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# The importance of *MTHFR* C677T/A1298C combined polymorphisms in pulmonary embolism in Turkish population

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ABSTRACT

*Background and objective:* Pulmonary embolism (PE) is an important cardiovascular emergency with high mortality. There are still problems related to the diagnosis of PE and genetic research may play a key role on diagnosis as well as determining risk stratification. In the present study, the aim was to evaluate *MTHFR* C677T and A1298C polymorphisms that play a role on folate metabolism in PE patients.

*Materials and methods:* A total of 118 PE patients and 126 controls were enrolled in the current study. Genomic DNA was isolated and genotyped using polymerase chain reaction (PCR) analyses for the *MTHFR* C677T and A1298C polymorphisms.

*Results:* There was no association between clinical and demographic characteristics of PE patients and both *MTHFR* C677T and A1298C polymorphisms. Allele frequencies showed a significant difference between patients and controls. T allele frequency was significantly higher in the patients' group than the control group. There was an association between PE and combined *MTHFR* C677T and A1298C polymorphisms.

*Conclusion:* We found an association between *MTHFR* C677T/A1298C combined mutations and PE in the Turkish population. Future genetic studies investigating combined mutations could be very helpful to identify risk population in PE.



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## 1. Introduction

Pulmonary embolism (PE), a serious clinical manifestation of venous thromboembolism (VTE), is commonly seen cardiovascular disease with high mortality rate [1]. PE is a multifactorial disease that includes genetic and environmental factors together on the pathogenesis. The importance of genetic factors was presented in recent years [2]. As an example; protein C, protein S and antithrombin deficiencies were identified in 1965 [3]. However, genetic screening has not been exactly clarified yet in clinics according to our knowledge.

The increased level of homocysteine (hyperhomocysteinemia) is a strong risk factor for thrombosis and it is influenced by genetic factors. Methylene-tetrahydrofolate reductase (*MTHFR*) is an important enzyme that regulates folate metabolism due to affect DNA methylation and nucleic acid synthesis [4]. *MTHFR* catalyzes the reduction of 5,10 methylenetetrahydrofolate (5,10-MTHF) to 5 methyltetrahydrofolate (5-MTHF). 5-MTHF is a methyl donor for the homocysteine-to-methionine remethylation and circulatory form of folate. The reduction of *MTHFR* activity causes hyperhomocysteinemia [5]. The gene for *MTHFR* is located on chromosome 1 at 1p36.3 and consists of 11 exons [6]. *MTHFR* gene includes two common polymorphisms – C677T (ALA222VAL) and A1298C (GLU429ALA) polymorphisms [7]. These polymorphisms are correlated with reduction on *MTHFR* enzyme activity. With this way, these polymorphisms play a role on pathogenesis of thrombosis. There are several studies that present influence of MTHFR polymorphisms on diseases such as ischemic stroke [8], obstetrical pathologies [9], arterial and venous thrombosis [10,11] and metabolic diseases [12].

There are still some problems in the diagnosis stage of PE due to variety of symptoms particularly in emergency departments [3]. CT scans are reliable on diagnosis but it includes some risks as allergic reactions or contrast-induced nephropathy. Also, it includes downsides as its cost and exposure of radiation [13]. Besides variety of symptoms, subclinical cases may be seen in PE [14]. Both of them lead to an increase suspicion of pulmonary embolism in ED. Some risk scoring systems as Wells and Geneva are identified due to risk stratification for PE [15]. Additionally, especially the negativity of D-dimer may estrange from doubt of PE [16,17]. Nonetheless, there is an increase to use CT scans in ED [13]. All these mentioned factors increase importance of genetic researches due to finding possible diagnosis marker or determine patients with genetic predisposition to PE. Additionally, it may provide prophylaxis to the risk population and it will help decrease the incidence of PE. To the best of our knowledge, in literature, the effects of *MTHFR* polymorphisms in PE were shown mostly in case reports and thus, there is limited information about this issue [18]. This study aimed to evaluate these polymorphisms in PE patients in our population.

## 2. Materials and methods

### 2.1. Subjects

The study group consisted of 118 unrelated patients with PE (61 male and 57 female; mean age: 58.21 ± 10.531 standard deviation [SD] years), and 126 (74 male and 52 female; mean age: 55.98 ± 7.773 SD years) unrelated healthy controls. PE patients were recruited consecutively and prospectively from those whom were treated and followed up in the Emergency Medicine Department of Gaziosmanpasa University Research Hospital, Tokat, Turkey. The diagnosis of PE was confirmed with thorax computed tomography pulmonary angiography (CTPA) by experienced emergency physicians and also radiologists. All control subjects were confirmed to be free from VTE, coronary artery disease, malignancy, pregnancy, previous surgery and stroke. Controls with family history of any evidence for thrombosis and women controls with prior history of abortions or other obstetric complications were excluded from the study. All participants, patients and healthy controls, were of Turkish origin, from the inner Central Black Sea region of Turkey. The healthy controls matched for age and gender with PE patients. The study protocol was approved by the Local Ethics Committee of Gaziosmanpasa University, Faculty of Medicine and written informed consent was obtained from the study participants.

### 2.2. Genotyping

Genomic DNA was extracted from whole venous blood samples using a commercial DNA isolation kit (Sigma–Aldrich, Taufkirchen, Germany). The *MTHFR* gene C677T (Ala222Val, rs1801133) and A1298C (Glu429Ala, rs1801131) polymorphisms were analyzed by polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assay. The *MTHFR* C677T polymorphism was analyzed by using forward (F) 5′-TGA AGG AGA AGG TGT CTG CGG GA-3′ and reverse (R) 5′-AGG ACG GTG CGG TGA GAG TG-3′ primers. The amplification conditions consisted of an initial melting step of 5 min at 94 °C; followed by 35 cycles of 30 s at 94 °C, 30 s at 61 °C, and 30 s at 72 °C; and a final elongation step of 5 min at 72 °C. After amplification, the 198 bp PCR product was digested with HinfI. The digestion products were separated on 3% agarose gels, and fragments stained with the ethidium bromide were photographed on an ultraviolet transilluminator. Wild type (CC) individuals were identified by only a 198 bp fragment, heterozygotes (CT) by both the 175/23 bp and 198 bp, and homozygote variants (TT) by the 175/23 bp. For *MTHFR* A1298C polymorphism, amplification was carried out using primers (F: 5′-CTT TGG GGA GGT GAA GGA CTA CTA C-3′ and R: 5′-CAC TTT GTG AGC ATT CCG GTT TG-3′) and the protocol described previously [19]. The amplified 256 bp product was digested with MboII. Wild type (AA) was identified by 4 fragments (176, 30, 28, and 22 bp), heterozygous AC by 5 fragments (204, 176, 30, 28, and 22 bp) and homozygous variant by 3 fragments (204, 30, and 22 bp). The major visible bands were 204 and 176 bp. Second PCR was performed to confirm samples whose results were not clear.

### 2.3. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (IBM SPSS Statistics, version 20) and OpenEpi Info software package version 3.01 (www.openepi.com). The chi-square ($\chi^2$) test was used to evaluate the Hardy–Weinberg equilibrium (HWE) for the distribution of

the genotypes of the patients and the controls. The relationships between *MTHFR* gene C677T and A1298C polymorphisms and the clinical and demographical characteristics of patients were analyzed by using $\chi^2$ test or analysis of variance (ANOVA) statistics. $\chi^2$ test and Fisher's exact test were used to compare categorical variables appropriately. Odds ratio (OR) and 95% confidence interval (CI) were used for the assessment of risk factors. All p values were 2-tailed and p values less than 0.05 were considered as significant.

## 3. Results

Clinical and demographical characteristics of PE patients (gender, age, SatO2, WBC, RDW, HTC, previous surgery, smoking, comorbitidy, ECG abnormalities, thorax CT) stratified according to *MTHFR* gene polymorphisms were shown in Tables 1 and 2. There were no significant association between clinical and demographical characteristics of PE patients and the *MTHFR* gene C677T and A1298C polymorphisms ($p > 0.05$) (Tables 1 and 2).

Allelic and genotypic distributions of the *MTHFR* gene C677T and A1298C polymorphisms are shown in Table 3. Genotype frequencies did not show any significant differences between patients and controls according to *MTHFR* gene C677T and A1298C polymorphisms ($p > 0.05$) (Table 3). However, allele frequencies of C677T polymorphism showed a statistically significant difference between patients and controls ($p = 0.042$, OR: 1.57, 95% CI: 1.01–2.43). T allele frequency was significantly higher in the patients' group than the control group. A significant difference was not observed for the allele frequencies of A1298C polymorphism between patients and controls (Table 2).

We also examined the risk associated with inheriting the combined genotypes for the two polymorphisms (Table 4). According to these results, individuals who were CC homozygous at C677T locus and AA homozygous at A1298C locus have a lower risk of developing PE ($p = 0.031$). The observed and expected frequencies of both the *MTHFR* gene polymorphism were in Hardy–Weinberg equilibrium in control and patient groups.

## 4. Discussion

In the present study, it was found that there was no association between clinical and demographical characteris-

**Table 1 – Clinical and demographical characteristics of PE patients stratified according to *MTHFR* gene C677T polymorphisms.**

| Characteristics | Total $n = 118$ | C677T | | | P value |
|---|---|---|---|---|---|
| | | CC $n = 69$ | CT $n = 38$ | TT $n = 11$ | |
| Gender, male/female, *n* (%) | 61/57 (51.7/48.3) | 38/31 (55.1/44.9) | 20/18 (52.6/47.4) | 3/8 (27.3/72.7) | 0.228 |
| Age, years | 58.21 ± 10.531 | 58.26 ± 10.681 | 58.74 ± 10.882 | 56.09 ± 8.814 | 0.766 |
| $SatO_2$, % | 90.41 ± 5.009 | 90.06 ± 4.810 | 90.50 ± 5.788 | 92.37 ± 2.446 | 0.479 |
| WBC, per $mm^3$ | 9932.7 ± 2815.3 | 9709.4 ± 2678.9 | 10,203.0 ± 3116.7 | 10,488.9 ± 2661.5 | 0.582 |
| RDW, % | 16.38 ± 2.580 | 16.35 ± 2.487 | 16.39 ± 2.819 | 16.52 ± 2.543 | 0.983 |
| Htc, % | 38.41 ± 4.163 | 38.54 ± 3.959 | 38.62 ± 4.465 | 36.68 ± 4.475 | 0.429 |
| Surgery, *n* (%) | 3 (2.5) | 3 (100) | 0 | 0 | 0.335 |
| Smoking, *n* (%) | 67 (56.8) | 40 (59.7) | 20 (29.9) | 7 (10.4) | 0.772 |
| Comorbitidy, *n* (%) | | | | | |
| Coronary artery disease | 43 (36.4) | 28 (65.1) | 12 (27.9) | 3 (7.0) | 0.523 |
| Diabetes mellitus | 42 (35.6) | 23 (54.8) | 15 (35.7) | 4 (9.5) | 0.816 |
| Hypertension | 62 (52.5) | 36 (58.1) | 21 (33.9) | 5 (8.1) | 0.844 |
| Chronic renal failure | 15 (12.7) | 11 (73.3) | 4 (26.7) | 0 | 0.299 |
| Cerebrovascular disease | 8 (6.8) | 7 (87.5) | 1 (12.5) | 0 | 0.215 |
| Malignancy | 10 (8.5) | 6 (60.0) | 3 (30.0) | 1 (10.0) | 0.987 |
| ECG abnormalities, *n* (%) | | | | | |
| RBBB | 19 (16.1) | 10 (52.6) | 8 (42.1) | 1 (5.3) | 0.543 |
| Sinusal tachycardia | 42 (35.6) | 23 (54.8) | 14 (33.3) | 5 (11.9) | 0.724 |
| S1Q3T3 | 33 (28.0) | 21 (63.6) | 10 (30.3) | 2 (6.1) | 0.676 |
| AF | 31 (26.3) | 19 (61.3) | 10 (32.3) | 2 (6.5) | 0.807 |
| Ischemia | 16 (13.6) | 8 (50.0) | 6 (37.5) | 2 (12.5) | 0.745 |
| Thorax CT[a] | | | | | 0.801 |
| Right main bronchus | 21 (18.8) | 12 (57.1) | 7 (33.3) | 2 (9.5) | |
| Right distal | 31 (27.7) | 20 (64.5) | 8 (25.8) | 3 (9.7) | |
| Left main bronchus | 7 (6.2) | 3 (42.9) | 4 (57.1) | 0 | |
| Left distal | 4 (3.6) | 3 (75.0) | 1 (25.0) | 0 | |
| Bilateral main bronchus | 45 (40.2) | 26 (57.8) | 15 (33.3) | 4 (8.9) | |
| Bilateral distal | 4 (3.6) | 3 (75.0) | 0 | 1 (25.0) | |

Data were analyzed by analysis of variance or $\chi^2$ test. Mean plus standard deviation values are presented for age, SatO2, wbc, RDW, HTC. PE: Pulmonary embolism, $SatO_2$: Oxygen saturation, WBC: White blood cell, RDW: Red blood cell distribution width, Htc: Haematocrit, ECG: Electrocardiography, RBBB: Right bundle-branch block, AF: Atrial fibrillation, CT: Computed tomography.

[a] Place of thrombus.

**Table 2 – Clinical and demographical characteristics of PE patients stratified according to *MTHFR* gene A1298C polymorphisms.**

| Characteristics | Total $n = 118$ | A1298C | | | |
|---|---|---|---|---|---|
| | | AA $n = 57$ | AC $n = 52$ | CC $n = 9$ | P value |
| Gender, male/female, *n* (%) | 61/57 (51.7/48.3) | 25/32 (43.9/56.1) | 29/23 (55.8/44.2) | 7/2 (77.8/22.2) | 0.868 |
| Age, years | 58.21 ± 10.531 | 58.02 ± 10.084 | 59.29 ± 10.440 | 53.22 ± 13.396 | 0.277 |
| $SatO_2$, % | 90.41 ± 5.009 | 91.14 ± 3.807 | 89.76 ± 6.020 | 89.43 ± 5.740 | 0.406 |
| WBC, per $mm^3$ | 9932.7 ± 2815.3 | 9790.7 ± 2512.4 | 10,294.7 ± 3153.3 | 8798.1 ± 2430.7 | 0.303 |
| RDW, % | 16.38 ± 2.580 | 16.16 ± 2.813 | 16.84 ± 2.346 | 15.14 ± 1.929 | 0.134 |
| Htc, % | 38.41 ± 4.163 | 37.56 ± 3.706 | 39.35 ± 4.495 | 38.33 ± 4.198 | 0.093 |
| Surgery, *n* (%) | 3 (2.5) | 2 (66.7) | 1 (33.3) | 0 | 0.767 |
| Smoking, *n* (%) | 67 (56.8) | 34 (50.7) | 27 (40.3) | 6 (9.0) | 0.592 |
| Comorbitidy, *n* (%) | | | | | |
| Coronary artery disease | 43 (36.4) | 21 (48.8) | 18 (41.9) | 4 (9.3) | 0.849 |
| Diabetes mellitus | 42 (35.6) | 20 (47.6) | 20 (47.6) | 2 (4.8) | 0.639 |
| Hypertension | 62 (52.5) | 32 (51.6) | 25 (40.3) | 5 (8.1) | 0.689 |
| Chronic renal failure | 15 (12.7) | 7 (46.7) | 8 (53.3) | 0 | 0.437 |
| Cerebrovascular disease | 8 (6.8) | 5 (62.5) | 2 (25.0) | 1 (12.5) | 0.513 |
| Malignancy | 10 (8.5) | 5 (50.0) | 5 (50.0) | 0 | 0.629 |
| ECG abnormalities, *n* (%) | | | | | |
| RBBB | 19 (16.1) | 7 (36.8) | 11 (57.9) | 1 (5.3) | 0.414 |
| Sinusal tachycardia | 42 (35.6) | 21 (50.0) | 17 (40.5) | 4 (9.5) | 0.764 |
| S1Q3T3 | 33 (28.0) | 17 (51.5) | 12 (36.4) | 4 (12.1) | 0.381 |
| AF | 31 (26.3) | 17 (54.8) | 10 (32.3) | 4 (12.9) | 0.198 |
| Ischemia | 16 (13.6) | 5 (31.2) | 9 (56.2) | 2 (12.5) | 0.314 |
| Thorax CT[a] | | | | | 0.383 |
| Right main bronchus | 21 (18.8) | 12 (57.1) | 6 (28.6) | 3 (14.3) | |
| Right distal | 31 (27.7) | 10 (32.3) | 17 (54.8) | 4 (12.9) | |
| Left main bronchus | 7 (6.2) | 4 (57.1) | 3 (42.9) | 0 | |
| Left distal | 4 (3.6) | 2 (50.0) | 2 (50.0) | 0 | |
| Bilateral main bronchus | 45 (40.2) | 22 (48.9) | 22 (48.9) | 1 (2.2) | |
| Bilateral distal | 4 (3.6) | 2 (50.0) | 1 (25.0) | 1 (25.0) | |

Data were analyzed by analysis of variance or $\chi^2$ test. Mean plus standard deviation values are presented for age, SatO2, wbc, RDW, HTC. PE: Pulmonary embolism, $SatO_2$: Oxygen saturation, WBC: White blood cell, RDW: Red blood cell distribution width, Htc: Haematocrit, ECG: Electrocardiography, RBBB: Right bundle-branch block, AF: Atrial fibrillation, CT: Computed tomography.

[a] Place of thrombus.

**Table 3 – Genotype and allele frequencies of *MTHFR* gene polymorphisms in patient and control groups.**

| Polymorphism | PE patients $n = 118$ (%) | Healthy controls $n = 126$ (%) | *p* | OR (95% CI) |
|---|---|---|---|---|
| **C677T** | | | | |
| *Genotypes* | | | | |
| CC | 69 (58.5) | 86 (68.2) | 0.137 | |
| CT | 38 (32.2) | 35 (27.8) | | |
| TT | 11 (9.3) | 5 (4.0) | | |
| CC: CT + TT | 69 (58.5): 49 (41.5) | 86 (68.3): 40 (31.7) | 0.113 | 1.52 (0.90–2.59) |
| CC + CT: TT | 107 (90.7): 11 (9.3) | 121 (96.0): 5 (4.0) | 0.091 | 2.48 (0.84–8.14) |
| Alleles | | | | |
| C | 176 (75.6) | 207 (82.1) | **0.042** | **1.57 (1.01–2.43)** |
| T | 60 (25.4) | 45 (17.9) | | |
| **A1298C** | | | | |
| *Genotypes* | | | | |
| AA | 57 (48.3) | 74 (58.7) | 0.133 | |
| AC | 52 (44.1) | 48 (38.1) | | |
| CC | 9 (7.6) | 4 (3.2) | | |
| AA: AC + CC | 57 (48.3): 61 (51.7) | 74 (58.7): 52 (41.3) | 0.103 | 1.52 (0.92–2.53) |
| AA + AC: CCI | 109 (92.4): 9 (7.6) | 122 (96.8): 4 (3.2) | 0.121 | 2.51 (0.76–9.61) |
| Alleles | | | | |
| A | 166 (70.3) | 196 (77.8) | 0.061 | 1.47 (0.98–2.22) |
| C | 70 (29.7) | 56 (22.2) | | |

Data were analyzed by $\chi^2$ or Fisher's exact test. PE: Pulmonary embolism, *MTHFR*: Methylene tetrahydrofolate reductase. The results that are statistically significant are typed in bold.

**Table 4 – Comparative analysis of combined genotypes of PE patients and controls.**

| Genotypes | Patient ($n$ = 118) | | Control ($n$ = 126) | | $p$ |
|---|---|---|---|---|---|
| | $n$ | % | $n$ | % | |
| **C677T-A1298C** | | | | | |
| CC-AA | 34 | 28.8 | 53 | 42.1 | **0.031** |
| CC-AC | 31 | 26.3 | 31 | 24.6 | 0.765 |
| CC-CC | 4 | 3.4 | 2 | 1.6 | 0.623 |
| CT-AA | 17 | 14.4 | 18 | 14.3 | 0.978 |
| CT-AC | 17 | 14.1 | 15 | 11.9 | 0.563 |
| CT-CC | 4 | 3.4 | 2 | 1.6 | 0.623 |
| CC-AA | 6 | 5.1 | 3 | 2.4 | 0.437 |
| CC-AC | 4 | 3.4 | 2 | 1.6 | 0.623 |
| CC-CC | 1 | 0.8 | 0 | 0 | – |

Data were analyzed by $\chi^2$ or Fisher's exact test. PE: Pulmonary embolism. The results that are statistically significant are typed in bold.

tics of PE patients and the *MTHFR* gene C677T and A1298C polymorphisms ($p > 0.05$). Similarly, Zappacosta et al. [20] reported no association between *MTHFR* genotypes and age, gender and smoking habits in PE patients in middle-southern Italian population.

Genotype frequencies did not show any significant differences between patients and controls according to *MTHFR* gene C677T and A1298C polymorphisms in this study. Lu et al. [21] suggested that there was no association between C677T polymorphism and patients with PE in Chinese population. *MTHFR* gene C677T mutation rate was reported as 12%–15% in European population [22]. In the literature, there are some studies on *MTHFR* C677T mutation in our population. The mutation rate was reported as 20.5% in a study of Kupeli et al. [18]. Akar et al. [23] reported it between 38.4% and 48.5% while Tug et al. [24] reported it as 37%. In line with these studies, our results were 41.5%. The frequency of *MTHFR* C677T mutation was found higher in PE patients than control group. Additionally, in control group, this mutation was determined as 31.7% and there was no statistical difference between two groups. Thus, this mutation may not be a specific risk factor for PE in the Turkish population. However, when the allele frequencies were evaluated, it was seen that T allele frequency was significantly higher in patients than controls ($p = 0.042$, OR: 1.57, 95% CI: 1.01–2.43). Inversely, Ivanov et al. [2] was reported that there was no association between carrying T allele and PE risk. In a study that evaluates severe CAD patients according to frequencies of *MTHFR* polymorphisms in Tunisian population, it was suggested that plasma homocysteine levels of patients with *MTHFR* C677T allele was significantly higher than others [5]. However, there is not enough data to compare allele frequencies in PE in the Turkish population.

In the present study, according to combined genotypes, individuals who were CC homozygous at C677T locus and AA homozygous at A1298C locus have a lower risk of developing PE ($p = 0.031$). It is a significant finding that shows the importance of combined genotypes in PE. As an example for effects of these polymorphisms' combination, Rahimi et al. [12] suggested that the risk of diabetic nephropathy increased in combination of MTHFR C677T and A1298C polymorphisms in Iranian population. Simsek et al. [25] suggested that single mutation of *MTHFR* was not a risk factor for tromboembolism. However, it was reported that there was an increase in plasma homocysteine levels in patients with homozygote *MTHFR* mutation. Bezemer et al. [26] reported that there was an increase in blood clotting together with plasma homocysteine in *MTHFR* C677T/A1298C combined heterozygote genotypes in patients with venous thrombosis. In the present study, *MTHFR* C677T/A1298C combined homozygote genotypes were found higher in controls. It is agreed that there is a direct association between PE and elevated plasma homocystein levels [21,22]. Also, it is known that mutations of *MTHFR* cause decrease on *MTHFR* enzyme activities due to hyperhomocycteinemia [26]. However, it is suggested that single mutation of *MTHFR* may not have strong effects on predisposing thrombus. In several studies, it was reported that *MTHFR* C677T mutations with additional genetic risk factors might increase the risk of thromboembolism and the importance of combined mutations was emphasized [18,25]. Thus, *MTHFR* C677T/A1298C heterozygote mutation may be a risk factor for PE due to hyperhomocysteinemia.

## 5. Conclusion

A single mutation in the *MTHFR* gene had no significant impact on PE. However, there was an association between *MTHFR* C677T/A1298C combined mutations and PE in the Turkish population. It is known that PE is not only affected by genetic factors, but also genetic predisposition has a powerful impact on the pathogenesis. The genetic research of *MTHFR* C677T/A1298C combined mutations may be used to determine the risk groups for PE.

## Conflict of interest

The authors state no conflict of interest.

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