Blood Coagulation, Fibrinolysis and Cellular Haemostasis

Catechol-O-methyltransferase genotype is associated with plasma total homocysteine levels and may increase venous thrombosis risk

Henkjan Gellekink[1,2]*, Jan-Willem Muntjewerff[4]*, Sita H. H. M. Vermeulen[2], Ad R. M. M. Hermus[2], Henk J. Blom[1], Martin den Heijer[2,3]

[1]Laboratory of Pediatrics and Neurology, [2]Department of Endocrinology and [3]Department of Epidemiology and Biostatistics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; [4]GGz Nijmegen, Mental Health Institute, Nijmegen, The Netherlands

Summary

A disturbed methylation has been proposed as a mechanism via which homocysteine is associated with diseases like vascular disease, neural tube defects and mental disorders. Catechol-O-methyltransferase (COMT) is involved in the S-adenosyl-methionine-dependent methylation of catecholamines and catecholestrogens and in this way contributes to homocysteine synthesis. COMT dysfunction has been related to schizophrenia and breast cancer. We hypothesized that COMT dysfunction by virtue of functional genetic polymorphisms may affect plasma total homocysteine (tHcy). Our primary objective was to study the association between common COMT polymorphisms and tHcy. Secondly, we evaluated these polymorphisms as a risk factor for recurrent venous thrombosis. We obtained genotype data from four polymorphisms in the COMT gene (rs2097603, rs4633, rs4680 [324G>A] and rs174699) from 401 population-based controls. We performed haplotype analysis to investigate the association between common haplotypes and tHcy. In addition, we assessed the rs4680 variant as a genetic risk factor in a case-control study on recurrent venous thrombosis (n= 169). We identified a common haplotype that was significantly associated with tHcy levels. This effect was largely explained by the rs4680 variant, resulting in an increase in tHcy of 10.4% (95% CI 0.01 to 0.21, p=0.03) for 324AA compared with 324GG subjects. Interestingly, we found that the 324AA genotype was more common in venous thrombosis patients (OR 1.61 [95% CI 0.97 to 2.65], p=0.06) compared to control subjects. We show that the COMT rs4680 variant modulates tHcy, and might be associated with venous thrombosis risk as well.

Introduction

A disturbed homocysteine metabolism has been associated with diseases of the vascular system, both of arterial and venous origin (1–3). In addition, high plasma total homocysteine (tHcy) increases the risk of spina bifida, and mental disorders like schizophrenia and Alzheimer's disease (4–6). The mechanism of how homocysteine is related to disease is still obscure (7), but there are strong indications that a disturbed transmethylation may partly explain this association (8–11). Several studies show that plasma total homocysteine levels (tHcy) correlate well with plasma S-adenosylhomocysteine (AdoHcy) (12, 13), a strong inhibitor of S-adenosylmethionine (AdoMet)-dependent methyl-

ation. Given the importance of methylation of nucleic acids, proteins, lipids (8, 10, 14, 15), but also hormones and neurotransmitters (16, 17), it seems plausible that the inhibition or dysfunction of specific methyltransferases affect critical processes and hence confer a higher risk of disease.

The enzyme Catechol-O-methyltransferase (COMT, E.C. 2.1.1.6) is one of the methyltransferases that is highly susceptible to inhibition by AdoHcy (18). COMT represents a major pathway in the degradation of catecholamine neurotransmitters, like dopamine and (nor)adrenaline, and catecholestrogens. Hence, COMT dysfunction has been implicated in complex diseases like schizophrenia, Parkinson's disease and breast cancer (17, 19–21).

Correspondence to:
Martin den Heijer, MD, PhD
Department of Endocrinology (471)
Radboud University Nijmegen Medical Centre
P. O. Box 9101, 6500 HB Nijmegen, The Netherlands
Tel.: +31 24 3614599, Fax: +31 24 3618809
E-mail: m.denheijer@endo.umcn.nl

COMT enzyme exists as a membrane-bound (MB) and soluble (S) isoform, the expression of which is regulated by two different promoters. A common 324G>A polymorphism (rs4680) in the COMT gene, resulting in a valine-to-methionine substitution at position 108 (S-isoform) and 158 (MB-isoform), has been studied extensively for its effect on enzyme activity and expression, although the data is non-consistent (22, 23).

The metabolic pathways of COMT and homocysteine are interconnected as the O-methylation of catecholamines and catecholestrogens catalyzed by COMT produces homocysteine (Fig. 1). Therefore, functional variants within the COMT gene may influence tHcy levels and possibly reflect disturbed transmethylation capacity implicated in COMT-related diseases. This is illustrated by the fact that we did find an interaction between MTHFR and COMT in relation to schizophrenia risk (25).

The primary objective of our study was to investigate the effect of genetic variations within the COMT gene on tHcy by means of haplotype analysis. Considering the controversy whether the 324G>A variant is causally related to decreased enzyme activity or expression, we included three other variants (rs2097603) [22], rs4633 and rs174699) in or directly adjacent to the COMT gene in our analyses. Secondly we evaluated these polymorphisms as a risk factor for recurrent venous thrombosis.

Material and methods

Subjects used in the present study
The study group was recruited via a general practice in The Hague, the Netherlands, and has been described in more detail elsewhere (25). Recurrent venous thrombosis cases were selected from the files of the anticoagulant clinic in Leyenburg Hospital in The Hague, The Netherlands, and control subjects were recruited via a general practice; pregnancy was the only exclusion criterion. For the current association study, DNA was available from 438 control subjects and 169 recurrent venous thrombosis patients. With these numbers we had power of 50 to 78% to detect a difference of 10 to 15% in tHcy (two sided α=0.05). The power to detect an effect of COMT genotype on venous thrombosis was 54%.

Biochemical parameters
Blood samples were drawn after an overnight fast from the antecubital vein in 5 ml Vacutainer tubes and 4.5 ml EDTA vacuum glass tubes for determination of total plasma homocysteine and for DNA extraction. EDTA samples for homocysteine measurement were placed on ice immediately and centrifuged at 3,500 g for 5 minutes (min) with minimal delay. The plasma was separated and then stored at –20ºC. Total plasma homocysteine concentrations were measured by an automated high-performance liquid chromatography method with reverse phase separation and fluorescent detection (Gilson 232–401 sample processor, Spectra Physics 8800 solvent delivery system and LC 304 fluorometer) (26). DNA extraction was performed as described previously (27), and the DNA was stored at 4ºC.

Genotype analysis
We genotyped four single nucleotide polymorphisms (SNPs) distributed over the gene of interest: rs2097603, rs4633, rs4680 (sometimes referred to as rs165688) and rs174699. The SNPs were chosen based on frequency, functionality and location within the gene. All four created or abolished an enzyme restriction site allowing a simple screening based on restriction-fragment length polymorphism (RFLP) analysis. Screening conditions were similar in all analyses. For details see Table 1. PCR conditions: 4 min at 94°C, 35 cycles of 94°C/60 seconds (s), 52–60°C/60 s, and 72°C/30 s, and a final extension of 7 min at 72°C. Each PCR reaction mixture contained 50 ng of both forward and reverse primer (Biolegio BV, The Netherlands), 200 µM dNTPs, 10 mM Tris-HCl buffer (pH 8.2), 50 mM KCl, 2.0–4.0 mM MgCl$_2$, 0.5 U of recombinant Taq polymerase, 5% DMSO (Invitrogen, The Netherlands) and 75 ng DNA. The resulting PCR product was digested by at least 10 units of restriction enzyme (all from New England Biolabs, Inc.) and incubated for 3 hours or overnight at 37°C. The digests were analyzed by gel electrophoresis on a 3% or 4% agarose gel, stained with ethidiumbromide and visualized by UV. In all PCR amplifications and restriction analyses, DNA samples from which the genotype had been identified by sequence analysis served as positive controls. From 401 population-based controls, genotype data was available for all four SNP's (although the total number of genotyped individuals were different for each polymorphism).

Figure 1: Simplified representation of homocysteine metabolism and the location of Catechol-o-methyltransferase (COMT) enzyme within this metabolism.
Abbreviations: Met, methionine; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine; Hcy, homocysteine; MS, Methionine Synthase; THF, tetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase.
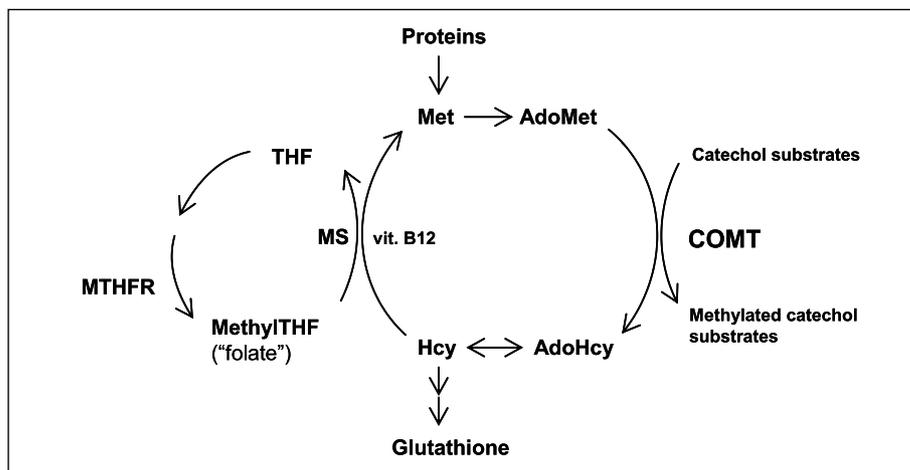
Variable	Cases (n = 185)	Controls (n = 500)	P-value
Age (year)	62 (42–79)	50 (34–69)	<0.01
Sex (men)	94 (51%)	208 (42%)	0.03
Post-menopausal women	64 (35%)	138 (28%)	<0.01
Time between 1st event and study (years)	17 (range 1 to 58)	-	-
Time between last event and study (years)	7 (range 1 to 30)	-	-
Type of venous thrombosis – Only pulmonary embolism (PE) – Only deep vein thrombosis (DVT) – Both DVT and PE	43 73 69	-	-
Smoking (yes/no)	61 (33%)	167 (34%)	0.9
B-vitamin (yes/no)	23 (12%)	76 (15%)	0.9
Homocysteine (µM)	12.2 (8.3–20.8)	10.7 (6.7–15.5)	<0.01
Creatinine (µM)	81 (61–112)	74 (55–99)	<0.01
Folate (nM)	13.5 (7.7–23.5)	12.7 (7.0–23.6)	0.2
Vitamin B12 (µM)	241 (118–466)	217 (125–389)	0.04
Vitamin B6 (nM)	25.8 (12.7–55.7)	27.6 (15.7–54.8)	0.03
Vitamin B2 (nM)	10.2 (4.9–37.1)	9.1 (4.0–25.9)	0.07
Factor V Leiden (MAF)[†]	14.6%	3.3%	<0.01
Prothrombin G20210A (MAF)[†]	2.9%	1.6%	0.12
MTHFR C677T (MAF)[†]	33.3%	29.1%	0.21

*Data are given as medians with 10th to 90th percentiles in parenthesis for continuous variables and as numbers with perecentages in parenthesis for categorical variables, † MAF= minor allele frequency.

Table 1: Baseline characteristics of patients with recurrent venous thrombosis and control subjects*.

Table 2: Relevant data of analyzed polymorphisms and method of screening.

Variant	Reported MAF[a]	DNA	Protein[b]	HW P-value	Primers (5'à 3')	Validation method	[Mg2+] (mM)	T$_{ann}$ (°C)
rs2097603	0.39 (G)	5' region A>G (P2 promoter)	-	0.87	F aatttggctattgccgtgtc R gtcccataaaaggggattcg	+HindIII	2.0	52
rs4633	0.39 (T)	36T>C	H12H	0.52	F acaacctgctcatgggtgac R tcctgtaagggctttgatgc	+PmlI	4.0	60
rs4680	0.36–0.52 (A)	324G>A	V108M	0.40	F tactgtggctactcagctgtc R gtgaacgtggtgtgtgaacacc	+NlaIII	2.0	59
rs174699	0.19 (C)	Intron 3 T>C	-	1.0	F cctctgtgtgaggctccaactc R gaaagaaggcagcacctgtc	-Ncol	3.0	59

[a] MAF, minor allele frequency (source: NCBI), [b] amino acid change using the (short) soluble form as reference, [c] Hardy-Weinberg (HW) p-value.

In addition, data of four other reported variants (i.e. rs6267 [Ala22Ser] [28], rs740602, rs13306281 and rs3218737) was obtained from 150 subjects from the same population by means of sequence analysis using the ABI Prism 3130XL automated sequencer according to the instructions of the manufacturer (Applied Biosystems, The Netherlands). However, three of them appeared non-polymorphic, while rs740602 was found only once in the heterozygous state (minor allele frequency of <0.01) and all were excluded from further analysis. For the case-control study, genotype data (rs4680) from 169 cases with a history of venous thrombosis was obtained.

Statistical analyses

Haploview (29) was used to evaluate linkage disequilibrium (LD) and correlations between the SNPs. Haplotype association analyses for homocysteine levels were performed using Whap. Whap (http://pngu.mgh.harvard.edu/purcell//whap) takes the ambiguity in individual haplotype estimations into account by applying a weighted likelihood approach. Prior to the haplotype analyses, the log transformed Hcy values were standardized. We used permutation testing as implemented in the Whap program to correct for multiple testing in all analyses. Haplotypes with a frequency of less than 2% were excluded from the analysis. Single-locus genotype effects on (log-transformed) metabolites were evaluated by linear regression analysis (SPSS 12.0). In the haplo-

Table 3: Linkage disequilibrium coefficient (D') between SNPs across the COMT gene in controls. Above the diagonal the D'-values are shown and below the diagonal their corresponding squared correlation coefficients (in italics).

Variant	Rs number	Distance from rs2097603 (bp)	rs2097603	rs4633	rs4680	rs174699
1	rs2097603	0	-	0.427	0.438	0.50
2	rs4633	22143	*0.149*	-	0.964	0.627
3	rs4680	23179	*0.165*	*0.89*	-	0.695
4	rs174699	26366	*0.016*	*0.033*	*0.038*	-

type analysis, changes in homocysteine levels are expressed relative to the most frequent haplotype group. Odds ratios (ORs) were estimated using logistic regression analysis (SPSS 12.0).

Results

Population charateristics and genotyping

Baseline characteristics of the study populations are shown in Table 1.

For details about these SNPs regarding location, function and validation method, see Table 2. The four COMT SNPs under study were all in Hardy-Weinberg equilibrium (p>0.4). Minor allele frequencies for rs2097603, rs4633, rs4680 and rs174699 were 0.48 (G allele), 0.47 (C allele), 0.49 (G allele) and 0.068 (C allele), respectively. Of note, the 324G allele (which is presumed to be the wild-type allele of rs4680) had a lower frequency than the 324A allele, which is also observed in other studies (Table 2). Linkage disequilibrium (D') and squared correlation coefficients between each of the genotyped variants are shown in Table 3. The SNP pair rs4680 (324G>A) and rs4633 (36T>C) was in almost complete LD, with a D' of 0.96 and a squared correlation (r^2) of 0.89. No strong LD was observed for the other SNP pairs.

Haplotype associations in the general population

Genotype data for all four polymorphisms was obtained from 401 individuals (mean age 50.6 ± 13.3 years), from which 41.0% was male (n=164). Mean fasting tHcy was 10.4 (95% CI 10.0 to 10.8) µM, serum creatinin was 74.3 (95% CI 72.7 to 75.9) µM, vitamin B12 was 221 (95% CI 208 to 234) pM and folate was 13.1 (95% CI 12.3 to 13.9) nM. tHcy was correlated with plasma folate, red blood cell folate and vitamin B12 (Spearmann ρ values were –0.21, –0.24 and –0.31, respectively, with p<0.01 at a two-tailed level).

The haplotype frequencies and their relative effects on tHcy are presented in Table 4. By omitting the haplotypes with a frequency of less than two percent, 95% of the haplotypes was covered. The omnibus association test using all haplotypes for crude fasting tHcy showed a bordeline significant effect (p=0.05). Haplotype specific analysis, i.e. analysis of the effect of the haplotype relative to the most common haplotype, showed that the effect was mainly due to the G-C-G-T haplotype that was statistically significant associated with low tHcy levels (-13.3% [95% CI –23.6 to –3.1], p=0.01). Adjustment for age, sex, serum creatinine, MTHFR 677C>T polymorphism and plasma folate did not change this point estimate (not shown). The omnibus haplotype test was no longer significant when the analysis was conducted conditional on rs4680 or on rs4633 (p=0.28 and p=0.26, respectively). This means that the haplotype analysis did not reveal major additional effects besides

that observed for the single-loci. Indeed, an increase of 10.4% (95% CI 0.01 to 0.21, p=0.03) and 8.8% (95% CI –0.00 to 0.18, p=0.06) in tHcy was observed for COMT 324AA and 324GA (rs 4680) individuals, respectively, when compared with 324GG subjects (Table 5). None of the genotypes was associated with folate, red blood cell folate or vitamin B12 levels (not shown). Moreover, the fact that adjustment for folate status (an important determinant of tHcy) did not influence the association between the COMT haplotype and tHcy suggests that the observed genotype-phenotype association is not confounded by differences in vitamin status.

Furthermore, by comparing a model in which the effects of the three haplotypes containing allele C and G at the second and third locus were constrained to be equal to a model in which all haplotype effects were estimated separately, we found that the effects of the N-C-G-N haplotypes (where N represents one of the alleles observed at the first and fourth locus) were not different (p=0.18). This suggests that the haplotype background on which these variants appear had no major influence on tHcy.

The COMT 324G>A variant and recurrent venous thrombosis risk

Genotype data of the COMT 324G>A (rs4680) polymorphism was obtained from 424 controls and 169, from which 41% (n=174) and 51% (n=86) were male, respectively. For the controls, mean age and blood parameters were similar as those individuals included in the haplotype analysis. For the recurrent venous thrombosis cases (mean age 61.5 ± 14.3 years) geometric mean (95% CI) tHcy was 12.6 (11.6 to 13.6) µM, 83.1 (78.6 to 87.6) µM for serum creatinin, 237 (207 to 267) pM for vitamin B12 and 13.7 (12.4 to 15.0) nM for folate.

The genotype frequencies were 34.9% (n=59), 45.6% (n=77), 19.5% (n=33), and 27.6% (n=117), 47.6% (n=202), 24.8% (n=105) for AA, AG, GG genotypes in cases and controls,

Table 4: Haplotype frequencies and relative change in tHcy in the general population using the G-T-A-T haplotype (corresponding with SNP 1, 2, 3 and 4, respectively) as a reference.

Haplotype	Frequency	Crude change tHcy % [95% CI]
G-T-A-T	0.355	0[a]
A-C-G-T	0.312	–3.2 [-9.7 to 3.4]
A-T-A-T	0.167	1.3 [-7.3 to 9.8]
G-C-G-T	0.120	–13.3 [-23.6 to –3.1] [b]
A-C-G-C	0.046	–3.3 [-15.8 to 9.2]

[a] Reference category, [b] *p*-ANOVA = 0.01.

Table 5: COMT single-locus genotype effects on tHcy concentrations in population-based controls.

Variant	Genotype	n (%)	MAF[b]	Crude mean tHcy [95% CI] (μM)	Crude change % [95% CI]	P-value
rs2097603	AA	117 (27.7)		10.7 [10.0 to 11.5]	0[a]	
(n=422)	AG	208 (49.3)		10.3 [9.8 to 10.8]	–4.1 [-12.1 to 4.7]	
	GG	97 (23.0)	0.48 (G)	10.3 [9.5 to 11.1]	–4.2 [-13.6 to 6.3]	0.474
rs4633	TT	120 (29.1)		10.8 [10.4 to 11.5]	0[a]	
(n=413)	TC	199 (48.2)		10.4 [9.9 to 11.0]	–3.0 [-11.0 to 5.6]	
	CC	94 (22.8)	0.47 (C)	10.0 [9.2 to 10.8]	–7.3 [-16.3 to 2.6]	0.143
rs4680	GG	105 (24.8)	0.49 (G)	9.6 [8.7 to 10.7]	0[a]	
(n=424)	GA	202 (47.6)		10.5 [9.6 to 11.5]	8.8 [-0.00 to 0.18]	
	AA	117 (27.6)		10.8 [10.0 to 11.5]	10.4 [0.01 to 0.21]	0.036
rs174699	TT	365 (86.9)		10.4 [10.0 to 10.8]	0[a]	
(n=420)	TC	53 (12.6)		10.2 [9.2 to 11.3]	–2.4 [-12.7 to 9.1]	
	CC	2 (0.5)	0.07 (C)	10.7 [6.3 to 18.3]	2.8 [-39.8 to 75.1]	0.748

[a] reference category, [b] MAF, minor allele frequency.

respectively. These correspond to minor (G) allele frequencies of 0.42 and 0.49 for cases and controls, respectively. We found that individuals with the 324AA genotype, corresponding with those having the highest tHcy, were at higher risk for recurrent venous thrombosis compared with 324GG subjects, although this estimation did not reach statistical significance (OR 1.61 [95%CI 0.97 to 2.65], p=0.06). The OR for individuals with the 324GA genotype was 1.21 (95% CI 0.76 to 1.94, p=0.18). The other genetic variants were not associated with recurrent venous thrombosis risk (not shown)

Discussion

We screened for the rs4680 variant as well as three other SNPs dispersed over the COMT gene (rs2097603, rs4633 and rs174699) and performed haplotype analysis in order to identify whether a specific haplotype was related to tHcy. We show that rs4680 was singularly associated with tHcy levels in a Dutch population study, and was responsible for the observed haplotype effect. The small, but non-significant, effect on tHcy that was observed for variant rs4633 (not shown) is likely to be explained by its high correlation with rs4680 (Table 2) (20, 30). Interestingly, the 324AA genotype (rs4680) was more prevalent in venous thrombosis cases suggesting that this variant may affect recurrent venous thrombosis risk as well.

The 324G>A (rs4680) polymorphism has been extensively studied for its effect at the molecular level, mostly because of its potential role in schizophrenia susceptibility. Functional studies showed that the COMT 324AA genotype is associated with decreased enzyme activity *in vitro* and in human brain extracts (22, 31) although the Val-allele was expressed at a slightly lower level in human brain (23). In the past, Goodman et al. (19) showed that the COMT 324G>A variant affected tHcy in controls, while Geisel et al. did not find such an effect in elderly subjects (32). In addition, it has been suggested that other variants might explain the observed associations (20). Our results show that the 324AA ge-

notype is significantly associated with increased tHcy levels, which may support the observation of higher expression of the Met-allele by Bray et al. (23). The higher levels of tHcy associated with the 324AA genotype may explain why these subjects tend to have a higher risk for venous thrombosis. However, a disturbed methylation by COMT in itself may also be involved, especially since the vascular system is constantly exposed to circulating catecholamines and catecholestrogens. It has been shown that catecholestrogens may have beneficial effects on the vascular system by reducing fibrinogen and overall fibrinolysis potential (33), although negative effects have also been reported (16). The measurement of plasma AdoMet and AdoHcy levels, the ratio of which is a marker of methylation capacity, and in-vitro methylation studies could provide additional evidence for disturbed methylation in subjects carrying this variant. It should be noted that we included patients with a history of venous thrombosis, which may give an overestimation of the relative risk. Additional studies are required to study whether the COMT 324G>A polymorphism is related to a first thrombotic event.

One may raise the question whether it is plausible that the flux through the COMT enzyme is high enough to generate a relatively large difference in tHcy (about 10%) between subjects having the 324GG and 324AA genotype. Studies with Parkinson's disease patients whose tHcy levels rose upon L-DOPA treatment (34–36), indicate that a higher COMT flux is reflected in plasma tHcy levels. In addition, a recent genome-wide linkage scan performed by Souto et al. identified another methyltransferase, Nicotinamide N-methyltransferase (NNMT), as a possible major determinant of tHcy (37). This shows that not only methyltransferases with a high flux-rate, like guanidinoacetate- and phosphatidylethanolamine methyltransferase (15), contribute to homocysteine synthesis, but also methyltransferases with an apparently modest contribution to overall methyltransferase activity. In conclusion, the 324AA genotype (rs4680) is associated with increased tHcy in the general population. Subjects with the 324AA genotype also tend to have a higher risk for recurrent ve-

nous thrombosis compared to subjects with the 324GG genotype. These data may give a hint as to what is the high-risk allele in COMT-related disorders, like cardiovascular disease and schizophrenia in particular.

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