UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
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PhD THESIS

Abstract

THE ROLE OF THE INNATE IMMUNE GENE POLYMORPHISMS IN MYCOBACTERIUM TUBERCULOSIS INFECTION

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INTRODUCTION

Tuberculosis remains a major health problem, worldwide, being the second leading cause of death from an infectious disease, the first place belonging to the human immunodeficiency virus (HIV) (Johanneke et al., 2011).

Infection with Mycobacterium tuberculosis is present in one third of the world's population and is responsible for approximately 2 million deaths per year (Dye et al., 1999).

The study of this disease comes with a distinct value as Romania is the leading country in the EU in terms of number of TB cases. Control over the Mycobacterium tuberculosis infection by the human body is a process involving pathogen recognition and activation, of both the innate immune system and the adaptive one.

Identifying the genes, which contain the mutations responsible for the susceptibility to tuberculosis, has allowed the essential components of the immune system's defense against mycobacteria to be discovered.

In this context, our research aims to assess the association main polymorphisms located in genes encoding cytokines and susceptibility or resistance to pulmonary tuberculosis in Eastern Europe (Romania), a region where these genetic variants have not been investigated so far, and identification of new polymorphisms possibly associated with pulmonary tuberculosis.

Keywords: tuberculosis, polymorphism, genotype, cytokines, susceptibility.

I STATE OF KNOWLEDGE

CHAPTER 1. EPIDEMIOLOGY OF MYCOBACTERIUM TUBERCULOSIS INFECTION

Recent data on incidence and mortality rates for pulmonary tuberculosis in different regions of the world and then to the European continent are presented in this chapter. Romania ranks first among EU countries and 3rd in Europe.

CHAPTER 2. MYCOBACTERIUM TUBERCULOSIS

In the second chapter is presented classification and global distribution of strains of Mycobacterium tuberculosis. Also, information about latent TB diagnostic methods. In the same chapter there are presented dibates on multidrug-resistant tuberculosis and BCG vaccination.

CHAPTER 3. HOST GENETICS AND SUSCEPTIBILITY TO MYCOBACTERIUM TUBERCULOSIS INFECTIONS

In this chapter references are made to the main polymorphisms localized in cytokines and their receptors, polymorphisms that have been studied in other geographic regions to highlight their potential association with susceptibility to pulmonary tuberculosis.

Subjects with a particular genetic profile based on a combination of marker genes that encode cytokines / chemokines (eg, IL-1B, IL-10, IL-8, TNF-α) and components of the innate immune system (e.g., TLR2) may respond excessively to infection with Mycobacterium tuberculosis, which can cause increased susceptibility to tuberculosis.

II. PERSONAL CONTRIBUTIONS

CHAPTER 4. MOTIVATION AND OBJECTIVES

The aim of our study was to assess the main polymorphisms located in genes encoding cytokines, Toll-like receptors and correlation of these genetic variants with susceptibility to pulmonary tuberculosis in Eastern Europe / Romania, a region where these genetic variants have not been investigated.

Fulfilling the purpose was achieved through the following objectives;

- determining the frequency of major genes genotypes encoding cytokines,
- determining the frequency of genotypes for genes encoding Toll-like receptors,
- establish whether these cytokine gene polymorphisms are associated with susceptibility to pulmonary tuberculosis in this region.

To achieve the above objectives, the following steps were made:

- Establishment of study groups and establishing a database identifying the main parameters of interest: gender, age, ethnicity, area of origin , occupation, number of family / household , number of family members with tuberculosis , smoking status , history TB treatment, signs and symptoms , physical examination , severity of signs on chest radiographs, microscopic examination of sputum , sputum culture results , drug resistance test results , laboratory test results, comorbidity.

- Investigate proposed genetic polymorphisms and identification of polymorphic variants of genes encoding cytokines, TLR family receptors (Toll-like receptors) - molecular pattern recognition receptors (non- self) specific infectious agents (PAMPs - pathogen - Associated Molecular Patterns) both in the group of patients with TB and the control group.
- Statistical analysis.

CHAPTER 5. MATERIAL AND METHOD

5.1. Setting lots and inclusion of patients in the study

This study included 388 patients diagnosed with pulmonary tuberculosis based on TB history, clinical examination and radiological examination. All cases were confirmed by microscopic examination of sputum and obtaining sputum cultures of Mycobacterium tuberculosis. Patients included in this study were diagnosed at the Clinical Hospital of Infectious Diseases and Pneumology "Victor Babes" of Craiova - Dolj Pneumology Hospital Leamna - Dolj and Pneumology Hospital “Tudor Vladimirescu" - Runcu, Gorj, from March 2011 - March 2013. Comparison was made with a control group selected mainly in hospital units above and in the Emergency Hospital Craiova. Both groups were based on the following criteria. Both TB patients and control subjects signed an informed consent for inclusion in the study, and a detailed questionnaire to obtain important demographic data.

I. The group of patients with tuberculosis

a. criteria for inclusion in the study:
   - patients diagnosed with active tuberculosis or TB history;
   - residing in Oltenia region;
b. exclusion criteria from the study:
   • fever greater than 38.5 ° C ;
   • significant weight loss;
   • productive cough and night sweats for more than 2 weeks .

II. The control group

a. criteria for inclusion:
   - healthy patients with no active pulmonary tuberculosis or TB history;
   - socio -economic environment identical to that of patients in the study group;
   - suitable in terms of age and male / female ratio with pulmonary tuberculosis group;
b. exclusion criteria from the study:
   • patients with a history of tuberculosis, regardless of location;
   • TB patients who have had contact;
   • patients with suspected pulmonary tuberculosis in the radiographic image .

Sampling and biological material

Regarding the group of patients with pulmonary tuberculosis, the biological material was whole blood (about 2.5 to 5 ml of venous blood) collected on EDTA and kept at 4 ° C until DNA isolation was performed. For the subjects in the

control group, the biological material, whole blood was collected on EDTA. Samples were coded with letters and numbers in the collecting order.

5.2. Identification of genetic polymorphisms

The identification of polymorphisms was performed at the Laboratory of Biological Cellular and Molecular, University of Medicine and Pharmacy Craiova (genes IL-1B, IL-1RN, IL-4R, IL-10, TNF-A and TLR2).

Detection of genetic polymorphisms followed the next steps:

- isolation of genomic DNA from blood,
- spectophotometric assessment,
- identification by Real Time PCR technique with TaqMan probes,
- visualization and interpretation of results.

5.3. Statistical analysis:

- Hardy-Weinberg equilibrium (HWE)– X^2 test,
- Effects of cytokine alleles on the risk of diseases - OR with 95% CI.

CHAPTER 6. RESULTS

6.1. Characteristics of the studied groups

In this study we included a total of 750 patients: 388 patients diagnosed with pulmonary tuberculosis and 362 healthy control subjects. The characteristics of the two batches are summarized in Table 1.

Were selected patients diagnosed with active pulmonary tuberculosis or TB history. The diagnosis was based on positive sputum examination, positive culture for Mycobacterium tuberculosis, as well as on x-ray changes.

The control group was selected both within hospital units involved in enrolling patients and in the Emergency Hospital Craiova.

Table 1. Subjects characteristics

	Pulmonary tuberculosis N=388	**Control N=362**
Male/Female	306/82	240/122
Age (years), mean ±SD	48.42 (14.62)	57.77 (14.78)
Provenance		
- **urban**	103	46
- **rural**	285	52
- **unknown**		264

Tabel 1 continued	Pulmonary tuberculosis N=388	Control N=362
Occupation		
- **student**	13	1
- **employee**	47	10
- **social support**	126	12
- **unemployed**	9	1
- **pensioner**	185	74
- **unknown**	8	264
TB history		
- **ever**	174	
- **last year**	45	
- **never**	118	
- **don't know**	51	
Smoking		
- **current**	182	28
- **ever**	45	21
- **never**	154	46
- **nd**	7	267
BCG vaccination		
- **yes**	209	33
- **no**	4	3
- **nd**	175	326
Diagnosis criteria		
- **positive microscopy/culture**	230	
- **x-ray**	208	

Regarding the relationship between genders, there was a value nearly four times higher in men than women (figure 1).

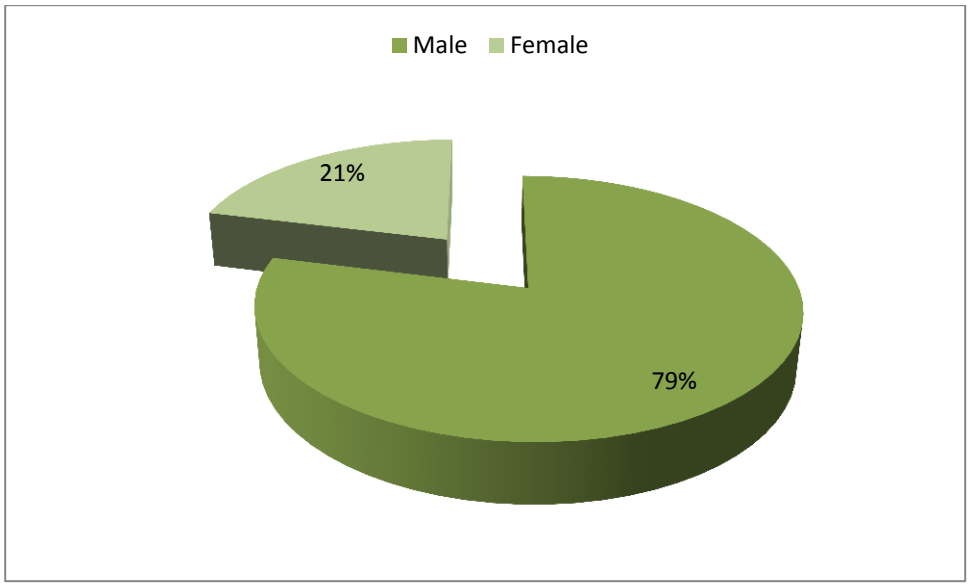

Figure 1. Gender distribution of TB patients

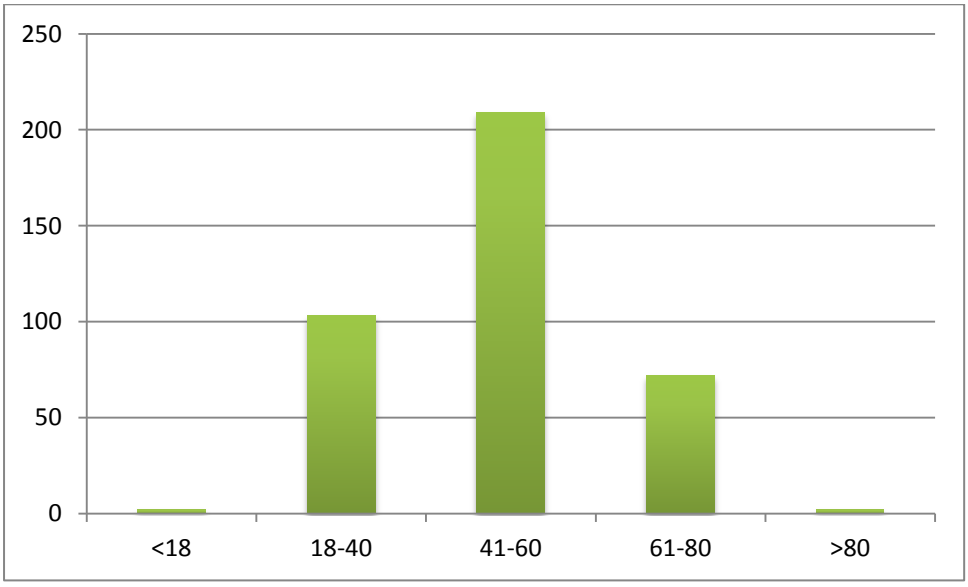

Figure 2. Age distribution of TB patients

Age distribution of TB patients shows the maximum value for patients aged between 41 and 60 years (figure 2).

The report on provenance shows a value almost three times higher in rural areas than urban areas (figure 3).

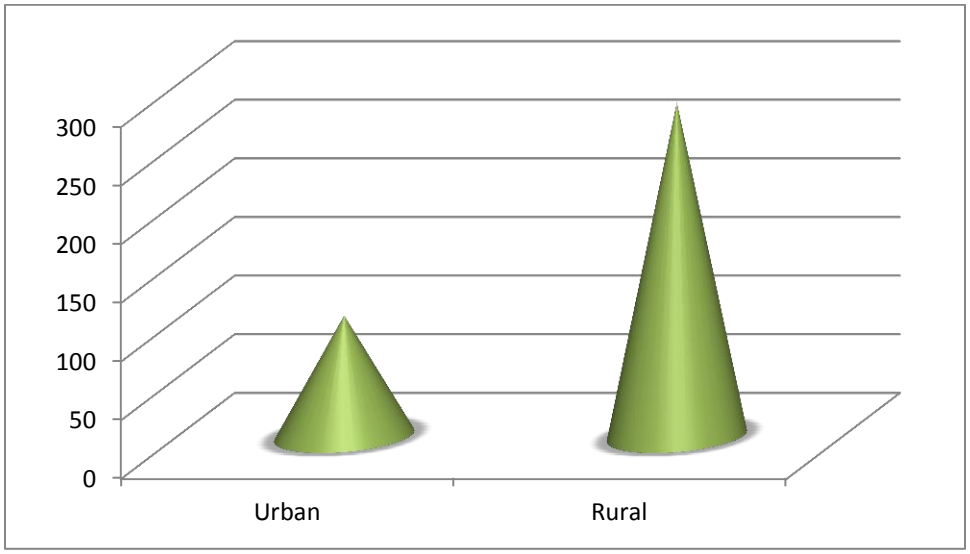

Figure 3. Provenance distribution of TB patients

6.2. Hardy-Weinberg equilibrium relationship (HWE)

Regarding the investigated polymorphisms in the control group, there were no deviations from Hardy-Weinberg equilibrium (p> 0.05, x2 <3.84), except for TLR2 polymorphism 1892C> A in which there was a significant deviation from Hardy-Weinberg equilibrium (p <0.05, x2> 3,84).

The following cytokine polymorphisms were genotyped by allelic discrimination TaqMan PCR assay (5' nuclease assay) using predesigned *TaqMan SNP Genotyping Assays:*

- IL-1B -31T>C (rs1143627),

- IL-1B -511C>T (rs16944),

- IL-1B +3954C>T (rs1143634),

- IL1-RN +2018T>C (rs419598),

- IL-8 -251T>A (rs4073),

- TNF-A -308G>A (rs1800629),

- IL-4R -3223C>T (rs2057768),

- IL-10 -1082A>G (rs1800896),

- TLR2 1892C>A (rs5743704).

The genotyping assays were performed in the Molecular and Cellular Biology Department, University of Medicine and Pharmacy from Craiova.

According to fluorescent emissions of the TaqMan probe, we identified Allele 1–FAM and Allele 2 -VIC, and consecutively the genotypes (figure 4).

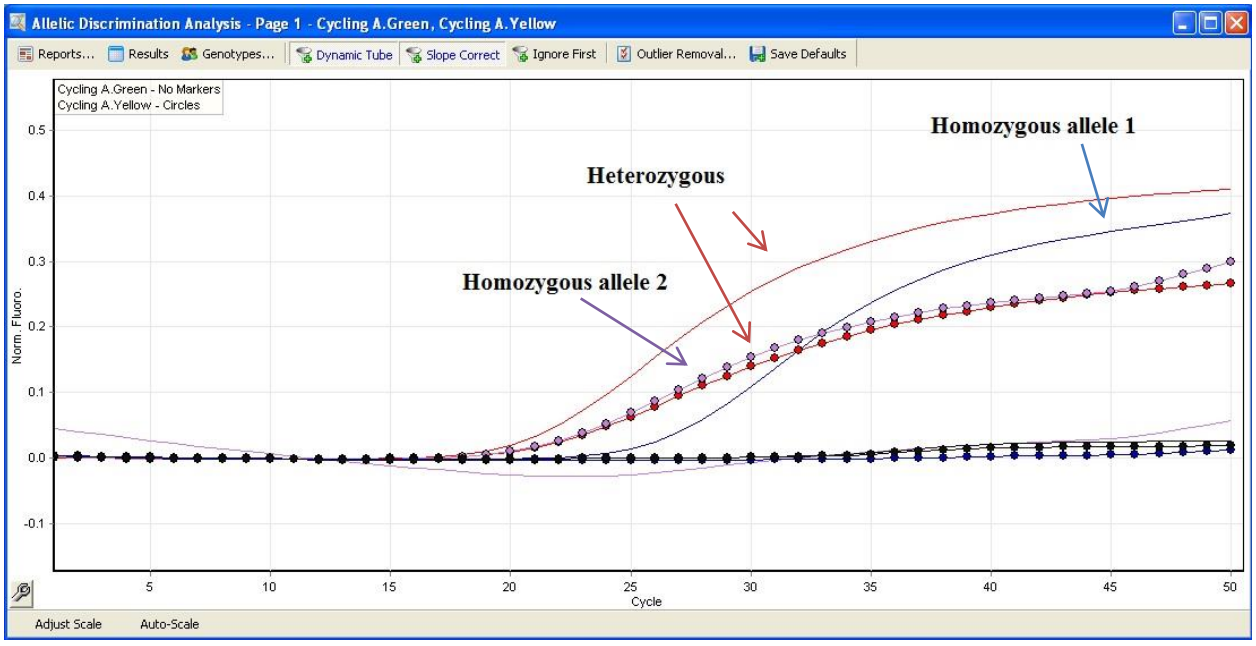

Figure 4. The genotype discrimination according to TaqMan probe

6.3. Pro-inflammatory cytokine gene polymorphisms

The genotype frequencies for all tested pro-inflammatory cytokine polymorphisms in pulmonary tuberculosis and control groups are showed in Table 2.

Table 2. Pro-inflammatory cytokine polymorphisms and risk of pulmonary tuberculosis

Polymorphism	Pulmonary tuberculosis n=321	Control n=331	OR (95%CI)	p
IL-1B -31T>C				
TT	148 (46.10%)	156 (47.12%)	Ref	
TC	135 (42.05%)	140 (42.29%)	1.02(0.73-1.41)	0.92
CC	38 (11.83%)	35 (10.57%)	1.14(0.69-1.91)	0.6
Carriers for allele C	173 (53.89%)	175 (52.87%)	1.04 (0.76-1.42)	0.8
IL-1B -511C>T				
CC	142 (38.06%)	153 (43.83%)	Ref	
TC	153 (41.01%)	145 (41.54%)	1.13(0.82-1.57)	0.43
TT	78 (20.91%)	49 (14.04%)	**1.71(1.12-2.62)**	**0.012**
Carriers for allele T	231 (61.93%)	194 (55.90%)	1.28(0.95-1.73)	0.10
IL-1B +3954 C>T				
CC	114 (62.29%)	156 (52%)	Ref	
TC	56 (30.60%)	121 (40.33%)	**0.63 (0.42-0.94)**	**0.023**
TT	13 (7.10)	23 (7.66%)	0.77 (0.38-1.6)	0.48
Carriers for allele T	69 (37.70%)	144 (48%)	**0.65 (0.45-0.95)**	**0.027**

Tabel 2 continued

Polymorphism	Pulmonary tuberculosis n=321	Control n=331	OR (95%CI)	p
IL1-RN +2018T>C				
TT	144 (51.79%)	185 (58.17%)	Ref	
TC	76 (27.33%)	109 (34.27%)	0.89(0.62-1.29)	0.55
CC	58 (20.86%)	24 (7.54%)	**3.1 (1.84-5.24)**	**0.00001**
Carriers for allele C	134 (48.20%)	133 (41.82%)	1.29(0.93-1.79)	0.11
IL-8 -251T>A				
TT	47 (31.12%)	93 (34.70%)	Ref	
TA	74 (49.00%)	121 (45.14%)	1.21 (0.77-1.91)	0.41
AA	30 (19.86%)	54 (20.14%)	1.1 (0.62-1.94)	0.74
Carriers for allele A	104 (68.87%)	175 (65.29%)	1.17(0.77-1.80)	0.45
TNF-A -308G>A				
GG	295 (76.03%)	289 (79.83%)	Ref	
AG	87 (22.42%)	71 (19.61%)	1.20 (0.84-1.71)	0.31
AA	6 (1.54%)	2 (0.55%)	2.94(0.59-14.68)	0.17
Carriers for allele A	93 (23.96%)	73 (20.16)	1.24(0.88-1.76)	0.21

IL- 1B- 511C > T polymorphism is associated with an increased risk of pulmonary tuberculosis, the result being obtained when compared one genotype to the other (the largest served as reference -CC). The TT genotype carriers have an increased risk of pulmonary tuberculosis, about 1.71 times higher than those with the CC genotype.

Statistical analysis shows that IL -1B - 3954 C > T polymorphism affects the risk of pulmonary tuberculosis, carriers of the T allele conferring protection (OR 0.65, 95 % CI: 0.45-0.95). This result was confirmed by comparing one genotype to the other (OR 0.63, 95 % CI: 0.42-0.94, the largest served as reference -CC).

IL1 -RN 2018 T > C polymorphism is associated with an increased risk of pulmonary tuberculosis, the result was obtained when one genotype was compared to the other genotype (the largest served as reference -TT). The CC genotype carriers had an increased risk of pulmonary tuberculosis, about 3.1 times higher than those with the TT genotype.

For IL- 1B- 31T > C, IL -8- 251T > A, TNF -A- 308G > A polymorphisms we found no significant differences between pulmonary tuberculosis cases and controls.

6.4. Anti-inflamatory cytokine gene polymorphisms: IL-4R and IL-10.

The genotypes frequency for IL- 4R - 3223C > T and IL -10- 1082 > G polymorphisms is shown in Table 3.

Tabel 3. Anti-inflammatory cytokine polymorphisms and risk of pulmonary tuberculosis

Polymorphism	Pulmonary tuberculosis n=351	Control n=327	OR (95%CI)	p
IL-4R -3223C>T				
CC	168 (47.86%)	186 (56.36%)	Ref	
CT	150 (42.73%)	118 (35.75%)	**1.41 (1.023-1.94)**	**0.035**
TT	33 (9.40%)	23 (6.96)	1.59 (0.89-2.81)	0.11
Carriers for allele T	183 (52.13)	141 (43.11)	**1.43(1.06-1.94)**	**0.02**
IL-10 -1082A>G				
AA	113 (33.53%)	136 (40.23%)	Ref	
AG	165 (48.96%)	156 (46.15%)	1.27(0.91-1.77)	0.15
GG	59 (17.50%)	44 (13.01%)	**1.61(1.01-2.56)**	**0.042**
Carriers for allele G	224 (66.46)	200 (59.52)	1.34(0.98-1.84)	0.06

The result of statistical analysis showed that IL-4R-3223C>T polymorphism is associated with an increased risk of pulmonary tuberculosis. The CT genotype carriers have an increased risk of pulmonary tuberculosis about 1.41 times higher than those with genotype CC, also T allele carriers have an increased risk of pulmonary tuberculosis.

IL-10-1082> G polymorphism is associated with an increased risk of pulmonary tuberculosis, carriers of GG genotype having an increased risk of pulmonary tuberculosis about 1.61 times higher than those with genotype AA.

6.5. TLR2 1892C>A (rs5743704) polymorphism

The genotypes frequency for TLR2 1892C>A polymorphism is shown in table 4.

Tabel 4. TLR2 1892C>A polymorphism and risk of pulmonary tuberculosis

TLR2 1892C>A	**Pulmonary tuberculosis n=379**	**Control n=251**	**OR (95%CI)**	**P**
CC	335 (88.39%)	234 (93.22%)	Ref	
AC	43 (11.34%)	16 (6.37%)	2.68(0.15-45.57)	0.5
AA	1 (0.26%)	1 (0.39%)	1.43(0.09-23.00)	0.8
Carriers for allele A	44 (11.60%)	17 (6.77%)	1.51(0.09-24.28)	0.77

The result of statistical analysis showed that TLR2 polymorphism 1892C> A is associated with an increased risk of pulmonary tuberculosis when a genotype was compared to the other (the largest served as reference-CC) or when comparing allele carriers (OR 1.51, 95% CI 0.09-24.28, p =0 .77).

CHAPTER 7. DISCUSSION

This chapter analyses the results presented in Chapter 6, comparing our data with existing data in the literature for each group of polymorphisms: polymorphisms of pro-inflammatory cytokines , anti -inflammatory cytokine polymorphisms and TLR2 polymorphism 1892C > A.

For the Romanian population we evaluated for the first time, a possible association between pulmonary tuberculosis and polymorphisms of these genes.

The recurrence of active tuberculosis despite treatment may occur many years after primary infection with the same strain, or a reinfection with a new strain of Mycobacterium tuberculosis (van Rie et al., 1999).

Factors that predispose people to a new re-infection are still unclear. Factors related to the host, especially genetic factors, will influence susceptibility and severity of the infection and the patient's recovery. Polymorphisms of the cytokines may influence the effectiveness of the immune response to infection with such an important role in the response of the host organism.

TNF gene promoter region is highly polymorphic. Several allelic polymorphisms have been Identified at positions - 863, -857, -308, -238 and -1031 (Merza et al., 2009, Sharma et al., 2010), which are considered potential susceptibility factors for TB.

Until now, there were a relatively large number of published studies, but the results are inconsistent and limited in statistical terms. Most studies have focused on TNF polymorphism - 308G > A.

Our results show that there is no association between TNF's polymorphism -308G > A and the increased risk of pulmonary tuberculosis, results also confirmed by the research of: Delgado et al. , 2002 JH Oh et al. , 2007; Amirzargar et al. , 2006; Henao et al. , 2006 Vejbaesya et al. , 2007).

There are many studies which have examined the association of polymorphisms located in the promoter region of the IL - 10 with the severity and susceptibility to tuberculosis, but there is a disparity in those results from one study to another. In some studies, the allele of IL - 10 -1082 A polymorphism and IL -10 -1082 heterozygosis have been associated with susceptibility to TB (Delgado et al., 2002 , Oh et al . , 2007) , while in other studies who targeted a large number of subjects, polymorphism IL - 10 -1082 A> G was not associated with TB (Bellamy et al. , 1998; Lopez - Maderuelo et al. , 2003, Tso et al. , 2005; Shin et al . , 2005). Another study showed that allele IL - 10 -1082 G was found significantly more often in patients with TB than in healthy subjects group (Oral HB , et al. , 2006).

A study by Ioana M. et al., in 2012, showed that TLR2 polymorphisms are associated with tuberculosis or that exerts specific effects on susceptibility to various mycobacterial strains such as Mycobacterium tuberculosis Beijing type (Kleinnijenhuis et al., 2011). Beijing type strains have a clear geographical distribution (Kleinnijenhuis et al., 2011; Parwati et al., 2010) which makes it possible the hypothesis that human receptors TLR2 co- evolved in different populations, depending on the pressure of various infections in a given region.

To further support this hypothesis, Ioana M. et al., (2012) evaluated the distribution of population and functional consequences of three of the most common polymorphisms in the TLR: 1892C > A, rs5743704, 2029C > T, rs121917864 and 2258G > A. These SNPs of TLR2 have been reported to influence the susceptibility to various infections, but little is known regarding their

geographical distribution, and this may explain, at least in part, differences in susceptibility to infections in different populations.

In this study were evaluated a number of 941 subjects grouped geographically and seven ethnic populations : Romanian - Romania , Vlax - Rome - Romania , Netherlands - Netherlands European population , Han - Chinese (China - Asia) , Dogon (Mali - Africa) , Fulani (Mali - Africa) , and Trio - Indians (Suriname - South America).

For these three groups was performed an analysis, genotyping TLR2 polymorphisms. Two of the three polymorphisms , namely 1892C > A and 2258G > A were evaluated in terms of their impact in stimulating the secretion of pro-inflammatory cytokines IL- 1β , IL -6, TNF , IFN- γ and IL -17 peripheral mononuclear blood cells.

Regarding the geographical distribution of the three polymorphisms, allelic variant 1892 were identified only in the European population. 2029T polymorphism was absent in both the European population and the non -European except Vlax group - Roma. 2258 allelic variant was present only in the European population and a very low frequency in Vlax group - Roma. No differences were observed in the ability of peripheral blood mononuclear cells to produce pro-inflammatory cytokines IL - 1β, IL -6, TNF a, IFN - γ and IL -17 under the action of the various genotypes of TLR2.

A possible explanation for this apparent discrepancy between the studies could be that, specific ethnic genetic variations may significantly influence host immunity to tuberculosis, causing tuberculosis susceptibility in different ethnic groups studied. Another possible explanation could be the number of subjects in the study population.

CONCLUSIONS

- Our study is the first to evaluate the main polymorphisms located in genes encoding cytokines and risk of pulmonary tuberculosis in Eastern Europe (Romania).

- IL- 1B- 511C > T polymorphism is associated with an increased risk of pulmonary tuberculosis, a result obtained when compared one genotype to the other (the largest served as reference -CC). The TT genotype carriers have an increased risk of pulmonary tuberculosis about 1.71 times higher than those with the CC genotype.

- Polymorphism of IL -1B +3954 C>T affects the risk of pulmonary tuberculosis, carriers of the T allele conferred protection (OR 0.65, 95 % CI: 0.45-0.95). This result was confirmed by a comparison to the other genotypes (OR 0.63, 95 % CI: 0.42-0.94, served as the largest reference -CC).

- IL1 -RN 2018 T > C polymorphism is associated with an increased risk of pulmonary tuberculosis; a result was obtained when one genotype was compared to the other (the largest served as reference -TT). The CC genotype carriers had an increased risk of pulmonary tuberculosis about 3.1 times higher than those with the TT genotype.

- The result of statistical analysis showed that IL- 4R -3223C > T is associated with an increased risk of pulmonary tuberculosis when a genotype was compared to the other (the largest served as reference -CC) or when

comparing risk allele carriers of T. The CT genotype carriers have an increased risk of pulmonary tuberculosis about 1.41 times higher than those with genotype CC, also T allele carriers had an increased risk of pulmonary tuberculosis.

- The polymorphism of IL -10- 1082A>G is associated with an increased risk of pulmonary tuberculosis; a result was obtained when compared to the other genotype (the largest served as reference -AA). The GG genotype carriers had an increased risk of pulmonary tuberculosis about 1.61 times higher than those with genotype AA.

- To polymorphisms IL-1B -31T > C, IL-8- 251T > A, TNF –A -308G > A, TLR2 1892C>A was not observed any association with pulmonary tuberculosis in the population of Romania. Lack of association of these polymorphisms with risk of pulmonary tuberculosis could be a reflection of genetic heterogeneity in the pathogenesis of pulmonary tuberculosis.

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