Original Article

Association of Mycobacterium infections in patients with Mendelian Susceptibility to Mycobacterium Disease (MSMD) with Venous Thromboembolism

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Running title: *MSMD in mendelian- Thromboembolism patients*

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, Typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1348-0421.12442

Abstract

Hypercoagulable state in Mendelian Susceptibility to Mycobacterium Disease (MSMD) patients who are prone to mycobacterium infection has been established in a few studies and thrombosis considered as a rare. In a case-control study, the prevalence of factor V Leiden (FVL), Prothrombin (PTH) G20210A and Methylenetetrahydrofolate Reductase (MTHFR) C677T, A1298C mutations, were investigated among mycobacterium infected patients. The study were compromised 30 patients with mycobacterium infections (Invasive, disseminated and/ or recurrent infections with Bacille Calmette-Guerin (BCG) or non Tuberculosis Mycobacterium (NTM) and Mycobacterium Tuberculosis (MTB), with positive result for Acid Fast Bacilli (AFB), and Tuberculin skin test (TST)) and 30 normal healthy controls. In this study, 66.7% (40 individual) of female and 33.3% (20 individual) of male were recruited for study. The age of patients was 3-70. The genotyping of targeted genes were done by Real-Time PCR and cytokine Tumor Necrosis factor (TNF)-α concentration was quantitated by a commercially available ELISA kit. Significant association was seen between mycobacterium infections and TNF-α production after stimulating Peripheral Blood Mononuclear cells (PBMCs) with Lipopolysacharid (LPS) alone, and Interferon (IFN)-γ plus LPS. Moreover, the genotyping analysis in studied population revealed a significant association between MTHFR c.677C>T (OR 3.28 95% CI 1.35 to 7.92 P value<.05), MTHFR c.1298A>C (OR 2.33 95% CI 1.10 to 4.93 P value<.05) with Mycobacterium infection in affected patients and as a result base on previously studies susceptibility to Venous Thromboembolisms (VTE). However, increased prevalence of the MTHFR C677T, A1298C mutations in mycobacterium infected patients were statistically significant compared to controls.

Key Words: MSMD, MTHFR A1298C, MTHFR C677T, TNF-α

Introduction

Studies of Mendelian Susceptibility to Mycobacterium Disease (MSMD) Syndrome demonstrated a complex molecular network involved in the immunity to mycobacterium infection. Thus understanding the molecular mechanisms underlying this complex process is critical for the development of effective prevention and therapeutic strategies due to controlling infection [1, 2]. Mycobacterium products can cause thrombosis by various mechanisms such as local invasion, venous compression [3] or by producing a transitory hypercoagulable state secondary [3-5]. Thrombotic risk is determined both by circumstancial factors (age, surgery, pregnancy, oral contraception, Air pollution, Metabolic syndrome and smoking and infections [5, 6], as well as by genetic predisposition [5, 7-9]. Recent studies have shown that haemostatic changes [4, 10] due to mutations in related genes such as C677T and A1298C in Methylenetetrahydrofolate Reductase (MTHFR); Factor V Leiden (FVL) and G20210A in Prothrombin (PTH) [11] are associated with deep vein thrombosis (DVT) in pulmonary tuberculosis [6, 12]. Congenital thrombophilia was account for 35% of the etiology of intra abdominal vein thrombosis in TB patients [13].These mutations increased the risk of thrombosis by diverse mechanisms. While the majority of known genetic defects within the blood coagulation cascade (protein S, protein C, antithrombin III) is rare, a G>A mutation (also known as R506Q or rs6025) at nucleotide position 1691 in the gene for coagulation FVL that causes resistance to activated protein C [11] is found at high frequency (20- 60%) in thrombosis patients [7-9]. Similarly, a further common point mutation in the 3´-untranslated region of the PTH-factor II gene (20210: G>A, rs1799963) has been reported to be associated with elevated plasma prothrombin levels and is estimated to increase the risk for venous thrombosis by 3 to 5-fold. Mutations in both genes result in an increased susceptibility to develop venous thrombosis (DVT) [11]. Another inherited risk factor for incidence of congenital thrombophilia is point mutations in MTHFR. Considering the central role of MTHFR in folate metabolism and in control of homocysteine (Hcy) level, Hcy is an important biomarker for vascular diseases [14]. It has shown that MTHFR mutations (C677T (rs1801133), A1298C (rs1801131)) correlate with reduced MTHFR enzyme activity [PMID 18483342]. MTHFR enzyme is involved in folate metabolism and can catalyzes 5, 10 methylenetetrahydrofolate to 5 metyltetrahydrofolate which necessary cofactor for the re-methylation of homocysteine [14]. Reduced enzyme activity due to these mutations able to increases level of plasma Hcy, and lead to vascular diseases (arterial thrombosis) [14].

Compound heterozygosity for both mutations of MTHFR (C677T, A1298C) associated with increased plasma Hcy levels and increased risk for arteriosclerotic coronary disease and VTE [11, 14] But heterozygoty for either c.677C>T, c.1298 A>C not accounts to increased risk factor for incidence of premature cardiovascular and venous thrombosis disease (VTE). In patient's carrying the 677T variant, 1298C has been shown increase in blood Hcy level and reduce enzyme activity to 40% of normal [15]. Additionally, mycobacterium infections one of the risk factors that can activate a clotting cascade and causes to development of the DVT as a state secondary [3] in people who have genetic background of thrombosis. Other hypothesis favoring a hypercoaguable state in patients with mycobacterium infections are a high frequency of anti-phospholipid antibodies, prothrombin deficiency, Rifampicin containing regimen [3].

The aim of present study was to investigate the role of a genetic background of VTE and co- incidence of mycobacterium infection (MTB, NTM) and their products to increase the risk for development of VTE in affected patients. In this study, we investigated whether common Single Nucleotide Polymorphisms (SNPs) in the thrombosis genes influence susceptibility mycobacterium infected patients to development of VTE. Recently, several studies have shown that inherited and acquired thrombophilias markedly increased the risk of VTE in MTB infected patients [3, 6]. However, all investigations did not confirm the association between the VTE and adverse infections with other mycobacterium species outcome.

For this reason, in the present study the frequency of 4 common thrombophilic mutations and their association with incidence, and recurrence of VTE among mycobacterium infected patients was examined.

Materials& methods

Subjects and study groups

The population studied consisted of 60 Iranian subjects including 30 MSMD suspected patients, aged ≤14 yrs (6 female, 7 male) and aged >14 yrs (14 female, 3 male) compare to 30 normal healthy controls, aged ≤14 yrs (7 female, 4 male) and aged >14 yrs (13 female, 6 male). Blood samples were collected in EDTA tubes. Informed consent was obtained from each subject. The samples were coded and stored at -20°C.

In vitro cytokine assays

A peripheral blood mononuclear cell (PBMCs) of the patients and healthy controls was prepared from EDTA isolated blood sample by differential centrifugation over Ficoll-Pague (Pharmacia) methods. PBMCs (10^6/ml) was plated in 1 ml of complete RPMI-1640 (Gibco, Invitrogen, Paisley, UK) containing penicillin-streptomycin (Life Thechnologies, USA) and 10% heat- inactivated human serum, onto 24-well plates. Selected plates were stimulated with 200 ng/ml Lipopolysaccharide (LPS; Sigma) alone and 5000 ul/ml Interferon-γ (IFN-γ; Gibco, USA) plus 200 ng/ml LPS for 18 h at 37°C, and then supernatants were collected in 1.5 ml tubes and frozen at -70°C until performing the cytokine assay [16].

Enzyme-Linked Immunosorbent Assay (ELISA)

Cultured supernatants were thawed, and the level TNF-α, was measured by use of ELISA kit. Paired antibodies were used to detect TNF-α (Invitrogen, R&D systems, USA). Optical density was determined for each well, at 450 nm for TNF-α, by use of an automated MPR4+ Micro plate ELISA reader (Hyperion, Germany).Standard curve was performed in parallel for determination of cytokine concentration.

DNA extraction

Human genomic DNA was extracted from PBMCs (5×10^6 cultured cells) by MagCore® Cultured cells DNA Kit (RBC Bioscience Corp, UK, Cartridge Code 110, Cat.No.MCC-01 // MCC-02). The quantity and quality of the extracted DNA were checked using UV absorption measurement at 260 nm and agarose gel electrophoresis, respectively.

Genotyping by Real- Time PCR

The genotyping of 4 SNPs including MTHFR c.677C>T and c.1298A>C, and FVL, PTH G20210A was performed in a single PCR reaction tube using AB system Real Time machine (ANALITICA Real quality RS-MTHFR C677T, A1298C (code RQ-S29, S31), FVL, PTH G20210A (code RQ-S25, S27)) according to the manufacturer`s instructions. The Real quality kits were capable of correctly determining the

genotype of all tested samples. The diagnostic sensitivity and specificity of the Real quality device was 100 %.

Statistical analysis

Some of data were collected in excel database and were converted for analysis using the SPSS statistical software package version 22. A p value of less than .05 (p-value <.05) was considered statistically significant. The other parameters reported in this paper were calculated using the web-based program SNP analyzer, which is freely available at http://www. Exonprimer.com / SNP analyzer/ case- control study, and http://www. Odds ratio calculator.com / med calc. For determination of significant differences in the allele and genotype frequencies, the odds ratio Altman, 1991 test was done. Odds ratio and 95% confidence intervals were calculated to assess quantitatively the degree of association between the gene polymorphisms in thrombosis factors and mycobacterium infections in Iranian patients [14].

Result

TNF –α level increased in MSMD patients

In this study, 66.7% (40 individual) of female and 33.3% (20 individual) of male were studied. The age of 24 participants (40%) were under 14 years and 36 (60%) were up to 14 years. Significant association was detected in (supernatants of cultured PBMCs) between mycobacterium infected patients and TNF-α concentration (P value 0.001).

PBMCs from MSMD patients when stimulated with LPS produced lower levels of TNF-α in comparison to healthy controls. Interestingly we show that the release of TNF-α in co-stimulation of LPS plus Interferon (IFN)-γ (P value 0.001) substantially in MSMD patients are comparable with healthy controls (Table 1).

Changes of gene phenotype of coagulator factor in Mycobacterium infected patients

There was no significant correlation between gender of the study population and mutations in FVL, PTH G20210A, and MTHFR C677T (TT genotype) and A1298C (CC genotype). On the other hand, there was no significant correlation between incidences of VTE in certain ages (Table 2). Furthermore, our data show that significant association between mycobacterium infections and age of patients (P value 0.001) (Table 3).

Also, according to negative correlation index significant association between MTHFR C677T and A1298C mutations in case-control group were seen but not in PTH G20210 and FVL (Table4). 58.3% of patients and controls were normal for MTHFR C677T, 66.7% have heterozygote mutation for MTHFR A1298C, 95%, 91.7% normal for FVL and PTH G20210A, respectively. The Hardy-Weinberg equilibrium analysis for MTHFR A1298C, (p value= 0.010, p value= 0.018) revealed that both of case and control group have an unbalanced distribution as result of small population size and sampling limitations. Although for the other three SNPs including MTHFR C677T, FVL, PTHG20210A, Hardy-Weinberg equilibrium analysis shows a balanced distribution in case and control group (Supp. Table S1 and Table 5). Evidence suggested that the MTHFR polymorphisms might contribute to increase VTE in mycobacterium infected patients. A significant increase in VTE risk was found among carriers of the 677CT (C>T), 1298 AC (A>C) mutations (odds ratio 3.28, 95% confidence interval 1.35 to 7.92 with a significant trend p value= 0.006 and odds ratio 2.33 95% confidence interval 1.10 to 4.93 p value= 0.025), respectively compared with those with the 677CC, 1298AA (normal) genotypes. Statistically significant odds ratios were also found in patients homozygous for MTHFR 677TT, 1298CC, who have a 19.2, 25 fold higher risk of developing VTE (95% confidence interval=1.00 to 369.22; p value= 0.006, 1.11 to 562.82; p value= 0.006) compared with the normal genotypes. In order to evaluate the allelic dominant-recessive interaction according to Armitage's trend test & Hardy-Weinberg equilibrium (Munich Test), allele frequency for both of MTHFR mutations (CT677, AC1298), shows significant association with mycobacterium infections (p value= 0.012, p value= 0.0042) but this association was not seen in FVL, PTH G20210 mutations (Supp. Table S1 and Table 5).

The evidence suggested that females more than males were normal for MTHFR C677T, FVL, PTH G20210 mutations in case – control group (≤14,>14). Most common mutation was seen in MTHFR A1298C (mean 0.80) in two groups and heterozygous MTHFR A1298C mutation was found in 30 female

which 23 up to 14 (Table 6). Also, there was no significant association (p value >0.05) between this mutation and sex of MSMD suspected patients and controls. A/C MTHFR1298 mutation has seen in 80% of individuals in two groups. Factor V Leiden, PTH 20210 was normal in groups and there was no significant association between coagulation factors and gender of subjects as described earlier (Table 2).

Surprisingly, we have found in mycobacterium infected patients simultaneously mutations of coagulation factors. In this line, two of 13 MTB patients simultaneously had compound heterozygotyfor MTHFR C/T677, A/C 1298 and 4 MTB patients simultaneously had heterozygote mutations for MTHFR C/T677, A/C 1298, and PTH G20210A. Besides 2 MTB patients had heterozygote mutation for MTHFR A/C 1298, mutant mutation for MTHFR 677 T/T and 1 patient have compound heterozygote mutations for MTHFR A/C 1298 and FVL. Among 9 MSMD patients with NTM infections (6 cases with BCGosis, 1 case with M. Chelonei, 1 case with M.Fortitum and 1 case with M. Simiyaei), two (1 with M. Simiyaei, 1 with BCGosis) were compound heterozygote for both MTHFR A/C 1298, C/T 677 and one (BCGosis) had MTHFR (C/C 1298, C/T 677) genotype. Among of 3 MSMD patients with TB, one patient has compound heterozygote for both mutation of MTHFR. One of 4 MSMD patients with dual infection (MTB, NTM), was compound heterozygote for MTHFR A/C 1298 and C/T 677 and one patient was heterozygote for MTHFR A/C 1298 and mutant genotype (T/T) for MTHFR 677. Compound heterozygoty for MTHFR A/C 1298 and C/T 677 was seen in one of 30 healthy controls (Table 7).

Discussion

Deficiencies of thrombosis factors including; Antithrombin III, Protein C and Protein S, and the FVL mutation, the PTH gene variant and bi-allelic mutations in the MTHFR are responsible for about 35% percent of the etiology of intra abdominal vein thrombosis in mycobacterium infected patients [13]. Thus, the incidence of congenital thrombophilia in TB and other mycobacterium infected patients with defect in the production of Interferon (IFN)-γ or Tumor Necrosis factor (TNF)-α, as well as in the Interleukin-12 Receptor (IL-12R)-β1 and Interferon γ Receptor (IFN-γR) have to study [13, 17]. Clinical mycobacterium infection has now been described in a number of patients with IL-12/IL-23 & IFN-γ system defects [13].Mycobacterium products could be able to increase the risk for development of VTE (3-10% due to

homeostatic changes induced by the acute inflammatory response) [12]. Besides, emerging data indicated to co-incidence of Mycobacterial infection have several possibility mechanisms that can induce a hypercoagulable state secondary may lead to thromboembolic complications by various pathway. In addition, in the pathogenesis of coagulation disorders in mycobacterium-infected patients pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α induces the healthy non-thrombogenic internal surface and alter endothelial cell surface to the thrombogenic surface, which leads to development of local thrombosis [18]. It has been reported that MTB induces the expression of tissue factor in monocyte –macrophages, that is, a primary activator of clotting cascade for induction of thrombophilia complication [6]. This activation could able to stimulate the hepatic synthesis of coagulation proteins as well.

However, in this line, systemic hematological complications, such as disseminated intra vascular coagulation (DIC) and DVT, association to pulmonary tuberculosis (PTB) has been reported. Because of Thromboembolic complications associated with infection by MTB have been reported in the literature that occurred in 1.5–3.4% of TB patients [3]. In-Hospital morbidity and mortality of patients with both active TB and DVT (11/72 [15%]) was higher than mortality of patients with only active TB [6, 19]. The results of studying on common polymorphisms in morocco population showed that a significant association of 2 SNPs of the IL-12R-β1 gene (c.-2C>T,c.-111A>T) with PTB in MSMD patients [20]. This result suggests that IL12RB1 polymorphisms might play role as a risk factor for the development of PTB as we saw PTB in 3 IL-12Rβ1 deficiency patients who had (c.-2C>T,c.-111A>T) polymorphisms exception with 1 patient had compound heterozygote for both mutation of MTHFR. Moreover, the incidence of pulmonary infections with NTM in HIV negative patients due to these is increasing. Some researchers found 35 PTB patients and DVT. In 33 of them, DVT occurred 7 days after the diagnosis of TB, while only 2, DVT was the presenting feature [3, 4]. Besides of loop, increased plasma fibrinogen and impaired fibrinolysis in active PTB associated with decreased levels of antithrombin III and reactive thrombocytosis causes to development of the DVT [18].

Other hypothesis favoring a hypercoaguable state in TB is a high frequency of anti-phospholipid antibodies [3]. Some studies indicate that PTH deficiency occurs in approximately one third of TB patients which leads to the hyperactivity of prothrombin [3].

Additionally, it has been shown that Rifampicin containing regimen in mycobacterium-infected patients should be considered. It seems that Rifampicin effects on cytochrome P450 pathways which triggering to

the development of hyper-coagulable state (a relative risk of 4.74) [3, 19] with decreasing production and increasing clearance of anticoagulant hepatic proteins [4].

As VTE can be fatal (Nearly two thirds of all VTE events result from hospitalization, and approximately 300,000 of these patients die), early diagnosis and institute prompt treatments is crucial. Thus, now in mycobacterium-infected patient's development of the VTE much more appreciated. In the current study, we aimed to investigate a role of coagulation dysregulation in the mycobacterium species infected patients who were suspected to MSMD. In this line, we determined the functional and genetically of IFN-γ/IL-12 axis infected patients. Besides, the mutations of some important coagulation factors including MTHFR, FVL, and PTH were studied. There is now accumulating data for a role of thrombotic mechanism in the development of VTE. In other words, all these conditions associated with thrombotic complications in MTB infected patients. Since our series does not include adequate number of case in each subgroup (≤14,>14), we decided to collect all cases associated with mycobacterium diseases under a common title: MSMD suspected patients. Although mycobacterium species involves a stimulation of hepatic synthesis of coagulation proteins, but their association with VTE in other mycobacterium species remains controversial.

To our knowledge, MTHFR c.677C > T and c.1298A > C, and FVL, PTH G20210A in the MSMD suspected patients were not previously genotyped in Iranian mycobacterium infected patients. After 4-year follow-up in a case-control study, 30 newly diagnosed of patients with disseminated and local mycobacterium infections and 30 healthy controls conducted in one geographical area of Iran. Sixteen of the 30 infected patients were found suffered from MSMD (IL-12Rβ1, IFN-γR1, IFN-γR2, TYK-2 deficiencies) syndrome, 4 of 16 MSMD patients simultaneously have dual infection (NTM, MTB), the rest of them, just were infected with one of infectious agents (NTM or MTB) (Table3).

Generally, the analysis results show that MTHFR polymorphisms including (rs1801133 (MTHFR C677T), rs1801131 (MTHFR A1298C)), P value <.05 (0.012, 0.0042), have significant contribution in VTE risk rising in mycobacterium-infected patients (Supp. Table S1 and Table 5). The Altman and Armitage's trend test odds ratio, revealed that there is a significant association between VTE incidence due to MTHFR c.677C>T (OR 3.28 95% CI 1.35 to 7.92, P value <0.05), MTHFR c.1298A>C (OR 2.33 95% CI 1.10 to 4.93, P value <0.05) mutations and Mycobacterium infections in affected patients. (Supp. Table S1 and 8-

9). It should be noted, all of these analyses represent a significant increase in VTE risk due to MTHFR polymorphisms. On the other hand, the different amounts of p-values < .05 in (Table 5,8-9) are dependent on the parameters power and since in each of these analyses, parameters are different so the emphasis on each method of parameters will determine the amount of significant different p-values.

Furthermore, some of infected patients simultaneously have several mutations in coagulations factors. In conclusion, our findings indicate that VTE was increased ~1.6x and ~3.6x for heterozygous (P value= 0. 372, p value= 0.042) MTHFR rs1801133 C677T, rs1801131 A1298C and ~19.2x and ~25x for homozygous rs1801133 (T), Rs1801131(C) genotypes (P value=0.006, P value=0.006), respectively (Supp. Table S1). If a carrier of an rs1801133 (T), Rs1801131(C) SNPs risk allele were also to be a carrier of another risk alleles of SNPs including rs6025 (FV L) or Rs1799963 (PTH G20210A), as a predictable outcome the risk for VTE will be even higher. Generally, the VTE risks of infected patients were increased with genotypes associated with reduced MTHFR activity. In conclusion we could drive statement about mycobacterium tuberculosis beyond mysteries whether considered in pathogenesis of pulmonary disease such as sarcoidosis [21], or as we show here has association with Venous Thromboembolism.

Acknowledgement

The authors would like to thanks all of patients and their families, healthy controls, and all clinicians and physicians who collaborated with Iranian society of immunology and genetic department, Shahid Beheshti medical University of Science, Tehran, Iran. Dr Maryam Alinejad received personnel assistance from Iranian society of immunology and genetic department, Shahid Beheshti medical University of Science, Tehran, Iran.

Disclosure

The authors declare that they have no conflict of interest.

References

1. Qu, H.-Q., Fisher-Hoch, S. P. & McCormick, J. B. (2011) Molecular immunity to mycobacteria: knowledge from the mutation and phenotype spectrum analysis of Mendelian susceptibility to mycobacterial diseases. Int J Infec Dis, **15**: e305-e313.

2. Tabarsi, P., Marjani, M., Mansouri, N., Farnia, P., Boisson-Dupuis, S., Bustamante, J., Abel, L., Adimi, P., Casanova, J.-L. & Mansouri, D. (2011) Lethal tuberculosis in a previously healthy adult with IL-12 receptor deficiency. J clin immunol, **31**: 537-539.

3. Kouismi, H., Laine, M., Bourkadi, J.-E. & Iraqi, G. (2013) Association of deep venous thrombosis with pulmonary tuberculosis. Egypt J Chest Dis Tuberc, **62**: 541-543.

4. Goncalves, I. M., Alves, D. C., Carvalho, A., do Ceu Brito, M., Calvario, F. & Duarte, R. (2009) Tuberculosis and Venous Thromboembolism: a case series. Cases J, **2**: 9333.

5. Previtali, E., Bucciarelli, P., Passamonti, S. M. & Martinelli, I. (2011) Risk factors for venous and arterial thrombosis. Blood Transfus, **9**: 120-138.

6. Dentan, C., Epaulard, O., Seynaeve, D., Genty, C. & Bosson, J.-L. (2014) Active tuberculosis and venous thromboembolism: association according to international classification of diseases, ninth revision hospital discharge diagnosis codes. Clin infect dis, **58**: 495-501.

7. Koster, T., Rosendaal, F. R., Dirven, R. J., de Ronde, H., van der Velden, P. & Reitsma, P. (1994) Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature, **369**: 6467.

8. Poort, S. R., Rosendaal, F. R., Reitsma, P. H. & Bertina, R. M. (1996) A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. Blood, **88**: 3698-3703.

9. Frosst, P., Blom, H. J., Milos, R., Goyette, P., Sheppard, C. A., Matthews, R. G., Boers, G. J., den Heijer, M., Kluijtmans, L. A. & Van den Heuvel, L. (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet, **10**: 111–113.

10. Sarkar, S., Saha, K., Maikap, M. K. & Jash, D. (2012) Deep Vein Thrombosis: A Rare Association with Tuberculosis. J Med, **13**: 106-108.

11. Ulukus, M., Eroglu, Z., Yeniel, A., Toprak, E., Kosova, B., Turan, O. & Ulukus, M. (2006) Frequency of Factor V Leiden (Gc1691G> A), prothrombin (G20210A) and methylenetetrahydrofolate reductase (C677T) genes variants in woman with adverse pregnancy outcome. J Turkish-German Gynecol Assoc, **7**: 195-201.

12. Akpek, G., Hematologic findings in mycobacterial infections among immunosuppressed and immunocompetent patients. 2004: Springer Publisher pp. 213-230.

13. Akar, H., Kose, M., Ceylan, O., Patiroglu, T., Bustamante, J., Casanova, J., Akyildiz, B. & Doganay, S. (2014) Congenital IL-12R1B receptor deficiency and thrombophilia in a girl homozygous for an IL12RB1 mutation and compound heterozygous for MTFHR mutations: A case report and literature review. Eur J Microbiol Immunol, **4**: 83-87.

14. Lajin, B., Alachkar, A. & Sakur, A. A. (2012) Triplex tetra-primer ARMS-PCR method for the simultaneous detection of MTHFR c. 677C> T and c. 1298A> C, and MTRR c. 66A> G polymorphisms of the folate-homocysteine metabolic pathway. Mol cell probes, **26**: 16-20.

15. Rozycka, A., Jagodzinski, P. P., Kozubski, W., Lianeri, M. & Dorszewska, J. (2013) Homocysteine level and mechanisms of injury in Parkinson's disease as related to MTHFR, MTR, and MTHFD1 genes polymorphisms and L-Dopa treatment. Curr genomics, **14**: 534.

16. Holland, S. M., Dorman, S. E., Kwon, A., Pitha-Rowe, I. F., Frucht, D. M., Gerstberger, S. M., Noel, G. J., Vesterhus, P., Brown, M. R. & Fleisher, T. A. (1998) Abnormal regulation of interferon-γ, interleukin-12, and tumor necrosis factor-α in human interferon-γ receptor 1 deficiency. J Infect Dis, **178**: 1095-1104.

17. Edeer Karaca, N., Boisson-Dupuis, S., Aksu, G., Bustamante, J., Kandiloglu, G., Ozsan, N., Hekimgil, M., Casanova, J.-L. & Kutukculer, N. (2012) Granulomatous skin lesions, severe scrotal and lower limb edema due to mycobacterial infections in a child with complete IFN-γ receptor-1 deficiency. Immunother, **4**: 1121-1127.

18. Muley, p., Shah, U., Shah, V., Gandhi, D. . (2014) Deep vein thrombosis with tuberculosis: a rate presentation of common disease. GJMEDPH, **3**: 1-4.

19. Marjani, M., Tabarsi, P., Baghaei, P., Shamaei, M., Biani, P. G., Mansouri, D., Masjedi, M. R. & Velayati, A. A. (2012) Incidence of Thromboembolism in Hospitalized Patients With Tuberculosis and Associated Risk Factors. Archives of Clinical Infectious Diseases, **7**: 56-9.

20. Vosse, E., Haverkamp, M. H., Ramirez-Alejo, N., Martinez-Gallo, M., Blancas-Galicia, L., Metin, A., Garty, B. Z., Sun-Tan, Ç., Broides, A. & Paus, R. A. (2013) IL-12Rβ1 Deficiency: Mutation Update and Description of the IL12RB1 Variation Database. Hum mutat, **34**: 1329-1339.

21. Mortaz, E., Adcock, I. M. & Barnes, P. J. (2014) Sarcoidosis: Role of non-tuberculosis mycobacteria and Mycobacterium tuberculosis. International journal of mycobacteriology, **3**: 225-229.

Supplemental Table1. Evaluation of the allelic Dominant-Recessive interaction according to Armitage's trend test.

Abbreviation list

DIC: Disseminated Intra vascular Coagulation

DTB: Disseminated Tuberculosis

DVT: Deep Venous Thromboembolism

FVL: Factor V Leiden

IFN-γ R: Interferon –γ Receptor

IL-12Rβ1: Interlukin-12 Receptor β1

MSMD: Mendelian Susceptibility to Mycobacterium Disease

MTB: Mycobacterium Tuberculosis

MTHFR: Methylenetetrahydrofolate Reductase

NTM: Non Tuberculosis Mycobacterium

PBMC: Peripheral Blood Mononuclear Cell

PTB: Pulmonary Tuberculosis

PTH: Prothrombin

TNF-α: Tumor Necrosis Factor- α

VTE: Venous Thromboembolism

Table1. TNF-α production from stimulated peripheral blood mononuclear cells (PBMCs) in patients and controls group[†]

		patients(n = 30)‡	Controls (n = 30)	*P* value*
		Mean ± SD	**Mean ± SD**	
TNF-α§ (pg/ml)	LPS¶ (ng/ml)	521± 167.25	697 ± 162.901	0.001
	LPS+IFN-γ‼ (ul/ml)	857± 178.60	1218± 118.49	0.001

† Peripheral blood mononuclear cells (10^6) were co-culture with LPS (200 ng/ml) alone, and stimulated LPS (200 ng/ml) plus IFN-γ (5000 ul /ml) for 18 hr at 37 °C, then supernatants were collected and analyzed by ELISA.

‡ Data are presented as the number of individuals. Results are shown as mean± SD.

§ TNF-α, Tumor Necrosis Factor; ¶ LPS, Lipo polysaccharide; ‼IFN-γ, Interferon-γ

*P value <.05 was considered to be statistically significant (Student's t- test's)

Table2. Association of Thrombosis factors with gender and age of study population

	MTHFR† C677T	MTHFR A1298C	FVL‡	*PTH*§ *G20210A*

*P value for gender	.273	.076	1.00	.322
**P value for Age of onset (≤14, >14)	.318	.074	.558	.077

† MTHFR, Methylenetetrahydrofolate Reductase; ‡ FV-L, Factor V Leiden; § PTH, Prothrombin

* P value from multivariate analysis of thrombosis factor with gender of the study population.

**P value from multivariate analysis of thrombosis factor with age of the study population.

P value <.05 was considered to be statistically significant.

Table3. Association of Mycobacterium infections with Age of patients

	≤14	>14	Total
†NTM, ‡MTB, §DEFICIENCY	4	0	4
MTB,DEFFICIENCY	1	2	3
NTM, DEFFICIENCY	7	2	9
NTM	1	0	1
MTB	0	13	13
HEALTHY	11	19	30
*P Value			**.001**

† NTM, Non Tuberculosis Mycobacterium; ‡ MTB, Mycobacterium Tuberculosis; §Deficiencies like IL-12 receptor β1, IFN-γ receptor1&2,...deficiency in Mendelian Susceptibility to Mycobacterium Diseases(MSMD) patients**.**

*p-Value < .05 was considered as a significant.

Table4. Association of Thrombosis factors in case - control group

	MTHFR C677T	MTHFR	FVL	*PTH G20210A*

		A1298C		
P value*	**.024**	**.015**	1.000	.353

* P value from multivariate analysis of thrombosis factor in case and control group by using a chi -square test. P Value < .05 considered as a significant.

Bold numbers are considered as a significant.

Table 5. Genotype and allele frequencies for c.677C>T, c.1298A>C, and FVL, PTH G20210A in case and control group based on Armitage's trend test & Hardy-Weinberg equilibrium (Munich Test)

	MTHFR-C677T			MTHFR-A1298C			FV- Leiden			PTH-G20210A		
	Control NO=30	Case NO=30	Total [¶], No. (%)	Control NO=30	Case NO=30	Total, No. (%)	Control NO=30	Case NO=30	Total, No. (%)	Control NO=30	Case NO=30	Total, No. (%)
Normal[†]	21	14	35(58.3)	12	4	16(26.7)	28	29	57(95)	29	26	55(91.7)
Heterozygote[‡]	9	10	19(31.7)	18	22	40(66.7)	2	1	3(5)	1	4	5(8.3)
Mutant[§]	0	6	6(10)	0	4	4(6.7)	0	0		0		0
P value*		**0.012**			**0.0042**			0.55			0.16	
OR (95%CI)**		**7.86**			**12.4**			0.48			4.4	
HW*** value	**Case**	0.122			**0.010**			0.926			0.695	
	Control	0.333			**0.018**			0.850			0.926	

† Normal homozygote: MTHFR 677(C; C), MTHFR 1298(A; A), FV L (G; G), PTH (G; G)

‡ Heterozygote: MTHFR 677(C; T), MTHFR 1298(A; C), FV L (G; A), PTH (G; A)

§ Mutant homozygote: MTHFR 677(T; T), MTHFR 1298(C; C), FV L (A; A), PTH (A; A)

¶ Data are presented as the number of individuals (%)

[*]p value from multivariate analysis including normal homozygote, heterozygote (Hetero) and mutant homozygote Genotype of Thrombosis factors in study population based on Armitage's trend test& Hardy-Weinberg equilibrium (Munich Test). P Value < .05 considered as a significant. Bold numbers are considered as a significant.

[**]Odds ratio; 95% Confidence interval

[***]Tests for deviation from Hardy-Weinberg equilibrium (Munich Test). HWE shows the distribution and sampling accuracy.

Table6. The frequency of SNPs based on range of age and gender of study population

			Case-Control											
			Case				Control				Total			
			Range Of Age (Yr)				Range Of Age(Yr)				Range Of Age (Yr)			
			≤14		>14		≤14		>14		≤14		>14	
			Sex		sex		Sex		Sex		Sex		Sex	
			Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
SNPs														
MTHFR[¶]-677	Normal[†]		3	3	8	0	4	2	11	4	7	5	19	4
	Hetero[‡]	Count	1	2	4	3	3	2	2	2	4	4	6	5
	Mutant[§]		2	2	2	0	0	0	0	0	2	2	2	0
MTHFR-1298	Normal		2	2	0	0	3	3	2	4	5	5	2	4
	Hetero	Count	3	4	12	3	4	1	11	2	7	5	23	5
	Mutant		1	1	2	0	0	0	0	0	1	1	2	0
FVL[‼]	Normal		6	7	13	3	6	3	13	6	12	10	26	9
	Hetero	count	0	0	1	0	1	1	0	0	1	1	1	0
	Mutant		0	0	0	0	0	0	0	0	0	0	0	0
PTH[⁼]-20210	Normal		6	7	12	1	7	4	13	5	13	11	25	6
	Hetero	Count	0	0	2	2	0	0	0	1	0	0	2	3
	Mutant		0	0	0	0	0	0	0	0	0	0	0	0

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† Normal homozygote : MTHFR 677(C; C), MTHFR 1298(A; A), FV L (G; G), PTH (G; G)

‡ Heterozygote: MTHFR 677(C; T), MTHFR 1298(A; C), FV L (G; A), PTH (G; A)

§ Mutant homozygote: MTHFR 677(T; T), MTHFR 1298(C; C), FV L (A; A), PTH (A; A)

[¶] MTHFR, Methylenetetrahydrofolate Reductase; [‼] FV-L, Factor V Leiden; [¨]PTH, Prothrombin

Table7. The frequency of multiple thrombosis genes involved in Mycobacterium infected patients and healthy controls

Groups	MTHFR[¶] C/T 677, A/C 1298	MTHFR C/T 677, C/C 1298	MTHFR T/T 677 ,A/C 1298	MTHFR C/T 677, A/C 1298, PTH [‼] G20210A	MTHFR A/C 1298, FVL[¨] G/A	Total
NTM[†], MTB[‡], Deficiency[§]	1	-	1	-	-	4
MTB, Deficiency	1	-	-	-	-	3
NTM, Deficiency	2	1	-	-	-	9
NTM	-	-	-	-	-	1
MTB	2	-	2	4	1	13
Healthy control	1	-	-	-	-	30

[†] NTM, Non Tuberculosis Mycobacterium; [‡] MTB, Mycobacterium Tuberculosis; [§] Deficiencies like IL-12 receptor β1, IFN-γ receptor1&2,...deficiency in Mendelian Susceptibility to Mycobacterium Diseases (MSMD) patients; [¶] MTHFR, Methylenetetrahydrofolate Reductase; [‼] PTH, Prothrombin; [¨]FV-L, Factor V Leiden.

Table8. The frequency of various genotypes in Mycobacterium infected patients and controls according to Armitage's trend test (Munich)

GROUP	MTHFR[¶]-C677T			MTHFR-A1298C			FVL“			PTH[‼] 20210			Total
	CC	CT	TT	AA	AC	CC	GG	GA	AA	GG	GA	AA	
NTM[†],MTB[‡],deficiency[§]	2	1	1	0	4	0	4	0	0	4	0	0	4
MTB, deficiency	1	1	1	1	1	1	3	0	0	3	0	0	3
NTM, deficiency	4	3	2	3	4	2	9	0	0	9	0	0	9
NTM	1	0	0	0	1	0	1	0	0	1	0	0	1
MTB	6	5	2	0	12	1	12	1	0	9	4	0	13
HEALTHY	21	9	0	12	18	0	28	2	0	29	1	0	30
TOTAL	35	19	6	16	40	4	57	3	0	55	5	0	60
OR* (95% CI)	**3.28 (1.35-7.92)**			**2.33 (1.10 -4.93)**			0.49 (0.043-5.57)			4.21(0.457-38.86)			
P value**	**0.006**			**0.025**			1.03			0.369			

[†] NTM, Non Tuberculosis Mycobacterium; [‡] MTB, Mycobacterium Tuberculosis; [§] Deficiencies like IL-12 receptor β1, IFN-γ receptor1&2,...deficiency in Mendelian Susceptibility to Mycobacterium Diseases(MSMD) patients; [¶] MTHFR, Methylenetetrahydrofolate Reductase; ‼ PTH, Prothrombin; “FV-L, Factor V Leiden

*OR (95% CI) ‡ Odds ratio; 95% Confidence interval. Data in parentheses are 95% confidence intervals. Calculation of the 95% confidence interval was performed using the Armitage's trend test.

**P Value < .05 considered as a significant.

Bold numbers are considered as a significant

Table9. Genotype and allele frequencies of FVL, PTH G20210A, MTHFR C677T and A1298 C Mutations according to Altman Odds ratio 1991.

SNPs	Allele frequencies	Mycobacterium infected patients (%) †	Control (%)	OR (95% CI) ‡	*P* Value*
	C	38 (63.3)	51 (85)		
MTHFR §C677T	T	22 (36.7)	9 (15)	**3.28 (1.35 to 7.92)**	**0.008***
	A	30 (50)	42 (70)		
MTHFR A1298C	C	30 (50)	18 (30)	**2.33 (1.10 to 4.93)**	**0.026***
	G	59 (98.3)	58 (96.6)		
FVL¶	A	1 (1.7)	2 (3.4)	0.49 (0.043 to 5.57)	0.566
	G	56 (93.3)	59 (98.3)		
PTH‖G20210A	A	4 (6.7)	1 (1.7)	4.21 (0.457 to 38.86)	0.20
Genotype frequencies					
	CC	14 (46.7)	21 (70)		
MTHFR C677T	CT	10 (33.3)	9 (30)		
	TT	6 (20)	0	16.18 (0.86 to 301.6)	0.06
	AA	4 (13.3)	12 (40)		
MTHFR A1298C	AC	22 (73.3)	18 (60)		
	CC	4 (13.3)	0	10.3 (0.532 to 201.4)	0.12
	GG	29 (96.7)	28 (93.3)		
FVL	GA	1 (3.3)	2 (6.7)		
	AA	0	0	1.00 (0.019 to 52.03)	1.00
	GG	26 (86.7)	29 (96.7)		
PTH G20210A	GA	4 (13.3)	1 (3.3)		
	AA	0	0	1.00 (0.019 to 52.03)	1.00

† Data are presented as the number of individuals (%)

‡ Odds ratio; 95% Confidence interval. Data in parentheses are 95% confidence intervals. Calculation of the 95% confidence interval was performed using the Altman Odds ratio.

§ MTHFR, Methylenetetrahydrofolate Reductase; ¶FV-L, Factor V Leiden; ‼ PTH, Prothrombin

*P Value < .05 considered as a significant. Bold numbers are considered as a significant