


# Synergistic effect of genetic polymorphisms in *TLR6* and *TLR10* genes on the risk of pulmonary tuberculosis in a Moldavian population

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## Abstract

Polymorphisms in genes that control immune function and regulation may influence susceptibility to pulmonary tuberculosis (TB). In this study, 14 polymorphisms in 12 key genes involved in the immune response (*VDR*, *MRI*, *TLR1*, *TLR2*, *TLR10*, *SLC11A1*, *IL1B*, *IL10*, *IFNG*, *TNF*, *IRAK1*, and *FOXP3*) were tested for their association with pulmonary TB in 271 patients with TB and 251 community-matched controls from the Republic of Moldova. In addition, gene–gene interactions involved in TB susceptibility were analyzed for a total of 43 genetic loci. Single nucleotide polymorphism (SNP) analysis revealed a nominal association between *TNF* rs1800629 and pulmonary TB (Fisher exact test  $P = 0.01843$ ). In the pairwise interaction analysis, the combination of the genotypes *TLR6* rs5743810 GA and *TLR10* rs11096957 GT was significantly associated with an increased genetic risk of pulmonary TB (OR = 2.48, 95% CI = 1.62–3.85; Fisher exact test  $P$  value =  $1.5 \times 10^{-5}$ , significant after Bonferroni correction). In conclusion, the *TLR6* rs5743810 and *TLR10* rs11096957 two-locus interaction confers a significantly higher risk for pulmonary TB; due to its high frequency in the population, this SNP combination may serve as a novel biomarker for predicting TB susceptibility.

## Keywords

Gene polymorphisms, host genetics, immunity, pulmonary tuberculosis, susceptibility/resistance

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## Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a major cause of morbidity and mortality in many developing countries and is a significant threat to health in the developed world. One-third of the world's population is infected with *M. tuberculosis*, but only a minor fraction (5–10%) develop an active form of the disease.<sup>1</sup> Although several environmental and clinical conditions, such as diabetes mellitus, malnutrition, alcohol abuse, smoking, age, AIDS, and immunosuppressive therapies, are known to promote the development of the disease, many TB patients have no obvious risk factors. Evidence from animal and human studies indicates the importance of host genetic factors in the development of TB.<sup>2</sup>

To date, a considerable number of genetic candidates for TB susceptibility have been detected and

characterized across populations by means of candidate gene approaches and genome-wide association studies (GWAS).<sup>3,4</sup> Particular interest has focused on

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genetic factors of the immuno-inflammatory response that modulate infectivity and the progression of infection, including TLRs (*TLR1–TLR10*), natural resistance-associated macrophage protein (*NRAMP1*; also called solute carrier 11a1, *SLC11A1*), vitamin D receptor (*VDR*), cytokines TNF- $\alpha$  (*TNF*), IL-1 (*IL1B*), IFN- $\gamma$  (*IFNG*), and IL-10 (*IL10*). Polymorphisms in these and other genes involved in the immune response have been reported to be associated with TB in different populations, although the results are inconsistent.<sup>3–5</sup>

We previously evaluated the effect of common genetic variations in the TLR pathway on the risk of pulmonary TB in a Moldavian population and identified variants in *TLR2*, *TLR8*, and *TLR9* as being associated with TB.<sup>6</sup> In the present work, we extended the analysis to 14 additional polymorphisms from 12 TB immune response candidate genes and investigated all pairwise genetic interactions for these and the previous genetic variants (43 polymorphisms altogether). The selected polymorphisms in this study have previously been shown to change the level or function of corresponding gene products and influence susceptibility/resistance to infections (Table 1).

The Republic of Moldova is a country with an unfavorable epidemiological situation regarding TB, ranking first for TB incidence (86/100,000 in 2018) in the European region.<sup>29</sup> The identification of host genetic factors may provide a biological and theoretical basis for better understanding the high prevalence of TB within the Moldavian population and ultimately for the development of effective TB prevention and control strategies.

## Materials and methods

### Samples

The investigated case-control cohort included 272 unrelated patients (120 women and 152 men; mean age at recruitment =  $40.7 \pm 12.7$  yr; mean age at diagnosis =  $39.1 \pm 12.2$  yr) with infiltrative pulmonary TB. The diagnosis was based on clinical symptoms and chest radiographic findings and was confirmed by bacteriological assessment. None of the patients had a clinical history of diabetes mellitus, HIV infection, or receipt of immunosuppressive therapy. All patients were Caucasian and predominantly Moldavians (91.5%). The control group included 251 unrelated and ethnically matched (Moldavians, 87%) healthy individuals (150 women and 101 men; mean age at recruitment =  $47.6 \pm 14.1$  yr) without history of TB and with normal radiographic examination findings. Control group subjects were recruited from the same high-burden TB-affected community as patients.

Venous blood samples (EDTA anticoagulant added) were collected from TB patients and controls, and genomic DNA was extracted from peripheral blood leukocytes using a standard salting-out method.<sup>30</sup> All cases and controls were vaccinated with the BCG vaccine, which was confirmed by the presence of a scar in the left shoulder. This study was approved by the Ethics Committee of the Institute of Phthisiopneumology (Republic of Moldova), and the Declaration of Helsinki protocols were followed. Each participant was thoroughly informed and provided written informed consent prior to enrollment in the study.

### Polymorphism selection and genotyping

The following polymorphisms, *VDR* rs7975232, *VDR* rs1544410, *VDR* rs2228570, *MRI* rs1052632, *TLR1* rs5743618, *TLR2* rs111200466, *TLR10* rs11096957, *SLC11A1* rs2276631, *IL1B* rs1143643, *IL10* rs1800896, *IFNG* rs2430561, *TNF* rs1800629, *IRAK1* rs1059703, and *FOXP3* rs2232365 were investigated. Polymorphisms were selected based on previously published associations with TB, thus increasing the chance of selecting polymorphisms with phenotypic effects (Table 1). All polymorphisms except the insertion/deletion variant rs111200466 in *TLR2* (–196 to –174 ins/del) were single nucleotide polymorphisms (SNPs).

The genotypes of the 12 SNPs (*VDR* rs7975232, *VDR* rs1544410, *VDR* rs2228570, *MRI* rs1052632, *TLR10* rs11096957, *SLC11A1* rs2276631, *IL1B* rs1143643, *IL10* rs1800896, *IFNG* rs2430561, *TNF* rs1800629, *IRAK1* rs1059703, and *FOXP3* rs2232365) were determined using Agena iPLEX assays using a MALDI-TOF mass array spectrometer (Agena, San Diego, CA) following the manufacturer's recommendations. Primers were designed using the Assay Design Suite v2.0 (<https://agenacx.com>). Two of the SNPs were not in Hardy–Weinberg equilibrium (HWE) in the control group: rs11096957 in *TLR10* ( $P = 0.0071$ ) and rs2430561 in *IFNG* ( $P = 0.013$ ). Genotyping errors were excluded by random re-genotyping of the respective SNPs. Confirmatory genotyping of *TLR10* rs11096957 was performed in 52 control samples (21%) using PCR–RFLP. PCR products were digested with the restriction endonuclease *NlaIII* (New England Biolabs; Arundel, Australia) and subjected to 2% agarose gel electrophoresis.

Confirmatory genotyping of *IFNG* rs2430561 was carried out by PCR with sequence-specific primers in 105 control samples (42%), and amplified SNP products were electrophoresed on 2% agarose gel. Additionally, SNPs *TNF* rs1800629 and *TLR6* rs5743810 (the latter from our previous study)<sup>6</sup>, which exhibited a relationship with TB, were

**Table 1.** Candidate genes and polymorphisms that are of interest in the present study.

| Gene           | Locus   | Protein  | Gene class                | Protein function   | Polymorphism | Nucleotide substitution | Location               | Frequency <sup>a</sup> | Reference   |
|----------------|---------|--|---------------------------|--|--------------|-------------------------|------------------------|------------------------|---|
| <i>TLR1</i>    | 4p14    | TLR 1  | PRR                       | Recognizes bacterial lipopeptides, activates macrophages and initiates immune response                       | rs5743618    | C>A                     | Missense (p.Ser602Ile) | 0.172-0.500            | Uciechowski et al., 2011, <sup>7</sup><br>Naderi et al., 2016 <sup>8</sup>  |
| <i>TLR2</i>    | 4q31.3  | TLR 2  | PRR                       | Recognizes bacterial lipopeptides, activates macrophages and initiates immune response                       | rs11200466   | 23 bp del               | 5' UTR                 | 0.116-0.172            | Velez et al., 2010 <sup>9</sup>   |
| <i>TLR10</i>   | 4p14    | TLR 10   | PRR                       | Recognizes bacterial ligands, activates macrophages and initiates immune response                            | rs11096957   | T>G                     | Missense (p.Asn241His) | 0.324-0.486            | Oosting et al., 2014, <sup>10</sup><br>Bulat-Kardum et al., 2015 <sup>11</sup>                                    |
| <i>IRAK1</i>   | Xq28    | IL-1 receptor-associated kinase 1                  | Serine/threonine kinase   | Regulates TLR and IL-1R mediated signaling   | rs1059703    | G>A                     | Missense (p.Ser532Leu) | 0.088-0.211            | Sampath et al., 2013, <sup>12</sup><br>Hu et al., 2015 <sup>13</sup>  |
| <i>IFNG</i>    | 12q15   | IFN- $\gamma$                                      | Cytokine                  | Activates macrophages to eliminate intracellular pathogens, including Mtb                                    | rs2430561    | A>T                     | Intron                 | 0.354-0.528            | Pravica et al., 2000, <sup>14</sup><br>Wei et al., 2017 <sup>15</sup>   |
| <i>TNF</i>     | 6p21.3  | TNF- $\alpha$                                      | Cytokine                  | Activates macrophages to eliminate intracellular pathogens, attracts immunity cells to the site of infection | rs1800629    | G>A                     | Promoter               | 0.094-0.187            | Correa et al., 2005, <sup>16</sup><br>Elahi et al., 2009, <sup>17</sup><br>de Arellano et al., 2020 <sup>18</sup> |
| <i>IL1B</i>    | 2q14.1  | IL-1 $\beta$                                       | Cytokine                  | Activates T-cells and promotes the production of IFN- $\gamma$   | rs1143643    | C>T                     | Intron                 | 0.286-0.388            | Hall et al., 2015 <sup>19</sup>   |
| <i>IL10</i>    | 1q32.1  | IL-10  | Cytokine                  | Inhibits IFN- $\gamma$ production and MHC class II expression on macrophages                                 | rs1800896    | A>G                     | Promoter               | 0.456-0.601            | Turner et al., 1997, <sup>20</sup><br>Areeshi et al., 2017 <sup>21</sup>  |
| <i>SLC11A1</i> | 2q35    | Natural Resistance-Associated Macrophage Protein 1 | Metal ion transporter     | Influences Mtb survival by regulating cation levels in the macrophage  | rs2276631    | C>T                     | Synonymous             | 0.202-0.332            | Freidin et al. 2006, <sup>22</sup><br>Yuan et al., 2017 <sup>23</sup>   |
| <i>FOXP3</i>   | Xp11.23 | Forkhead box protein P3 (FoxP3)                    | Transcriptional regulator | Inhibits cytokine production (IFN- $\gamma$ ) and T-cell effector function                                   | rs2232365    | T>C                     | Intron                 | 0.338-0.450            | Beiranvand et al., 2017 <sup>24</sup>   |

(continued)

Table 1. Continued.

| Gene | Locus    | Protein                          | Gene class                            | Protein function   | Polymorphism           | Nucleotide substitution | Location                       | Frequency <sup>a</sup>     | Reference  |
|------|----------|----------------------------------|---------------------------------------|--|------------------------|-------------------------|--------------------------------|----------------------------|--|
| VDR  | 12q13.11 | Vitamin D receptor               | Ligand activated transcription factor | Regulates expression of a number of genes involved in killing of Mtb | rs7975232<br>rs1544410 | C>A<br>C>T              | Intron<br>Intron               | 0.404-0.539<br>0.338-0.470 | Uitterlinden et al., 2004, <sup>25</sup><br>Xu and Shen, 2019 <sup>26</sup><br>Uitterlinden et al., 2004, <sup>25</sup><br>Xu and Shen, 2019, <sup>26</sup><br>Chen et al., 2013 <sup>27</sup> |
| MR1  | 1q25.3   | MHC class I-related gene protein | Ag-presenting molecule                | Presents metabolites of microbial vitamin B to MAITs                 | rs2228570<br>rs1052632 | A>G<br>G>A              | Missense (p.Met Thr)<br>Intron | 0.327-0.429<br>0.266-0.303 | Uitterlinden et al. 2004, <sup>25</sup><br>Xu and Shen 2019, <sup>26</sup><br>Chen et al. 2013 <sup>27</sup><br>Seshadri et al., 2017 <sup>28</sup>  |

<sup>a</sup> Allele frequency range in European populations (CEU, FIN, GBR, IBS, TSI) according to 1000 Genomes (<http://www.1000genomes.org>); minor allele in CEU was used as the reference. Minor allele in CEU population is underlined.  
 PRR: pattern recognition receptor. SNP rs2276631 in *SLC11A1* is non-functional (synonymous) variant, which is in strong LD with the nearby TB associated SNP rs3731865 (INT4) ( $D' = 0.9788$  and  $r^2 = 0.9479$  in European populations).

Sanger-sequenced (primers are in Supplemental Table S1) in 50 (~10%) randomly selected samples, to confirm the quality of MALDI-TOF genotyping. All sequenced SNPs were 100% consistent with those obtained by MALDI-TOF.

Two polymorphisms, *TLR1* rs5743618 and *TLR2* rs111200466, could not be included in the Agena iPLEX assay, and therefore, were genotyped using PCR and PCR-RFLP. Genotyping of rs5743618 was performed using PCR-RFLP. PCR products were digested with the restriction endonuclease *AluI* (New England Biolabs; Arundel, Australia) and subjected to 2% agarose gel electrophoresis. Genotyping of the rs111200466 polymorphism was conducted by standard PCR and agarose gel electrophoresis (2%).

The primer sequences, PCR conditions, and restriction enzymes for genotyping/sequencing of *TLR2* rs111200466, *IFNG* rs2430561, *TLR1* rs5743618, *TLR10* rs11096957, *TNF* rs1800629, and *TLR6* rs5743810 are outlined in Supplemental Table S1. The methodology used for the genotyping of *TLR2* rs111200466, *IFNG* rs2430561, and *TLR1* rs5743618 polymorphisms has been previously described.<sup>14,31,32</sup>

### Statistical analysis

HWE tests were performed in controls and cases using Fisher's exact test. Fisher's exact test was also used to compare differences in allele frequencies between the groups. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to measure the associations between SNPs and the risk of pulmonary TB. Minor alleles were assumed to be effect-associated alleles in all the tests. In addition, logistic regression was used to assess the association of each SNP with the risk of pulmonary TB using a log-additive genetic model. To account for possible bias due to statistically significant differences in age and sex distributions between the case and control groups ( $P < 0.0001$  and  $P = 0.0004$ , respectively), the variables age and sex were included as covariates in the logistic regression analysis. All genetic tests described above were based on single SNP tests and were performed using the PLINK software package (version 1.9, <http://pngu.mgh.harvard.edu/purcell/plink/>).<sup>33</sup> For *IRAK1* rs1059703 and *FOXP3* rs2232365 SNPs located on the X chromosome, HWE  $P$  values were calculated in females only and association tests were performed separately in men and women; therefore, 16 tests were conducted in total. To correct the significance level for multiple testing, Bonferroni correction was applied, and the significance level for all statistical tests was set at  $P < 0.003125$  (0.05/16).

Haplotype analysis was performed for five polymorphisms in the *TLR1-TLR6-TLR10* gene cluster, two



polymorphisms in the *TLR2* gene and three *VDR* gene polymorphisms using Haploview version 4.2 software.<sup>34</sup> SNPs *TLR1* rs4833095, *TLR2* rs3804099, *TLR6* rs5743810, and *TLR10* rs11466657 were taken from the previous study conducted on the same cohort of TB patients and healthy controls. The solid spine of the linkage disequilibrium (LD) algorithm was applied to define the haplotype blocks. Differences in haplotype frequencies were tested using the  $\chi^2$  test. Haplotypes with frequencies below 1% were excluded from the analyses. Multiple comparisons of haplotype frequencies were corrected by generating empirical *P* values via 10,000 permutations.

Pairwise epistatic interactions associated with susceptibility to TB were investigated via an exhaustive search of all pairwise combinations. There were 1065 combinations (741 common and  $2 \times 162$  sex-specific) and an exhaustive search was the easiest way to find the best combination. Nonparametric methods such as multifactor dimensionality reduction (MDR) or *k*-nearest neighbors (KNN) check only a small part of the entire search space to be computationally effective and are applied mostly when a large number of markers are to be tested (e.g. in GWAS experiments).<sup>35,36</sup> Another drawback of such methods is the interpretation of results, for example, when a strong marginal effect of one SNP makes the effects of several other polymorphisms in the interaction negligible.<sup>35,37</sup> To address the latter, we restricted the search to the interactions of only two SNPs and evaluated the added value of the pairwise effects over individual effects.

Overall, pairwise epistatic analysis was performed as follows. First, we found SNP combinations associated with TB using logistic regression adjusted for sex and age under log-additive genetic model (implemented in software package SNPstats)<sup>38</sup> for all pairs of the 43 genetic markers from this and our previous study.<sup>6</sup> We used the log-additive model because it is a generalized model that is the best choice when the true inheritance pattern is unknown. Second, we found the exact genotype combination(s) that most contributed to the associations revealed in the previous step. Two methods were applied to all significant pairs ( $P < 0.05$ ): logistic regression adjusted for sex and age, and Fisher's exact test.<sup>39,40</sup> Here, no genetic model was required for regression, since in both methods, one specific combination was tested against all other genotypes. The advantage of the Fisher test is its clear biological interpretation, but it is not trivial to adjust it to sex and age differences in the datasets. Therefore, logistic regression was used, which could easily incorporate such adjustment parameters.

To account for multiple testing, we used the Bonferroni correction and counted all tests of the

first and second steps as independent, yielding a multiplication factor of  $1/(1065 + 543) = 0.000622$ .

Power calculations were done using CaTS<sup>41</sup> with a log-additive genetic model, assuming significance of 0.05 and the reported TB disease prevalence of 0.0020.<sup>29</sup> The study had sufficient power ( $> 80\%$ ) to detect effect sizes (ORs) of 1.7 and 1.5 for high-risk allele frequencies of  $> 0.10$  and  $> 0.20$ , respectively.

## Results

### Single polymorphism analysis

The overall genotyping performance in the study population was 83.2–100.0% depending on the SNP (Supplemental Table S2). Distributions of alleles of the investigated SNPs were in accordance with HWE in both groups except for *TLR10* rs11096957 (Supplemental Table S2), which showed some deviation from HWE in controls ( $P = 0.0078$ ). The minor allele frequencies of all polymorphisms in our controls were similar those in the populations of European descent from the 1000 Genomes Project database (Table 1). Association analysis between individual SNPs and TB revealed a moderate association between rs1800629 in *TNF* and pulmonary TB risk (Fisher exact test: OR = 0.63,  $P$  value = 0.01843; logistic regression: OR = 0.64,  $P = 0.03643$ ) (Table 2), which did not remain significant after Bonferroni correction for multiple testing ( $P > 0.003125$ ). None of the other loci showed an association with pulmonary TB risk (Table 2).

### Haplotype analysis

Nine common haplotypes in the gene cluster *TLR1-TLR6-TLR10*, four in *TLR2*, and three in *VDR* were identified at a frequency  $\geq 1\%$  by LD analysis (Figure 1). The rs11466657-rs11096957-rs5743618-rs4833095-rs5743810 haplotype A-T-G-T-A of the gene cluster *TLR1-TLR6-TLR10* was significantly higher in the TB group compared with the controls ( $P = 0.0485$ ; Table 3). However, after 10,000-fold permutation testing, this haplotype was not significant ( $P = 0.4463$ ). In addition, marginal differences were observed between cases and controls for the haplotype A-T-G-T-G (rs11466657-rs11096957-rs5743618-rs4833095-rs5743810) in the gene cluster *TLR1-TLR6-TLR10* ( $P = 0.0688$ ) and haplotypes ins-T and ins-C (rs111200466-rs3804099) in *TLR2* ( $P = 0.0732$  and  $P = 0.0987$ , respectively). None of the remaining haplotypes were associated with the risk of pulmonary TB in this study (Table 3).

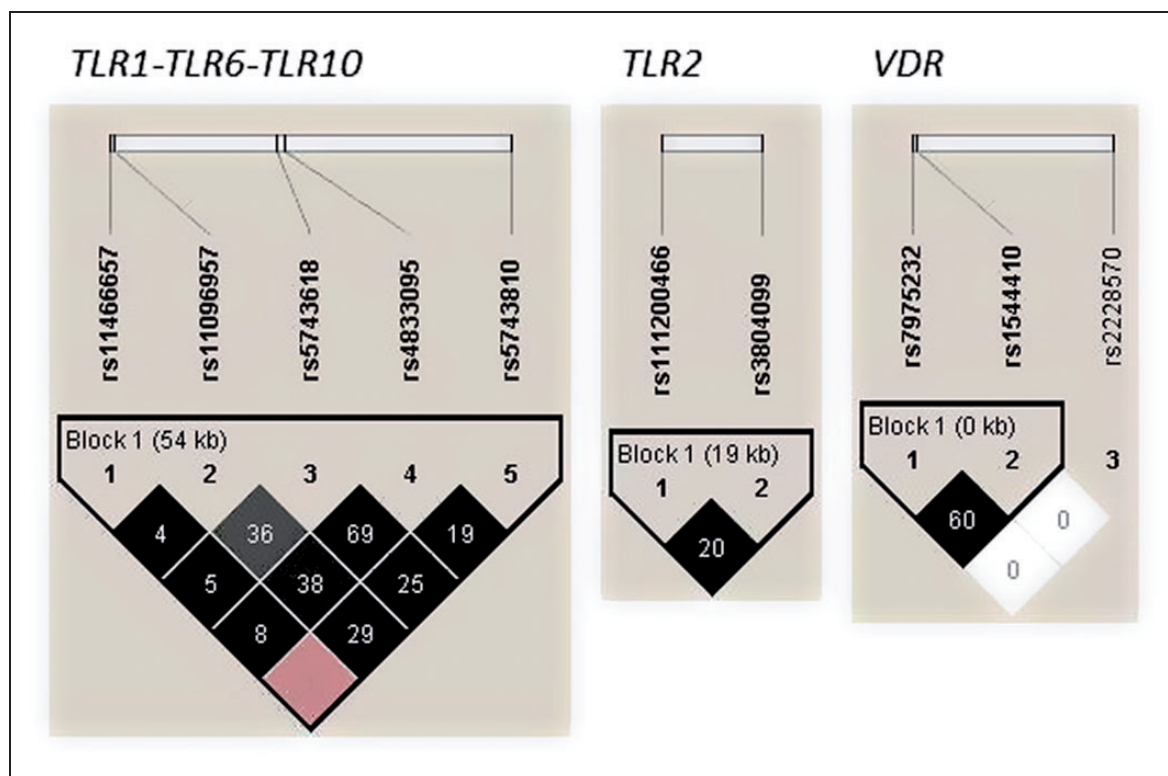
**Table 2.** Association analysis of individual polymorphisms with pulmonary TB risk. For genotype data, see Supplementary Table 2.

| Polymorphism      | Major/Minor allele | MAF in controls | MAF in patients | Fisher exact test |                         | Logistic regression <sup>a</sup> |                         |
|-------------------|--------------------|-----------------|-----------------|-------------------|-------------------------|----------------------------------|-------------------------|
|                   |                    |                 |                 | P Value           | OR (95% CI)             | P Value                          | OR (95% CI)             |
| <b>Both sexes</b> |                    |                 |                 |                   |                         |                                  |                         |
| VDR rs7975232     | C/A                | 0.481           | 0.509           | 0.3797            | 1.12 (0.87–1.43)        | 0.1819                           | 1.20 (0.92–1.57)        |
| VDR rs1544410     | C/T                | 0.379           | 0.374           | 0.8977            | 0.98 (0.76–1.26)        | 0.9695                           | 0.99 (0.76–1.30)        |
| VDR rs2228570     | G/A                | 0.438           | 0.400           | 0.2262            | 0.85 (0.66–1.10)        | 0.3169                           | 0.88 (0.67–1.14)        |
| MRI rs1052632     | G/A                | 0.271           | 0.259           | 0.7118            | 0.94 (0.70–1.26)        | 0.7854                           | 0.96 (0.71–1.30)        |
| TLR1 rs5743618    | G/T                | 0.402           | 0.407           | 0.8991            | 1.02 (0.80–1.31)        | 0.9359                           | 1.01 (0.78–1.32)        |
| TLR2 rs11200466   | 23 bp ins/del      | 0.175           | 0.165           | 0.6813            | 0.93 (0.68–1.29)        | 0.5575                           | 0.90 (0.65–1.27)        |
| TLR10 rs11096957  | T/G                | 0.474           | 0.472           | 0.9504            | 0.99 (0.78–1.27)        | 0.7115                           | 1.05 (0.82–1.35)        |
| SLC11A1 rs2276631 | C/T                | 0.242           | 0.249           | 0.8266            | 1.04 (0.78–1.39)        | 0.9478                           | 0.99 (0.73–1.35)        |
| IL1B rs1143643    | C/T                | 0.363           | 0.343           | 0.5127            | 0.92 (0.71–1.18)        | 0.2367                           | 0.85 (0.65–1.11)        |
| IL10 rs1800896    | T/C                | 0.447           | 0.447           | 1.0000            | 1.00 (0.77–1.31)        | 0.9803                           | 1.00 (0.75–1.32)        |
| IFNG rs2430561    | T/A                | 0.464           | 0.442           | 0.4925            | 0.92 (0.72–1.17)        | 0.8409                           | 1.02 (0.79–1.32)        |
| TNF rs1800629     | G/A                | 0.138           | 0.091           | <b>0.01843</b>    | <b>0.63 (0.42–0.92)</b> | <b>0.03643</b>                   | <b>0.64 (0.43–0.97)</b> |
| <b>Males</b>      |                    |                 |                 |                   |                         |                                  |                         |
| IRAK1 rs1059703   | A/G                | 0.192           | 0.242           | 0.4356            | 1.34 (0.72–2.51)        | 0.3563                           | 1.34 (0.72–2.51)        |
| FOXP3 rs2232365   | C/T                | 0.404           | 0.456           | 0.4353            | 1.24 (0.74–2.07)        | 0.4156                           | 1.24 (0.74–2.07)        |
| <b>Females</b>    |                    |                 |                 |                   |                         |                                  |                         |
| IRAK1 rs1059703   | A/G                | 0.250           | 0.233           | 0.686             | 0.91 (0.61–1.36)        | 0.6544                           | 0.91 (0.61–1.36)        |
| FOXP3 rs2232365   | C/T                | 0.463           | 0.413           | 0.2562            | 0.81 (0.58–1.15)        | 0.2431                           | 0.81 (0.58–1.15)        |

MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.

<sup>a</sup> Log-additive model adjusted for sex and age at recruitment.

Bold: significant associations (nominal *P* value < 0.05).



**Figure 1.** Linkage disequilibrium (LD) plots of *TLR2*, *VDR* and *TLR1-TLR6-TLR10* polymorphisms in cases and controls. The colours of the squares represent  $D'$  values, with black being  $D'=1$ , and white  $D'=0$ .  $r^2$  values (%) are indicated on the squares.

**Table 3.** Haplotype frequencies and associations with pulmonary TB.

| Haplotypes <sup>a</sup> | Frequency in Patients <sup>b</sup> | Frequency in Controls | P Value <sup>c</sup> | Permutation P value |
|-------------------------|------------------------------------|-----------------------|----------------------|---------------------|
| <i>TLR2</i>             |                                    |                       |                      |                     |
| insT                    | 0.591                              | 0.536                 | 0.0732               | 0.6576              |
| insC                    | 0.244                              | 0.289                 | 0.0987               | 0.7665              |
| delC                    | 0.154                              | 0.160                 | 0.7951               |                     |
| delT                    | 0.012                              | 0.016                 | 0.5744               |                     |
| <i>TLR1-TLR6-TLR10</i>  |                                    |                       |                      |                     |
| ATGTA                   | 0.298                              | 0.243                 | <b>0.0485</b>        | 0.4463              |
| AGTCG                   | 0.255                              | 0.263                 | 0.7784               |                     |
| ATGTG                   | 0.170                              | 0.215                 | 0.0688               | 0.6201              |
| AGGTG                   | 0.110                              | 0.121                 | 0.5933               |                     |
| AGTTG                   | 0.045                              | 0.041                 | 0.7844               |                     |
| GGTCG                   | 0.044                              | 0.028                 | 0.1705               | 0.9125              |
| ATTTG                   | 0.030                              | 0.033                 | 0.7762               |                     |
| ATTCCG                  | 0.023                              | 0.034                 | 0.2712               | 0.9818              |
| AGGTA                   | 0.010                              | 0.018                 | 0.27                 | 0.9816              |
| <i>VDR</i>              |                                    |                       |                      |                     |
| CC                      | 0.492                              | 0.515                 | 0.4556               |                     |
| AT                      | 0.375                              | 0.371                 | 0.8939               |                     |
| AC                      | 0.133                              | 0.113                 | 0.3484               | 0.9917              |

<sup>a</sup>Haplotypes: *TLR2* [rs111200466, rs3804099]; *TLR1-TLR6-TLR10* [rs11466657, rs11096957, rs5743618, rs4833095, rs5743810]; *VDR* [rs7975232, rs1544410]

<sup>b</sup>Only haplotypes with a frequency > 0.01 were included in this table.

<sup>c</sup>P Values were calculated using  $\chi^2$  test.

Bold: significant differences (nominal P value < 0.05) in haplotype distribution.

### Gene-gene interaction analysis

Analysis of SNP-SNP combinations using a logistic regression model revealed 59 significant ( $P$  value < 0.05) genetic interactions (Supplemental Table S3), with the smallest  $P$  values observed for the marker pairs *TOLLIP* rs3793964-*IRAK2* rs3844283 ( $P=0.0025$ ), *TLR2* rs111200466-*TOLLIP* rs5743899 ( $P=0.0044$ ), and *TLR10* rs11096957-*TLR6* rs5743810 ( $P=0.0052$ ). Although none of these associations exceeded the conservative Bonferroni-corrected threshold of  $P$  value =  $4.7 \times 10^{-5}$  (0.05/1065), they suggest a potential role in conferring TB risk and therefore were pipelined into the in-depth genotype combination analysis using Fisher's exact test and logistic regression adjusted for sex and age differences in the datasets. Genotype combinations reported by both methods were considered significant.

This resulted in 62 nominally significant associations ( $P$  value < 0.05 for either logistic regression or Fisher's exact test) out of 543 possible genotype combinations (Supplemental Table S4). The strongest association was observed between TB and the combination of *TLR6* rs5743810 GA and *TLR10* rs11096957 GT genotypes (Fisher exact test  $P$  value =  $1.5 \times 10^{-5}$ ; logistic regression  $P$  value =  $1.9 \times 10^{-5}$ ; Table 4). This association remained significant after Bonferroni correction ( $P$  value =  $0.05/(1065 + 543) = 3.5 \times 10^{-5}$ ).

Remarkably, the effect of this genotype combination on TB risk (OR = 2.48) was much greater than that of the individual genotypes (heterozygous model, OR = 1.72,  $P=0.0042$  and OR = 1.33,  $P=0.13$  for rs5743810 and rs11096957, respectively), implying a strong synergistic interaction between the two polymorphisms. None of the other genotype combinations were significant after the Bonferroni correction.

### Discussion

In the present study, we applied a candidate gene approach and tested the association of 14 polymorphisms in genes *VDR*, *MRI*, *TLR1*, *TLR2*, *TLR10*, *SLC11A1*, *IL1B*, *IL10*, *IFNG*, *TNF*, *IRAK1*, and *FOXP3* with the risk of pulmonary TB in Moldavian population. Although these genes are critical components of human immunity and their polymorphisms have been implicated in susceptibility to TB, we could not find convincing statistical evidence for their association with the risk of pulmonary TB. A borderline association was revealed for polymorphism rs1800629 in the *TNF* gene only, but this was not significant after corrections for multiple testing by the Bonferroni method. Overall, the results presented here do not support a major role of the analyzed common variants in

**Table 4.** Association of combined genotypes *TLR6* rs5743810 and *TLR10* rs11096957 with pulmonary TB.

| Genotype       |                  | Counts, n (%) |           | Association (P value)        |                                  |                          |
|----------------|------------------|---------------|-----------|------------------------------|----------------------------------|--------------------------|
| TLR6 rs5743810 | TLR10 rs11096957 | Controls      | Patients  | Fisher test                  | Logistic regression <sup>a</sup> | OR (95% CI) <sup>a</sup> |
| GG             | TT               | 58 (23.8)     | 56 (21.1) | 0.56                         | 0.83                             | 0.94 (0.60–1.47)         |
| GG             | GT               | 57 (23.4)     | 43 (16.2) | 0.059                        | 0.033                            | 0.61 (0.38–0.96)         |
| GG             | GG               | 19 (7.8)      | 16 (6.0)  | 0.55                         | 0.59                             | 0.80 (0.37–1.72)         |
| GA             | TT               | 7 (2.9)       | 2 (0.8)   | 0.14                         | 0.094                            | 0.25 (0.025–1.36)        |
| GA             | GT               | 42 (17.2)     | 91 (34.3) | <b>1.5 × 10<sup>-5</sup></b> | <b>1.9 × 10<sup>-5</sup></b>     | <b>2.48 (1.62–3.85)</b>  |
| GA             | GG               | 42 (17.2)     | 38 (14.3) | 0.45                         | 0.34                             | 0.79 (0.48–1.30)         |
| AA             | TT               | 17 (7.0)      | 18 (6.8)  | 0.94                         | 0.86                             | 0.91 (0.43–1.91)         |
| AA             | GT               | 2 (0.8)       | 1 (0.4)   | 0.94                         | 0.60                             | 0.45 (0.0077–8.86)       |

<sup>a</sup> Adjusted for sex and age at recruitment.

Bold: significant association after Bonferroni correction (P value < 3.10945 × 10<sup>-5</sup>).

conferring susceptibility or resistance to pulmonary TB in the Moldavian population.

The inability to reach significance level after Bonferroni correction may be explained by a fairly small sample size and inadequate statistical power to produce convincing associations for polymorphisms with low and moderate effects (OR < 1.5). For this reason, we cannot completely rule out the possibility of true functional effects for variants with nominal associations, particularly for the *TNF* rs1800629 polymorphism. The *TNF* gene encodes a multifunctional pro-inflammatory cytokine, TNF- $\alpha$ , which is mainly produced by activated monocytes, macrophages, and T-lymphocytes when stimulated by mycobacterial antigens. TNF- $\alpha$  acts synergistically with IFN- $\gamma$  to induce macrophage killing of *M. tuberculosis*.<sup>42</sup> In addition, TNF- $\alpha$  is involved in the recruitment of leukocytes to the site of infection and contributes to the formation of TB granuloma, preventing the spread of infection.<sup>42</sup> It is reported that treatment with TNF- $\alpha$  inhibitors leads to reactivation of latent TB infection, indicating TNF- $\alpha$  is a key cytokine toward resistance to *M. tuberculosis*.<sup>43</sup> SNP rs1800629 (-308G>A) is located within regulatory hotspot region and thus influences transcription critically.<sup>44,45</sup> The minor allele A of rs1800629 has been associated with increased expression of *TNF* and higher plasma levels of TNF- $\alpha$ .<sup>17</sup> In agreement with published functional studies, our results demonstrated a higher frequency of allele A in controls than in cases, suggesting its protective role against TB (OR = 0.63). Similarly, allele A was protectively associated with TB in the Colombian<sup>16</sup> and Mexican<sup>18</sup> populations. However, other genetic epidemiologic studies involving patient cohorts from various population groups, including Malawi,<sup>46</sup> Iran,<sup>47</sup> Indian,<sup>48</sup> Cambodian,<sup>49</sup> Chinese Han, and Tibetan,<sup>50</sup> did not confirm these findings. The disparity in results across studies may be explained by certain factors, such as inadequate sample sizes and differences in environmental,

demographic, cultural, host genetic, and bacterial characteristics of *M. tuberculosis* strains.

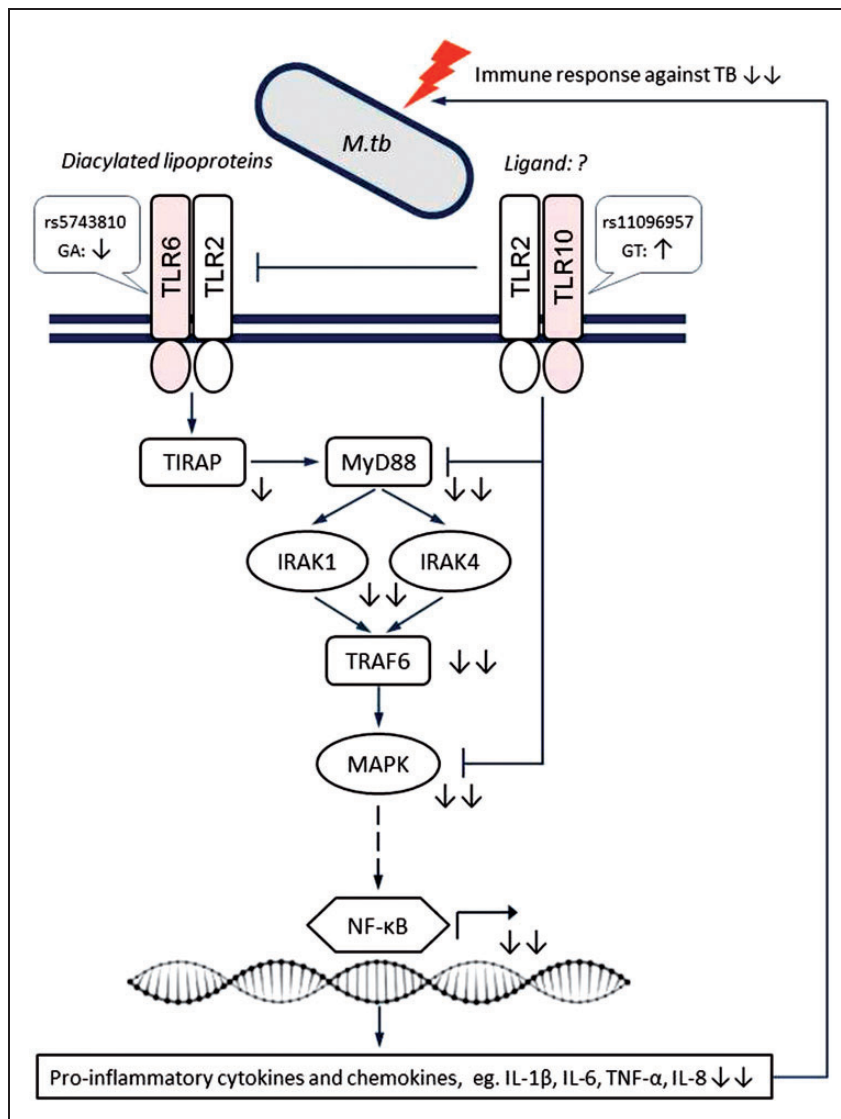
Additionally, a nominal yet suggestive association was demonstrated for haplotype A-T-G-T-A of the block rs11466657-rs11096957-rs5743618-rs4833095-rs5743810 in the cluster *TLR1-TLR6-TLR10*. *TLR1*, *TLR6*, and *TLR10* are located in a 54-kb genomic region on chromosome 4p14 and encode proteins that share a high degree of homology in their amino acid sequences. All three genes belong to the TLR2 subfamily of TLRs, which plays a critical role in the early recognition of *M. tuberculosis* and subsequent activation of immune responses.<sup>51</sup> Individual polymorphisms and haplotypes within the *TLR10-TLR1-TLR6* locus have been associated with altered susceptibility to infectious diseases, including mycobacterial infections of leprosy and TB.<sup>11,52–55</sup> Unfortunately, different sets of SNPs used in this and other studies complicate direct comparisons of the results. Even so, the identified haplotype A-T-G-T-A and, more generally, variations in genes *TLR10*, *TLR1*, and *TLR6* could be promising replication targets for future studies in larger cohorts.

Genetic interactions are thought to underlie susceptibility or resistance to TB,<sup>5,56</sup> so they could explain some of the missing heritability in this study. Therefore, we also analyzed the impact of allele combinations on the risk of TB. The strongest evidence for interaction in our data was between SNPs rs5743810 and rs11096957 located in *TLR6* and *TLR10*, respectively. Interestingly, the two SNPs showed no or only a weak effect on TB susceptibility when evaluated alone, indicating a synergetic mechanism of *TLR6* rs5743810 and *TLR10* rs11096957 in conferring risk for pulmonary TB. The interactions between these SNPs are biologically plausible. First, TLRs are key players in host defense against infections. Specifically, TLR6 functionally interacts with TLR2 to mediate the cellular response to bacterial lipoproteins and activate the NF- $\kappa$ B pathway and inflammatory events through



MyD88-dependent signaling.<sup>10,51,55</sup> TLR10 also can form heterodimers with TLR2, but its specific ligands have not yet been identified and its downstream signaling is not fully understood. It is thought to act through both MyD88-dependent and -independent signaling pathways with inhibitory effects on inflammation.<sup>10</sup> The genetic interaction between *TLR6* and *TLR10* observed in this study may reflect their mutual functional contribution to *M. tuberculosis* recognition and subsequent downstream signaling (Figure 2). Second, the SNPs in *TLR6* and *TLR10* genes were previously

shown to be of functional significance. In fact, the two SNPs are non-synonymous variants located in the extracellular (leucine-rich repeat) domains of the encoded proteins. Both *ex vivo* and *in vitro* experiments showed that SNPs rs5743810 (Ser249Pro) and rs11096957 (Asn241His) may influence pro-inflammatory cytokine production in humans.<sup>10,57,58</sup> In addition, rs5743810 was observed to affect NF- $\kappa$ B signaling activity, thereby modulating inflammatory responses.<sup>59</sup> Furthermore, these two polymorphisms have been associated with several immune-



**Figure 2.** Schematic representation of the proposed epistatic/synergistic interaction between SNPs *TLR6* rs5743810 and *TLR10* rs11096957 in conferring susceptibility to TB based on the present results. TLR6 and TLR10 have largely opposite effects on the immune response, with TLR10 mainly having a suppressive function. Simultaneous suppression of TLR6 signaling and activation of TLR10 signaling caused by genetic variations may result in decreased pro-inflammatory responses against *M. tuberculosis*, thus increasing the risk of TB. Symbols indicate the following: sharp arrow – positive interaction; blunt arrow – negative interaction; solid line – direct interaction; dashed line – indirect interaction; question mark – unknown ligand partner; up/down arrows – activation/suppression; double arrows – synergistic effects of two mutations. Figure adapted from Oosting et al. (2014).<sup>10</sup>

related pathologic conditions and infectious diseases, including TB.<sup>11,54,55,60</sup> These data support the relevance of additive interaction between SNPs rs5743810 and rs11096957 and suggest a molecular mechanism by which genetic variations in *TLR6* and *TLR10* genes might increase susceptibility to TB (Figure 2).

The present study is the first to identify an interaction between *TLR6* rs5743810 and *TLR10* rs11096957 gene variants in TB risk. Further larger case-control studies followed by functional tests are warranted to validate this initial finding and eventually translate it into clinical practice. Particularly, given the high spread of the combined *TLR6* rs5743810 GA-*TLR10* rs11096957 GT genotype in the European population (~15–20%), it might be used as a novel predictive biomarker for identification of individuals at high risk for active TB disease.

Some limitations of our study should be considered. First, it was limited in power to detect weak association signals, so our negative results should be treated with caution. Second, healthy controls were not tested for latent *M. tuberculosis* infection, and therefore, it was not possible to discriminate between TB-infected and TB-uninfected individuals. However, as mentioned in the “Materials and methods,” the controls were recruited from TB communities where they were permanently exposed to TB and were therefore expected to be infected. Third, the number of polymorphisms in the immune system genes analyzed was limited. Given their key role in TB pathogenesis, additional TB risk variants, haplotypes, and allele combinations may exist.

A potential limitation of this study is the significant deviation from the HWE of the interacting SNP *TLR10* rs11096957 (Asn241His) in the controls. Such deviations can result from genotyping errors, recruiting biases, natural selection, or simply chance. We excluded genotyping errors by random re-genotyping of *TLR10* rs11096957 in 21% of the samples. In addition, our study design prevented the recruitment of relatives. Natural selection could be the reason for the observed deviation, which may be supported by the evidence of similar heterozygosity deficiency in Toscani in Italy, TSI (HWE *P* value = 0.02; 1000 Genomes Project data), and considerable intra-population variation of SNP *TLR10* rs11096957 within Europe (G allele frequency range: 32.4% in British, GBR to 48.6% in Iberian population, IBS; 1000 Genomes Project data). Moreover, the recruitment of healthy controls from TB communities used in this project may have a similar impact on *TLR10* to natural selection, contributing to the deficiency of rs11096957 heterozygotes. Taken together, these arguments justify the inclusion of *TLR10* rs11096957 in association tests.

## Conclusion

In the current study, we again found a significant association between rs1800629 in the *TNF* gene and pulmonary TB. In addition, haplotypes in the gene cluster *TLR1-TLR6-TLR10* showed a weakly yet suggestive association. Furthermore, we provide convincing statistical evidence for a synergistic effect between polymorphic variants in the *TLR6* and *TLR10* genes on TB risk, which further supports the importance of TLR signaling in the genetic basis of TB and the concept of genetic interactions accounting for missing heritability. Further studies are warranted to validate the interaction between *TLR6* and *TLR10* and to elucidate its role in TB risk, which would be beneficial for human health.

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## Declaration of conflicting interests

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.


## Ethics approval and consent to participate

The study was approved by the Research Ethic Committee of the Institute of Phthisiopneumology (Republic of Moldova) and was conducted in accordance with the Declaration of Helsinki. All participants gave their written informed consent.

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## Supplemental material

Supplemental material for this article is available online.

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