. Determination and comparison of the genotype of *Blastocystis* isolates from patients with acute and chronic spontaneous urticaria and from a control group of carriers

We used conventional PCR to genotype *Blastocystis* isolates from 45 patients with acute and chronic spontaneous urticaria and from 24 healthy parasite carriers. The median age of the group of urticaria patients is 47 years (5, 80), while the median age of the healthy carrier group is 47 years (2, 78).

The group of patients with allergic symptoms includes 19 males (42.22%) and 26 females (57.78%). The healthy parasite carriers group includes 13 males (54.17%), and 11 females (45.83%).

Depending on the type of allergic reaction the patients with urticaria are divided into 20 subjects with acute urticaria (44.44%) and 25 with chronic spontaneous urticaria (55.56%).

The figures № 6 and № 7 show the gel electrophoresis step of PCR typing with primer sets for subtype 1 (ST1) and subtype 3 (ST3) of the parasite based on Yoshikawa et al.

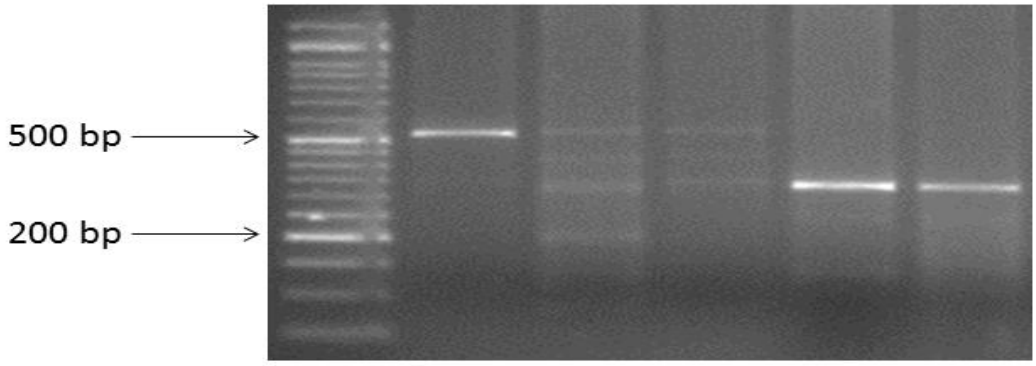

Fig. №6 *Results of PCR typing using primer set SB83, which corresponds to subtype 1 (ST1).*

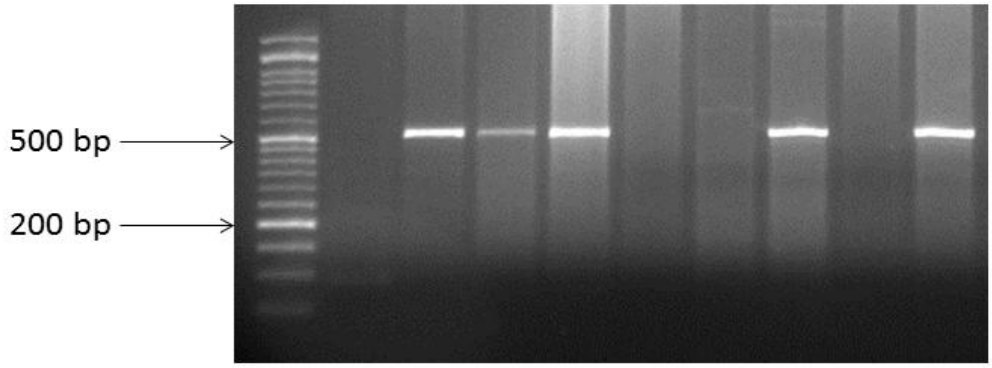

Fig. №7 *Results of PCR typing using primer set SB227, which corresponds to subtype 3 (ST3).*

Out of 45 samples from patients with urticaria and blastocystosis we isolated *Blastocystis sp.* DNA from 88.89% (40 isolates). In the healthy carrier control group, we successfully isolated *Blastocystis sp.* DNA from 23 out of the 24 samples (95.84%).

In the samples of 9 patients with urticaria and 6 subjects from the control group, there was no DNA amplification. We presume that this is due to either the isolation of DNA from subtypes for whom primers were not used in the study or due to genetic variability in the targeted gene sequences.

The distriburion of the isolated subtypes among the two studied groups is shown in table № 2.

Out of 45 studied samples from patients with acute and chronic spontaneous urticaria, we determined the subtype of *Blastocistis sp.* Of 31 isolates. From 25 patients (80.65%) we isolated subtype 3 (ST3), while subtype 1 (ST1) was isolated from 5 patients (16.13%). In a sample from a single patient (3.22%) we determined the presence of two subtypes – ST1 and ST3.

In the healthy parasite carrier group, we isolated ST3 from 11 subjects (64.71%) and ST1 from 6 people (35.29%). Despite using primers for 7 human subtypes of *Blastocystis sp*., during the PCR typing we did not isolate any DNA belonging to ST2, ST4, ST5, ST6 or ST7.

The most common subtype of *Blastocystis sp*. from urticaria patients was ST3. Subtype 3 was isolated from 11 patients with acute urticaria (80.65%). Subtype 3 was isolated from 11 patients with acute urticaria (42.31%), and from 15 patients with chronic spontaneous urticaria (57.69%). Subtype 3 was also the most commonly isolated subtype from healthy parasite carriers (64.71 % of the studied subjects).

Table. № 2 *Frequency of isolated Blastocystis sp. subtypes from patients with allergic symptoms and from healthy parasite carriers*

Studied group	DNA could not be isolated	No amplification	Subtype 1 (ST1)		Subtype 3 (ST3)		Subtype 1 + Subtype 3 (ST1 + ST3)		Sum of isolated subtypes	
	n	n	n	%	n	%	n	%	n	%
Patients with acute urticaria	**3**	**5**	**1**	**8.33%**	**11**	**91.67%**	**0**	**0%**	**12**	**100%**
Patients with chronic spontaneous urticaria	**2**	**4**	**4**	**21.05%**	**14**	**73.68%**	**1**	**5.27%**	**19**	**100%**
All patients with urticaria	**5**	**9**	**5**	**16.13%**	**25**	**80.65%**	**1**	**3.22%**	**31**	**100%**
Healthy parasite carriers	**1**	**6**	**6**	**35.29%**	**11**	**64.71%**	**0**	**0%**	**17**	**100%**

Our study is on a small contingent of allergic patients and as such we cannot reach any definite conclusions. Due to the high percentage (80%) of expected frequencies <5 we could not perform an analysis on the statistical significance of our results.

3. Comparison of the frequency of antitoxocara IgG antibody carriership in patients with acute and chronic spontaneous urticaria and in a control group

For a six-year period (2018 - 2023) in the parasitology laboratory of DKC – 2 Pleven, 297 patients with urticaria and 50 people with no allergic symptoms were tested for the presence of antitoxocara IgG antibodies. The distribution of the subjects of the study based on type of the allergic reaction, sex and age is shown on table №3.

Out of 297 patients with urticaria, 164 were female (55.22 %) and 133 (44.78 %) were male. The median age of the urticaria patients was 48 (1, 85). The median age of patients with AU was 45 years (1, 82), the median of patients with CSU was 47 years (2, 85) and the median of the control group was 56 years (9, 86). Among the urticaria patients, females predominate - 54.5% of the patients with AU and 55.4% of the ones with CSU.

Table № 3 *Distribution of subjects based on the type of allergic reaction, sex and age*

Type of allergic reaction	**Acute urticaria**	**Chronic spontaneous urticaria**	**Controls**
	n (%)	**n (%)**	**n (%)**
Sex			
Male	30 (45.5)	103 (44.6)	24 (48.0)
Female	36 (54.5)	128 (55.4)	26 (52.0)
Total	*66 (100.0)*	*231 (100.0)*	*50 (100.0)*
Age Mdn (Min, Max)	*45 (1, 82)*	*47 (2, 85)*	*56 (9, 86)*

Figure № 8 shows data on the frequency of antitoxocara IgG antobody carriership among subjects with urticaria and in the control group.

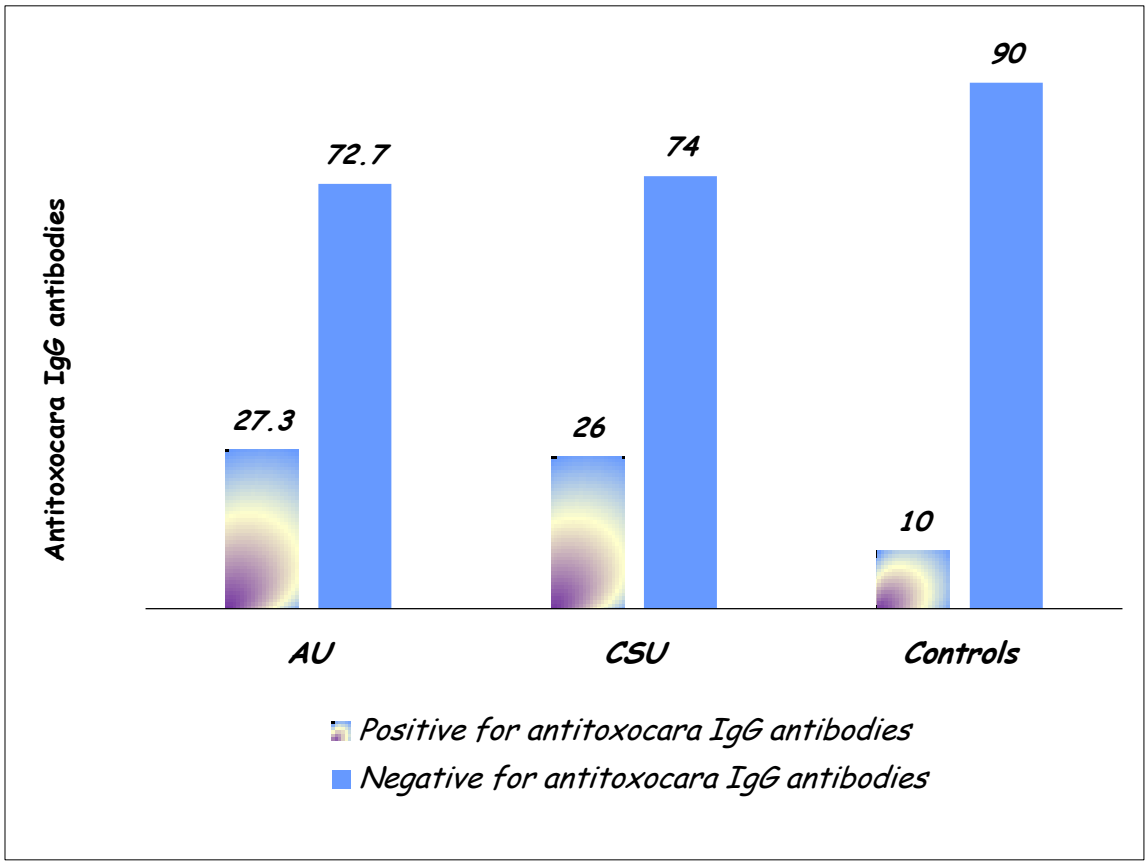

Fig. № 8 *Frequncy of carriership of antixocara IgG antibodies in the compared groups (%)*

Out of 297 patients with acute and chronic spontaneous urticaria 78 showed a positive serological result for antitoxocara IgG antibodies (26.3%). In the control group 5 people had antitoxocara IgG antibodies (10%).

Out of the 66 cases of acute urticaria 18 (27.3%) showed a positive serological result. Sixty (26%) of the patients with chronic spontaneous urticaria had a positive serological result.

Patients with chronic spontaneous urticaria were more commonly tested for the presence of antitoxocara IgG antibodies. Out of the toal 297 subjects with urticaria 231 had CSU. We consider that the higher relative share of patients with a positive result for antitoxocara IgG antibodies in the group of chronic spontaneous urticaria patients (76.9%) compared to the share in patients with

acute urticaria (23.1%) is due to the unequal number of tested patients in the two studied urticaria groups (Figure № 9).

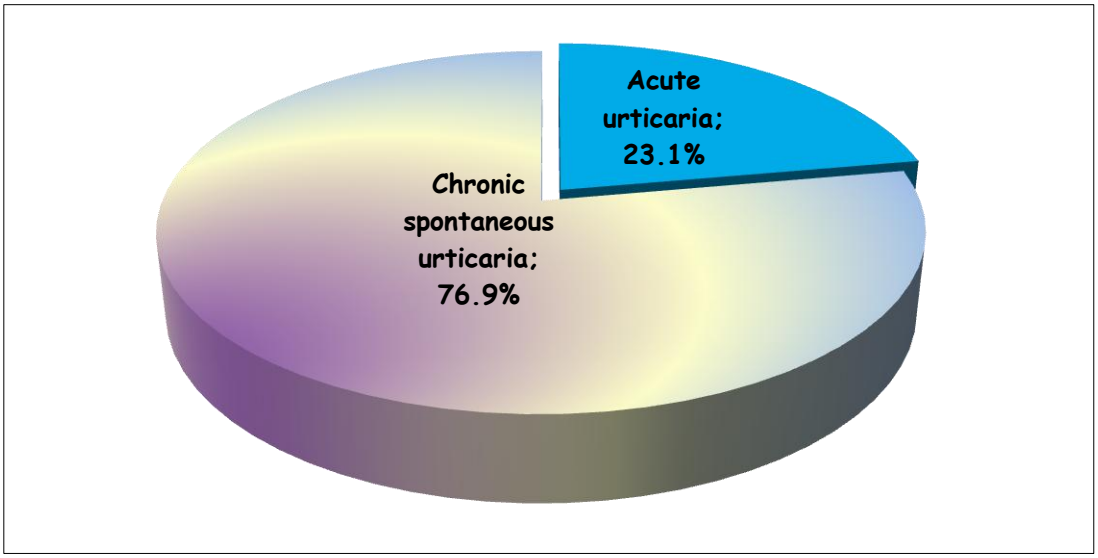

Fig. № 9 *Relative share of patients with urticaria positive for antitoxocara IgG antibodies, based on the type of allergic reaction.*

Table №4 shows the rate of antitoxocara IgG antobody carriership and the levels of antitoxocara antibodies in each of the compared groups.

Table № 4 *Frequency of antitoxocara IgG antobody carriership and levels of antitoxocara IgG antibodies among the tested patients with urticaria and in subjects from the control group*

Diagnosis	Positive for antitoxocara IgG antibodies		Levels of antitoxocara IgG antibodies (SR)		Median levels of antitoxocara IgG antibodies (SR)
	n	%	Minimum value	Maximum value	
Acute urticaria	18	27.3	1.2	7.0	2.07
Chronic spontaneous urticaria	60	26	1.2	6.8	1.95
Control group	5	10	0.03	3.5	0.3

We found a statistically signifact higher rate (χ^2=6.267, df=2, p=0.044, V=0.193, V=0.134) positive results for antitoxocara IgG antibodies in patients with AU and CSU compared to the control group, though the strength of the effect is low.

Figure № 10 shows the results from a regression analysis. The singlular multinomial regression confirms the predictive value of antitoxocara IgG antibodies regarding allergic reactions.

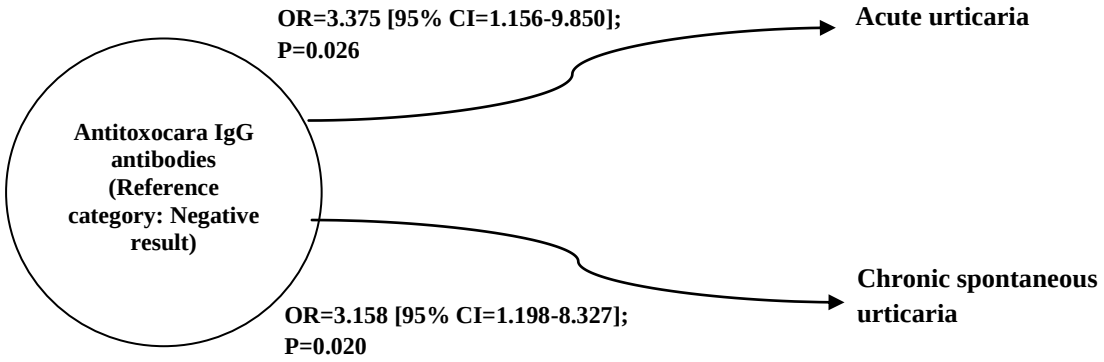

Fig. №10 *Results of the logistic regression analysis*

The patients with AU have a roughly 3.4 times higher risk (OR=3.375; 95% CI=1.156-9.850; P=0.026) to be antitoxocara IgG antibody carriers, compared to the control group. For the patients with CSU the risk is 3.3 times higher (OR=3.158;95% CI=1.198-8.327; P=0.020).

4. Comparison of the levels of total IgE in patients with acute and chronic spontaneous urticaria and carriership of antitoxocara IgG antibodies and a control group of healthy individuals.

We studied and compared the levels of total IgE in three groups: patients with urticaria and antitoxocara IgG antibodies (n=46), patients with urticaria and a negative result for antitoxocara IgG antibodies (n=46) and a group of healthy controls without allergic symptoms and without antixocara IgG antibodies (n=50). All subjects that were part of the study showed a negative result for intestinal protozoans and helminths.

Out of 46 patients with urticaria positive for antitoxocara antibodies, 12 (26.1%) were male and 34 (73.9%) were female. The median age is 65.50 (21, 80). Depending on the type of urticaria, the patients from this group are divided into 20 subjects with acute urticaria (43.5%) and 26 patients with chronic spontaneous urticaria (56.5%).

The median age of the urticaria group with a negative result for antitoxocara IgG antibodies is 52 (10, 78). Twelve were male (26.1%), 34 (73.9%) were, 17 (37%) had acute urticaria and 29 (63%) had chronic spontaneous urticaria.

In the control group out of 50 healthy individuals, 23 were male (46%) and 27 (54%) were female, while the median age is 57.5 (9, 86).

The total IgE levels in the three compared groups is shown in table № 5.

Table. № 5 *Levels of total IgE in patients with urticaria positive and negative for antitoxocara IgG antibodies and in helathy controls*

Group	Diagnosis	Total IgE level (IU/ml)		Median levels of total IgE (IU/ml)
		Minimum value	Maximum value	
Patients with urticaria, positive for antitoxocara IgG antibodies	Acute urticaria	5	787.70	76.94
	Chronic spontaneous urticaria	1.21	846.30	43.40
	Total	1.21	846.30	73.39
Patients with urticaria, negative for antitoxocara IgG antibodies	Acute urticaria	5.60	804.50	59.20
	Chronic spontaneous urticaria	0.9	449.10	75.20
	Total	0.9	804.50	61.47
Healthy control group		1.62	518.23	23.99

The results of the Kruskal-Wallis test (Figure № 11) show statistically significant higher median levels of total IgE (73.39, 1.2-846.3) in patients with urticaria and carriership of antitoxocara IgG antibodies and in patients with urticaria with a negative result for antitoxocara IgG antibodies (61.47, 0.9-804.5) compared to the control group (H=12.480, df=2, p=0.002).

We did not find a statistically significant difference between the levels of total IgE between the two urticaria groups – with and without antitoxocara IgG antobodies (H=0.568, df=1, p=0.451). We also did not find a significant difference in total serum IgE levels between patients with acute and chronic spontaneous urticaria (H=1.069, df=1, p=0.295).

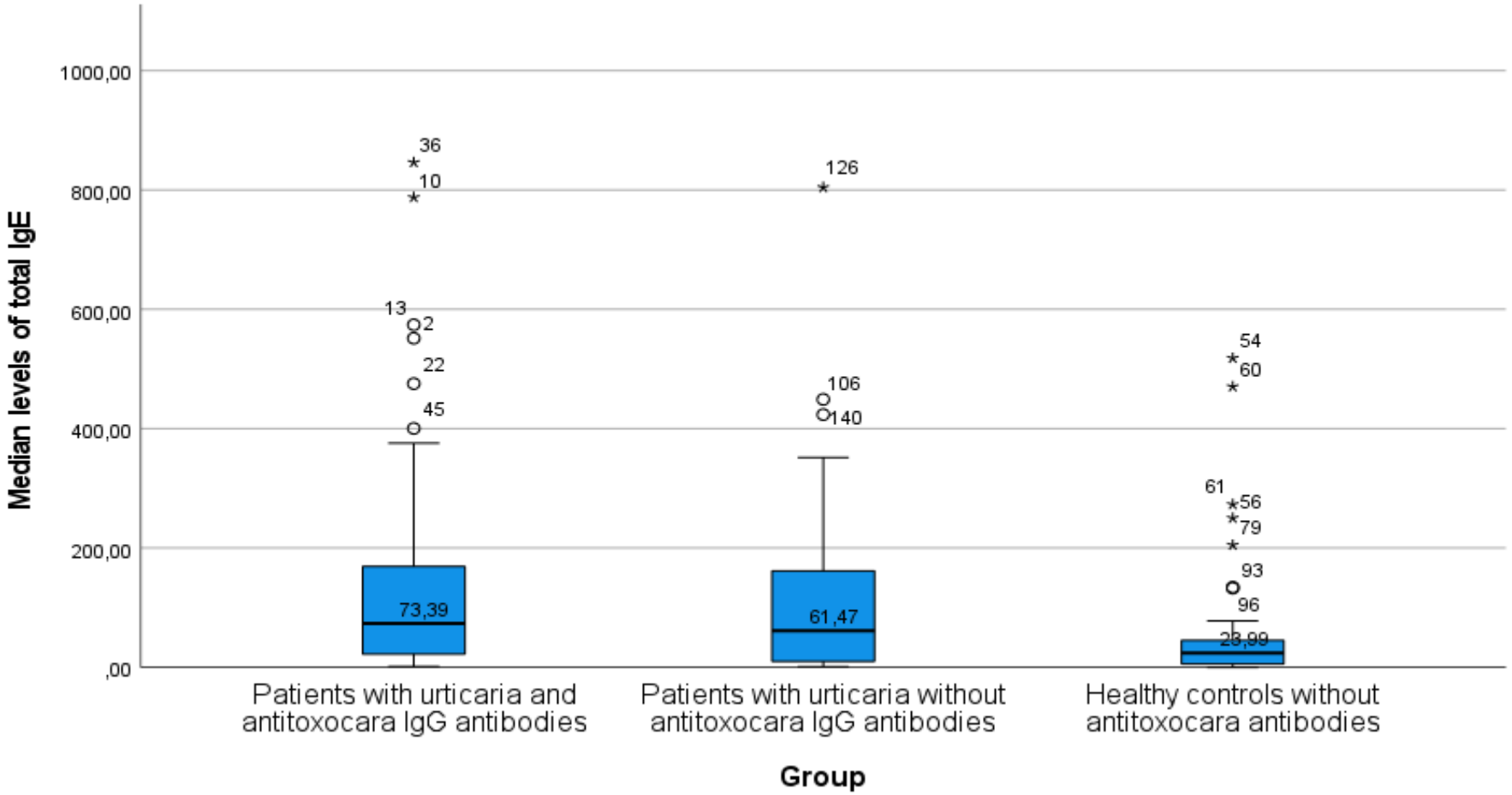

Fig. №11 *Median levels of total serum IgE in the compared groups*

We found a statistically significant positive correlation between the levels of antitoxocara IgG antibodies and the levels of total serum IgE (ρ=0.235; p=0.005) i.e. as antitoxocara IgG antibodies increase, a proportional increase in total swerum IgE is also found (Figure №12).

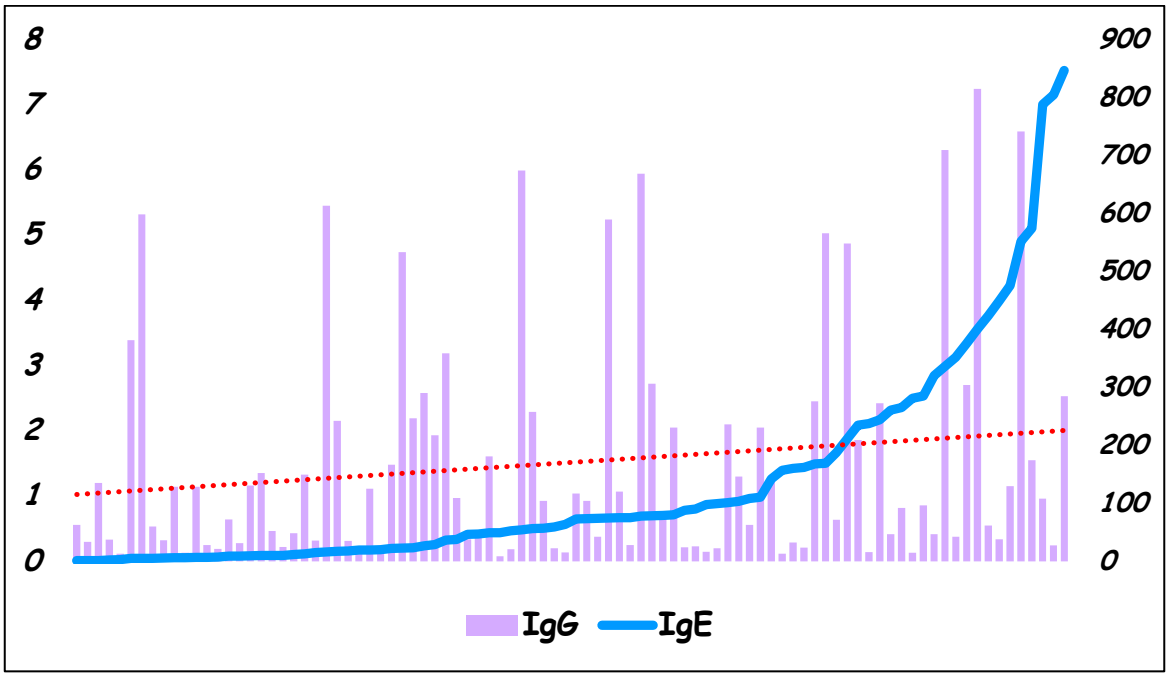

Fig. ***№ 12*** *Comparison between the levels of antitoxocara IgG antibodies and total serum IgE in patients with acute and chronic spontaneous urticaria*

5. Comparison of the levels ECP in patients with acute and chronic spontaneous urticaria and carriership of antitoxocara IgG antibodies and a control group of healthy individuals

We studied the levels of ECP in three groups. The first one is made up of 48 patients with acute and chronic spontaneous urticaria and have a positive result for antitoxocara IgG antibodies. The second group contains 45 subjects with urticarial symptoms and they do not carry antitoxocara antibodies. The third group includes 50 healthy individuals, with no patient history of prior allergic reactions and without antitoxocara IgG antibodies.

The median age of the patients with antitoxocara antibodies and urticaria is 64 (21, 76). Dpending on the type of allergic reactions they are divided into 21 subjects with acute urticaria (43.8%) and 27 patients with chronic spontaneous urticaria (56.3%). Twelve of the tested subjects were male (25%) and 36 (75%) were female.

In the group of patients with urticaria with a negative serological result for antitoxocara antibodies, 18 had acute urticaria (40%) and 27 had chronic spontaneous urticaria (60%). The median age is 53 years (12, 78), while 15 of the group were male (33.3%) and 30 were female (66.7%).

In the control group, out of 50 subjects, 23 were male (46%) and 27 were female (54%). The median age of the group is 57.50 years (9, 86).

Table № 6 shows the distribution of tested individuals for eosinophil cationic protein, based on sex and type of allergic reaction.

Table № 6 *Distribution of the subjects in the compared groups based on the type of urticaria sex and age.*

Type of allergic reaction	**Acute urticaria**	**Chronic spontaneous urticaria**	**Controls**
	n (%)	**n (%)**	**n (%)**
Sex			
Male	15 (38.5)	12 (22.2)	23 (46.0)
Female	24 (61.5)	42 (77.78)	27 (54.0)
Total	*39 (100.0)*	*54(100.0)*	*50 (100.0)*
Age Mdn (Min, Max)	*56 (17, 76)*	*58 (12, 78)*	*57.50 (9, 86)*

Table № 7 shows the levels of ECP in the three compared groups.

Table. № 7 *Levels of ECP (ng/ml) among the studied subjects based on the type of allergic reaction.*

Group	Diagnosis	ECP level (ng/ml)		Median levels of ECP (ng/ml)
		Minimum value	Maximum value	
Patients with urticaria, positive for antitoxocara IgG antibodies	Acute urticaria	11.21	90.80	39.51
	Chronic spontaneous urticaria	13.35	121.31	36.60
	Total	11.21	121.31	36.77
Patients with urticaria, negative for antitoxocara IgG antibodies	Acute urticaria	1.56	43.37	28.96
	Chronic spontaneous urticaria	1.56	119.71	28.48
	Total	1.56	119.71	28.48
Healthy control group		1.56	62.27	25.48

We found statistically significant higher median value of ECP in patients with urticaria and a positive result for antitoxocara IgG antibodies and in patients with urticaria with no antitoxocara IgG antibodies compared to the healthy controls (H=9.867, df=2, p=0.007) (Figure №13). Additionally, we also found a significant difference in the levels of eosinophil cationic protein between the two urticaria groups - with and without the presence of antitoxocara IgG antibodies (H=6.841, df=1, p=0.009).

The levels of ECP were statistically higher in patients with acute urticaria that were carriers of antitoxocara IgG antibodies, compared to patients with acute urticaria with a negative result for antitoxocara IgG antibodies (H=4.232, df=1, p=0.040). We did not find a significant different in the levels of ECP between patients with chronic spontaneous urticaria with and without antitoxcara antibodies (H=3.213, df=1, p=0.073).

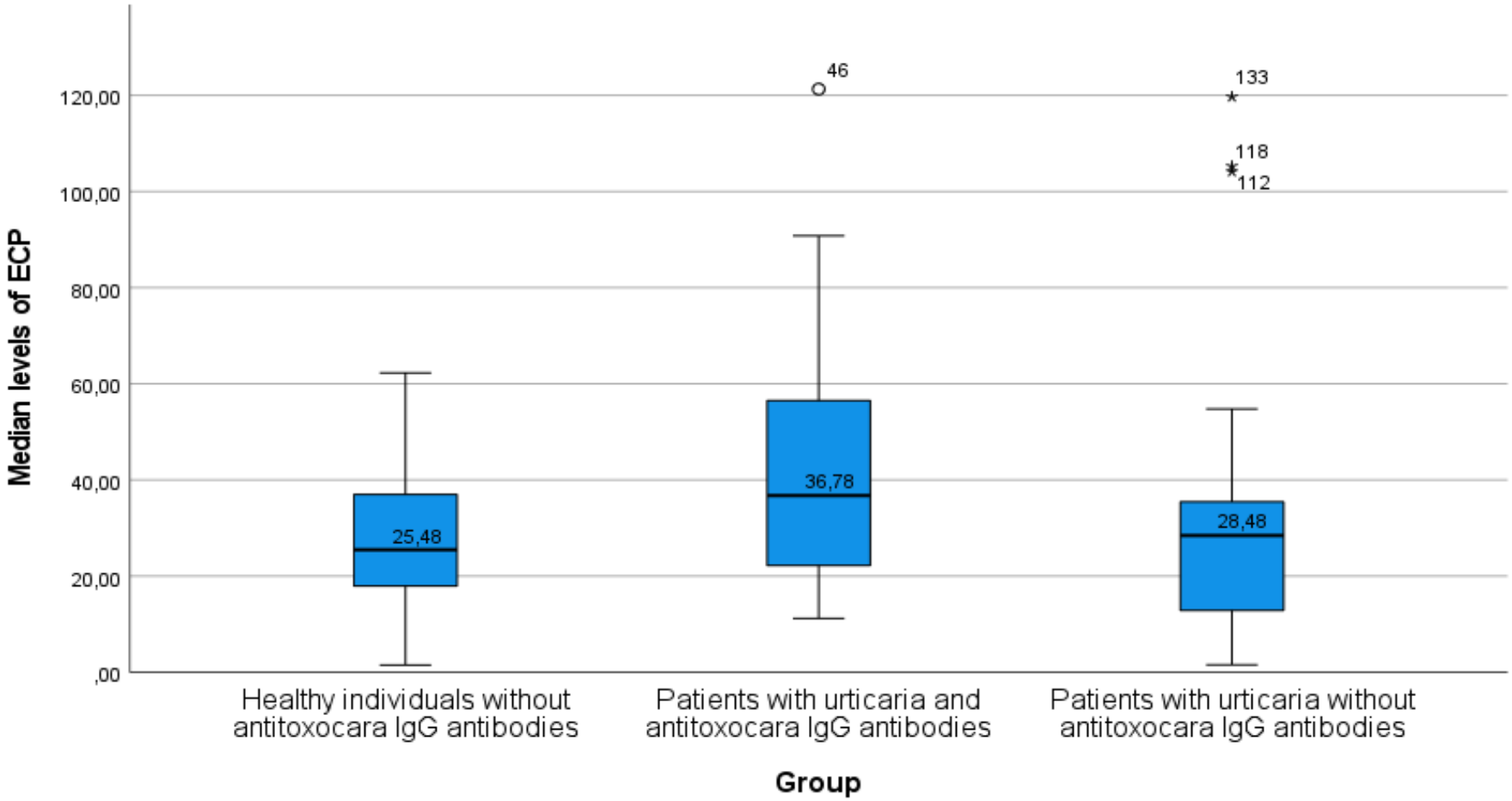

Fig. № 13 *Median values of ECP in the compared groups*

The difference in ECP levels between patients with acute and chronic urticaria is not significant (H=0.146, df=1, p=0.703).

We found a positive correlation between the levels of ECP and the levels of antitoxocara IgG antibodies in patients with AU (ρ = 0.360; p = 0.024), (Figure №14).

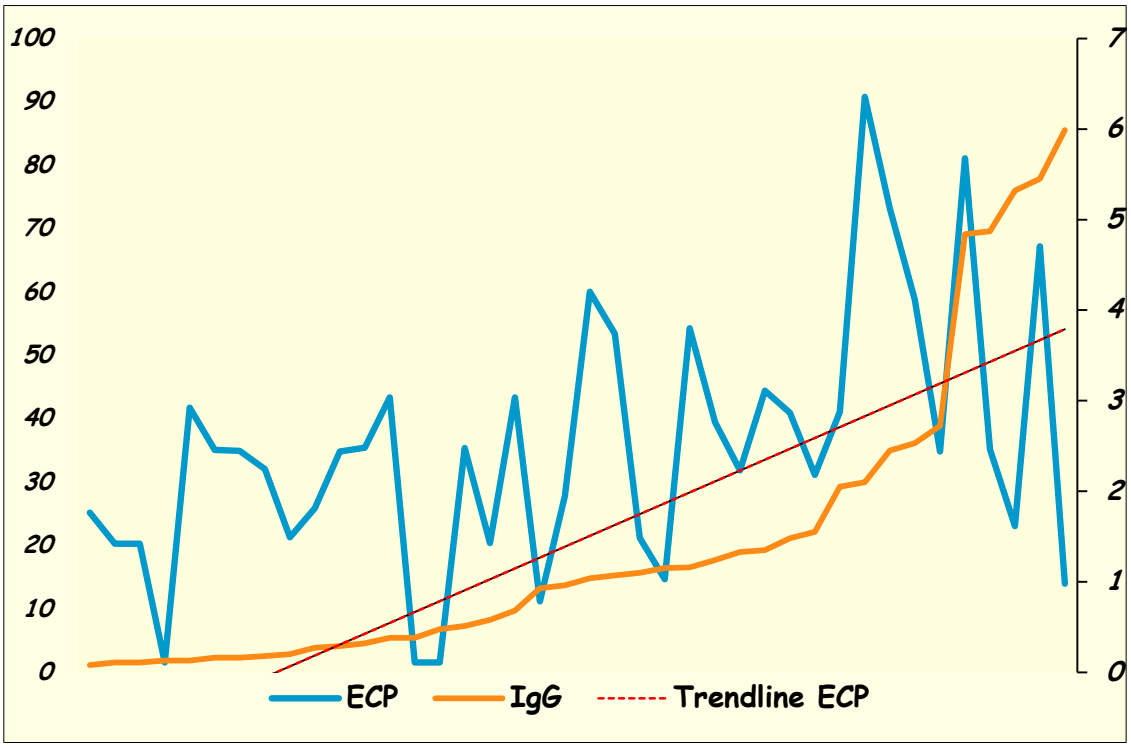

Fig. № 14 *Comparison between the levels of ECP and antitoxocara IgG antibodies in patients with acute urticaria.*

As the levels of antitoxocara IgG antibodies increase, so do the levels of ECP in patients with acute urticaria also increase. We did not discover a statistically significant correlation between the levels of ECP and antitoxocara antibodies in patients with chronic spontaneous urticaria (ρ = 0.181; p = 0.191).

VI. ANALYSIS OF THE RESULTS AND DISCUSSION

In the last decades the cases of allergic reactions have increased as a part of human pathology and this is why they are socially important. According to literature the frequency of allergic diseases varies between 10% and 30%, which depends on geography, economic development, lifestyle and nutrition (Grammatikos, 2008; Gutowska-Ślesik et al., 2023).

Clinical allergy is a polyetiological condition that depends on both endogenous factors (such as genetic predisposition and immune response) a as well is on exogenous factors which provoke the formation of allergic symptoms. Multiple exogenous allergens are known in humans – food, air, animal, drug allergens, insect stings and others. Parasite allergens are also a type of exogenous allergen (Caraballo et al., 2018; Björkander et al., 2019). Parasites migrate and reproduce in the organism of the infested human and release a multitude of metabolic products as a result of their life cycle. This causes allergization and sensibilization of the host and leads to the development of systemic allergic reactions (Петров и сътр., 2016). Allergic reactions can develop in both the acute and the chronic stage of parasitic invasion such as urticaria, angioedema, asthma-like symptoms and others which persist and affect the patient's quality of life (Kolkhir et al., 2016).

Knowing the etiology of a disease are are decisive for a physician's diagnostic and therapeutic behavior. In this sense clarifying the exact role of parasites in the pathogenesis of allergic diseases would allow proper diagnosis and treatment for both parasitic and allergic conditions.

Researchers have developed several theories while studying the conection between invasions with intestinal or tissue parasites and allergic reactions in modern humans. The first is the so called "hygiene hypothesis", created by Stracjan in 1989, according to which the increase of the number of cases of allergic disease is due to the lower number of children in modern families, better quality of life and better personal hygiene. According to Strachan children that

live in unhygienic conditions are more likely to be infected with parasites in early childhood and as a result develop atory more rarely. He considers that the lower rate of asthma among the children of farmers and in families with pets as proof for the theory (Strachan, 1989).

Another theory is the so called “old friends hypothesis”. According to it the increased rate of allergic diseases is caused by a lack of infection with “useful” parasites in early childhood. Or in other words, the host requires immunomodulation caused by parasitic invasions in order for the immune system to properly develop and to avoid the future development of immunomediated disease, including allergies (Bilbo et al., 2011; Lambrecht et al., 2017; McSorley et al., 2019).

Other authors consider that the mechanism of immunomodulation of the host’s immune system by helminths is an epigenetic process, which works through temporary modification of gene expression and the “protective” effect is lost after several generations of no helminth invasion (Rook et al., 2017; Maizels, 2020; Rook, 2023). This may explain why some studies show a protective effect in terms of allergic diseases (in people living in endemic regions with high incidence of helminthoses), while other studies show allergization (studies done in areas with low or lacking helminthosis incidence). According to a meta-analysis by Arrais et al, despite all the studies that show a protective effect, helminth invasions are associated with an increase in allergic responses due to the characteristic strong Th2-type immune response (Arrais et al., 2022).

In our study our goal was to look for a possible connection between acute and chronic spontaneous urticaria and two somewhat understudied parasites in Bulgaria - *Blastocystis sp.* and *Toxocara spp.* We chose them specifically because of the relatively high rate of their discovery among allergic patients hospitalized in the Allergology clinic of University Hospital – Pleven and

among ambulatory patients passing through the Parasitology Cabinet of DKC-2 Pleven.

Blastocystis sp. is a widely distributed zooanthroponotic unicellular microorganism, which is considered to be the most commonly isolated protozoan from human feces (Wawrzyniak et al., 2013; Skotarczak, 2018; Stensvold et al., 2020). It was long considered to be a commensal, but now most authors consider that its disease-causing capabilities depend on its subtype and on the immune response of the host (Tan et al., 2010; Ajjampur et al., 2016). Infection with *Blastocystis sp.*, regardless if it causes symptoms or not, is termed blastocystosis. The most common form found in prophylactic diagnosis is asymptomatic carriership (Vielma, 2019).

Epidemiological studies in Europe and Asia show that the incidence rate of c *Blastocystis sp.* infection varies wildly: 4.2% among children in Switzerland; among a contingent made up of various age groups in Slovakia - 10.7%; in China – 3.37%; North Cyprus – 10.5%; Iran – 24.46% (Seyer et al., 2017; Deng et al., 2019; Shirvani et al., 2020; Kantzanou et al., 2021).

Blastocystis has a local distribution in Bulgaria. Data from the National Center for Infectious and Parasitic Diseases in Sofia, based on yearly analysis of parasitoses incidence, allows us to see the rate of infection on a national level. In 2020, 515 cases of blastocystosis were registered (incidence of 0.17%), in 2021 – 1596 cases (incidence of 0.44%), in 2022 – 1593 cases (incidence of 0.39%) and in 2023 – 1800 cases (incidence of 0.53%) (Harizanov et al., 2022, 2023, 2024). A local study in the Pleven region, done over a period of 7 years, among a contingent of 14402 prophylactically tested subjects shows an incidence rate of 3.4% The same study shows a statistically significant higher rate of of 4.3% in patients tested for Blastocystis based on clinical indications (Ангелов и сътр., 2009).

The link between blastocystosis and urticaria has been studied in multiple publications with varying conclusions. Regardless it is a fact, however, that

urticaria is the most common skin reaction associated with *Blastocystis sp.* infection and multiple studies confirm this connection (Kantardjiev et al., 2019; Vezir et al., 2019; Aykur et al., 2022; Bahrami et al., 2022). It has also been shown that the skin symptoms dissipate after etiological treatment with an antiparasitic drug (Bahrami et al., 2020; Fonte Galindo et al., 2023). Tuzer et al. have studied the influence of treatment on clinical presentation and lab results of 70 subjects with blastocystosis and CSU, treated with antihistamines and antiparasitic drugs. Compared to 70 subjects with CSU without blastocystosis, the first group showed a significant reduction of urticaria severity, total serum IgE and peripheral eosionophilia (Tuzer et al., 2022).

Our study found *Blastocystis sp.* in 115 fecal samples out of 1197 patients with urticaria. The incidence of 9.6% likely does not represent the actual rate of infection, especially since the diagnosis was made based on a single examination. In the control group the incidence was 1.2% out of 1300 healthy subjects. The difference between the two groups is significant (χ^2=92.793, df=2, *P*=0.001, V=0.193) and it allows us to consider *Blastocystis sp*. as a co-factor for the development of urticaria.

In the available scientific literature, we find similar data in Aykur et al.'s study – 31.9% incidence of *Blastocystis sp.* in oatients with CSU and in Jafari A. et al's study, which shows a rate of 21.3% in patients with CSU (Aykur et al., 2022; Jafari et al., 2024).

We found a higher risk of infection with *Blastocystis sp.* – 8 times higher than normal in patients with AU (95% CI=7.806-14.269; P=0.001) and 10 times higher in patients with CSU (95% CI=5.694-17.535; P=0.001). The higher probability of infection with *Blastocystis sp.* and its higher rate among urticaria patients confirm its allergizing potential for humans.

The advancement of molecular methods over the years has allowed for important discoveries to be made in the field of medical parasitology. The molecular analysis of *Blastocystis* has continued for more than 20 years.

Multiple ribosome strains called subtypes (ST) have been isolated based on the polymorphism of the small unit of SSrRNA gene (Stensvold, 2020; Maloney et al., 2021). *Blastocystis sp.* is divided into around 34 subtypes, 22 of which are considered valid. Fourteeen of the subtypes have been isolated from human feces – from ST1 to ST10, from ST12 to ST14 and ST16 (Stensvold et al., 2016; Cian et al., 2017; Lalev et al., 2020; Stensvold et al., 2020; Jiménez et al., 2022).

Almost 90% of the *Blastocystis sp.* isolates belong to ST1, ST2, ST3 and ST4 (Stensvold et al., 2016; Zanetti et al., 2020). Among these four ST3 is the most commonly isolated subtype and because of this is called a "human subtype". ST3 isolates show genetic difference from ST3 isolated from animals (Stensvold et al., 2012). Epidemiological studies have shown that in Europe ST1, ST3 and ST4 are the most common, which in the Americas ST1 and ST2 predominate; in Asia and Australia - ST1 и ST3 (Stensvold et al., 2016; Skotarczak, 2018; Jiménez et al., 2019).

Subtype 3 is the most commonly isolated one from allergic patients in our study. We found ST3 in 11 patients with AU and in 15 with CSU. ST3 was also the most isolated from healthy parasite carriers (64.71%).

Our results on ST3 correspond to the ones from a precious study on subtypes in the Pleven region on hospitalized patients in University Hospital – Pleven (Lalev et al., 2020). ST3 is also the most isolated subtype worldwide in both allergic patients and in asymptomatic people (Cian et al., 2017).

ST1 is usually isolated from patients with intestinal symptoms and from asymptomatic subjects, but studies have shown that it is also found in CSU patients (Bahrami et al., 2020). In our study we isolated ST1 from 5 patients with allergy (1 with AU and 4 with CSU) and in 6 healthy carriers.

These results are similar to the results of European studies which show that ST1 is the second most common sybtype in Europe after ST3 (Skotarczak, 2018).

In this study, similar to previous Bulgarian studies on subtype, no ST4 was isolated, despite it being the 3rd most common subtype in Europe (Popruk et al., 2021). We also did not isolate any ST2, even though it's among the four most common human subtypes in the world (Jiménez et al., 2019). There were also no isolates belonging to ST5, ST6 and ST7. ST5 is a zoonotic subtype usually isolated from pigs and rarely isolated from people with blastocystosis in Europe. ST6 and ST7 are isolates from birds and are only isolated from people in Central and Southeast Asia (Süli et al., 2021). ST6 is rare in Europe, though 4 cases of blastocystosis with intestinal symptoms caused by it have been described in Serbia. Sporadic cases of ST6 and ST7 have also been found among poultry farm workers in Poland (Rudzińska et al., 2023).

In our study ST1 and ST3 were roughly isolated equally from both allergic and healthy subjects. Our study, however, is on a small contingent of people and as such we cannot come to any categorical conclusions. We did not find any difference in *Blastocystsis* subtype based on the type of allergic reaction as posited by some authors. Additional analysis is required, perhaps through multilocus typing or DNA sequencing to discover whether any subvariants of subtypes exist and what their role is for the clinical presentation of blastocystosis. The answer to these questions remains to be revealed. We consider that since *Blastocystis sp.* has a statistically higher frequency in patients with AU and CSU, it shows, albeit indirectly, that parasite antigens act as allergens for the host and this requires allergology specialists to treat not just the allergic symptoms but also the parasitic disease.

In our multidirectional study in both hospitalized and ambulatory patients, we often find the presence of antitoxocara IgG antibodies. Due to the lack of characsteristic symptoms of classic clinical forms of toxocariasis, we consider the patients with allergic symptoms that carry antitoxocara antibodies as cases of "covert" toxocariasis.

Covert toxocariasis, also known as common toxocariasis often presents subclinically with just allergic symptoms such as urticaria, pruritus and asthma-like symptoms (Lee et al., 2014; Henke et al., 2023; Patterson, 2023).

The diagnosis of toxocariasis is difficult due to *Toxocara spp.* not reaching secual maturity in human hosts and this not releasing any infective elements (Петров и сътр., 2016; Ma et al., 2018). Because of this, diagnosis of toxocariasis is based on immunological reactions that can detect the existance of antitoxocara IgG antibodies in serum samples of the host. Routine diagnosis is sone via ELISA, while Western blot, because of its higher sensitivity, is used to confirm positive ELISA results for toxocariasis (Yariktas et al., 2007; Rudzińska et al., 2017).

The serological diagnosis for toxocariasis has several disadvantages. False positives are possible, due to cross reactions between the excretory-secretory antigens of *Toxocara spp.* and antigen determinants of helminths from the genera *Trichinella, Ascaris, Strongyloides* and *Ancylostoma*. (Rudzińska et al., 2017; Noordin et al., 2020). The presence of antitoxocara IgG antibodies reflects the infection of the human host with the parasite, howver it cannot determine the stage of invasion – acute, chronic, latent or persistence of antibodies. It is known that antitoxocara IgG antibodies detected with ELISA persist for up to 2.7 years. While the ones detected with Western blot persist for up to 5 years (Fillaux et al., 2013). This creates diagnostic and therapeutic difficulties in clinical practice and requires the development of additional criteria for the evaluation of each case. We consider that the levels of total serum IgE and eosinophil cationic protein would help the diagnosis of toxocariasis, since they are part of the pathogenesis of both allergic and parasistic diseases. The studies that are part of this dissertation thesis include the rate of invasion with *Toxocara spp.* and the levels of total serum IgE and ECP in patients with urticaria that are carriers of antitoxocara IgG antibodies.

The statistical analysis shows a significant difference between the levels of antitoxocara IgG antibodies in both patients with AU and with CSU, compared to the levels in the control group (χ^2=6.267, df=2, p=0.044, V=0.193, V=0.134). We also determined that the risk of carriership of antitoxocara IgG antibodies is 3.4 times higher than usual in subject with AU (95% CI=1.156-9.850; P=0.026) and 3.2 times in subjects with CSU respectively (95% CI=1.198-8.327; P=0.020). We consider that the higher risk reflects the role of Toxocara spp. for the development of allergization in the host.

A similarly high rate of carriership of antitoxocara IgG antibodies has been described by various other authors. In a Brazilian study, the researchers determined that the seropositivity rate for antitoxocara antibodies was 16% among children with urticaria (Yoon et al., 2018). Humbert et al. describe a carriership rate for antitoxocara IgG antibodies of 19.5% among the urticaria patients they tested. (Humbert et al., 2020). The first studies on the frequency of toxocariasis in Bulgaria were done by Rainova et al. It was determined that the rate of antitoxocara IgG antibody carriership was 10.9% among subjects suspect for toxocariasis and 4% among healthy blood donors (Райнова, 2006). During the period 2000-2017 660 people with clinical allergy were tested in Bulgaria and 12% of them showed a positive serological result for toxocariasis (Райнова, 2020). During the period 2018-2020, 386 people passed through the National reference laboratory "Diagnosis of parasitoses" at NCIPD – Sofia and antitoxocara IgG antibodies were found in 22.3% of the tested individuals and 17.6% of them had allergic symptoms (Кънева и сътр., 2022). The statistically significant difference in the carriership of antitoxocara IgG antibodies between allergic patients and healthy individuals is confirmed by several other similar studies (Humbert et al., 2000; Mohammadzadeh et al., 2018; Yoon et al., 2018; Darvish et al., 2021).

Reflecting over our results we consider that toxocara antigens are allergens for humans, however the type of allergic reaction and its presentation

over time presumably depends on the type and stage of the invasion process and on the condition and sensitivity of the host's immune response.

Like other researchers we consider that the link allergy – toxocariasis is not a coincidence, despite the fact that in literature they are reports with the opposite conclusion (Kaneva et al., 2015; Maizels, 2016; Kim et al., 2017). The high rate of carriership of antitoxocara IgG antibodies in patients with different types of allergies in our study gives us reason to recommend serological testing in every allergic patient. An important moment for the clinical assessment of cases with antitoxcara IgG antibodies in patients with urticaria is whether both antiallergic and antiparasitic therapy is required. The long persistence of antitoxcara antibodies leads to irrational polypragmasy. In order to determine whether antiparasitic therapy is necessary in these patients, additional clinical, serological and laboratory criteria are required. Possible additional criteria could be the changes in levels of total serum IgE, the levels of eosinophil cationic protein, as both are part of the local immune response in toxocariasis.

Total serum IgE's potential as a diagnostic marker during toxocara invasion derives from its role in the immunopathogenesis of parasitic and allergic diseases (Caraballo et al., 2023). As early as 1008 Obwaller et al. described higher levels of total serum IgE in patients with toxocarisis (Obwaller et al., 1998). Abd El Wahab er al. found a statistically significant connection between the carriership of antitoxocara IgG antibodies and above norm levels of total IgE in children with bronchial asthma (Abd El Wahab et al., 2023). A Spanish study on the children of migrants infected with *Toxocara spp*. determined that heightened levels of total IgE were found in 50% of the studied subjects. They also described that the titres of IgE fell after etiological therapy with Albendazole (Bustamante et al., 2022). In Bulgaria total IgE levels in people with toxocariasis were studied by Kaneva et al in 2019. Among 120 patients with visceral and ocular toxocariasis they found a significantly higher

mean level of total IgE (153.7 IU/ml), compared to mean levels in healthy controls (49.40 IU/ml) (Kaneva et al., 2019).

In our study we found statistically significant higher median levels of total IgE among patients with urticaria (with positive and negative serological result for antitoxocara IgG antibodies) compared to a control group of healthy individuals. Similar to the study conducted by Kaneva et al. the median levels of total IgE in patients with AU and CSU were around three times higher than in individuals without allergic symptoms (Kaneva et al., 2019). Darvish et al. described a significant difference in the levels of total serum IgE between children with asthma with antitoxocara IgG antibodies and children with asthma without antitoxocara antibodies (Darvish et al., 2021). Datolli et al. found above the norm levels of total IgE among Brazilian blood donors with a positive result for antitoxocara IgG antibodies (Dattoli et al., 2021).

The significant correlation between the levels of total IgE and antitoxocara IgG antibodies in our study is interesting. The high levels of total IgE as well as the correlation could be a sign of a fresh invasion with *Toxocara spp.* and that the disease is active – a condition for etiological treatment. Our results on the connection between the levels of total IgE and antitoxocara IgG antibodies in patients with AU and CSU give us reason to recommend that every patient with allergic symptoms and high levels of total IgE should be tested for antitoxocara IgG antobodies. The early discovery and treatment of covert toxocariasis cases would favor not only the reversal of the allergic symptoms, but also the effect of the antiparasitic therapy.

Eosionophils are a fraction of granulocytes that are part of the pathogenesis of both allergic and parasitic diseases (Rosenberg et al., 2007; Amoani et al., 2019; Sakyi et al., 2022). As a part of the local antiparasitic immune response, eosinophil secrete cytotoxic proteins from the granules, one of which is eosinophil cationic protein (ECP). ECP is a protein from the ribonuclease family, which has been shown in vitro to be able to paralyze or kill

migrating parasites in the host's tissue (Hotz et al., 2022). Despite its short half-life of 45 minutes, it is considered that it reflects the total mass of activated eosionphils in the organism of the host. Because of this it would be a more valid criterion than peripheral eosinophilia for the eosinophilic reaction in toxocariasis, due to aneosinophilic forms of toxocariasis being fairly common (Shah et al., 2021). It is now known that ECP can be successfully used diagnostically to determine the severity of invasions with the helminths *Ancylostoma duodenale* and *Necator americanus,* in which the levels of ECP correlate with the intensity of the invasion (Sakyi et al., 2022).

In our study the median levels of ECP were statistically higher in both groups of patients with urticaria, with positive and negative result for antitoxocara antibodies, compared to the ECP levels in healthy individuals (H=9.867, df=2, p=0.007).

Similar results have been described before. Kaneva et al. found a significant difference in the levels of ECP between patients seropositive for antitoxocara IgG antibodies and in clinically healthy blood donors (Kaneva et al., 2020). A French study among patients with covert toxocariasis found a statistical difference in the mean levels of ECP between patients with allergies and those without allergic symptoms (Magnaval et al., 2020).

We found a statistical difference in the levels of ECP between patients with urticaria, carriers of antitoxocara IgG antibodies and patients with urticaria but whith no antitoxocara antibodies (H=6.841, df=1, p=0.009).

In literature there are various studies that have followed up the levels of ECP in patients with toxocariasis both before and after etiological treatment. Magnaval et al. described a case of an 11-year-old girl with asthma-like symptoms and a positive serological result for antitoxocara IgG antibodies with elevated levels of ECP of 28 mg/l. After a 21-day course with the antihelminthic drug diethylcarbamazine, they described a drop in the levels of ECP down to 8 mg/l (Magnaval, 2011). Niedworok et al. found a significant lowering of ECP

levels in patients with antitoxocara antibodies 6 months after therapy with Mebendazole (Niedworok, 2008). In our work, we could not follow up the levels of ECP due to the singular nature of study.

We found significantly higher levels of ECP in patients with AU that had antitoxocara antibodies, compared to AU patients with a negative serological result for antitoxocara antibodies (H=4.232, df=1, p=0.040). We did not find a similar relation in ECP levels between patients with CSU (H=3.213, df=1, p=0.073). Since eosinophil cationic protein is part of the secreted cytotoxic eosinophil proteins, we assume that the higher levels described in our study in AU patients reflect a fresh invasion with *Toxocara spp*. and coincide with a strong local eosinophil reaction. Similar results have been described before. A study in Korea found a positive correlation between ECP levels and the intensity of tissue eosinophilia in toxocariasis patients (Choi et al., 2003).

Similarly to our study on total IgE levels, we also found a statistically significant positive correlation between the levels of ECP and the levels of antitoxocara IgG antibodies in patients with acute urticaria ($\rho = 0.360$; $p = 0.024$). We did find any similar studies in literature, in order to compare them to our result.

We should, however, mention that changes in ECP levels can be found not just in helminthic diseases. Elevated ECP levels are also found in some atopic diseases like bronchial asthma and allergic rhinitis, in cases of eosinophilic neoplastic processes and in a few bacterial diseases such as tuberculosis (Bystrom et al., 2011; Lu, 2021). In this sense our results in patients with acute and chronic spontaneous urticaria have to be carefully interpreted in diagnostic practice.

CONCLUSION

In our study we found a significantly higher incidence rate of infection with *Blastocystis sp.* and *Toxocara spp.* among patients with acute and chronic spontaneous urticaria, in comparison to healthy individuals. This fact allows us

to recommend parasitological testing for intenstinal and tissue parasites in all cases of clinically presented allergies. The changes in total IgE and ECP levels im patients that were carriers of antitoxocara IgG antibodies, albeit carefully interpreted, can ba use to determine cases of covert toxocariasis among cases of clinical allergy. We consider that our study and conclusions will be of use for clinical practice and would help physicians in making decisions regarding diagnostic behavior in order to clarify the etiology of allergic disease and the necessity of paralel treatment with antialergic and antiparasitic drugs.

VI. CONCLUSIONS AND CONTRIBUTIONS

Conclusions:

1. In our study we found a *Blastocystis sp.* infection incidence rate of 9.6% in patients with acute and chronic spontaneous urticaria.
2. When a comparing roughly similarly sized groups of patients with clinical allergy and no allergic symptoms respectively, we found that the rate of infection with *Blastocystis sp.* is statistically higher in patients with urticaria.
3. Genotyping of *Blastocystis* isolated from patients with acute and chronic spontaneous urticaria and helathy carriers shows only two subtypes - ST1 и ST3, despite using primer sets for subtypes from ST1 to ST7.
4. We found a statistically higher carriership of antitoxocara IgG antibodies in patients with urticaria, compared to healthy individuals.
5. The mean levels of total serum IgE in patients with urticaria with antitoxocara IgG antibodies are statistically higher than the mean levels in the healthy control group.
6. We found a significant poisitive correlation between the levels of antitoxocara IgG antibodiesand the levels of total serum IgE. As antixocara antibodies increase, so do the levels of total serum IgE proportionally
7. ECP levels in patients with urticaria are statistically higher than in healthy individuals.
8. ECP is significantly higher in patients with urticaria and antitoxocara IgG antibodies, compared to patients with urticaria that don’t carry antitoxocara antibodies.
9. We found a statistically significant positive correlation between the levels of ECP and antitoxocara IgG antibodies in patients with acute urticaria.

Contributions:

Scientific and theoretical contributions of an original nature:

1. We tested and applied a molecular-genetic method for the determination of the subtype of *Blastocystis sp.*, isolated from patients with acute and chronic spontaneous urticaria.

2. With the help of immunological and statistical methods we determined the significance of the difference in ECP levels between patients with urticaria and antitoxocara IgG antibodies and patients with urticaria without antitoxocara antibodies. This allows ECP to be used as an indicator for the presence of a parasitic invasion.

3. The positive correlation between the levels of total serum IgE, ECP and antitoxocara IgG antibodies in patients with acute urticaria allows the cause of the allergic symptoms to be determined indirectly.

Scientific contributions of an applied nature:

1. As a result of this study the incidence of infection with *Blastocystis sp.* was determined in a significant contingent of hospitalized patients with acute and chronic spontaneous urticaria.

2. By analyzing the rates of infection with *Blastocystis sp.* in patients with clinical allergy, we determined that it is necessary to test each patient with acute and chronic spontaneous urticaria for the presence of intestinal parasites.

3. We studied the frequency of antitoxocara IgG antibody carriership, using the ELISA method, in a large contingent of patients with acute and chronic spontaneous urticaria.

4. Using statistical methods, we showed that there exists a significant difference in the frequency of antitoxocara IgG antibody carriership between patients with urticaria and healthy individuals. This requires that every patient with clinical allergy should be serologically tested for the presence of tissue parasites.

5. The statistical changes in the levels of total serum IgE and ECP that correlate with antitoxocara IgG antibody levels can be used in medical practice as additional criteria for the discovery of “covert” cases of toxocariasis among patients with urticaria.

VII. List of publications, reports and projects related to the dissertation thesis

Publications related to the topic of the dissertation thesis

1. Lalev M, Nankov V, **Stoyanov L**, Angelov I. Polymerase chain reaction-based subtype diversity of Blastocystis species isolates obtained from hospitalized patients with blastocystosis. Archives of the Balkan Medical Union. 2020;55(3):431-436. ISSN 2558-815X. (SJR - 0,128; Q4)

2. Kaneva E, **Stoyanov L**, Lalev M, Angelov I, Harizanov R. *Blastocystis sp.* and *Toxocara spp.* Coinfection in Patients with Clinically Manifested Skin Allergy. Acta Microbiologica Bulgarica. 2023;39(4):423-427. ISSN 2603 - 3755. (SJR - 0,136; Q4)

3. Stoyanov L, Angelov I. Carriership of anti-Toxocara IgG antibodies among patients with clinical allergy. *J of IMAB.* 2024;30(2):5490-5494. ISSN 1312 - 773X. (IF - 0,1; Q4)

Scientific reports at congresses and conferences, related to the topic of the dissertation thesis

1. Stoyanov L, Angelov I. Clinical allergy and its connection to the presence of antiToxocara IgG antibodies. XIth national scientific conference on HIV/AIDS and exotic infectious and parasitic disease. May 30th – July 2nd 2024, Cigov chark.

2. Stoyanov L, Dragomirova P, Angelov I. Levels of total IgE and anti-Toxocara IgG antibodies in patients with clinical allergy. XIth national scientific conference on HIV/AIDS and exotic infectious and parasitic disease. May 30th – July 2nd 2024, Cigov chark.

3. Stoyanov L, Nankov V, Lalev M, Angelov I. Genotyping of *Blastocystis sp.* isolates from patients with clinical allergy and healthy carriers. XIth national scientific conference on HIV/AIDS and exotic infectious and parasitic disease. May 30th – July 2nd 2024, Cigov chark.

4. Stoyanov L, Kaneva E, Angelov I. Eosinophil cationic protein as an additional marker for diagnosis of covert toxocariasis in patients with clinical allergy. Jubilee scientific conference with international participation "50 years of medical education and science in Pleven" 1st – 3rd November 2024, Pleven

Scientific projects related to the topic of the dissertation thesis

1. Project № D2/2023г „Changes in the levels of total IgE, eosinophil cationic protein and eosinophiles in patients with toxocariasis and clinical allergy", financed by Medical University – Pleven

ABSTRACT

Parasitic diseases often present with clinical allergy, due to the allergizing effect of the parasite's life cycle inside the host. We studied the role of *Blastocystis sp.* and *Toxocara spp.* as allergens due to how often they are isolated from patients with acute and chronic spontaneous urticaria.

We found an incidence rate for blastocystosis of 9.6% in 1197 patients with urticaria and a rate of 1.2% among 1300 healthy individuals with no prior history of allergies. The frequency of *Blastocystis sp.* infection in patients with acute urticaria was 8.4% and in patients with chronic spontaneous urticaria it was 10.4%. We found that age and sex were confounding factors, in terms of *Blastocystis sp.* infection. We found that the risk of blastocystosis was 8 times higher in patients with acute urticaria and 10 times higher in patients with chronic spontaneous urticaria compared the healthy individuals.

We studied the subtype diversity of *Blatsocystis* isolates among 45 patients with acute and chronic urticaria and 24 healthy parasite carriers. We isolated the subtypes 1 and 3, which correspond to results from previous studied done in Bulgaria and in Europe. We found that both subtypes were roughly evenly distributed among both urticaria patients and healthy carriers.

We determined that out of 297 patients with acute and chronic spontaneous urticaria, 26.3% were carriers of antitoxocara IgG antibodies, while only 5% of healthy non-allergic subjects were carriers. The risk of carriership of antitoxocara antibodies was 3.4 times higher in patients with acute urticaria and 3.2 times higher in patients with chronic spontaneous urticaria compared to healthy controls.

We found that mean total serum IgE levels were significantly higher patients with urticaria with or without antitoxocara IgG antibody carriership, compared to healthy individuals with no history of allergic reactions and with no

antitoxocara antibodies. We also found a positive correlation among urticaria patients, between the levels of total serum IgE and antitoxocara IgG antibodies.

We found statistically higher levels of eosinophil cationic protein in patients with urticaria, carriers of antitoxocara IgG antibodies, compared to patients with urticaria and no antitoxocara antibodies and healthy controls. We also found a correlation between the levels of ECP and antitoxocara IgG antibodies in patients with acute urticaria.

The results of our study are of practical use and will aid the disgnostic behaviour of physicians with allergic patients. It will also help evaluate the necessity of additional etiological antiparasitic treatment.