Homozygous Mthfr C677t Carriers Develop Idiopathic Portal Vein Thrombosis Twenty Years Earlier Than Wild Type

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Research Article

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Abstract

Objective: To compare age at occlusion and plasma homocysteine (HC) in patients with idiopathic portal vein thrombosis (PVT) genotyped for methylene tetrahydrofolate reductase (MTHFR) rs1801133 (C®T667 transition), to identify which clinical or laboratory variables influenced age at first PVT, including the prothrombin rs1799963 PT (G®A transition at position 20210) (PT) mutation.

Methods: Retrospective cross-sectional cohort survey on 17 MTHFR TT, 45 MTHFR TC and 24 MTHFR CC participants with idiopathic PVT who contributed age, sex, age at PVT, smoking status, plasma HC and natural anticoagulant concentrations.

Results: Age at PVT was lower in MTHFR TT than MTHFR TC and CC (31±8 vs. 46±16 vs. 52±14 years, respectively, p=0.001); median (IQR) plasma HC was higher in MTHFR TT than in the other groups [17 (12.6,25.0) vs. 10 (7.4,13.1) vs. 11.9 (8.1,5.8) μmol/l, respectively, p=0.002)]. MTHFR TT and protein C independently predicted age at PVT (p<0.0001 and p=0.04 respectively); MTHFR TT independently predicted plasma HC (p=0.05). Smoking and sex predicted cavernoma (p=0.002 and p=0.02 respectively). Compound MTHFR TT with PT GA had no lowering effect on age at first VTE compared to MTHFR TT alone (37±7 vs. 30±8 years).

Conclusions: MTHFR TT anticipates age at PVT by an average of 20 years compared to other MTHFR genotypes. PT GA had no age lowering effect combined with MTHFR TT. Smoking predicts cavernoma formation, suggesting that smoking may associate with recurrent subclinical PVT before the symptomatic presentation.

Introduction

The intracellular and plasma concentrations of homocysteine (HC) are finely tuned by three enzymes: cystathionine beta synthase, methionine synthase and methylene tetrahydrofolate reductase (MTHFR); inherited deficiencies or genetic polymorphisms may reduce their enzymatic activity and impair the metabolism of HC that reaches toxic concentrations favouring premature vascular disease [1].

Up to 25% of the general Italian population carries a polymorphism of the MTHFR gene (C →T nucleotide transition at position 677) (rs1801133) that codes for an enzyme with 70% reduced activity [2]: as a consequence of this laboratory phenotype, plasma HC raises into the mild to moderate concentration range favouring an increased risk of venous thromboembolism in Caucasians [2]. Indeed, carriership of the MTHFR TT genotype from other cohort studies is associated with a 10-year earlier age of onset of venous thromboembolism [3] and with an 8-year earlier presentation of acute peripheral artery occlusion [4] compared to the heterozygous or wild type counterparts, suggesting that the clinical phenotype of MTHFR TT is the anticipation of the age at thrombosis.

Inherited thrombophilia [5], including the MTHFR TT genotype [6], partly explains non-cirrhotic idiopathic portal vein thrombosis (PVT), a rare but potentially life-threatening occlusion, particularly if left untreated.
[7]. Because elevated plasma HC is associated with oxidative stress [8] that may induce post-translational modifications in proteins [9] and because plasma prothrombin is susceptible of oxidative activation in vitro [10], we investigated whether idiopathic PVT carriers of the MTHFR TT genotype underwent an anticipated age at occlusion than heterozygous and wild type patients and whether compound MTHFR TT + the heterozygous or homozygous rs1799963 PT (G→A transition at position 20210) (PT GA) anticipated the age at PVT presentation compared to MTHFR TT in isolation.

**Participants And Methods**

**Study type and participants**

Retrospective cohort study of patients with PVT referred to the Haemostasis Unit of the Ospedali Riuniti di Foggia (Foggia, Italy) for an extensive thrombophilia screen consisting of antithrombin, protein C, protein S, anticardiolipin antibodies, lupus anticoagulant, homocysteine, factor V Leiden, PT 20201 and MTHFR genotypes. All samples for thrombophilia screens were taken after a period of six months of anticoagulation to minimize the impact of any possible acute phase on the laboratory determinations and after three weeks of oral anticoagulation cessation whereas samples for plasma HC were taken at PVT presentation.

For the purpose of this retrospective cohort survey the inclusion criteria were: 1) genotyping for MTHFR as part of the thrombophilia screen performed by the Haemostasis Units; 2) PVT confirmed by imaging (doppler ultrasound, computerised tomographic scanning, magnetic resonance and and/or their angiographic counterparts); 3) complete demographic data.

We identified 298 patients presenting with a first PVT from our electronic records who had been genotyped for MTHFR between January 2000 and July 2011; at this stage we excluded 6 records with insufficient data (missing date of birth or date of diagnosis); then, to focus on idiopathic PVT, we excluded 164 records of patients who developed PVT in the context of liver disease, liver cirrhosis in 126 and chronic hepatitis in 38. The latter two conditions were excluded on the basis of abnormal clinical, laboratory and imaging data and/or from liver histology.

We then excluded patients whose PVT developed in association with persistent and/or circumstantial risk factors (in decreasing order of frequency): myeloproliferative disorders n = 9, acute pancreatitis n = 6, cancer of gallbladder or biliary tract n = 3, post liver transplant n = 3, ulcerative colitis n = 2, acute cholangitis n = 1 and pregnancy n = 1.

We finally excluded 12 PVT patients with inherited thrombophilia (protein S deficiency n = 1, protein C deficiency n = 2, heterozygous factor V Leiden n = 9) and 2 patients with primary antiphospholipid syndrome. The remaining 86 PVT participants were the object of this survey.

**Genetic, immune and clotting assays**
DNA was extracted from peripheral blood leukocytes and submitted to polymerase chain reaction for the detection of the MTHFR, factor V Leiden and prothrombin mutation 20210 previously described [11]. Plasma HC was measured via a commercially available ELISA (Bio-Rad, Oslo, Norway); according to this method protein-bound HC is hydrolysed to free HC that is enzymatically converted to S-adenosyl-L-homocysteine (SAH); the sample SAH competes with SAH immobilized on the walls of the microtiter plate for binding sites on a monoclonal anti-SAH antibody. The cut-off for positivity was set at the 95th percentile, 12.5 umol/L; this derived from 90 blood samples of 40 apparently normal subjects and 50 healthy hospital employees who underwent periodical health screens [male 51, female 49, median age (IQR) 33 (22, 54)]; inter and intra-assay coefficient for plasma HC was 3.7% and 4.1% respectively. The thrombophilia screen was completed by the measurement of antithrombin, protein C (chromogenic assays from Behring, Marburg, Germany) and free protein S antigen (ELISA, Diagnostica Stago, Asnieres, France). Forty-six healthy hospital employees [(male 23, female 23, median age (IQR) 37 (9, 18)] contributed plasma to generate the reference ranges (mean ± 2 SD) that were: antithrombin, 66–112 U/dl, protein C 60–118 U/dl, free protein S 60–122 U/dl.

**Outcomes**

Our first null hypothesis was that MTHFR TT participants showed similar age at incident and first PVT as MTHFR CT and CC participants; our second null hypothesis was that mean age at first PVT in compound MTHFR TT and PT GA or PT AA patients was similar to that of MTHFR TT in isolation. Relationships between clinical and laboratory variables were also investigated.

**Statistics**

Mean and standard deviation summarised normally distributions, median and interquartile ranges (IQR) summarised non-normal distributions; analysis of variance, Kruskal-Wallis or Mann-Whitney tests were used accordingly whereas χ-square test compared frequencies across groups. Multivariable analysis with backward elimination was employed to minimize issues with variable selection [12]. For all statistical analyses, a p < 0.05 (two-tailed) was considered statistically significant. All calculations were performed using the MedCalc Statistical Software (MedCalc Software, version 19.2.6, Ostend, Belgium).

**Results**

**Comparative demographics and laboratory variables across the MTHFR groups**

The demographics of our cohort is shown in Table 1: the proportion of smokers was not different across the groups and none of the participants indulged in alcoholic beverages. MTHFR TT participants were younger than participants with the other MTHFR genotypes and were more likely to have a plasma HC above the cut-off of normality (12.5 μmol/L). Median (IQR) plasma HC was higher in MTHFR TT than in CT & CC participants (Table 1). According to the prevalence of elevated HC, the relative risk of developing PVT in MTHFR TT compared to MTHFR CC was 1.66 (95% CI 1.033 to 2.774; p = 0.04). The average
plasma concentration of the natural anticoagulants was relatively similar across the MTHFR genotypes but for protein C that was non significantly lower in the MTHFR TT group (Table 1).
Table 1
Demographics, clinical and laboratory variables by genotypes

<table>
<thead>
<tr>
<th>MTHFR</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>24</td>
<td>45</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>M/F</td>
<td>13/11</td>
<td>21/24</td>
<td>10/7</td>
<td></td>
</tr>
<tr>
<td>Age years ((\bar{x} \pm \sigma))</td>
<td>69 ± 15</td>
<td>64 ± 15</td>
<td>48 ± 11</td>
<td>0.001</td>
</tr>
<tr>
<td>Age at PVT, years ((\bar{x} \pm \sigma))</td>
<td>52 ± 14</td>
<td>46 ± 16</td>
<td>31 ± 8</td>
<td>0.001</td>
</tr>
<tr>
<td>No</td>
<td>%</td>
<td>No</td>
<td>%</td>
<td>No</td>
</tr>
<tr>
<td>Smokers</td>
<td>7</td>
<td>29.1</td>
<td>17</td>
<td>68.0</td>
</tr>
<tr>
<td>Cigarettes per day ((\bar{x} \pm \sigma))</td>
<td>8 ± 3</td>
<td>7 ± 3</td>
<td>8 ± 4</td>
<td></td>
</tr>
<tr>
<td>PVT in isolation</td>
<td>13</td>
<td>54.1</td>
<td>19</td>
<td>42.2</td>
</tr>
<tr>
<td>+ SVT</td>
<td>1</td>
<td>4.1</td>
<td>4</td>
<td>8.8</td>
</tr>
<tr>
<td>+ IHVT</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>6.6</td>
</tr>
<tr>
<td>+ SVT + IHVT</td>
<td>1</td>
<td>4.1</td>
<td>2</td>
<td>4.4</td>
</tr>
<tr>
<td>+ MVT</td>
<td>9</td>
<td>45.8</td>
<td>11</td>
<td>24.4</td>
</tr>
<tr>
<td>+ SVT + MVT</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>13.3</td>
</tr>
<tr>
<td>+ SVT + MVT + IHVT</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2.2</td>
</tr>
<tr>
<td>Cavernoma</td>
<td>2</td>
<td>8.3</td>
<td>14</td>
<td>31.3</td>
</tr>
<tr>
<td>PT G20210A</td>
<td>2</td>
<td>8.2</td>
<td>10</td>
<td>22.2</td>
</tr>
<tr>
<td>AT IU/dl ((\bar{x} \pm \sigma))</td>
<td>92 ± 15</td>
<td>92 ± 12</td>
<td>91 ± 15</td>
<td></td>
</tr>
<tr>
<td>PC IU/dl ((\bar{x} \pm \sigma))</td>
<td>90 ± 17</td>
<td>89 ± 21</td>
<td>80 ± 18</td>
<td></td>
</tr>
<tr>
<td>PS IU/dl ((\bar{x} \pm \sigma))</td>
<td>84 ± 16</td>
<td>84 ± 19</td>
<td>83 ± 14</td>
<td></td>
</tr>
<tr>
<td>HC No</td>
<td>24</td>
<td>44</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>%</td>
<td>No</td>
<td>%</td>
<td>No</td>
</tr>
<tr>
<td>HC &gt; 12.5 µmol/L</td>
<td>11</td>
<td>45.8</td>
<td>13</td>
<td>29.5</td>
</tr>
<tr>
<td>HC µmol/L median (IQR)</td>
<td>11.9 (8.1, 5.8)</td>
<td>10 (7.4, 13.1)</td>
<td>17 (12.6, 25.0)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Abbreviations. MTHFR: methylene tetrahydrofolate reductase; No: number; M/F: male/female; PVT: portal vein thrombosis; SVT: splenic vein thrombosis; IHVT: intrahepatic vein thrombosis; MVT: mesenteric vein thrombosis; PT: prothrombin; AT: antithrombin; PC: protein C; PS: protein S; HC: homocysteine; IQR: interquartile range
Table 2
Independent predictors of age at portal vein occlusions, plasma homocysteine and cavernoma

| A) Independent predictors of age at portal vein occlusion |
|---------------------------------|-------|-------|------|-----|
| Independent variables          | β     | SE    | t    | P   |
| Categorical                    |       |       |      |     |
| MTHFR TT                       | -10.380 | 2.2844 | -4.544 | < 0.0001 |
| Continuous                     |       |       |      |     |
| Protein C                      | 0.1789 | 0.0890 | 2.010 | 0.04 |

B) Independent predictors of plasma homocysteine

<table>
<thead>
<tr>
<th></th>
<th>β</th>
<th>SE</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Categorical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTHFR TT</td>
<td>2.6287</td>
<td>1.3679</td>
<td>1.922</td>
<td>0.05</td>
</tr>
</tbody>
</table>

C) Independent predictors of cavernoma

<table>
<thead>
<tr>
<th></th>
<th>β</th>
<th>SE</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Categorical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>0.2634</td>
<td>0.0854</td>
<td>3.083</td>
<td>0.002</td>
</tr>
<tr>
<td>Gender</td>
<td>0.1893</td>
<td>0.0827</td>
<td>2.287</td>
<td>0.02</td>
</tr>
<tr>
<td>Continuous</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>0.0418</td>
<td>0.00896</td>
<td>4.662</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Abbreviations. SE: standard error; MTHFR: methylene tetrahydrofolate reductase.

Effect of genotypes and other variables on age at portal vein thrombosis

The mean age at PVT presentation was lowest in the MTHFR TT group than in the other MTHFR genotype groups (Table 1). A first regression model having sex, MTHFR, PT 20210, HC, and smoking as categorical dependent variables, identified MTHFR TT as an independent predictor of age at PVT (Table 2A). A second regression model having natural anticoagulants, HC and cigarettes smoked per day as continuous independent variables identified protein C as a predictor of age at PVT after adjusting for sex (Table 2A).

Compound MTHFR TT + PT GA (n = 4) did not have lower age at PVT compared to MTHFR TT in isolation (n = 13) (35 ± 7 vs 30 ± 8 years) though their median (IQR) plasma HC was slightly higher [19.4 (14,23.2) vs 14.6 (12.3,27.2) p = 0.7)].

Effect of genotype and other variables on plasma homocysteine
A first regression model that included sex, MTHFR, smoking and type of occlusion as categorical independent variables, and HC as dependent variable, identified only MTHFR TT as an independent predictor of plasma HC (Table 2B). A second regression model that employed age at diagnosis and cigarettes smoked per day as continuous independent variables failed to enter any variables in the model after adjustment for sex. Median (IQR) plasma HC was slightly higher in males than females [(12.8 (9.1,17.2) vs 9.8 (7.4,13.9) µmol/L, p = 0.04] but was similar between smokers and non-smokers [11.6 (8.8, 16.7) vs 11.1 (7.3,15.6) µmol/L, p = 0.4]). While there was no relationship between plasma HC and protein C in the total cohort, plasma HC inversely related to protein C in the MTHFR CC and TT groups (Fig. 1A and B), but not in the MTHFR CT group.

**Effect of genotypes and other variables on cavernoma**

We explored any possible clinical and laboratory relation with cavernomas (n = 19): a regression model having cavernoma as the dependent variable and sex, MTHFR, PT 20210, smoking and plasma HC as independent categorical variables identified female gender and smoking as independent predictors of cavernoma (Table 2C); by entering age at occlusion, number of cigarettes smoked per day, plasma HC and natural anticoagulant concentrations as continuous variables after correction for sex, smoking again predicted cavernoma (Table 2C).

**Effect of genotypes and other variables on extent of vessel involvement**

To relate the extent of PVT with any of the demographic and laboratory variables, we adopted the anatomical categorisation of Yerdel et al [13]: accordingly, 39 participants had an isolated PVT, 3 had PVT extending into the intrahepatic vein, 3 has PVT extending into the intrahepatic vein with splenic vein occlusion, 23 had PVT associated with mesenteric vein occlusion, 5 had PVT associated with splenic vein occlusion, 11 had PVT associated with mesenteric and splenic vein occlusions, and 2 had PVT with intrahepatic extension combined with mesenteric and splenic vein occlusion.

Two regression models were used: the first included sex, MTHFR, PT 20210, smoking, and HC as independent categorical variables and the second included HC and number of cigarettes smoked per day as continuous independent variables, both with type of vessel involvement as the dependent variable; neither model retained any predictive variables.

**Discussion**

The mean age at PVT in carriers of MTHFR TT was 31 years, 15 years earlier than the MTHFR CT and a 21 years earlier than MTHFR CC, strikingly wide differences that refute our null hypothesis. Interestingly, the MTHFR TT genotype and protein C predicted age at PVT, whereas smoking had no effect, probably because of the low representation of smokers (35%) in our cohort.

Although MTHFR TT has been found in association with PVT in all age groups, a previous meta-analysis included case-controls studies that had to correct for a number of additional risk factors for PVT [14]
whereas our survey is the first to compare age at presentation of PVT of an entire cohort genotyped for MTHFR and devoid of all possible circumstantial factors for non-cirrhotic PVT, in order to investigate the isolated effect of the MTHFR genotypes: indeed the MTHFT TT genotype independently predicted not only age at PVT but also plasma HC concentration, as expected.

The very early age at PVT presentation of our MTHFR TT carriers is unusual and several factors may contribute to this prematurity: leaking of bacterial lipopolysaccharide from the intestine into the portal vein may cause inflammasome activation [15, 16] and thrombin generation [17, 18], the latter further supported by the oxidation of lipids, abundant in the portal circulation [19, 20]; on this background, the additional oxidative effect of elevated HC may anticipate acute thrombosis in MTHFR TT carriers [8].

Strangely, low, but within range, plasma protein C concentration related to age at PVT presentation. In a series of 23 non-cirrhotic PVT patients, up to 43.5% had a natural anticoagulant deficiency [21], and in another series of 29 patients, 62% presented with one or more natural anticoagulant deficiencies [22]: however, the median delay between diagnosis and thrombophilia testing in this study was 5 years [22], whilst in our cohort patients were tested after an average of six months of anticoagulation and after three weeks of anticoagulation cessation. Reduced hepatic blood flow or local intravascular consumption may partly account for some of these lower values and surgical correction of blood flow restored to normal the low concentration of natural anticoagulants [23]. Moreover, the high concentration of HC in MTHFR TT patients may induce post-translational modifications in protein C that becomes unable to digest factor Va and VIIIa in vivo, leading to thrombosis, and unable to digest its substrate in the chromogenic assay, leading to low functional levels [24, 25, 26]; indeed, we found an inverse relation between plasma HC and PC in the MTHFR CC and TT groups.

Cavernoma was present in almost 21% of our patients, predicted by gender and smoking: gender was not a risk factor in the largest [27] and the longest [28] studies on cavernomas, and smoking may represent a chance finding in our cohort given the low proportion of smokers, though smoking is a recognised a risk factor for porto-mesenteric occlusion after sleeve gastrectomy [29] and after liver transplantation [30].

However, several toxic compounds inhaled with cigarette smoke may induce oxidative [31] and nitrative stress [32] as well as endothelial dysfunction [33]. Nitrative stress may inhibit the activity of cystathionine beta synthase (CBS), the enzyme that converts HC to cystathionine then to cysteine [34], favouring an increase in intracellular and plasma concentrations of HC, that in turn may cause disulphide redox inhibition of CBS [35], perpetuating thus the elevated HC that could contribute to repetitive occlusions hence cavernoma formation in smokers.

By similar mechanisms, we postulated that the higher concentration of plasma prothrombin in the portal circulation of patients with the PT GA genotype would be more susceptible to activation by the oxidation of low-density lipoprotein [8] that parallels the elevated homocysteine and/or the MTHFR TT status [34, 35, 36]; but the PT GA did not predict age at PVT and the compound MTHFR-TT + PT GA did not lower the age at PVT compared to MTHFR TT alone, confirming our second null hypothesis.
Our survey has several limitations: 1) the retrospective cross-sectional design; 2) the lack of B12/folate measurement and of the genotyping of other genes involved in HC disposal, as 48% of our MTHFR CC had an unexplained elevated plasma HC, though at low concentration; 3) the lack of follow-up data that would have provided insight into the status of the portal vein after oral anticoagulation.

Conversely, our cohort had a stringent exclusion process that led us to study a neat population of idiopathic PVT devoid of all circumstantial factors involved in PVT that allowed us to better assess the role of MTHFR and plasma HC; indeed, our retrospective survey shows that: 1) MTHFR TT carriers developed PVT almost 21 years earlier than MTHFR CC; 2) a low and possible dysfunctional protein C predicted age at PVT; 2) smoking predicted cavernomas.

The knowledge that idiopathic PVT thrombosis may occur with an anticipation of around 20 years in MTHFR TT carriers has no real practical application in the general asymptomatic population, and in the latter sense supports the uselessness of MTHFR testing as part of a thrombophilia screen [37], but, if our data were replicated in PVT related to liver cirrhosis [38] primary thromboprophylaxis could be individually considered after identification of the risk factors for PVT in the cirrhotic setting.

While lowering elevated plasma HC with folic acid supplementation seems intuitive, folic acid does not reduce intracellular HC [39] and failed to prevent primary [40] and secondary VTE [41]; the consumption of foodstuffs fortified with folic acid could be recommended, but this should take into account the MTHFR genotypes, as MTHFR TT carriers respond less well to increased dietary folate compared to other genotypes [42]; no such information is available for PVT whether or not in association with liver cirrhosis.

The marginal 11.6% reduction of plasma HC after smoking cessation [43] should not deter clinicians to encourage this life style change, as it could reduce the risk of cavernoma formation in people with a previous PVT, though the major recommendation, in keeping with public health campaigns, is not to take up smoking at all [44].

**Declarations**

**Conflict of interest**

None of the authors has any financial or non-financial competing interest to declare.

**Author contributions**

All authors contributed to the study design. Luigi Iannaccone, Giovanna D'Andrea and Alessia Arcaro prepared and performed the analysis; Paul RJ Ames, Fabrizio Gentile and Vincenzo Marottoli collected the data; Paul RJ Ames and Maurizio Margaglione run the statistical analysis; Maurizio Margaglione wrote the first draft of the manuscript that was subsequently reviewed by all authors and finalised by Paul RJ Ames. All authors read and approved the final manuscript.

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**Declarations Conflict of Interests**

None of the authors has any relevant financial or non-financial interests to disclose.

**Ethics approval and consent to participate**

At the time of their first attendance patients gave informed and written consent to the use and storage of their genetic material and of their anonymised clinical information as per approval of the local Ethics Committee.

**Consent to publish**

The data collected for the study do not reveal any individual person's data

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Figures
Figure 1

Correlation between plasma homocysteine (HC) and protein C (PC) in patients with methylene tetrahydrofolate reductase CC (A) and TT (B) genotypes.