

# Genetic Profiling to Assess Susceptibility to Tuberculosis Disease in a Pilot Study with Bulgarian Patients

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## Abstract

The research project aims to perform genetic profiling in Bulgarian patients with latent or active tuberculosis (TB) and assess the influence of genetic polymorphisms on disease susceptibility and severity. The gene NRAMP1 (Natural resistance-associated macrophage protein 1), also known as SLC11A1 (solute carrier family 11 proton-coupled divalent metal ion transporter membrane 1), encodes a divalent ion channel (Fe<sup>2+</sup> and Mn<sup>2+</sup>) in the lysosomal membrane. Mutations in this gene have been associated with susceptibility to infectious diseases, including tuberculosis. The genetic profiling conducted in the current research includes the isolation of high-molecular-weight genomic DNA from venous blood, amplification of target genetic markers by polymerase chain reaction (PCR), followed by restriction fragment length polymorphism (RFLP) analysis, and genotype interpretation. Statistical analysis of the data was performed using SPSS v.20, along with case-control and family-based association studies utilizing Plink version 1.07. The study includes 45 samples from 10 families: 27 children and 16 parents. Among them, 13 were healthy, while 32 had active tuberculosis or relapsed. An additional 50 healthy controls were included. The main forms of pulmonary involvement observed were tuberculosis of intrathoracic lymph nodes (TITLV), primary tuberculosis complex (PTK), infiltrative-pneumonic form (IPF), tuberculous meningitis (TM), and latent tuberculosis infection. Of the investigated NRAMP1 polymorphisms—INT4 (rs3731865), D543N (rs17235409), and 3'UTR (rs17235416)—D543N and 3'UTR were found to be normal. For INT4, 17 patients (39.5%) exhibited a normal genotype, 19 (44.3%) were heterozygous (INT4 GC), and

7 (16.2%) were homozygous (INT4 CC). No statistical significance was found for the predisposing effect of NRAMP1 INT4 polymorphism on the development of tuberculosis ( $p = 0.335$ ). However, the greater frequency of the INT4 GC heterozygous genotype in the patient group ( $GF = 0.4$ ) compared to the healthy control group ( $GF = 0.3$ ) indicated a probable predisposing effect.

## Keywords

Tuberculosis, Polymorphisms, NRAMP1

## 1. Introduction

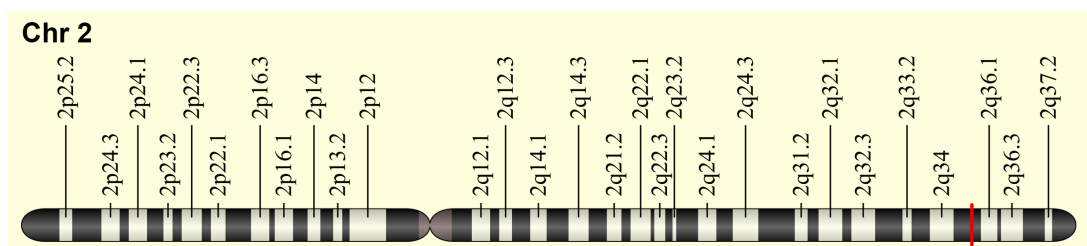
Tuberculosis (TB) is an infectious disease, yet one-third of the human population remains asymptomatic when infected. Only 5% - 10% of individuals with latent tuberculosis infection develop an active pulmonary or extrapulmonary form of the disease.

The aim of this study was to identify genetic polymorphisms associated with predisposition or protection against the development of tuberculosis in a cohort of Bulgarian patients. The involved genes encode molecules responsible for the immune response through which the human body interacts with Mycobacterial species [1].

In the current study, we focused on NRAMP1 (Natural resistance-associated macrophage protein 1), also known as SLC11A1 (solute carrier family 11 proton-coupled divalent metal ion transporter membrane 1) [2]. This gene is located on the long arm of the second chromosome (2q35) (Figure 1) and encodes a divalent ion channel ( $Fe^{2+}$  and  $Mn^{2+}$ ) in the lysosomal membrane [3].

*INT4*, *D543N* and *3' UTR* polymorphisms of NRAMP1 were chosen for this study because *NRAMP1* plays a role in the formation of the immune response to pulmonary tuberculosis. Mutations in the *NRAMP1* gene affects the phagosomal function of alveolar macrophages. The polymorphisms were chosen because there are scientific papers on various populations indicating their significance in predisposition to and protection from tuberculosis.

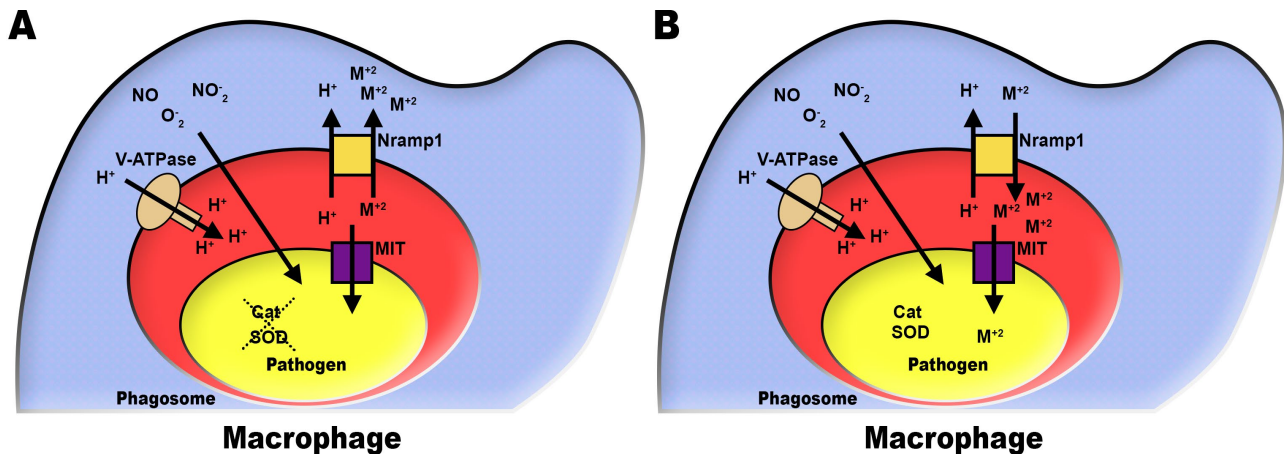
NRAMP1 plays a critical role in the formation of the immune response to pulmonary tuberculosis. It is associated with several infectious diseases caused by pathogens such as *Leishmania*, *Salmonella*, and *Mycobacteria*. NRAMP1 exhibits a pleiotropic effect on macrophage activation, including increased expression of HLA class II molecules, enhanced production of reactive oxygen species (ROS),



**Figure 1.** Genomic localization of the NRAMP1 (SLC11A1) gene. Source: <https://www.genecards.org/cgi-bin/carddisp.pl?gene=SLC11A1>.

and elevated levels of cytokines like IL1- $\beta$  and TNF $\alpha$ . These effects contribute to the intracellular killing of pathogens [4].

Early in the infection, two NRAMP genes play significant roles in killing *Mycobacteria* [6]. NRAMP1, localized in endosomal vesicles that fuse with phagosomes, facilitates the transport of metal ions and reduces the pH of the phagosome to levels toxic to bacteria (Figure 2). NRAMP2, on the other hand, functions as an efflux pump, exporting iron and other cations from the phagosome to the plasma membrane.



Legend: MIT—transports metal ions; M<sup>2+</sup>—divalent metal ion [5].

**Figure 2.** Hypotheses about the function of NRAMP1 in the interaction of macrophages with pathogens: (A) NRAMP1 functions as a symporter of metal ions and protons, transporting them from the phagosomal lumen to the cytoplasm. This extraction of metal ions limits the pathogen's ability to produce protective enzymes, such as catalase and superoxide dismutase, thereby preventing the reproduction of engulfed microorganisms; (B) NRAMP1 acts as an antiporter of protons and metal ions, transporting metal ions from the cytoplasm to the phagosome. The accumulated metal ions generate free radicals that contribute to the destruction of pathogens.

Mutations in the NRAMP1 and NRAMP2 genes impact the phagosomal function of alveolar macrophages [7]. In NRAMP1-deficient patients, the phagolysosome pH remains close to 6.8, which supports bacterial growth. Furthermore, defective NRAMP2 production results in iron accumulation in the phagosome, which is utilized for synthesizing bacterial heme-containing cytochromes during replication [8].

The aim of the current research project is to perform genetic profiling in patients with latent or active tuberculosis (TB) and to assess the influence of genetic polymorphisms on the propensity to develop the disease and its severity.

## 2. Materials and Methods

### 2.1. Selection of Genetic Markers Determining the Immune Response and Associated with Susceptibility to Tuberculosis, Based on International Studies

In recent years, scientific interest has focused on the genetic study of predisposition as a prognostic factor for the course of tuberculosis. Numerous genetic polymorphisms have been examined in relation to their association with the infectious

agent *Mycobacterium tuberculosis*.

Research has primarily concentrated on the following genes, which are involved in the immune response to *Mycobacterium* infection: CCL2 (chemokine ligand 2), NRAMP1 (natural resistance-associated macrophage protein 1), IRGM (immunity-related GTPase family), IL8 (interleukin 8), TLR (toll-like receptor), NOD2 (nucleotide-binding oligomerization domain-like receptor family), TYK2 (tyrosine kinase 2), and SP110 (nuclear body protein), among others.

Based on an extensive literature review [9]-[29], we aimed to analyze the protective and predisposing potential of polymorphisms in the NRAMP1 gene, as described above, in patients from the Bulgarian population.

## 2.2. Selection of a Group of Patients with Latent and Active Tuberculosis

We collected 45 samples from 10 families. Twenty-seven of these were from children, and 16 were from parents. Among all participants, 13 individuals (both parents and children) were healthy, while 32 were either sick or had relapsed. Pediatric patients ranged in age from 1 to 16 years, with a mean age of  $9 \pm 5$  years and a male-to-female gender distribution of 3:7.

Our sample size is 45 individuals. For the case-control study we chose 10 children that are not relatives. For controls we chose 50 healthy children with the same mean age and sex. Detection of significant associations is observed by determining odd ratio and p values.

The disease was confirmed through clinical, radiological, and immunological tests conducted at the Clinic of Pediatric Pneumology and Phthisiology, University Hospital "St. Ivan Rilski," Sofia.

### Inclusion criteria

Families with children who were treated in our clinic in 2023, to whom the essence and benefits of the study were explained, and who gave their consent to be included. The families have different numbers of children. Some have an active form of tuberculosis, others with latent tuberculosis infection. Some of the parents are healthy, others have been ill or are currently being treated for tuberculosis.

All included with active tuberculosis, or treated for such, have had a clinical picture of tuberculosis: intoxication and consummative syndrome, auscultatory changes specific to the different forms of the disease, X-ray data, and positive immunological tests specific to the disease: Mantoux test, T-SPOT TB test, QuantiFERON-TBC Gold Plus.

The treatment is carried out according to the standards for the treatment of tuberculosis introduced by the World Health Organization. It is carried out in two phases: intensive and long-term. Those who have completed treatment are monitored for the next two years.

### Exclusion criteria

Families with a child or parent with active tuberculosis or a history of tuberculosis refused to be included in the study.

The main forms of pulmonary involvement included tuberculosis of intrathoracic lymph nodes (TBITLV), primary tuberculosis complex (PTK), infiltrative-pneumonic form (IPF), one case of tuberculous meningitis (TM), and several cases of latent tuberculosis infection.

All participants provided informed consent for genetic testing.

### 2.3. Isolation of DNA from Venous Blood

A total of 10 ml of venous blood was collected into a 50 ml sterile conical plastic tube and mixed with 30 ml of cold lysis buffer, followed by incubation for 30 minutes on ice with occasional shaking. The mixture was then centrifuged at 3000 rpm for 15 minutes. The supernatant was carefully poured off and discarded, and the tubes were allowed to drain on filter paper.

The pellet was resuspended in 10 ml of cold lysis buffer and centrifuged again under the same conditions. After removing the supernatant, the pellet containing nucleated cells was resuspended in 5 ml of SE buffer. Next, 25 ml of proteinase K (10 mg/ml) and 250 ml of 10% sodium dodecyl sulfate (SDS) were added, followed by mixing and incubation at 37°C overnight or at 55°C for 5 hours.

After proteolysis, 1.4 ml of pre-saturated (6 M) sodium chloride (NaCl) was added, and the mixture was vortexed. Centrifugation was performed at 3000 rpm for 15 minutes at room temperature to prevent SDS precipitation. The DNA-containing supernatant was carefully decanted into a new 50 ml sterile conical plastic tube, and two volumes of ice-cold absolute ethanol were added. Under these conditions, the DNA precipitated visibly, floated to the surface, and was scraped off using a sterile (burnt) cloth. The DNA was washed in 70% ethanol, air-dried, and dissolved in sterile TE buffer.

### 2.4. Evaluation of the Quality of the Isolated DNA

The concentration and purity of the isolated genomic DNA were measured spectrophotometrically. This method relies on the specific absorption of light at a wavelength of  $\lambda = 260$  nm by purine and pyrimidine bases in DNA. The ratio of absorbances measured at  $\lambda 260$  and  $\lambda 280$  indicates the purity of the DNA and should be between 1.8 and 2.0. Values below 1.8 indicate the presence of protein impurities, while values above 2.0 suggest RNA contamination.

The selected polymorphisms in the NRAMP1 gene for this study were INT4 (rs3731865), D543N (rs17235409), and 3'UTR (rs17235416).

The primers necessary for amplification of the indicated polymorphisms, were selected according to article [25] and are presented in **Table 1**.

After a series of optimizations, a protocol was developed for the optimal components of the amplification reaction (**Table 2**). The optimal conditions for amplification are presented in **Table 3**.

### 2.5. Analysis of Restriction Fragment Length Polymorphisms (RFLP)

The analysis is based on cutting DNA with specific restriction enzymes. Individual

**Table 1.** Primers used in the analysis of polymorphisms in the NRAMP1 gene.

<i>NRAMP1</i> gene	
<b>3'UTR</b> (1729 + 55del4) (rs17235416)	F: 5'-GCATCTCCCCAATTCATGGT-3' R: 5'-GGAAGTGTCCCACTCTATCCTG-3'
<b>INT4</b> (469 +14G/C) (rs3731865)	F: 5'-CTCTCTGGCTGAAGGCCTC-3' R: 5'-GTGCTATCAGTTTGAGCCTCAG-3'
<b>D543N</b> (codon 543, Asp to Asn) (1627G/A) (rs17235409)	F: 5'-GCATCTCCCCAATTCATGGT-3' R: 5'-GGAAGTGTCCCACTCTATCCTG-3'

**Table 2.** Concentration of reagents used in the present PCR reaction.

Reagent	Quantity
10X PCR buffer containing 200 mM Tris HCl, 500 mM KCl	1×
A solution of deoxyribonucleotide triphosphates (dNTPs) with a concentration of 10 mM, each dNTP with a conc. 2.5 mM	0.2 mM for each dNTP
Forward primer	10 μM
Reverse primer	10 μM
Taq DNA polymerase 5 U/μl	1 U
Genomic DNA (~50 ng)	100 - 300 ng
Deionized water	Up to 20 μl

**Table 3.** PCR program.

94°C	5 min	
95°C	30 sec	
65°C	30 sec	35 cycles
72°C	30 sec	
72°C	10 min	
12°C	∞	

fragments typically differ due to point mutations that either create or disrupt restriction sites or through deletions/insertions, which can alter the length of the restriction fragments. The restriction enzymes used and the RFLP reactions are presented in **Table 4**.

Following incubation at 37°C overnight (o/n) and visualization on a 3% agarose gel stained with ethidium bromide, the samples were analyzed using a molecular marker. The length of the amplified fragments was determined relative to the Quick-Load® Purple 1 kb Plus DNA Ladder (NEB, USA).

**Table 4.** Reagents used in RFLP analysis.

Restriction enzymes	Apa I (for INT4)	Fok I (for 3'UTR)	Ava II (for D543N)
PCR product	15 µl	10 µl	15 µl
rCutSmart™ Buffer (10×) (NEB)	1×	1×	1×
Enzyme (NEB)	5 U	5 U	5 U
Deionized water	Up to 30 µl	Up to 20 µl	Up to 30 µl

## 2.6. Statistical Analysis

Statistical analysis of the data was performed using SPSS-20 [30], and case-control and family-based association analyzes were performed with Plink version 1.07 [31]. Plink is genome-wide association analysis software with focus on genotype/phenotype data analysis.

## 3. Results

### 3.1. Family-Based Association Analysis

Of the NRAMP1 polymorphisms studied—INT4, D543N, and 3'UTR—both 3'UTR (1729 + 55del4) (rs17235416) and D543N (codon 543, Asp to Asn) (1627G/A) (rs17235409) exhibited normal genotypes.

For INT4 (469 +14G/C) (rs3731865), a total of 43 samples were analyzed. Among them:

- 17 individuals (39.5%) had the normal genotype (INT4 GG),
- 19 individuals (44.3%) were heterozygous (INT4 GC), and
- 7 individuals (16.2%) were homozygous for the mutation (INT4 CC).

In percentage terms, the distribution of the INT4 polymorphism was as follows:

- 39.5% normal (INT4 GG),
- 44.3% heterozygous (INT4 GC),
- 16.2% homozygous (INT4 CC).

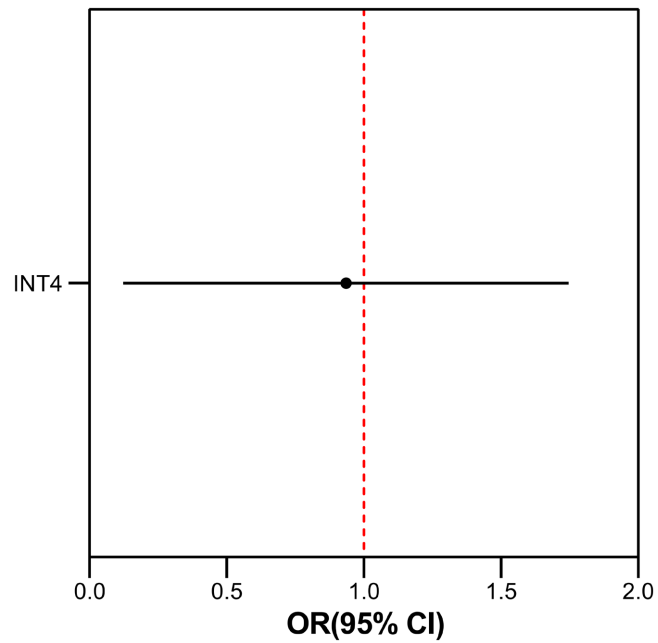
The results of the family-based association analysis for INT4 indicated that the increased frequency of heterozygotes and homozygotes for the mutation was not statistically significant (Table 5, Figure 3, and Figure 4).

**Table 5.** Results of family-based association analysis. OR (odd ratio)—odds ratio, CHISEQ—Chi-square, P—p-value, CI (confidence interval)—confidence interval.

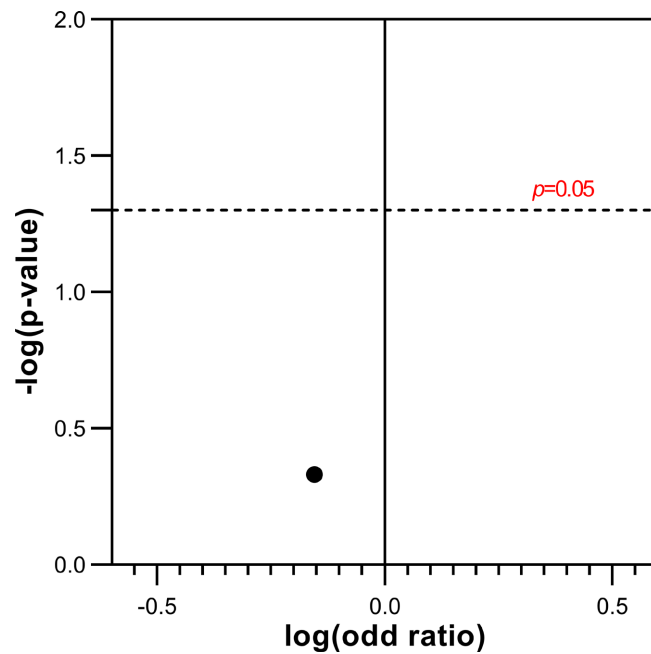
Chromosome	SNP	OR	95% CI	CHISQ	p
2	rs3731865	0.7	0.2665 - 1.839	0.5294	0.4669

### 3.2. Homo or Heterozygotes Occur in All Forms of TB

VGLV—1 homozygote and 8 heterozygotes; PTK—1 heterozygote; IPF. 1 homozygote and 4 heterozygotes; ME—1 heterozygote; FCF—one homo- and heterozygote each, LTBI—one homo or heterozygote each. Of note is the fact that



**Figure 3.** The forest plot depicts the odds ratio (OR) and lower and upper bounds of the confidence interval (95% CI).

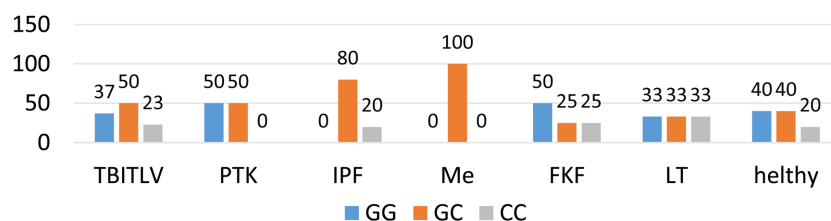


**Figure 4.** The volcano plot represents the distribution of the NRAMP1 polymorphism in patients and parents. X axis—odds ratio (OR) versus p values (Y os—p values). Dashed red line,  $p = 0.05$ .

2 homo- and 4 heterozygotes are also observed in the healthy.

In the most common childhood form, VGLV tuberculosis, 53% are heterozygotes, 6.6% homozygotes.

In infiltrative-pneumonic, one of the severe forms of tuberculosis, 80% of the included patients are hetero- and 20% are homozygous for the mutation.



**Figure 5.** Polymorphism by disease form in %. Hetero or homozygous forms are observed in all forms of active tuberculosis. The most severe forms—IPF and meningitis are represented only by heterozygotes or homozygous.

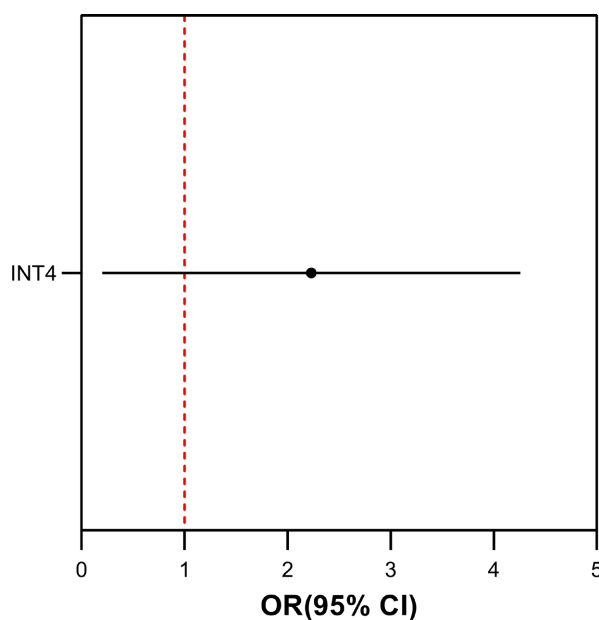
Isolation of *Mycobacterium tuberculosis* by direct microscopy or culture is essential for determining the form and severity of tuberculosis. Of particular interest is the frequency of bacillus detection in relation to the genetic phenotype. Notably, in children, who represent a significant proportion of the studied group, bacillus separation was observed in only 20% - 25% of the examined cases.

Among all tested individuals with detected bacilli ( $n = 8$ ), 50% were heterozygotes and 12.5% were homozygotes. The percentage of heterozygotes was notably high in samples from bacilli-secreting patients.

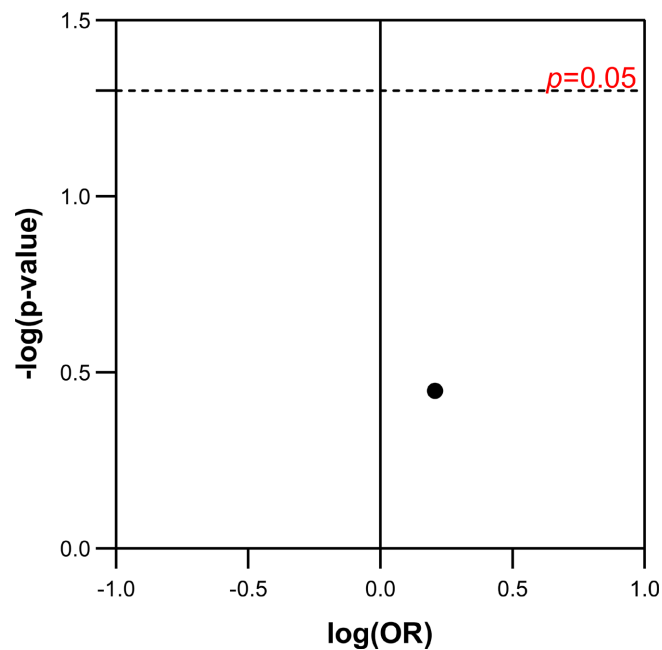
The results of the case-control association analysis further indicated that no statistical significance was found for the TB-predisposing effect of NRAMP1 INT4 (Table 6, Figures 5-7).

**Table 6.** Results of the case-control association analysis. OR (odd ratio)—odds ratio, CHISEQ—Chi-square, p—p-value, CI (confidence interval)—confidence interval.

Chromosome	SNP	CHISEQ	P	OR	95% CI
2	rs3731865	0.8523	0.3559	1.615	0.58 - 4.499



**Figure 6.** The forest plot depicts the odds ratio (OR) and lower and upper bounds of the confidence interval (95% CI).



**Figure 7.** The volcano plot represents the distribution of the NRAMP1 polymorphism in patients and healthy controls. X axis—odds ratio (OR) versus p values (Y axis—p values). Dashed red line— $p = 0.05$ .

#### 4. Discussion

Despite the small number of participants included, it is noteworthy to examine the distribution of the INT4 polymorphism (469 + 14G/C) across different regions. This polymorphism occurs in either homozygous or heterozygous form in all regions where patients were examined. The largest number of cases ( $n = 13$ ) was observed in the Northwestern region, comprising 66% of the total participants, while the highest proportion of homozygotes ( $n = 3$ , 42%) was found in the South Central region. These regions also accounted for the majority of hospitalized patients in the clinic.

As expected, the share of homo- and heterozygosity was highest in regions with a larger number of patients. However, comparative analysis did not establish a statistical association between these genotypes and healthy controls.

The INT4 polymorphism (469 + 14G/C) was detected in homozygous or heterozygous form across all areas where patients were examined (five out of six regions).

Similarly, this polymorphism was observed in all forms of tuberculosis (TB) documented in the clinic.

Based on the available data, no significant association was found between the INT4 polymorphism and specific forms of TB.

Half of the patients with bacillary shedding were heterozygotes.

However, the INT4 GC heterozygous genotype was more frequent in the patient group compared to healthy controls, suggesting a potential disease-predisposing effect.

The other two studied single nucleotide polymorphisms (SNPs)—D543N and

3'UTR—exhibited normal genotypes in all studied patients and healthy controls. This may be attributed to the reduced frequency of mutant alleles in the European population, as reported in dbSNP data. Considering the small sample size, the current data do not support a significant role of the NRAMP1 polymorphisms (INT4, D543N, and 3'UTR) in tuberculosis susceptibility [25] [31]-[34]. These findings align with a similar study conducted in the Serbian population [31], which reported comparable results.

## 5. Conclusions

Following this pilot study, the next step is to expand the patient cohort and examine additional polymorphisms with established relevance to tuberculosis development, such as VDR and TYK2, among others.

The possibility of introducing more markers will be excellent. The study of Tumor Necrosis Factor-Alpha (TNF)-308G/A and Interleukin 8(IL-8)-251C/T would allow for the presentation of more distinct characteristics, such as laboratory indicators, compared to only determining the clinical form of the disease or both [35] [36].

Expanding the scope of the current research will provide a clearer understanding of the genetic factors involved in TB susceptibility and offer directions for future studies. This expansion could also pave the way for the introduction of novel research methods, such as genetic sequencing, into clinical practice.

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## Ethics Approval Statement

The principles of the declaration of Helsinki were strictly followed during the study.

## Patient Consent Statement

All patients and healthy controls provided informed consent for genetic testing as part of the institutional review board’s approved standard-operating procedures at our institutions.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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