Molecular genetic analysis of hyperhomocysteinemia

With a focus on remethylation and transmethylation

Gellekink, Henkjan
Molecular genetic analysis of hyperhomocysteinemia – With a focus on remethylation and transmethylation, Thesis Radboud University Nijmegen with summary in Dutch

ISBN-10: 90-9021331-7
ISBN-13: 978-90-9021331-6

Cover: Depiction of the Rosetta stone as a metaphor for the central dogma of molecular biology. The “clock” is an abstract representation of homocysteine metabolism
Design by Bart van der Linden (www.bartvdl.nl)

Printed by: Print Partners Ipskamp, Enschede, The Netherlands

Molecular genetic analysis of hyperhomocysteinemia

With a focus on remethylation and transmethylation

Een wetenschappelijke proeve op het gebied
van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de Rector Magnificus prof. dr. C.W.P.M. Blom
volgens besluit van het College van Decanen
in het openbaar te verdedigen op

donderdag 11 januari 2007
om 13:30 uur precies

door

Henkjan Gellekink
geboren op 10 maart 1975 te Enschede

Promotor:	Prof. dr. A.R.M.M. Hermus
Co-promotores:	Dr. M. den Heijer
	Dr. H.J. Blom
Manuscriptcommissie:	Prof. dr. J.L. Willems (voorzitter)
	Prof. dr. Y.M. Smulders (VU Medisch Centrum, Amsterdam)
	Dr. B. Franke

The study described in this thesis was supported by a grant of the Netherlands Heart Foundation (NHF - 2002B068).

Financial support by the Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.

Aan mijn ouders,

Aan Nathalie

Table of contents

Part II: Pathophysiology – homocysteine and disturbed transmethylation

Abbreviations

AdoCbl	adenosylcobalamin
AdoHcy	S-adenosylhomocysteine
AdoMet	S-adenosylmethionine
Adox	periodate-oxidized adenosine
AHCY	S-adenosylhomocysteine hydrolase
ANOVA	analysis of variance
ATIC	aminoimidazole carboxamide ribonucleotide (AICAR) transformylase/inositol monophosphate (IMP) cyclohydrolase
BHMT	betaine-homocysteine methyltransferase
(k)bp	(kilo)basepairs
Cbl	cobalamin
CBS	cystathionine β-synthase
CI	confidence interval
COMT	catechol-O-methyltransferase
CTH	cystathionine γ-lyase
CV	coefficient of variation
(k)Da	(kilo)dalton
DHFR(P)	dihydrofolate reductase (pseudogene)
DMSO	dimethylsulfoxide
(c)DNA	(complementary) deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminotetraacetic acid
FR	folate receptor
FGCP	folylpoly-γ-glutamate carboxypeptidase
GCPII	glutamate carboxypeptidase II
HPLC	high-pressure liquid chromatography
HUVECs	human umbilical vein endothelial cells
LD / D'	linkage disequilibrium / coefficient of LD
MS	mass spectrometry
MeCbl	methylcobalamin
MMA	methylmalonic acid
MRM	multiple reaction monitoring
mRNA	messenger ribonucleic acid
MTHFR	methylenetetrahydrofolate reductase
MTR	methionine synthase
MTRR	methionine synthase reductase
m/z	mass-over-charge ratio
NAD^+/NADP	nicotinamide adenine dinucleotide / NAD phosphate
Nano-LC FTICR MS	nano-liquid chromatography Fourier transform ion cyclotron resonance mass spectrometry
OR	odds ratio
PCR	polymerase chain reaction

PML	post-methionine load
RBC	red blood cell
RFC1	reduced folate carrier 1
RFLP	restriction fragment-length polymorphism
SNP	single-nucleotide polymorphism
SD	standard deviation
LC ESI MS/MS	liquid chromatography electrospray injection tandem mass spectrometry
SPE	solid-phase extraction
SPSS	statistical package for the social sciences
tHcy	plasma total homocysteine
TYMS	thymidylate synthase
U	units (enzyme activity)
UTR	untranslated region

Chapter *1*

General introduction

and

objectives

Part of this chapter (1.5) is published as
Genetic Determinants of Plasma Total Homocysteine
Henkjan Gellekink, Martin den Heijer, Sandra G. Heil, Henk J. Blom
Seminars in Vascular Medicine 2005; 5: 98-109

1.1 Homocysteine metabolism

Homocysteine is a sulphur-containing intermediate produced during the conversion of the essential amino acid methionine to cysteine (Figure 1). Methionine metabolism is linked to the synthesis of glutathione and polyamines. Its unique function relates to a process called "transmethylation". First, methionine is converted into S-adenosylmethionine (AdoMet), the principle methyl donor in the human body, by the enzyme methionine adenosyltransferase (MAT). AdoMet is the methyldonor in over a hundred methylation reactions including the methylation of nucleic acids, proteins, lipids, hormones and neurotransmitters. A group of enzymes, called methyltransferases, are involved in the transfer of the methylgroup to its specific acceptor. By donating the methylgroup, S-adenosylhomocysteine (AdoHcy) is formed, which is readily hydrolyzed to homocysteine and adenosine in a reversible reaction catalyzed by AdoHcy hydrolase (AHCY).

Homocysteine can be remethylated to methionine or irreversibly degraded via the transsulfuration pathway. Transsulfuration is mainly restricted to the liver and kidney and produces cysteine (for protein or glutathione synthesis) or sulfate. These steps are catalyzed by the vitamin B6-dependent enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CTH). In the remethylation pathway, homocysteine accepts a methylgroup to form methionine again. In this ubiquitously present metabolic route, folate acts as an intermediate methylcarrier (Figure 1). Fully reduced folate (tetrahydrofolate, THF) accepts a one-carbongroup from the amino acid serine to produce 5,10-methyleneTHF. After reduction to 5-methylTHF, the main circulating form of folate in blood, by 5, 10-methylenetetrahydrofolate reductase (MTHFR), the methylgroup is transferred via cobalamin (vitamin B12) to homocysteine in a reaction catalyzed by methionine synthase (MTR). Importantly, folate is also essential for the synthesis of purine and thymidylate nucleotides. An alternative homocysteine remethylation pathway exists in which betaine (=trimethylglycine) donates the methylgroup in a reaction catalyzed by betaine-homocysteine methyltransferase (BHMT). Like CBS, its activity is mainly restricted to the liver and the kidney.

In the cell, homocysteine is mainly present as its precursor, AdoHcy. Under normal conditions, transsulfuration and remethylation activity determine intracellular homocysteine levels. If one of these pathways is compromized, for example due to enzyme dysfunction or low vitamin intake, the excess of homocysteine is presumed to be exported to the blood (reference range 5-15 μmol/L) and metabolized in other organs, such as the kidney and liver. Persisting higher concentrations of homocysteine in the blood (>15 μmol/L), also called "hyperhomocysteinemia", reflects a disturbance of the critical processes mentioned above.

1.2 Inborn errors of methionine metabolism

The discovery of the inborn error of metabolism called homocystinuria initiated great interest in homocysteine in the early 1960s. This rare inherited disease is caused by defects in either homocysteine transsulfuration or homocysteine remethylation. The most common cause of homocystinuria involves a block in the transsulfuration pathway due to CBS deficiency, which

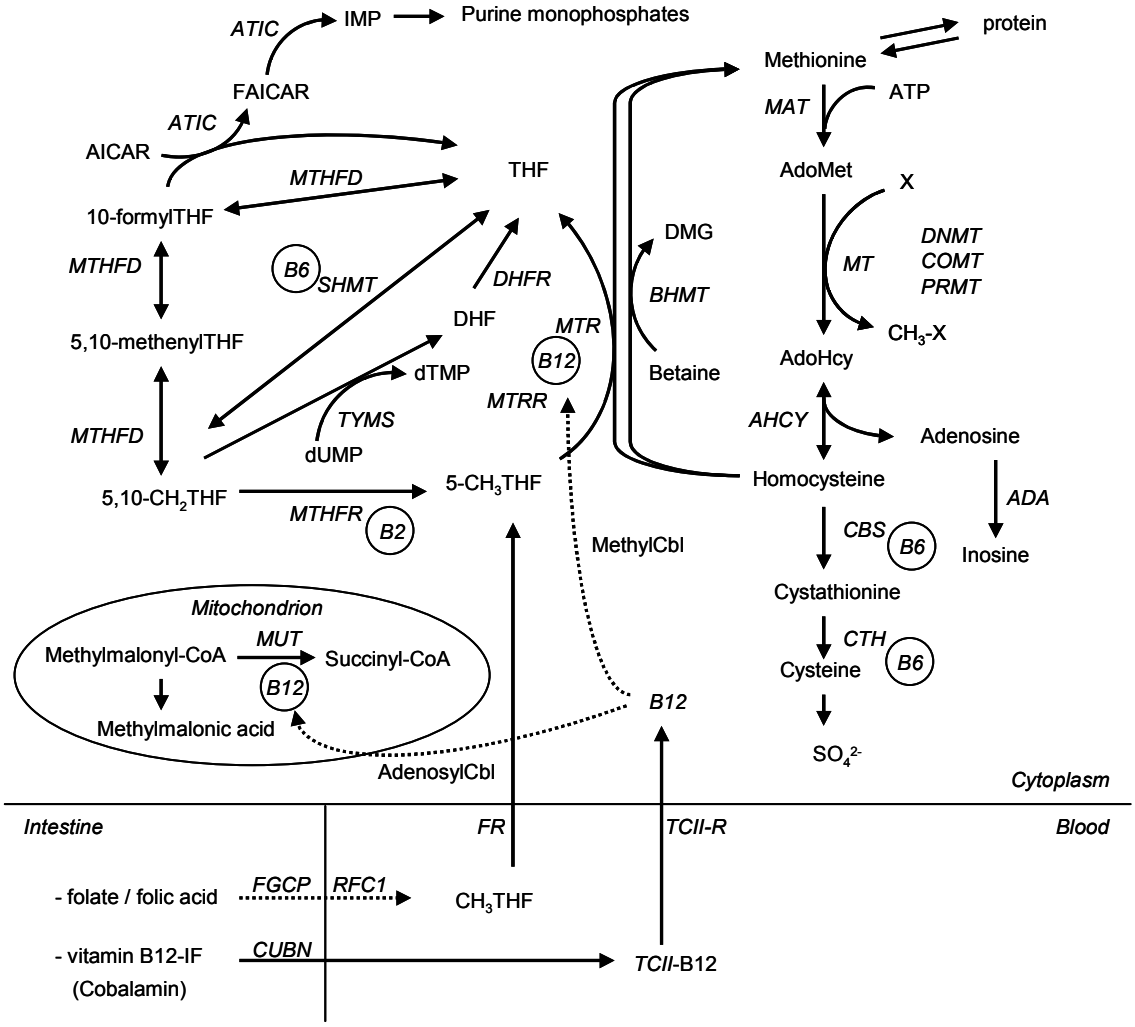

Figure 1. Relevant enzymes in folate, vitamin B12 and homocysteine metabolism

Abbreviations: ADA, adenosine deaminase; AdoMet, S-adenosylmethionine, AdoHcy, S-adenosylhomocysteine; AHCY, S-adenosylhomocysteine hydrolase; ATIC, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase / inosine monophosphate (IMP) cyclohydrolase; ATP, adenosine triphosphate; BHMT, betaine-homocysteine methyltransferase; CBS, cystathionine β-synthase; Cbl, cobalamin; COMT, catechol-O-methyltransferase; CTH, cystathionine γ-lyase; CUBN, cubulin; D(T)HF, di(tetra)hydrofolate; DHFR, dihydrofolate reductase; DNMT, DNA methyltransferases; DMG, dimethylglycine; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; FAICAR, formyl-AICAR; IMP, inosine monophosphate; FGCP, folyl-γ-glutamate carboxypeptidase; IF, intrinsic factor; FR, folate receptors; MAT, methionine-adenosyltransferase; MT, methyltransferases; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; MTR(R), methionine synthase (reductase); MUT, methylmalonyl-CoA mutase; PRMT, protein-arginine methyltransferase; RFC1, reduced folate carrier1; SHMT, serine-hydroxymethyltransferase; TCII, transcobalamin; TCII-R, transcobalamin receptor; TYMS, thymidylate synthase.

is often caused by the 833T>C mutation (minor allele frequency 0.2% to 0.7% in Caucasians) [1-3] (unpublished results). Although only sporadically seen, the two most common remethylation defects are MTHFR deficiency and MTR dysfunction (1:1.000.000 and 20-100 cases worldwide, respectively). There is a large variation in clinical presentation among "classical" homocystinuric patients, but they all share an excessive accumulation of

homocysteine in blood (>50 μmol/L) and, hence, excretion of homocystine (a disulfide of homocysteine) in urine. Irrespective of the underlying cause, thromboembolic and arterial vascular disease are major clinical findings in homocystinuria. Hence, McCully postulated that the premature vascular complications were a consequence of the elevated homocysteine [4,5]. This raised the question whether mild elevation of plasma total homocysteine (tHcy), called hyperhomocysteinemia, also increases the risk of vascular disease [6-8]. This is of clinical importance considering the high prevalence of hyperhomocysteinemia (about 10-15%) in the general population.

1.3 Hyperhomocysteinemia and disease

1.3.1 Arterial vascular disease and venous thrombosis

Cardiovascular disease is the leading cause of death in the Western industrialized world and a major cause of death throughout the world. Cardiovascular disease contributes to approximately 40% of all deaths in the United States and at least 20% in Europe [9,10]. The increasing prevalence of obesity and diabetes among children and adults will lead to an additional increase in cardiovascular disease and related mortality. A common type of vascular disease is atherosclerosis, which results from progressive narrowing of the blood vessels that supply oxygen to the heart, brain or other parts of the body. It develops when deposits (called plaques) build up on the inner lining (endothelium) of the vessel wall. Ultimately, this may lead to a rupture of the atherosclerotic lesion and cause coronary artery, cerebral artery or peripheral artery disease. Hypertension, high LDL-cholesterol, obesity (body mass index >25), diabetes and smoking are among the most important risk factors for atherosclerosis.

Arterial vascular disease and venous thrombosis are important clinical findings in classical homocystinuria [11,12]. Although in the general population the incidence of venous thrombosis with respect to the overall cardiovascular burden is relatively low, thromboembolic disease accounts for substantial morbidity and mortality [13]. Venous thrombosis occurs when a blood clot is formed in the veins due to (a combination of) tissue damage, hemostasis and a hypercoagulable state, also known as Virchow's triad. In the general population, the most common genetic cause of venous thrombosis is activated protein C resistance due to the factor V Leiden mutation and hypercoagulability due to the prothrombin 20210G>A mutation [14-16]. Other, less frequent, risk factors for venous thrombosis include protein S-, protein C- and antithrombin deficiency [13].

In the past two decades both retro- and prospective studies identified hyperhomocysteinemia as an independent risk factor for arterial vascular disease and venous thrombosis [17-19]. The current data show that the risk associated with a 5 μmol/L higher tHcy ranges from 30% to 60% (odds ratio 1.3 to 1.6) for venous thrombosis, stroke and ischemic heart disease [18,19]. Interestingly, homocysteine-lowering treatment in homocystinuric patients reduces the risk of vascular events by 90% (relative risk 0.09 [95% CI 0.036 to 0.228], P<0.0001) [20].

1.3.2 Hyperhomocysteinemia and other diseases

The increased disease risk associated with a disturbed homocysteine metabolism is not only confined to cardiovascular complications. In general, three other major domains of clinical conditions have been related to a disturbed homocysteine metabolism. These comprise pregnancy complications (including congenital defects), cancer and neuro(psycho)logical diseases (Figure 2).

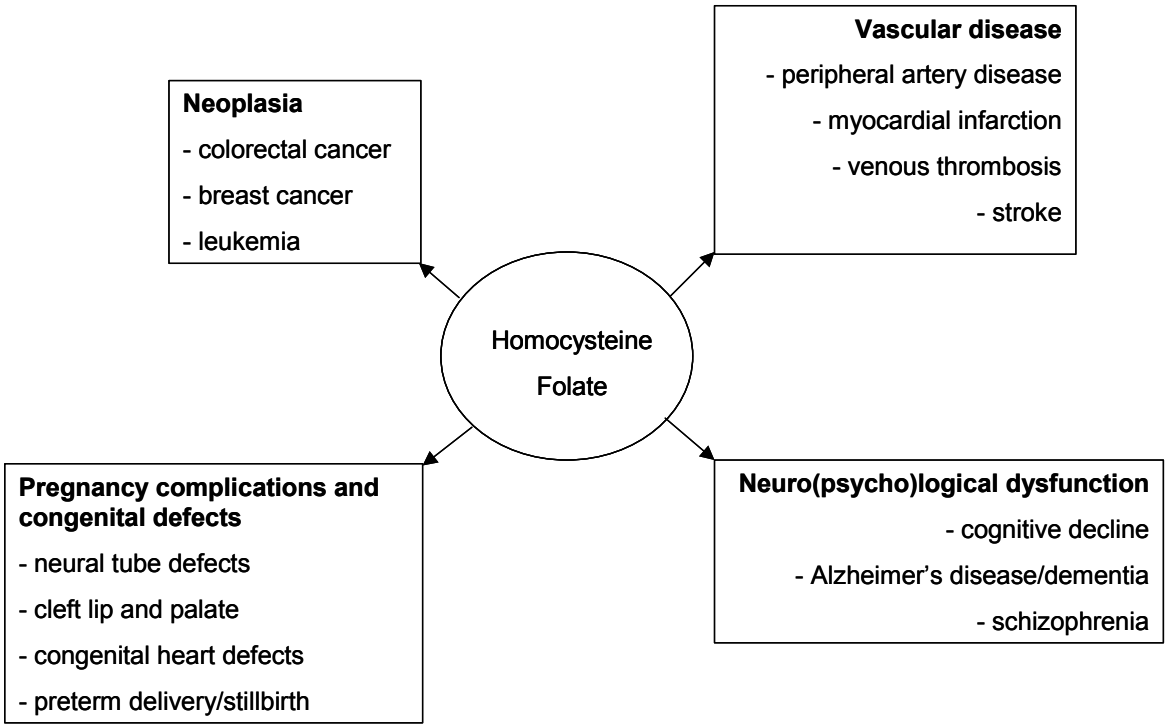

Figure 2. Clinical conditions that have been related to a disturbed homocysteine/folate metabolism

Low folate status and the concomitant increase in tHcy are known risk factors for neural tube defects. Already in the nineties of the previous century the preventive effect of folate supplementation regarding the occurrence and recurrence of neural tube defects has been established [21-23]. Also the risk for other pregnancy complications, like congenital heart defects [24,25], cleft lip and palate [26], stillbirth [27], preterm delivery and preeclampsia [28], is increased in hyperhomocysteinemic subjects. Folate deficiency and hyperhomocysteinemia have been related to neoplasia, such as leukemia, colorectal and breast cancer. This may be related to increased uracil misincorporation, disturbed methylation of DNA or catecholestrogens [29-35]. Finally, data is emerging that low B-vitamin status and hyperhomocysteinemia are more prevalent in dementia and Alzheimer's disease [36,37]. Together with the finding of higher tHcy and disturbed transmethylation in schizophrenic patients [38,39], these data suggest the involvement of homocysteine-related disturbances in neuro(psycho)logical disease as well.

The reported involvement of homocysteine in cardiovascular disease and other diseases encouraged many groups to search for genetic determinants of tHcy. Initial studies suggested that, in most cases, hyperhomocysteinemia resulted from heterozygosity for CBS deficiency [11,40]. Others could not reproduce these findings and a role for thermolabile MTHFR

in hyperhomocysteinemia was proposed [41,42]. In 1995, Frosst and co-workers [43] identified the 677C>T polymorphism in the MTHFR gene as the genetic cause of thermolabile MTHFR. However, the MTHFR 677C>T variant only partly explained the high homocysteine levels. This led to the search for other determinants of homocysteine (see section 1.5). Heritability studies indicate that the genetic contribution to the variation of homocysteine levels ranges from 20% to almost 50% [44-46], of which the MTHFR 677C>T polymorphism may explain about 10% [47]. Also "non-genetic" factors contribute to hyperhomocysteinemia, such as low vitamin intake, use of certain medication/drugs, smoking, renal dysfunction and coffee consumption [48]. The interaction between these environmental and genetic factors, as well as gene-gene interactions is thought to contribute to the complex mechanisms leading to hyperhomocysteinemia, and hence increase the risk of disease.

1.4 Pathogenesis of hyperhomocysteinemia

Several mechanisms have been proposed to explain how homocysteine may lead to disease. Regarding cardiovascular disease, these include impaired (nitric oxide-mediated) vasodilation due to endothelial dysfunction [49], oxidative stress [50], asymmetric dimethylarginine (ADMA) accumulation [51,52] and hemostatic changes resulting in hypercoagulability [53]. A more general mechanism was recently reviewed by Jacobsen, who suggests that molecular targeting of proteins by homocysteine (called "homocysteinylation") may disrupt protein function and contribute to the pathogenesis of cardiovascular disease [54]. Much research has focused on the process of transmethylation, as high homocysteine levels may reflect a disturbed transmethylation. Under hyperhomocysteinemic conditions the equilibrium of the (reversible) AHCY reaction favors AdoHcy synthesis, rather than hydrolysis. Several studies have shown that hyperhomocysteinemia leads to an increase in AdoHcy [55-57], a potent inhibitor of AdoMet-dependent transmethylation reactions. Because of the importance of methylation of various macromolecules (nucleic acids, proteins, lipids) and smaller molecules (neurotransmitters, hormones), it has been suggested that hypomethylation may partly explain homocysteine-induced pathology of the vascular and central nervous system [57-60].

1.5 Genetic variation and hyperhomocysteinemia

The identification of the MTHFR 677C>T polymorphism as an important determinant of plasma total homocysteine (tHcy) in the general population, has encouraged many groups to search for additional genetic variants that modulate tHcy. The majority of genes that were studied are involved in folate metabolism, which illustrates the presumed role of a disturbed homocysteine remethylation as a contributor to hyperhomocysteinemia. Given the importance of folate and vitamin B12 (cobalamin) in homocysteine remethylation, genes involved in their uptake and transport were studied as well. In this section, the variants in genes that have been assessed for their effect on tHcy are described (see Figure 1 and Table 1), which reflects the status of research in this field at the time the studies described in this thesis were initiated.

Table 1. Genetic variants studied for their effect on plasma total homocysteine relating to non-fortified populations

Gene	Variant	Amino acid substitution	Chromosomal Location	Allele frequency	Effect on tHcy (mutant vs wild type)
MTHFR	677C>T	A222V	1p36.3	0.30-0.40 (T)	+14% to +70%
	1298A>C	E429A		~0.30 (C)	No effect
MTR	2756A>G	D919G	1q43	~0.20 (G)	0% to -20% (ns)
MTRR	66A>G	I22M	5p15.31	0.46-0.59 (G)	0% to +10%
GCPII	1561C>T	H475Y	11q11.2	~0.06 (T)	-9% (ns)
RFC-1	80G>A	R27H	21q22.3	0.38-0.51 (A)	0% to +11% (ns)
TCN	776C>G	P259R	22q12.2	0.35-0.47 (G)	0% to +15% (ns)
	67A>G	I23V		~0.13 (G)	-35%
	280G>A	G94S		0.01 (A)	No effect
	1043C>T	S348F		0.11-0.17 (T)	No effect
	1196G>A	R399Q		~0.02 (A)	No effect
cSHMT	1420C>T	L474F	17p11.2	~0.30 (T)	+/-
mSHMT	7121del4	-	12q13.2	0.02 (del)	No effect
BHMT	595G>A	G199S	5q13.1-15	0.01 (A)	No effect
	1218G>T	Q406Hs		0.01 (T)	No effect
	716G>A	R239Q		0.22-0.31 (A)	No effect
MTHFD	2011G>A	R653Q	14q24	0.40-0.45 (A)	No effect
TYMS	28bp rpt	-	18p11.32	0.17-0.47 (2x rpt)	No effect
	1494del6	-		0.36 (del)	No effect
CBS	1080C>T	A360A	21q22.3	~0.36 (T)	No effect
	699C>T	Y233Y		~0.36 (T)	No effect
	14037 31 bp VNTR	-		~0.77 (18x rpt)	+10% (18/18 vs 17/17)
	-5707 GT STR	-		0.67 (16x rpt)	No effect
	844ins68	-		~0.09 (ins)	0% to -23% (ns)
CTH	1364G>T	S403I	1p31.1	0.29 (T)	+17%

* ns: non-significant

1.5.1 Genes involved in homocysteine remethylation

Methylenetetrahydrofolate reductase

The most studied polymorphism is the 677C>T (A222V) transition in the MTHFR gene, yielding a thermolabile variant of the enzyme with decreased activity [43]. Consequently, synthesis of 5-methyltetrahydrofolate (5-CH_3THF), the co-substrate for the MTR-driven remethylation of homocysteine, may be decreased resulting in a mean increase of tHcy of 25% in the general population [43,61-67]. This effect is observed in most populations throughout Europe and other continents [68,69], especially when folate status is low [47,62,70]. The average frequency of the TT genotype in Caucasians is 12% but may range from 1% to as high as 30% in different ethnic populations [68,71-74].

By direct sequencing of the MTHFR gene of MTHFR-deficient individuals our group detected a second variant in this gene, the 1298A>C polymorphism (E429A) [75]. This variant was associated with decreased enzyme activity *in vivo* and *in vitro* [75-77] although others observed an effect on enzyme activity only when the MTHFR 677C>T polymorphism was taken into account [78,79]. No effect of this variant alone on tHcy was observed [64,65,75,76,79], except for one

group, who found increased tHcy in 1298CC compared with 1298AA/AC individuals [80]. In addition, the combination with the MTHFR 677CC [81] or 677CT [75] genotype was shown to affect tHcy.

Methionine synthase and methionine synthase reductase

Both the MTR and MTRR enzymes are involved in folate-dependent homocysteine remethylation and the common MTR 2756A>G and MTRR 66A>G variants have been studied in relation to homocysteine. MTR uses cobalamin as cofactor and is directly involved in methyl transfer from 5-CH_3THF, via cobalamin, to homocysteine. In addition, cobalamin may be oxidized (i.e. inactivated) and requires the activity of MTRR to be reduced in order to re-enter the catalytic cycle and to maintain MTR activity.

By sequencing the coding region of the MTR gene of sixteen individuals (who were hyperhomocysteinemic, had a history of vascular disease or were mothers with children suffering from neural tube defects [NTD]), we detected a common variant in the MTR gene (i.e. 2756A>G) [82], although no clear effect on tHcy was observed [47,82-84]. Several other groups, however, reported that the 2756AA genotype was associated with higher tHcy levels [85-87].

The MTRR I22M (66A>G) variant was first described by Leclerc *et al.* [88] and because this polymorphism is very common (G allele frequency between 0.46 and 0.59) there is no consensus concerning the wild type allele at this locus. *In vitro* studies showed that this transition mildly decreased enzyme activity [89,90], although only one study showed the MTRR I22M polymorphism to be a determinant of tHcy in the general population [91,92]. The fact that other studies could not confirm this finding [47,93-97] may indicate that there is redundancy in the reductive reactivation of the MTR-cobalamin enzyme complex as reported by Olteanu *et al.* which attenuates the effects of the 66A>G polymorphism [98].

Glutamate carboxypeptidase, reduced folate carrier and folate receptors

FGCP, RFC1 and FR (α thru δ) are proteins involved in intracellular folate availability. FGCP, encoded by the GCPII gene, hydrolyses dietary folylpoly-γ-glutamates to monoglutamates, a process essential for cellular absorption of folates. Internalization of folate cofactors generally involves two primary systems in mammalian cells (i.e. the high-affinity FRs [for uptake of folic acid and CH_3THF] and the high-capacity transporter RFC1 [for uptake of reduced folates including the antifolate methotrexate (MTX)] [99-102].

GCPII harbors a rare 1561C>T (H475Y) polymorphism in the putative catalytic domain that may reduce enzyme activity by half and therefore compromise intestinal folate absorption [103]. In the same publication, Devlin *et al.* reported that heterozygosity for this polymorphism leads to reduced plasma folates and increased tHcy compared to individuals with the wild type genotype. In contrast, we reported increased plasma and/or red blood cell folates in individuals carrying the mutant allele but did not observe an evident effect on tHcy [104,105]. In addition, other groups reported no effects on plasma folate and tHcy in 1324 subjects of the Framingham Offspring Study [106] or mothers with children suffering from neural tube defects [107].

A common 80G>A (R27H) polymorphism has been described in the RFC1 gene, which is thought to affect carrier function. Whetstine and co-workers convincingly showed that this polymorphism had no effect on MTX and N^5-formylTHF uptake *in vitro* [108], which is supported by epidemiological data showing no effect of this polymorphism on folate and tHcy in renal patients [109] or mothers with NTD-offspring [107]. However, Chango *et al*. found a trend towards higher tHcy in 80GG individuals, which increased, and inversely affected RBC folate, when the MTHFR 677TT genotype was taken into account [110].

The human FR family includes several glycoproteins (α - δ) involved in the binding and internalization of 5-CH_3THF. The FRα and β isoforms are membrane proteins, the FRγ isoform is cytoplasmic and recently a FRδ isoform with a restricted expression profile has been reported [111]. Mutation-screening studies by our group and others have failed to identify common polymorphisms within the FRα or β gene [112,113], although some low frequency polymorphisms have been reported [114,115]. O'Leary and colleagues also screened the FRβ gene and identified one polymorphism but did not assess the effect on folate and tHcy [116]. In 1998, Wang *et al.* found the FRγ gene to be polymorphic due to a two-basepair deletion, resulting in a truncated protein (denoted FRγ') [117]. No reports concerning genetic variation in the FRδ [111] gene in relation to homocysteine have been published.

Transcobalamin

TCII, encoded by the transcobalamin (TCN) gene, is one of the three vitamin B12- binding proteins in humans, next to haptocorrin (binds B12 in stomach and blood) and intrinsic factor (binds B12 in intestine). Intestinal absorption of vitamin B12 is facilitated by an endocytotic process involving the intrinsic factor-B12 receptor (i.e. cubulin [118]). In the blood, only vitamin B12 bound to TCII (holo-TCII, 10-20%) is available for cellular uptake. The remaining 80 to 90% of circulating vitamin B12 is bound to HC, which functions as an alternative buffer for this vitamin in addition to storage in the liver. Holo-TCII is therefore said to be a better indicator of vitamin B12 status than total plasma vitamin B12.

By sequencing the TCN gene from mothers with NTD-offspring, we identified several polymorphisms including a 776C>G (P259R) transition which decreased holo-, apo- and/or total-TCII levels, possibly resulting from reduced B12 binding [119 120-125]. We observed a trend toward increased tHcy levels [119], whereas Namour *et al.* found that heterozygosity for this variant was associated with significantly higher tHcy compared with both homozygous genotypes [124]. Several other rare polymorphisms in the TCN gene have been reported by our group, such as 67A>G (I23V), 280G>A (G94S), 1043C>T (S348F) and 1196G>A (R399Q) [119]. Although we observed effects for some of these polymorphisms on TCII levels [119] only the 23VV genotype was associated with slightly reduced (p=0.05) tHcy levels [121].

Serine-hydroxymethyltransferase

SHMT catalyses the reversible transfer of the hydroxymethyl group of serine to THF to form 5,10-methyleneTHF and glycine. The enzyme is present in two isoforms, a mitochondrial (mSHMT) and cytoplasmic (cSHMT) form, and is thought to regulate the metabolic competition between the MTHFR and TYMS enzymes [126]. There is also indirect evidence that mSHMT synthesizes glycine while cSHMT may catalyze serine synthesis [127]. In 2001,

we performed single-strand conformation polymorphism (SSCP) analysis on DNA from 70 cases with a NTD to identify genetic variation within both SHMT genes [128]. Several variants were found including a 1420C>T transition in cSHMT and a 4-bp deletion in the 3' untranslated region (UTR) of the mSHMT gene (delTCTT 1721-1724). No effects of these two polymorphisms on tHcy were observed in the general Dutch population, although mothers of children with a neural tube defect with the 1420CC genotype had significant higher fasting tHcy, which was also reflected in a lower red blood cell and plasma folate [128]. Geisel *et al.* did not find an effect of the 1420C>T variant on tHcy in elderly individuals [129].

Betaine-homocysteine methyltransferase

BHMT, predominantly expressed in the liver and kidneys, catalyzes the alternative remethylation route of homocysteine by using betaine as methyldonor. Its importance is demonstrated by the fact that betaine treatment significantly reduces tHcy in homocystinuric patients and healthy volunteers [130]. In addition, the product of the BHMT reaction, dimethylglycine, is converted to sarcosine and further oxidized to glycine, introducing one-carbon units into the folate pool that may be used in folate-dependent remethylation of homocysteine. In 2000, we sequenced the BHMT gene of 16 hyperhomocysteinemic vascular disease patients and reported several variants in the coding region of this gene [131]. Two of them, i.e. 595G>A and 716G>A, were assessed for their effect on tHcy. However, these polymorphisms had no effect on fasting and post-load tHcy levels [131]. Recently, Weisberg and colleagues reported that the 716G>A transition had no effect on the binding properties of BHMT for betaine and did not affect tHcy in vascular disease patients [132].

Methylenetetrahydrofolate dehydrogenase

MTHFD is a trifunctional enzyme that catalyses three sequential reactions in the interconversion of one-carbon derivatives of THF (i.e. 10-formyl, 5,10-methenyl, and 5,10-methyleneTHF). We screened the MTHFD cDNA of 117 NTD cases for the presence of mutations by SSCP analysis and identified two common amino acid substitutions, i.e. R293H and R653Q [133]. However, no effect on tHcy was observed in our study [133] and in two other studies the effect on tHcy was not assessed [134,135]. The effect of these polymorphisms on folate distribution and tHcy needs to be further evaluated.

Dihydrofolate reductase and thymidylate synthase

DHFR is an essential enzyme in the human body as it reduces folic acid to dihydrofolate (DHF) and DHF to THF, with the latter serving as a substrate for 5,10-methyleneTHF synthesis. Theoretically, reduced DHFR activity may deplete THF leading to hyperhomocysteinemia. Kishi *et al.* reported that high-dose MTX, targeting DHFR but also other folate enzymes, induced a transient increase in tHcy [136]. Recently, a 19-bp deletion variant was described in intron 1 of the DHFR gene thereby removing a potential Sp1 transcription factor-binding site [137]. However, the effect on tHcy was not assessed, and additional studies are warranted.

TYMS catalyzes the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), a pyrimidine precursor, and competes with MTHFR

for the one-carbon unit of 5,10-methyleneTHF. Therefore, polymorphisms in these enzymes are potential determinants of tHcy. Two common polymorphisms have been described in the TYMS gene, i.e. a 28-bp repeat in the 5' UTR (yielding the common genotypes 2/2, 2/3 and 3/3) [138] and a 6bp deletion in the 3' UTR (denoted 1494del6) [139]. The 3-repeat allele is associated with enhanced translation efficiency [140] but not gene expression [141]. Several groups studied the effect of the 28-bp repeat on tHcy. Trinh *et al.* found reduced plasma folate and increased tHcy in the Chinese population for TYMS 28-bp repeat 3/3 individuals, an effect that became more pronounced when the MTHFR 677TT genotype was taken into account [142]. In contrast, others did not find an effect on tHcy nor on plasma folate [129,143].
The 1494del6 variant was identified by *in silico* screening of expressed sequence tags [139]. The deletion is said to cause mRNA instability *in vitro* and decreases mRNA levels intratumorally [144], but no studies regarding the effect of this polymorphism on tHcy have been reported.

1.5.2 Genes involved in homocysteine degradation

Cystathionine β-synthase

CBS catalyzes the first step in the transsulfuration pathway (i.e. the condensation of homocysteine and serine to form cystathionine). Deficiency for this enzyme results in classical homocystinuria, and is in many cases caused by the 833C>T mutation. Transsulfuration is the only way to dispose homocysteine from the body. The enzyme is predominantly expressed in the liver and kidneys and hence important in the clearance of tHcy originating from peripheral tissues. The CBS gene harbors many polymorphisms, including a common 68 bp insertion (844ins61) [145], a 31-bp variable number of tandem repeats (14037 31-bp VNTR), a GT short tandem repeat (-5707 GT STR) and single-nucleotide polymorphisms like 1080C>T (A360A) and 699C>T (Y233Y) [146].
The 31 bp VNTR was first identified by Kraus *et al.* [146] and further characterized in our laboratory [147]. The repeat consists of 15 to 21 repeats, the 18 repeat being the most common [146-148]. Because it is located at the exon 13-intron 13 boundary the repeat can, theoretically, create multiple alternate splice sites [148]. We were able to show that the repeat results in alternative splicing and reduces CBS activity in extracts of cultured fibroblasts [147]. We also demonstrated that a higher repeat-length increased tHcy, in particular after methionine loading, by about 10% (18/18 vs. 17/17 genotype). In addition, we found that individuals homozygous for the 18 repeat had higher tHcy than their 17-18 and 17-19 peers when the MTHFR 677TT genotype was taken into account [149]. However, Yang *et al.* observed significantly decreased tHcy levels in individuals carrying the 16-17 and 17-18 genotype when compared to 17-17 individuals [148].
In 1996 a 68 bp insertion (844ins68 bp), located at the junction of intron 7-exon 8 of the CBS gene, was described in American and European populations [145,150,151]. The insertion is surprisingly common in some [150,151], but not all [152], populations, with a frequency ranging from less than 1% up to about 17%. No effect of this insertion on fasting or post-load tHcy was observed in North Americans or young Irish adults [47,150], but DeStefano *et al.* showed that heterozygosity for this variant abolished the tHcy-increasing effect of the MTHFR 677TT genotype [153]. Another group observed increased post-load tHcy in heterozygous individuals

[85], especially when vitamin B6 (<38 nmol/L) was low [154], whereas we found decreased fasting and post-methionine load tHcy in Dutch individuals carrying the mutant allele [155]. The 844ins68 bp variant was found *in cis* with the pathogenic 833T>C mutation. The *in cis* double mutation appeared not to be pathogenic since the splicing of intron 7 eliminates both the insertion and the pathogenic 833T>C mutation, leaving a normal mRNA [150,155].
A GT-dinucleotide STR has been identified upstream of the –1a promoter region of the CBS gene (position -5707 bp) displaying between 14 and 20 repeats [146,156], the 16 repeat being the most common. No data on the effect of this repeat on CBS expression is available, but we could not reveal an association of this STR with fasting or post-load tHcy in the Dutch population [156].
Two silent mutations, i.e. 699C>T and 1080C>T, have been studied for their effect on tHcy. Most studies did not reveal an association of these two polymorphisms with fasting tHcy [153,157] and post-load tHcy [156] although Aras *et al.* did find an effect of the 699C>T variant, though only on post-load tHcy values in cardiovascular disease patients. This lowering effect of the 699TT genotype on tHcy became more pronounced when individuals carrying the CBS 844ins68 bp and the CBS 1080T allele were excluded from the analyses. They observed a similar effect for the 1080C>T variant in which 1080TT individuals had lower post-load tHcy levels, but only after excluding individuals with the 844ins68 bp variant and 699T allele [158].

Cystathionine γ-lyase
Very recently, Wang *et al.* reported a 1364G>T polymorphism (S403I) in the CTH gene, encoding the second enzyme in the homocysteine transsulfuration pathway. They found that 1364TT individuals had significantly higher tHcy compared to their wild type peers [159].

1.5.3 Genes involved in homocysteine formation

Catechol-O-methyltransferase
Transmethylation is essential to cellular function and the activity of methyltransferases is the only mechanism for homocysteine synthesis. Goodman and colleagues reported a common polymorphism (i.e. 324 G>A, Val108Met) in the COMT gene resulting in reduced enzyme activity [160]. COMT is involved in methylation of catecholamines (inactivation) and uses AdoMet as a methyldonor. COMT transcripts originate from one gene but two distinct promoters regulate its expression. This results in a short transcript (soluble form) and a long transcript (membrane-bound form) of the COMT enzyme, both containing the 324G>A transition. Homozygosity for the mutant allele (also denoted as the low activity allele) resulted in lower tHcy compared to their wild type peers [160]. Another group could not confirm these observations in a group of elderly subjects and vegetarians [129].

In conclusion, although a large number of polymorphisms in genes involved in homocysteine, folate, and cobalamin metabolism have been identified and assessed for their effects on tHcy (see Table 1), the MTHFR 677C>T polymorphism is by far the strongest genetic determinant of tHcy in the general population. This leaves part of the genetic contribution in the variation of tHcy unexplained, which is the rationale to continue the search for determinants of tHcy as described in this thesis.

1.6 Objectives and outline of this thesis

The genetic contribution to the variation in homocysteine levels (heritability) is only partly explained. The main goal (Part I) of this thesis was to identify novel genetic determinants of tHcy in the general population, and especially those that are also associated with a higher risk of disease. In this thesis we focused on recurrent venous thrombosis. The identification of determinants of tHcy will also provide information about the pathways and, hence, pathophysiological mechanisms involved in homocysteine-related diseases. The second goal (Part II) was to develop the tools for exploring the role of disturbed transmethylation in homocysteine-related pathology. A detailed knowledge of the pathophysiological processes initiated by high homocysteine may lead to new therapies, other than folic acid and B-vitamin supplementation, as a means to lower homocysteine levels in healthy subjects and patients in order to prevent or treat hyperhomocysteinemia and its related diseases.

Part I of this thesis describes the search for novel genetic determinants of tHcy. Because the folate cycle plays an essential role in homocysteine remethylation we focused on variation in genes encoding folate-converting enzymes as potential determinants of homocysteine. These studies are described in Chapters 2 to 4. In **Chapter 2** we studied common variants in genes encoding homocysteine remethylation enzymes (methionine synthase and methionine synthase reductase) and their relation to venous thrombosis risk, tHcy and serum folate. Given the importance of vitamin B12 in homocysteine remethylation we also assessed vitamin B12 status in relation to venous thrombosis. In **Chapter 3** we describe the molecular genetic analysis of the dihydrofolate reductase gene and the effect of newly identified variants on tHcy, serum and red blood cell folate. The effect of common variants in three enzymes of folate metabolism (thymidylate synthase, reduced folate carrier and AICAR transformylase/IMP cyclohydrolase) on tHcy and venous thrombosis risk is shown in **Chapter 4**. There is accumulating evidence that disturbed transmethylation may partly explain hyperhomocysteinemia-related pathology. Therefore, we also studied polymorphisms in genes involved in transmethylation processes, and their association with tHcy levels and disease (Chapters 5 to 8). In **Chapter 5** we describe the molecular genetic analysis of the S-adenosylhomocysteine hydrolase gene and investigated the effect of newly identified variants on tHcy levels and venous thrombosis risk. In **Chapter 6** we performed haplotype analysis in order to examine the role of catechol-O-methyltransferase in hyperhomocysteinemia and risk for venous thrombosis.

Part II of this thesis describes the studies that were performed to enable the investigation of a disturbed homocysteine metabolism in relation to transmethylation. In response to the increasing interest in transmethylation parameters for research and (future) diagnostic purposes we developed a method for the determination of AdoMet and AdoHcy in plasma and other body fluids, which is described in **Chapter 7**. Because elevated homocysteine is thought to affect transmethylation reactions, we aimed to investigate protein methylation in cultured human umbilical vein endothelial cells (HUVECs). The preliminary results of these experiments are described in **Chapter 8**.

Part *I*

Genetic determinants of plasma total homocysteine

Chapter 2

Disturbed Vitamin B12 Metabolism, Variation in Homocysteine Remethylation Genes and Recurrent Venous Thrombosis Risk

Henkjan Gellekink, Leo A.J. Kluijtmans, Henk J. Blom, Martin den Heijer

Laboratory of Pediatrics and Neurology, Department of Endocrinology and Department of Epidemiology and Biostatistics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Submitted for publication

Abstract

Some studies have shown that a decreased vitamin B12 level is associated with a higher risk of venous thrombosis, independent of its relation with homocysteine. Variation in genes involved in cobalamin metabolism and/or redox status, e.g. methionine synthase (MTR) and methionine synthase reductase (MTRR), may modulate cellular processes and predispose to vascular disease as well. We examined whether low vitamin B12 status and genetic variants in the MTR and MTRR genes were associated with disease risk in a case-control study on recurrent venous thrombosis. We measured vitamin B12, methylmalonic acid (MMA), plasma total homocysteine (tHcy) and folate and screened for the MTR 2756A>G and MTRR 66A>G variants in cases and controls. High plasma MMA (>0.24 μmol/L), but not low plasma vitamin B12 (<156 pmol/L), was suggestive for an increased risk of recurrent venous thrombosis (crude odds ratio 1.44 (95% CI 0.96 to 2.17, p=0.07). The risk estimate for the top (>0.24 μmol/L) versus bottom quintile (<0.13 μmol/L) was 2.10 (95% CI 1.21 to 3.57; p=0.03). An interaction for MMA above the 80th percentile of the control range and the MTRR 66GG genotype was observed, increasing the risk of venous thrombosis five times (crude odds ratio 5.4 [95% CI 1.3 to 21.6], p=0.02). Our results suggest that high plasma MMA, indicative of low intracellular vitamin B12 status, may increase the risk of recurrent venous thrombosis, especially in combination with the MTRR 66GG genotype.

Introduction

Venous thrombosis is a complex disease that involves both genetic and environmental factors. Hyperhomocysteinemia is an established risk factor for cardiovascular disease, including venous thrombosis [18,19]. Several B-vitamins (such as B6, B12 and folate) are essential for homocysteine metabolism but recent studies suggest that low 5-methyltetrahydrofolate or vitamin B6 increases thrombosis risk, independent of homocysteine [161,162]. Some studies [163-167], but not all [168], show that low plasma vitamin B12 increases the risk of thrombosis as well. Vitamin B12 (cobalamin) is cofactor in two metabolic pathways either as adenosylcobalamin (AdoCbl) or methylcobalamin (MeCbl). AdoCbl is a cofactor of methylmalonyl-CoA mutase, an enzyme that converts methylmalonyl-CoA into succinyl-CoA, whereas MeCbl is a cofactor in the remethylation of homocysteine. Plasma vitamin B12 levels do not always reflect vitamin B12 status, because only the fraction bound to transcobalamin (about 10-20%) is available for cellular uptake. In that respect, plasma methylmalonic acid (MMA) is regarded a sensitive indicator of intracellular vitamin B12 status [169] and shows a positive correlation with plasma total homocysteine levels (tHcy) [170]. Given the multifactorial nature of cardiovascular diseases, variation in genes affecting the function of the encoded enzymes that need vitamin B12 as a cofactor (such as methionine synthase, MTR) or maintain cobalamin redox status (such as methionine synthase reductase, MTRR) may further increase disease risk, in addition to suboptimal vitamin B12 levels. In the past decade, polymorphisms in MTR (c.2756A>G, D919G) and MTRR (c.66A>G, I22M) have been described [82,93]. MTR catalyzes the remethylation of homocysteine by 5-methyltetrahydrofolate to methionine in which cobalamin serves as an intermediate methyl carrier. Oxidation of cobalamin, however, inactivates the MTR-cobalamin enzyme complex and needs reactivation by MTRR [88] (Figure 3). Both polymorphisms have been studied as a possible risk factor for cardiovascular diseases [83,84,87,95,96,171-174], although data are not consistent.

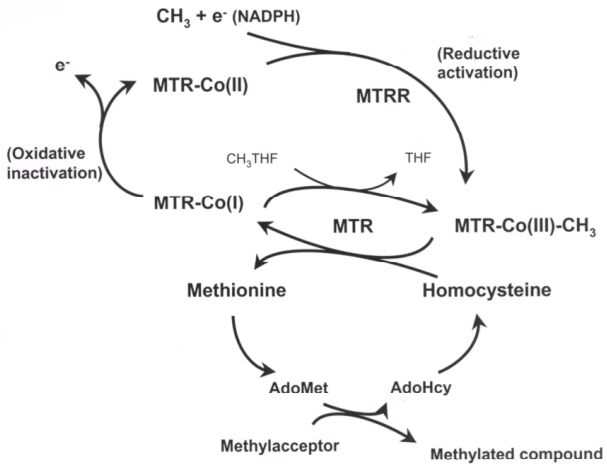

Figure 3. Role of methionine synthase reductase (MTRR) and the methionine synthase (MTR)-cobalamin complex in homocysteine remethylation. Homocysteine is remethylated by the MTR-cob(III)alamin enzyme complex. Occasionally, cob(I)alamin is oxidized to cob(II)alamin thereby inactivating the enzyme complex. MTRR catalyzes the reductive methylation of cob(II)alamin to methylcob(III)alamin thus rendering MTR into the active cycle. Co: cobalamin AdoHcy: S-adenosylhomocysteine, AdoMet: S-adenosylmethionine, NADPH: reduced nicotinamide adenine dinucleotide phosphate, (CH_3)THF: (methyl)tetrahydrofolate

In this study we examined plasma vitamin B12 and MMA as a risk factor for recurrent venous thrombosis. The MTRR 66A>G and MTR 2756A>G polymorphisms were assessed as

genetic risk factors for venous thrombosis as well. We also studied the effect of both genetic variants on tHcy, folate, MMA and vitamin B12.

Material and Methods

Patients and controls

We used data and DNA samples of a case-control study including 185 recurrent venous thrombosis patients and 500 control subjects. Patients were selected from the files of the anticoagulant clinic of The Hague and are described in more detail elsewhere [6]. In the Netherlands, virtually all patients with a history of recurrent venous thrombosis have long-term coumarin therapy and are registered at an anticoagulant clinic. It has been shown that coumarin therapy does not influence tHcy [175]. All patients between 20 and 90 years old, who had two or more episodes of venous thrombosis (ratio pulmonary embolism / deep-vein thrombosis is 1:1.5), were invited to take part and 185 patients were enrolled in this study. The control group was recruited via a general practice in The Hague [6]. We obtained a short medical history of all patients by interview and of all controls by questionnaire. DNA for genotyping was available from 178 patients and 446 population-based controls from whom relevant biochemical data had already been obtained [6]. Factor V Leiden and prothrombin 20210G>A mutation analysis has been described previously [176]. The medical ethics committee approved the study protocol and informed consent was obtained from all study participants.

Biochemical parameters

Blood samples were drawn from the antecubital vein in 5 mL Vacutainer tubes and 4.5 mL EDTA vacuum glass tubes for determination of plasma vitamin B12, MMA, tHcy and folate, and for DNA extraction. EDTA samples for plasma total homocysteine (tHcy) measurement were placed on ice immediately and centrifuged at 3500 g for 5 minutes with minimal delay. The plasma was separated and stored at -20 ºC until analysis. Plasma MMA levels were measured, using HPLC combined with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). The LC-ESI-MS/MS system consists of a Quattro LC (Waters Corporate, Milford, MA) connected to an HP1100 HPLC (Agilent Technologies, Palo Alto, CA) and a 232 XL autosampler (Gilson, Middleton, WI). Hundred µl of plasma together with 100 µl of 0.8 µM stable isotope-labeled methyl-d3-malonic acid (MMA-d3) (Cambridge Isotope Laboratories, Andover, MA) as an internal standard, was deproteinized using ultra filtration (Microcon YM30 filter, cut-off value 30 kDa, Millipore). Subsequently, 20 µl 1M formic acid was added to 150 µl of the ultra filtrate and was injected onto a Waters Symmetry column (2.1mmX100mm 3.5 µM). The mobile phase consisted of 15% Methanol / 0.3% formic acid and is eluted at 200 µl/min. Thereafter, 10 µl was analysed by tandem MS monitoring the carbonyl loss of MMA and MMA-d3; recording the transition of m/z 117 to m/z 73 and m/z 120 to m/z 76, respectively. This method was compared with an established LC-MS/MS method as published by Schneede *et al.* [177]. Deming linear regression formula (MMA range 0.10 to 1.60 µmol/L) Y = 1.013 (±0.011) X – 0.0218 (±0.0038), confidence interval of the slope 0.9919 to 1.035 (n=125). Within- and between-day coefficients of variation were below 6%, and the

limit of quantitation is 100 nmol/L. tHcy was measured using 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), as described earlier [178]. Vitamin B12 and folate concentrations were measured with the Dualcount Solid Phase No Boil Radioassay (Diagnostic Product Corporation, Los Angeles, CA, USA). DNA extraction was performed as described previously [179] and was stored at 4 ºC.

MTRR 66A>G and MTR 2756A>G genotyping

Since the MTRR 66A>G (I22M) polymorphism does not create or abolish a restriction site we used a PCR-heteroduplex generator (HG)-based technology to screen for this polymorphism, essentially as described earlier [91,180]. In short, a HG is a DNA molecule that is identical to a short sequence flanking the site of interest, except for a microdeletion close to the polymorphic site. This HG and the DNA under test are amplified and diluted 1:1. After a denaturation and reannealing step homoduplexes and heteroduplexes are generated. These products have a characteristic migration pattern on a polyacrylamide gel due to their differently-sized protruding loops. The HG used for MTRR 66A>G genotyping was synthesized by PCR-mediated site-specific mutagenesis (using the A allele as a template). A 3 bp deletion was introduced 3 bp downstream of the polymorphic site and the HG was cloned into the pGEM-T Easy vector (Promega, WI, USA), resulting in plasmid pMTRR66A>G. Positive clones were identified by sequence analysis and served as a positive control in every experiment. The primers MTRR1 (fw 5'-GAGGAGGTTTCTGTTACTATATGC-3') and MTRR4 (rv 5'-GTGAAGATCTGCAGAAAATCCATGTA-3') were used to amplify HG and genomic DNA separately under the following PCR conditions: an initial denaturation step for 5 minutes at 94ºC; 35 cycles of 1 minute at 95ºC, 1 minute at 55ºC and 30 seconds at 72ºC followed by a final extension for 7 minutes at 72ºC. PCR amplifications were performed in a total volume of 50 μL in an iCycler (Biorad, The Netherlands); each mixture contained 50 nmol/L of both the forward and reverse primer (Biolegio BV, Malden, The Netherlands), 200 μM dNTPs, 10 mM Tris-HCl buffer (pH 8.2), 50 mM KCl, 2.0 mM $MgCl_2$, 0.5 U of recombinant Taq polymerase, 5% DMSO (all from Invitrogen, The Netherlands) and 75 ng genomic DNA or 1 pg HG. To facilitate heteroduplex formation both PCR products were mixed (1:1), followed by denaturation for 5 minutes at 95ºC and allowed to re-anneal upon cooling to room temperature.

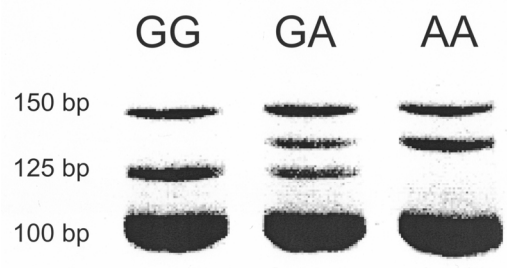

Figure 4. Migration pattern of positive controls for the MTRR 66 A>G polymorphism analysed by the heteroduplex generator method The primers were designed to amplify fragments of 103 and 106 bp from pMTRR66A>G and genomic DNA, respectively. Reannealing of both products results in duplex formation and hence an allele-specific migration pattern is established. In this case, the A and G alleles are identified by heteroduplexes that migrate as a 130 bp or 120 bp dsDNA fragment, respectively. The lower bands at ~100 bp are homoduplexes and the upper bands are non-specific heteroduplexes. The position of the molecular weight marker is shown on the left.

The products (diluted 1:1 with loading buffer; 2.5 g/L bromophenol blue, 0.36 v/v 87% glycerol and 10 mM Tris-HCl, pH 9.2) were analysed by electrophoresis on 17% polyacrylamide (19:1) gels at 150 V for 5-6 hours. To identify the MTRR 66A>G genotypes, the gels were stained with ethidium bromide (20 mg/L) and illuminated using UV-light (Figure 4). The MTR 2756A>G polymorphism was assayed as described earlier [82]. MTR 2756A>G genotype data was not available for six controls and five cases.

Statistics

Odds ratios and 95% confidence intervals were calculated to estimate the relative risk for venous thrombosis conferred by MMA and plasma B12 levels or genotypes using logistic regression analysis. Logistic regression analysis was also used to study interactions and linear regression analysis was applied to assess differences in (log-transformed) metabolite concentrations between different genotypes. A two-tailed $p<0.05$ was accepted as statistically significant. For all analyses SPSS 12.0 for Windows was used.

Results

Plasma vitamin B12 and MMA levels in cases and controls

The baseline characteristics of our study populations are presented in Table 2. Mean plasma vitamin B12 level (95% CI) was 228 (210 to 246) pmol/L and 218 (210 to 230) pmol/L for cases and controls, respectively (p=0.17). We stratified plasma vitamin B12 levels of both cases and controls into quintiles and calculated the relative risk for each stratum compared with the top quintile (Table 3). Plasma vitamin B12 concentrations were not related to recurrent venous thrombosis risk. The crude relative risk for recurrent venous thrombosis at plasma vitamin B12 below the 20th percentile (156 pmol/L) of the control distribution was 1.12 (95% CI 0.72 to 1.73) (Table 4).

Table 2. Baseline characteristics of recurrent venous thrombosis cases and population-based controls

Variable	Cases (n=185)	Controls (n=500)	p value
Age (range)	61 (21 to 81)	51 (23 to 87)	<0.01
Sex (men, %)	94 (50.8)	208 (41.6)	0.03
Factor V Leiden (MAF, %[a])	14.6	3.3	<0.01
Prothrombin G20210A (MAF, %[a])	2.9	1.6	0.12
Post-menopausal women, n (%)	64 (34.6)	138 (27.6)	<0.01
Time between 1st event and study	17 (range 1 to 58) y	-	
Time between last event and study	7 (range 1 to 30) y	-	
Creatinine (μmol/L)	82.4 (77.9 to 86.6)	74.0 (72.4 to 75.7)	<0.01
tHcy (μmol/L)	12.4 (11.5 to 13.3)	10.4 (10.0 to 10.8)	<0.01
Plasma folate (nmol/L)	13.0 (12.2 to 13.7)	13.8 (12.5 to 15.0)	0.25

[a] MAF, minor allele frequency

Mean plasma MMA levels (95% CI) were higher in cases than controls [0.20 (0.18 to 0.22) μmol/L and 0.18 (0.17 to 0.19) μmol/L respectively, p=0.03]. Stratification of MMA level into quintiles showed that the risk estimates increased with MMA concentration (p-trend=0.11, Table 3). The relative risk for recurrent venous thrombosis was 2.10 (95% CI 1.21 to 3.57, p=0.03) for subjects in the top quintile, using the bottom quintile as a reference. The crude relative risk for subjects with a plasma MMA level above versus below the 80th percentile (0.24 μmol/L) was 1.44 (95% CI 0.96 to 2.17, p=0.07) (Table 4).

Adjustment for possible confounders like age, sex, creatinine, or other risk factors of venous thrombosis, such as the factor V Leiden and Prothrombin 20210G>A mutation and menopausal status did not affect the risk estimates (Table 4). In addition, exclusion of factor V Leiden and prothrombin 20210G>A mutation carriers yielded an odds ratio of 1.69 (95% CI 1.05 to 2.73, p=0.03) (not shown).

Table 3. Recurrent venous thrombosis (RVT) risk for each quintile of plasma vitamin B12 and MMA

Variable	Percentile	Cut-off value	RVT Patients, n	Controls, n	OR (95% CI)	p value
Vitamin B12	0 – 20	< 156	33	98	1.48 (0.88 to 2.50)	0.14
	20 – 40	157 – 194	26	101	1.26 (0.74 to 2.15)	0.39
	40 – 60	195 – 239	32	99	0.96 (0.55 to 1.68)	0.89
	60 – 80	240 – 315	43	101	0.76 (0.43 to 1.37)	0.37
	80 – 100	> 316	50	100	1.0[a]	-
MMA	0 – 20	< 0.13	29	122	1.0[a]	-
	20 – 40	0.13 – 0.16	43	115	1.57 (0.92 to 2.69)	0.10
	40 – 60	0.16 – 0.19	36	93	1.63 (0.93 to 2.85)	0.09
	60 – 80	0.19 – 0.24	30	73	1.73 (0.96 to 3.11)	0.07
	80 – 100	> 0.24	45	91	2.10 (1.21 to 3.57)	0.008

[a] reference category

Table 4. Odds ratios (OR) of recurrent venous thrombosis (RVT) associated with low plasma vitamin B12 or high plasma MMA level

Variable	Cut-off value	RVT Patients n (%)	Controls n (%)	Crude odds ratio (95% CI)	Odds ratio (95% CI) [b]	Odds ratio (95% CI) [c]
Vitamin B12	<156 pmol/L	33 (17.9)	98 (19.6)	0.89 (0.58 to 1.38)	0.88 (0.57 to 1.37)	1.11 (0.52 to 2.38)
	>156 pmol/L	151 (82.1)	401 (80.4)	1.0[a]	1.0[a]	1.0[a]
				P=0.62	p=0.58	p=0.79
MMA	<0.24 μmol/L	138 (74.6)	403 (80.6)	1.0[a]	1.0[a]	1.0[a]
	>0.24 μmol/L	45 (24.3)	91 (18.2)	1.44 (0.96 to 2.17)	1.49 (0.98 to 2.25)	1.65 (0.80 to 3.42)
				p=0.07	p=0.06	p=0.17

[a] reference category, [b] risk estimates were adjusted for age, sex and creatinine, [c] risk estimates were adjusted for age, sex, creatinine, menopausal status, factor V Leiden and prothrombin 20210G>A mutations

MTR and MTRR genotypes and associated recurrent venous thrombosis risk

The genotype distributions of both the MTR 2756 A>G and MTRR 66A>G polymorphisms in the control population were in Hardy-Weinberg equilibrium (P>0.75) and similar in cases and controls (Pearson's χ^2, p=0.85 and p=0.52, respectively). MTR 2756GG, GA and AA genotype frequencies were 70.7 (n=118), 27.5 (n=46) and 1.8% (n=3) for cases and 72.3

(n=318), 25.7 (n=113) and 2.0% (n=9) for controls, respectively. The odds ratio for MTR 2756GG individuals compared to 2756AA individuals for venous thrombosis was 0.87 (95% CI 0.23 to 3.26). MTRR 66AA, AG and GG genotype frequencies were 19.1 (n=34), 44.4 (n=79) and 36.5% (n=65) for cases and 19.1 (n=85), 48.9 (n=218) and 32.1% (n=143), respectively (odds ratio 1.14 [95% CI 0.69 to 1.86] for MTRR 66GG compared with 66AA subjects). In addition, compound genotypes of MTR and MTRR variants did not affect risk of disease (not shown).

Interaction between plasma MMA and MTRR genotype

Because the cobalamin-MTR complex is reductively methylated by the MTRR enzyme, we assessed whether the MTR 2756A>G or MTRR 66A>G polymorphisms modified the effect of vitamin B12 status on disease risk. We observed an interaction between the MTRR genotype and high MMA levels. The adjusted odds ratios for recurrent venous thrombosis in subjects with the MTRR 66AG or 66GG genotype and high MMA (>80th percentile) were 2.4 (95% CI 0.6 to 9.5, p=0.18) and 5.1 (95% CI 1.3 to 20.5, p=0.03) (Table 5). No such association was observed for low plasma vitamin B12 and MTRR genotype. In addition, no interaction between high MMA (Table 5) or low vitamin B12 and MTR 2756A>G genotype on disease risk was observed.

Association between MTR and MTRR variants and metabolites

The separate effects of the MTR 2756A>G and MTRR 66A>G genotypes on tHcy, vitamin B12, MMA and folate in our control group are shown in Table 6. No evident effects of the two polymorphisms on tHcy, MMA and folate were observed. MTR 2756GG subjects had a decreased plasma vitamin B12 (-30%, p=0.007) compared to the 2756AA genotype, but this was not reflected in plasma MMA or tHcy levels. Creatinine levels were similar for the different genotypes in cases and controls (p-ANOVA=0.40 and 0.80 for the MTR and MTRR variant, respectively). In addition, low vitamin B12 status (B12 <156 pmol/L or MMA >0.24 μmol/L) did not affect tHcy in the different genotype groups defined by the MTRR and MTR variants (not shown).

Table 5. Interaction analysis for recurrent venous thrombosis (RVT) risk

Genetic variant	Genotype	MMA (>0.24 μmol/L) [a]	RVT cases, n	Controls, n	Crude OR (95% CI)	P value	OR (95% CI) [b]	P value
MTRR 66A>G	AA	No	28	69	1.0 [c]	-	1.0 [c]	-
	AG	No	61	176	0.85 (0.50 to 1.45)	0.56	0.74 (0.42 to 1.30)	0.29
	GG	No	44	116	0.94 (0.53 to 1.64)	0.81	0.80 (0.44 to 1.46)	0.47
	AA	Yes	5	16	0.77 (0.26 to 2.30)	0.64	0.38 (0.12 to 1.24)	0.11
	AG	Yes	17	38	1.68 (0.47 to 5.97)	0.43	2.43 (0.62 to 9.47)	0.20
	GG	Yes	21	25	2.88 (0.79 to 10.42)	0.10	5.12 (1.28 to 20.45)	0.02
MTR 2756A>G	AA	No	94	264	1.0 [c]	-	1.0 [c]	-
	AG	No	32	87	1.0 (0.65 to 1.65)	0.89	1.01 (0.61 to 1.68)	0.96
	GG	No	2	7	0.80 (0.16 to 3.93)	0.79	1.17 (0.23 to 5.92)	0.85
	AA	Yes	26	49	1.49 (0.88 to 2.53)	0.14	1.15 (0.64 to 2.04)	0.65
	AG	Yes	16	25	1.17 (0.47 to 2.92)	0.74	1.08 (0.40 to 2.92)	0.88
	GG	Yes	1	2	1.17 (0.06 to 21.72)	0.91	1.60 (0.08 to 31.42)	0.76

[a] MMA level of 0.24 μmol/L corresponds with the 80th percentile of the control population [b] Adjusted for age, sex and creatinine, [c] Reference category

Table 6. Association between the MTRR 66A>G and MTR 2756A>G polymorphisms and tHcy, plasma vitamin B12, MMA and folate in controls
Absolute changes are shown in the first row of the corresponding metabolite, and the relative changes are shown in the second row

Mean	MTRR 66A>G genotype			MTR 2756A>G genotype		
plasma level	66AA (n=85)	66AG (n=218)	66GG (n=143)	2756AA (n=318)	2756AG (n=113)	2756GG (n=9)
tHcy μmol/L [95% CI]	10.3 [9.5 to 11.2]	10.2 [9.3 to 11.2]	10.9 [9.8 to 12.0]	10.4 [10.0 to 10.9]	10.2 [9.4 to 11.1]	10.5 [8.1 to 13.5]
Relative change (95% CI)	0[a]	-1.2 % (-10.2 to 8.8)	5.1 % (-5.2 to 16.5)	0[a]	-2.5 % (-10.3 to 5.9)	0.2 % (-22.2 to 29.3)
Vitamin B12 pmol/L [95% CI]	216 [196 to 236]	227 [203 to 253]	215 [191 to 242]	225 [215 to 236]	217 [198 to 239]	152 [114 to 202] [b]
Relative change (95% CI)	0[a]	5.4 % (-5.5 to 17.6)	-0.3 % (-11.3 to 12.1)	0[a]	-3.4 % (-12.0 to 6.0)	-32.6 % (-49.3 to -10.3)
MMA μmol/L [95% CI]	0.17 [0.16 to 0.19]	0.18 [0.16 to 0.20]	0.17 [0.16 to 0.20]	0.17 [0.16 to 0.18]	0.18 [0.17 to 0.20]	0.19 [0.15 to 0.26]
Relative change (95% CI)	0[a]	6.0 % (-4.6 to 17.9)	1.5 % (-9.4 to 13.7)	0[a]	7.1 % (-2.2 to 17.4)	11.1 % (-15.9 to 46.8)
Folate nmol/L [95% CI]	13.2 [11.9 to 14.7]	12.5 [11.1 to 14.2]	13.6 [11.9 to 15.5]	12.7 [12.0 to 13.4]	13.6 [12.2 to 15.1]	15.4 [11.2 to 21.4]
Relative change (95% CI)	0[a]	-5.2 % (-16.2 to 7.3)	3.1 % (-9.6 to 17.7)	0[a]	7.5 % (-3.3 to 19.4)	21.9 % (-12.0 to 68.8)

[a] reference category, [b] P ANOVA <0.05

Discussion

Several studies have shown that low plasma vitamin B12 status increases the risk for venous thrombosis [163-167,181], although not all studies could confirm this finding [168]. We investigated vitamin B12 status and the MTR 2756 A>G and MTRR 66A>G polymorphisms as a risk factor for recurrent venous thrombosis.

In the present study, high plasma MMA seems a modest risk factor for recurrent venous thrombosis, independent of known risk factors for venous thrombosis. We observed that the MTRR 66A>G genotype modified the risk associated with high plasma MMA levels. Recurrent venous thrombosis risk increased more than five-fold in subjects with the MTRR 66GG genotype and a plasma MMA above the 80th percentile (>0.24 μmol/L). No such associations were observed for plasma vitamin B12 levels and disease risk.

Vitamin B12 status is difficult to assess, as low plasma vitamin B12 levels do not always indicate low vitamin B12 status and normal plasma vitamin B12 levels do not always exclude intracellular vitamin B12 deficiency [182,183]. Plasma MMA is regarded a better indicator of intracellular vitamin B12 status than total plasma vitamin B12 concentrations [169]. Holo-transcobalamin, the vitamin B12 carrier in the blood that is required for cellular uptake, could be measured in order to verify our observation that low vitamin B12 status may increase recurrent venous thrombosis risk. Although the cause of the lower vitamin B12 status is unclear, it may partly explain the high tHcy in our cases (Table 2). Possibly, mild dysfunction of one of the vitamin B12 carriers/transporters such as cubulin or low vitamin B12 intake may be involved.

We show that the MTRR 66A>G and MTR 2756A>G polymorphisms in themselves did not affect venous thrombosis risk in the general population. This is in agreement with some studies [96,171,172], but not all [83,95]. One group reported an interaction between these two variants on thrombosis risk in a small population [184], but we could not reproduce their finding. We did observe an interaction between MMA and the MTRR genotype on disease risk, suggesting that mild MTRR dysfunction due to the 66A>G polymorphism may affect disease risk but only at suboptimal vitamin B12 status. The functional effect of the MTR 2756A>G polymorphism is not known, but the group sizes in our study do not allow definite conclusions to be drawn as to whether this variant is a risk factor for recurrent venous thrombosis or not.

Like other groups, we did not observe an effect of the MTRR 66A>G variant on tHcy or other metabolites [94-96], although some studies did find an effect of the MTRR 66A>G variant on tHcy [91,92,174,185]. These inconsistencies may be attributed to differences in lifestyle or nutrition between these populations. We, and others, observed that the MTR 2756A>G variant did not alter tHcy, plasma MMA and folate [82,94]. The low plasma vitamin B12 level in MTR 2756GG subjects was not reflected by plasma MMA or tHcy levels, and may be influenced by chance (n=9).

The mechanism via which low vitamin status or hyperhomocysteinemia causes disease is not well understood. The observation that low vitamin B12 status affects transmethylation processes [186] may have implications for methylation of DNA, proteins or hormones. The measurement of methylation parameters like S-adenosylmethionine and S-adenosylhomocysteine, and methylation status may provide more insight into the association between suboptimal vitamin B12 status and venous thrombosis. In addition, vitamin B12

deficiency was shown to increase the levels of the pro-inflammatory cytokine, tumor necrosis factor α (TNF-α) [187]. TNF-α is known to affect coagulation via downregulating thrombomodulin expression, thus contributing to a pro-coagulable state [188,189]. The former association is likely to be mediated by homocysteine, while the latter may be independent from one carbon metabolism and directly due to the effects of low vitamin B12 status.

There are some limitations of this study to consider. Our study concerns subjects with two or more episodes of venous thrombosis, which may result in slightly higher risk estimates. An effect of low plasma vitamin B12 on a first thrombotic event has been demonstrated [181], but the investigation of plasma MMA as a risk factor for a first thrombotic event is awaited. Secondly, our study may be too small to detect small changes in metabolites or relative risk conferred by the separate polymorphisms. Our study demonstrated an overall effect of high MMA on recurrent venous thrombosis risk, which remained after adjustment for possible confounders. Although the risk profile may be different for specific subgroups (e.g. age, sex), the size of our study population is too small to perform proper subgroup analysis. The investigation of suboptimal vitamin B12 status as a risk factor for (recurrent) venous thrombosis in larger populations and specific subgroups is warranted.

In conclusion, our results show that high plasma MMA, indicative of low intracellular vitamin B12 status, may increase the risk of venous thrombosis, independent of established risk factors for venous thrombosis. Mild dysfunction of the homocysteine remethylation pathway, by virtue of the MTRR 66A>G polymorphism may further increase that risk.

Acknowledgments

This study was in part supported by grant 2002B68 from the Netherlands Heart Foundation. Martin den Heijer, MD, PhD, is supported by a VENI-grant from the Dutch Organization for Scientific Research (NWO). The authors greatfully acknowledge Sandra G. Heil, PhD, for suggestions and critical evaluation of the manuscript.

Chapter 3

Molecular Genetic Analysis of the Human Dihydrofolate Reductase Gene

Relation with plasma total homocysteine, serum and red blood cell folate levels

Henkjan Gellekink, Henk J. Blom, Ivon J.M. van der Linden, Martin den Heijer

Laboratory of Pediatrics and Neurology, Department of Endocrinology and Department of Epidemiology and Biostatistics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

European Journal of Human Genetics
(In press, doi 10.1038/sj.ejhg.5201713)

Abstract

Disturbances in folate metabolism may increase the risk of certain malignancies, congenital defects and cardiovascular diseases. The gene dihydrofolate reductase (DHFR) is primarily involved in the reduction of dihydrofolate, generated during thymidylate synthesis, to tetrahydrofolate in order to maintain adequate amounts of folate for DNA synthesis and homocysteine remethylation. In order to reveal possible variation that may affect plasma total homocysteine, serum folate and red blood cell folate levels, we sequenced the DHFR coding region as well as the intron-exon boundaries and DHFR flanking regions from twenty Caucasian individuals. We identified a 9-bp repeat in the 5' upstream region that partially overlapped with the 5' untranslated region, and several single-nucleotide polymorphisms, all in non-coding regions. We screened subjects for the 9-bp repeat (n=417), as well as the recently reported 19-bp deletion in intron 1 (n=330), and assessed their associations with plasma total homocysteine, serum and red blood cell folate levels. The 19-bp del/del genotype was associated with a lower plasma total homocysteine (-14.4% [95% CI -23.5 to -4.5], p=0.006) compared with the wild type genotype. This may suggest that intracellular folate levels are affected.

Introduction

Folate, a water-soluble B-vitamin, has gained increasing interest for its essential role in DNA synthesis/repair and the transmethylation pathway. The primary function of folate is as a carrier of one-carbon units for purine and thymidine synthesis and the conversion of homocysteine to methionine. Because of the close interrelation between folate and homocysteine, low folate status is reflected by elevated plasma total homocysteine concentrations (tHcy) [190,191]. A number of diseases have been linked to disturbances in the above-mentioned pathways. For example, low folate status increases the risk of certain cancers [29,32,33] and is related to congenital malformations, such as neural tube defects [22]. Moreover, an increase in tHcy increases the risk of cardiovascular disease and congenital abnormalities [18,19,192].

Dietary folates, present in reduced and partially reduced form [193], exist as polyglutamates and require hydrolysis to monoglutamates before absorption in the intestine. After release into the portal circulation, folate (mainly as 5-methyltetrahydrofolate) is distributed throughout the body and is used for homocysteine remethylation, leaving tetrahydrofolate (THF) and methionine (Figure 1). Methionine is used for protein synthesis or converted to S-adenosylmethionine (AdoMet), the principle methyl donor in the human body. THF is converted to methyleneTHF, among others, and used for thymidylate synthesis. The latter generates dihydrofolate (DHF) that requires the action of dihydrofolate reductase (DHFR) to be reduced to THF again. In addition, folic acid present in vitamin pills and fortified foods requires full reduction by DHFR via DHF into THF in order to become metabolic active. This makes DHFR an essential enzyme not only for the initial reduction of dietary folates (and folic acid) but also for the continuous reduction of DHF into THF generated during thymidylate synthesis [194].

DHFR (EC 1.5.1.3) is located on chromosome 5q11.2-q13.2 and spans about 30 kb. The gene consists of 6 exons, yielding a mRNA of about 3.9 kb [194]. DHFR enzyme (AC 008434.5) is a cytosolic protein of 21.3 kDa and requires NADP as a cofactor. At least three pseudogenes of DHFR are known, i.e. DHFRP1 (chromosome 18), DHFRP2 (chromosome 6) and DHFRP4 (chromosome 3, also denoted DHFRL1) [195,196], which are highly homologous to the corresponding DHFR coding regions. Outside these regions sequence homology is relatively low [197]. DHFR deficiency (OMIM 126060) is sporadically seen and results in severe megaloblastic anaemia and mild to moderate mental retardation [198,199]. The antifolate methotrexate (MTX) is well known for its inhibitory effect on DHFR thereby preventing DNA synthesis and, hence, tumor growth [200]. In addition, inhibition of DHFR by MTX and the antibiotic trimethoprim were shown to increase tHcy levels by 50% [136,201], suggesting that DHFR dysfunction due to genetic variants may affect tHcy as well. Recently, Johnson *et al.* described a 19-bp deletion that may affect gene expression [137].

In this study we aimed to identify genetic variation within the DHFR gene by sequencing the entire coding region, including the intron-exon boundaries and DHFR flanking regions. Newly identified variants, and the 19-bp deletion in intron 1 [137], were assessed for their effect on tHcy and serum and red blood cell folate concentrations in population-based controls.

Material and Methods

Study population

DNA from twenty individuals of Caucasian origin, who had participated in an earlier study [6], were used for DHFR sequence analysis. Functional polymorphisms in the DHFR gene are likely to be reflected in plasma folate and homocysteine levels. The subjects were selected based on these criteria: six subjects had low folate (<10th percentile = 7.0 nmol/L) and high homocysteine levels (>90th percentile = 15.5 μmol/L), nine subjects had a plasma total homocysteine above 15.5μmol/L and 5 subjects were selected randomly. Subjects with low vitamin B12 (<10th percentile = 124.7 pmol/L) or the MTHFR 677TT genotype were excluded. We aimed to identify common genetic variants that may contribute to changes in tHcy or folate at the population level. According to a binomial distribution, the chance of finding an allele with a frequency of 2.5% in 1 out of 40 alleles is 0.64. In addition, the minimum allele frequency required to find at least 1 out of 40 alleles (with 95% confidence) is 7.2%. The population used for the association study was recruited via a general practice in The Hague (The Netherlands) and has been described in more detail elsewhere [6]. In total 2812 people, 20-90 years of age, were invited to take part in a health survey on risk factors for cardiovascular disease. From the group that agreed to participate (n=532), the first 500 were included. For the current study, DNA was available of 438 individuals. All participants gave their informed consent.

Sequence analysis of the DHFR gene

Blood samples were drawn from the antecubital vein. DNA was extracted [179] and then stored at 4 ºC. Genomic DNA sequencing of the entire coding region (GenBank accession number NT_006713, AC008434.5) was performed in order to screen the DHFR gene for variation. We also sequenced 665 bp of the 5' upstream region, which included the 5' UTR of about 500 bp (NM_000791), and 1300 bp downstream of DHFR. Intron-based primers were developed to avoid amplification of pseudogenes and to reveal potential splice site variants. Concerning amplification of both 5' and 3' UTRs, primer sets were developed taking into account the homology with the known pseudogenes. In addition, alignment of these primer sets showed no matches with any of the known DHFR pseudogenes. PCR amplifications were performed in a total volume of 50 μL on an iCycler (Biorad, the Netherlands). For details see Table 7. The reaction mixture contained 200 nmol/L of both forward and reverse primer (Biolegio BV, The Netherlands); 200 μmol/L dNTPs, 0.5 U of recombinant Taq polymerase (both from Invitrogen, The Netherlands); PCR buffer containing 20 mmol/L Tris-HCl buffer (pH 8.4), 50 mmol/L KCl and 5% DMSO; 1.0-4.0 mmol/L $MgCl_2$ and 75 ng DNA. An alternative PCR buffer, containing 50 mmol/L Tris-HCl pH 9.2, 16 mmol/L $(NH_4)_2SO_4$, 2% DMSO and 0.1 % Tween-20 (Roche Applied Science, Switzerland), was used where necessary (see Table 7). PCR conditions were as follows: initial denaturation of 4 minutes at 94°C, 35 cycles of 94°C/60s, 55-65°C/30s, 72°C/30s, and a final extension of 7 minutes at 72 °C. The PCR products obtained were analysed on a 2% agarose gel and subsequently sequenced on an ABI Prism 3730 automated sequencer using the ABI Prism Big Dye Terminator cycle sequence kit according to the instructions of the manufacturer (PE

Biosystems, the Netherlands). Newly identified variants were confirmed on both DNA strands by direct sequencing.

Table 7. Primer sequences and PCR conditions used for amplification of the DHFR gene

Primer	Sequence (5'→ 3')	Product Size, bp (seq[b])	T_{ann} (°C)[c]	[$MgCl_2$] (mM)
5' UTR F [a]	CCTCAGCGCTTCACCCAATTTG	450 (-664 to -213)	65	4.0
5' UTR R	CCGGGCTGCCATCCTTGC			
Exon 1 F [a]	GGAGGAGGAGGTGGATTTC	591 (-358 to 233)	55	2.0
Exon 1 R	GCAGCAGAAAAGGGGAATC			
Exon 2 F	CCCTACCCACAGCGCTCCG	348 (343 to 691)	60	1.5
Exon 2 R	GCCTGATAATTTGCTCGTGCG			
Exon 3 F	GCATGCAGACTCCACACAGACG	372 (4833 to 5205)	65	1.5
Exon 3 R	GCAGCTTCATCAATAGCTCC			
Exon 4 F	GTTCTCTCCTGCCCTGTCCAAG	491 (16176 to 16667)	65	1.5
Exon 4 R	GGCAAGGAAGCTGAAAGTAGAAC			
Exon 5 F	GTAAGCAAACTGGAGGCCAGAC	490 (20245 to 20735)	65	1.5
Exon 5 R	GCACCCATCATCCTAGCAGTACAC			
Exon 6 F+3' UTR[a]	GAAACTGCTGACTGGTTTTTGAG	1666 (25036 to 26702)	65	4.0
Exon 6 R+3' UTR	GGTTCAAGCAACCATCATCCC			
ISP 3' UTR1 F	CTAGTTTAAGTTGTTCCCC	- (25417 to 25803)	58	-
ISP 3' UTR2 F	CGTGTATATCCAGAGGTTTGTAG	- (25780 to 26235)	66	-
ISP 3' UTR3 F	GGGAACAGTGAATGCCAAAC	- (26215 to 26702)	60	-

[a] An alternative buffer (see Materials and Methods section) was used for the PCR amplification reaction, [b] sequence covered by primer(set), positions are relative to the A in the translation initiation codon, [c] T_{ann} =annealing temperature, [d] ISP: internal sequence primer

Genetic analysis of DHFR 9-bp repeat

We identified a 9-bp repeat in the 5' upstream region of the DHFR gene. We obtained the most frequent alleles (3x, 6x and 7x repeat) by cloning PCR fragments (450 bp) into the pGEM-T vector system and transformation of *E. coli* JM109 High-efficiency Competent cells (Promega). After ampicillin selection on LB-plates (IPTG/X-gal screening) and plasmid DNA isolation (Wizard plus SV, miniprep kit, Promega), these alleles were used as positive controls in the genotype analyses. In order to facilitate genotyping, a new primer set was chosen for the analysis (F 5'-GCCCAGTCCCAGACAGAAC-3' and R 5'-GAGCCGATTCTTCCAGTCTAC-3'). The resulting PCR product was 180 bp (for the 6x repeat allele), allowing a clear distinction between the alleles. PCR conditions: 4 minutes at 94°C, 35 cycles of 94°C/60s, 59°C/30s, and 72°C/30s, and a final extension of 7 minutes at 72°C. The reaction mixture (50 μl) contained 200 nmol/L of both forward and reverse primer (Biolegio BV, The Netherlands); 200 μmol/L dNTPs, 0.5 U of recombinant Taq polymerase (both from Invitrogen, The Netherlands); PCR buffer containing 50 mmol/L Tris-HCl pH 9.2, 16 mmol/L $(NH_4)_2SO_4$, 2% DMSO and 0.1 % Tween-20 (Roche Applied Science, Switzerland); 2.0 mmol/L $MgCl_2$ and 75 ng DNA. The PCR products were analysed on large 3% agarose gels by running the samples at least four hours at 125V. In each PCR, DNA samples with a known (sequence confirmed) genotype were run as positive controls. When PCR products were not in control range or difficult to genotype, they were excised and purified from the agarose gel (Gel extraction kit, Qiagen) and sequenced to identify the length of the allele. Genotype data was obtained from 417 subjects.

Analysis of DHFR intron 1 19-bp deletion

Screening for the 19-bp deletion in intron 1 of the DHFR gene was essentially as described previously [137]. In short, the PCR reaction mix (25 µl) contained 75 ng of genomic DNA, 1x Ampli Taq Gold Buffer, 1.5 mmol/L $MgCl_2$, 250 µmol/L dNTPs, 1 µmol/L of each primers (FW1 5'-CCACGGTCGGGGTACCTGGG-3', FW2 5'-ACGGTCGGGGTGGCCGACTC-3' and RV 5'-AAAAGGGGAATCCAGTCGG-3'), 1 mol/L betaine and 0.5 U Taq Polymerase. PCR conditions were as follows: initial denaturation of 4 minutes at 94 °C, 35 cycles of 94°C/60s, 58°C/60s, 72 °C/60s, and a final extension of 7 minutes at 72 °C. The PCR products were analysed on a 3% agarose gel, stained with ethidium bromide and visualized by UV light. On each gel, DNA samples with a known (sequence confirmed) genotype (no del/no del, no del/del, del/del) were run as positive controls. Genotype data was obtained from 330 subjects.

Biochemical parameters

Blood samples were drawn from the antecubital vein for determination of total plasma homocysteine and folate. EDTA samples for homocysteine and folate measurement were placed on ice immediately and centrifuged at 3500 g for 5 minutes with minimal delay. The plasma or serum was separated and stored at -20 ºC. 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) [178]. DNA extraction was performed as described previously [179]. Serum and red blood cell folate concentrations were measured with the Dualcount Solid Phase No Boil Radioassay (Diagnostic Product Corporation, Los Angeles, CA, USA).

Statistics

Total homocysteine and folate concentrations were logarithmically transformed prior to all analyses, and are expressed as a geometric mean with a 95% confidence interval. Differences in metabolite concentrations were determined by linear regression analysis and expressed as changes relative to the wild type. A two-tailed $p<0.05$ was accepted as statistically significant. For all analyses SPSS 12.0 for Windows was used.

Results

Human DHFR sequence analysis

We sequenced the coding region, including intron-exon boundaries, and UTRs of the DHFR gene (nucleotide -664 to 26702 relative to the A in the translation initiation codon)(see Table 7 for details). We observed no genetic variation within the coding region, but did identify a 9-bp repeat in the highly GC-rich 5' upstream region of DHFR. The repeat was located 388 bp upstream of the first nucleotide of the translation initiation codon (-388 bp), and partially overlapped with the 5' UTR. We observed alleles ranging from 162 bp (i.e. 3x repeat) to 216bp (i.e. 9x repeat), of which the determined DNA sequences are shown in Figure 5. The most common genotypes (%) were 3/3 (10.6), 3/6 (30.9), 6/6 (41.0) and 6/7 (9.6). Less frequent genotypes (%) were 3/7 (4.1%), 6/9 (1.4%), 3/5, 3/8, 5/6, 6/8 (all 0.5%), 4/7 and 7/7

(both 0.2%). The general consensus sequence of the repeat was **A/C**GCTG**G/CG/A**G**G/C**. In addition, we found three single-nucleotide polymorphisms in the 5' UTR, one in intron IV and three in the 3' UTR (see Table 8).

Table 8. Observed sequence variants in the human dihydrofolate reductase gene in twenty subjects

Region	Location	Variant	Allele frequency
5' UTR [a]	-388 bp [b]	9-bp repeat [c]	0.29 (3x rpt), 0.62 (6x rpt)
			0.09 (7x rpt), <0.01 (4, 5, 8, 9x rpt)
	-204 bp	T>C	0.50 (C)
	-95 bp	G>A	0.13 (A)
	-82 bp	DelG	0.13 (delG)
Intron I	146 bp	19-bp deletion [c, d]	0.43 (del)
Intron IV	20404 bp	G>C	0.03 (C)
3' UTR	25508 bp	A>T	0.14 (T)
	25979 bp	C>T	0.13 (T)
	26095 bp	G>A	0.13 (A)
	26493 bp	delT	0.21 (delT)

[a] UTR: untranslated region, [b] Locations are relative to the A in the translation initiation codon,
[c] screened in the whole population, [d] First reported by Johnson *et al.* [18]

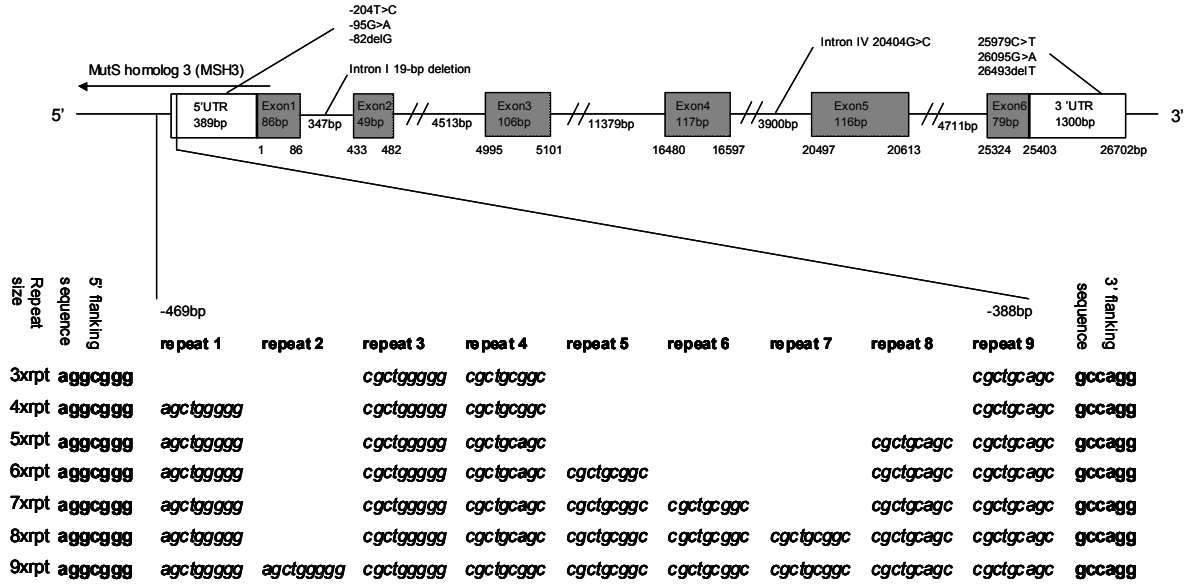

Figure 5. DHFR gene structure and DNA sequence of the 5' upstream region 9-bp repeat as observed for the different alleles

Association between DHFR variants and tHcy and folate

Because the 9-bp repeat is located in the promoter region and the 19-bp deletion is thought to affect gene expression [137], we only screened these potentially most important variants for their effect on tHcy, serum and RBC folate in our study population. As shown in Table 9, the 9-bp repeat was not associated with tHcy in our study population. Although the 3/6 and 3/7 repeat compared to the 3/3 repeat genotype showed a trend towards higher plasma folate

Table 9. Association between the DHFR variants and crude plasma total homocysteine and folate levels

DHFR Variant	Genotype	Controls, n (%)	Mean tHcy, μmol/L (95% CI)	Relative change tHcy, % (95% CI)	Mean folate, nmol/L (95% CI)	Relative change folate, % (95 % CI)	Mean RBC folate, nmol/L (95% CI)	Relative change RBC folate, % (95% CI)
9-bp repeat[a]	3/3	44 (10.6)	10.6 (9.4 to 11.8)	0[b]	11.9 (10.3 to 13.7)	0[b]	371 (325 to 425)	0[b]
	3/6	129 (30.9)	10.4 (9.2 to 11.9)	-1.3 (-13.2 to 12.4)	13.5 (11.5 to 16.1)	14.6 (-3.0 to 35.4)	423 (362 to 494)	13.9 (-2.6 to 33.1)
	3/7	17 (4.1)	10.2 (8.2 to 12.6)	-3.5 (-22.0 to 19.2)	15.1 (11.5 to 19.8)	27.1 (-3.1 to 66.9)	388 (302 to 498)	4.5 (-18.6 to 34.2)
	6/6	171 (41.0)	10.3 (9.1 to 11.7)	-2.2 (-13.8 to 10.8)	13.0 (11.0 to 15.2)	9.2 (-7.0 to 28.3)	405 (348 to 371)	9.0 (-6.3 to 26.7)
	6/7	40 (9.6)	11.0 (9.0 to 12.5)	0.4 (-14.7 to 18.1)	12.8 (10.4 to 15.8)	7.6 (-12.5 to 32.6)	416 (342 to 505)	12.0 (-7.9 to 35.9)
19-bp deletion	no del/no del	114 (34.5)	10.9 (10.2 to 11.6)	0[b]	13.5 (12.5 to 14.8)	0[b]	386 (357 to 418)	0[b]
	no del /del	150 (45.5)	10.6 (9.6 to 11.6)	-2.5 (-10.8 to 6.5)	13.5 (12.0 to 15.1)	-0.6 (-11.5 to 11.5)	398 (358 to 442)	+2.9 (-7.4 to 14.3)
	del/del	66 (20.0)	9.2 (8.0 to 10.4) [c]	-14.4 (-23.4 to -4.5) [c]	12.8 (11.1 to 14.8)	-5.8 (-18.5 to 8.8)	388 (340 to 442)	+0.4 (-12.0 to 14.3)

[a] only the genotypes with a frequency of >2.5% are shown, [b] reference category, [c] p-ANOVA <0.05

levels (p trend=0.015 for the 3/3, 3/6 and 3/7 repeat genotype), this increment did not persist in the 6/6 and 6/7 repeat genotypes. The 19-bp deletion was associated with a 2.5% (95% CI -10.8 to 6.5) and 14.4% (95% CI -23.5 to -4.5) decrease in tHcy for the heterozygous and homozygous genotype, respectively, compared with the wild type genotype (p ANOVA=0.016)(Table 9). Adjustment for age and sex did not change the association between this deletion and tHcy. Serum and red blood cell folate levels were similar between the genotypes. No interaction between the 9-bp repeat and 19-bp deletion or between low folate (<30th percentile, 10.0 nmol/L) and either variant on tHcy was observed (not shown).

Discussion

In this study we searched for genetic variation in the DHFR gene that may affect tHcy, serum and red blood cell folate levels. We identified a 9-bp repeat in the 5' upstream region of DHFR that partially overlapped with the 5' UTR. The 9-bp repeat may affect mRNA stability or translation efficiency, similarly as described for the 28-bp repeat in the functionally related thymidylate synthase [140]. The repeat appeared highly polymorphic with alleles ranging from 3 to 9 repeats. In 1995 Nakajima and co-workers [202] described, in Japanese subjects, a 9-bp repeat in exon 1 of the DNA repair gene mutS homolog3 (MSH3), which partially overlaps with the DHFR gene and may share promotor elements. Our repeat most likely represents the same repeat, as the consensus sequence highly resembled that reported by Nakajima, although higher repeat sizes were present in our, predominantly Caucasian, population. The repeat may have arisen from duplications of one (or more) ancestral allele(s) or result from DNA replication errors. Despite its location in the promoter region, the length of this repeat was not associated with tHcy, serum and red blood cell folate levels.

In 2001, Goto and co workers reported a functional variant (829C>T) in the 3' UTR of DHFR [203]. We did not observe this variant in the subjects that were included in the sequence analysis, suggesting that it is a population-specific polymorphism or has a minor allele frequency below 2.5%. Johnson and colleagues identified a 19-bp deletion in the highly conserved intron 1, that may be a maternal risk factor for having a child with spina bifida and preterm delivery [137,204]. It has been postulated that this variant deletes a putative SP1 transcription factor-binding site (required for transcriptional activation/repression) or may affect the splicing process. We show that the 19-bp del/del genotype is associated with a reduced plasma total homocysteine of about 1.5 μmol/L (14%) compared to wild type subjects. This may suggest that the 19-bp deletion indeed increases DHFR expression thereby facilitating homocysteine remethylation. Unfortunately, no RNA was available from our study population to support its effect at the expression level, which warrants further investigation.

We detected several other variants in non-coding regions of DHFR which, based on the sequence, could not be ascribed to known pseudogenes. The main part of the introns has not been screened, but these may contain functional variants as well. It is possible that intronic polymorphisms represent a functional variant although the intronic sequences, except for intron 1, are not well-conserved across species [205]. We assessed linkage disequilibrium (LD) between the 19-bp deletion and the most common alleles of the 9-bp repeat (3x and 6x), and found them to be in almost complete LD (D' 0.9). However, the

squared correlation coefficient was low (R^2 = 0.3). The HapMap consortium investigated LD between existing DHFR single nucleotide polymorphisms (http://www.hapmap.org/), and showed that one haplotype block of about 24 kbp almost completely covers the DHFR gene. This suggest that the number of allelic variants of the DHFR gene present in the population is limited and may be captured by genotyping just a small number of polymorphisms in the gene.

In conclusion, the coding region of DHFR harbours no variation in our population, which illustrates its importance in folate metabolism. Several variants were found within the non-coding regions of DHFR, potentially the most interesting being the 9-bp repeat in 5' UTR. The 19-bp deletion in intron 1 was associated with lower tHcy levels and may be a genetic risk factor for cardiovascular disease and cancer, in addition to its reported effect on neural tube defect risk.

Acknowledgments

This study was supported by grant 2002B68 from the Netherlands Heart Foundation. Martin den Heijer, MD, PhD, is supported by a VENI-grant from the Dutch Organization for Scientific Research (NWO).

Chapter 4

Effect of Common Polymorphisms in the Reduced Folate Carrier, Thymidylate Synthase and AICAR Transformylase/IMP Cyclohydrolase Genes on Folate and Homocysteine Levels and Venous Thrombosis Risk

Henkjan Gellekink, Henk J. Blom, Martin den Heijer

Laboratory of Pediatrics and Neurology, Department of Endocrinology and Department of Epidemiology and Biostatistics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Submitted for publication

Abstract

Introduction: Folate is important in processes like purine and thymidylate synthesis and S-adenosylmethionine-dependent transmethylation. Low availability of folate leads to hyperhomocysteinemia, which is a risk factor for arterial vascular disease and venous thrombosis. Genetic variation in folate metabolizing genes may affect folate availability and, hence, exert a greater risk for venous thrombosis.

Materials and Methods: We genotyped the thymidylate synthase (TYMS) 28-bp repeat and 6-bp deletion and the reduced folate carrier (RFC1) 80G>A and AICAR transformylase / IMP cyclohydrolase (ATIC) 346G>C polymorphisms in population-based controls, and assessed their effect on plasma total homocysteine (tHcy), serum and red blood cell (RBC) folate. We investigated whether these variants are risk factors for venous thrombosis as well.

Results: None of the genotypes, singularly or in combination, were associated with major changes in tHcy, serum or RBC folate levels. In addition, we found no evidence that the genetic variants were associated with recurrent venous thrombosis risk.

Conclusion: The TYMS 28-bp repeat and 6-bp deletion, RFC1 80G>A and ATIC 346G>C polymorphisms are not associated with tHcy, serum or RBC folate in the general population, nor did any of the polymorphisms affect venous thrombosis risk.

Introduction

Folate plays an essential role in the human body as carrier and donor of one-carbon groups in DNA synthesis and homocysteine remethylation. As such, folate is involved in the formation of purine and thymidine nucleotides, and S-adenosylmethionine (AdoMet) synthesis required for transmethylation processes. A disturbed folate and, the closely related, homocysteine metabolism has been implicated in several diseases, including arterial vascular disease and venous thrombosis [18,19], neurological and congenital disorders and cancer [22,29,36,39]. In addition, there is accumulating evidence that low B-vitamin levels, such as folate, vitamin B12 and B6, increase the risk of thrombosis independent of their effect on homocysteine levels [166,167,206]. Given the effect of the methylenetetrahydrofolate reductase (MTHFR) 677C>T polymorphism on folate distribution and homocysteine levels [47,207], one may speculate that other variants in genes encoding folate metabolizing enzymes also affect folate and homocysteine levels. In addition, based on segregation analysis, Jee *et al.* suggest the presence of other important determinants of tHcy besides the MTHFR 677 C>T polymorphism [45].

The main two mechanisms for cellular uptake of folates involves a relatively slow endocytotic process mediated by folate receptors while the second process involves the high-capacity uptake of reduced folates (and the antifolate methotrexate) by the reduced folate carrier (RFC1, or SLC19A1) [99]. After uptake, the folates need to be reduced and methylated (if not already) to become metabolically active. The enzymes thymidylate synthase (TYMS, EC 2.1.1.45) and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase / inosine monophosphate (IMP) cyclohydrolase (ATIC, EC 2.1.2.3 / 3.5.4.10) are two other enzymes involved in folate metabolism (Figure 1). TYMS uses 5,10-methylenetetrahydrofolate (THF) for the reductive methylation of deoxyuridine monophosphate (dUMP) into deoxythymidine monophoshate (dTMP), a pyrimidine nucleotide essential for DNA synthesis and repair. ATIC, requiring 10-formylTHF as a co substrate, catalyzes the successive transformylation and hydrolysis of AICAR into IMP, which are the two final steps in the *de novo* purine synthesis.

In the past decade, our group assessed variants in the majority of folate genes for their effect on plasma total homocysteine (tHcy) (for review, see Gellekink *et al.* [208]). Yet, some genes of folate metabolism have not been explored. The RFC1, ATIC and TYMS genes contain genetic variants, e.g. 80G>A in RFC1 (Arg27His), 346C>G (Thr116Ser) in ATIC, 5' untranslated region (UTR) 28-bp repeat and 3' UTR 6-bp deletion (1494del6) in TYMS. Higher repeat sizes of the TYMS 28-bp repeat were shown to increase translation efficiency [141] and tHcy in subjects with the MTHFR 677TT genotype [142]. The 6-bp deletion in TYMS is reported to decrease mRNA stability [144]. RFC1 represents the major internalization route for reduced folates and the 80G>A polymorphism, causing an arginine to histidine amino acid change at codon 27, was shown to affect thrombosis risk in one study [209], but not tHcy or folate [110,210]. The ATIC 346C>G polymorphism converts a threonine to serine at position 116, but the functional impact of this variant has not been studied. All four genetic variants may affect folate distribution and homocysteine levels and in this way confer a risk for thrombosis as well.

The main goal of the study was to assess these four genetic variants for their effect on tHcy and serum and red blood cell (RBC) folate concentrations in the general population. The second aim was to estimate the risk of disease for each of the variants in a case-control study on recurrent venous thrombosis. The effect of gene-gene combinations on tHcy and venous thrombosis risk is also studied.

Material and Methods

Patients and controls used in the case-control study

The control group was recruited via a general practice in The Hague (n=500). Patients (n=185) were selected from the files of the anticoagulant clinic of The Hague. All patients had two or more episodes of venous thrombosis (pulmonary embolism or deep-vein thrombosis). Both study populations participated in a case-control study on venous thrombosis and have been described in more detail previously [6]. We obtained a short medical history of all patients by interview and of all controls by questionnaire. DNA for genotyping was available from 178 patients and 446 population-based controls from which relevant biochemical data had already been obtained. The medical ethics committee approved the study protocol and informed consent was obtained from all study participants.

Biochemical parameters

Blood samples were drawn from the antecubital vein in 5 mL Vacutainer tubes and 4.5 mL EDTA vacuum glass tubes for determination of folate and total plasma homocysteine and for DNA extraction. EDTA samples for homocysteine measurement were placed on ice immediately and centrifuged at 3500 g for 5 minutes with minimal delay. The plasma was separated and stored at -20 ºC until analysis. Total plasma homocysteine concentration was 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) [178]. Samples for serum and RBC folate measurement were stored at -70 ºC and measured with the Dualcount solid-phase no boil Radioassay (Diagnostic Products Corporation, Los Angeles, CA, USA). RBC folate levels reflect long-term folate status whereas plasma folate reflects daily intake. DNA extraction was performed as described previously [179] and the DNA was stored at 4 ºC.

Genotype analyses

We genotyped four polymorphisms in three genes involved in folate metabolism: RFC 80G>A (Arg27His) [110], TYMS 28-bp repeat [141] and 6-bp deletion (1494del6, rs28365050) [139] and ATIC 348C>G (Thr116Ser, rs2372536). Three of these variants were screened for by means of PCR followed by restriction fragment-length analysis (PCR-RFLP). The TYMS 28-bp repeat was analysed by means of PCR amplification alone [211]. For details about the variants under study and validation method used, see Table 10.

Screening conditions were similar in all analyses. PCR conditions: 4 minutes at 94°C, 35-40 cycles of 94°C/60s, 51-63°C/60s, and 72°C/30s, and a final extension of 7 minutes at 72°C. Each PCR reaction mixture contained 50 ng of both forward and reverse primer (Biolegio BV, The Netherlands), 200 μM dNTPs, 0.5 U of recombinant Taq polymerase (Invitrogen, The

Table 10. Relevant data of analysed polymorphisms and method of screening

Gene	Variant	Location	Reported MAF[a]	Protein	Primers (5'→ 3')	Restriction enzyme	Mg^{2+} (mM)	Product size	T_{ann} (°C)
TYMS	28-bp repeat	5' UTR	0.17-0.47 (2 x repeat)	-	F aaggcgcgcggaaggggtcc R tccgagccggccacaggcat	-	1.0	114bp (2 x repeat)	63
TYMS	6-bp deletion	3' UTR	0.39 (del)	-	F caaatctgagggagctgagt R cagataagtggcagtacaga	+*Dra*I	1.0	152bp (del)	55
RFC1	60G>A	Exon 2	0.38-0.51 (A)	Arg27His	F atggtgccctccagcccagc R catgaagccgtagaagcaaagg	-*Hha*I	1.5	114bp	57
ATIC	346C>G	Exon 5	0.18-0.37 (G)	Thr116Ser	F cctagatagctgtaaaccac R gtaatcccaaaacacaatc	-*Hpy*CH4III	2.0	305bp	51

[a] MAF, minor allele frequency

Table 11. Association between variants in folate genes and fasting tHcy and folate levels in population-based controls

Variant	Genotype	Mean tHcy (μmol/l) [95% CI]	Adjusted[a] mean tHcy (μmol/l) [95% CI]	Mean folate (nmol/l) [95% CI]	Adjusted[a] mean folate (nmol/l) [95% CI]	Mean RBC folate (nmol/L) [95% CI]	Adjusted[a] mean RBC folate (nmol/L) [95% CI]
TYMS 28-bp rpt	2/2	10.5 [9.7 to 11.4]	10.0 [9.1 to 10.9]	13.7 [12.4 to 15.2]	13.8 [12.1 to 15.8]	444 [402 to 490]	429 [378 to 486]
	2/3	10.3 [9.3 to 11.3]	9.9 [9.1 to 10.8]	13.2 [11.7 to 14.9]	13.1 [11.5 to 14.9]	395 [352 to 445]	394 [349 to 444]
	3/3	10.6 [9.5 to 11.8]	10.3 [9.4 to 11.4]	12.1 [10.5 to 13.9][c]	12.2 [10.6 to 14.1]	387 [340 to 440][b]	384 [336 to 439]
TYMS 6-bp del	no del/no del	10.4 [9.8 to 11.0]	9.8 [9.1 to 10.6]	13.1 [12.2 to 14.1]	13.3 [12.0 to 14.8]	419 [391 to 449]	408 [369 to 451]
	no del/del	10.5 [9.7 to 11.3]	10.1 [9.4 to 10.8]	13.1 [11.9 to 14.5]	13.1 [11.8 to 14.5]	388 [352 to 426]	382 [346 to 421]
	del/del	10.3 [9.2 to 11.5]	9.9 [8.9 to 11.0]	12.4 [10.7 to 14.3]	12.2 [10.5 to 14.2]	404 [352 to 463]	403 [351 to 461]
RFC1 80 G>A	GG	10.9 [10.3 to 11.6]	10.3 [9.6 to 11.1]	13.5 [12.5 to 14.5]	13.6 [12.2 to 15.1]	422 [393 to 454]	427 [386 to 471]
	GA	10.3 [9.5 to 11.1]	9.8 [9.1 to 10.5]	12.7 [11.5 to 14.1]	12.8 [11.5 to 14.2]	381 [347 to 439]	370 [336 to 407]
	AA	9.6 [8.6 to 10.8][b]	9.8 [8.7 to 10.8]	12.3 [10.7 to 14.2]	12.4 [10.7 to 14.4]	405 [372 to 462]	390 [341 to 448]
ATIC 346 C>G	CC	10.5 [9.9 to 11.1]	10.0 [9.2 to 10.7]	13.3 [12.4 to 14.3]	13.6 [12.2 to 15.1]	401 [375 to 429]	394 [356 to 435]
	GC	10.3 [9.6 to 11.2]	9.9 [9.3 to 10.7]	13.2 [11.9 to 14.5]	12.9 [11.7 to 14.4]	405 [369 to 445]	397 [361 to 438]
	GG	9.9 [8.7 to 11.2]	9.7 [8.7 to 10.9]	11.7 [10.2 to 13.7]	11.8 [10.0 to 13.9]	402 [347 to 467]	375 [322 to 437]

[a] adjusted for age, sex, creatinine and MTHFR 677C>T genotype, [b] $P<0.05$, [c] $P=0.06$

Netherlands), 10 mM Tris-HCl buffer (pH 8.2), 50 mM KCl, 1.0-2.0 mM $MgCl_2$, 5% DMSO (Sigma, 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 (or PCR product in case of the TYMS 28-bp repeat) were analysed by gel electrophoresis on agarose gel, stained with ethidiumbromide and visualized by UV. DNA samples from which the genotype had been identified by sequence analysis served as positive controls. Due to poor PCR amplification, genotype data was only available from at least 413 control individuals and 130 venous thrombosis cases.

Statistics

Metabolite concentrations were logarithmically transformed prior to all analyses, and are expressed as a geometric mean with a 95% confidence interval. Differences in metabolite concentrations were determined by linear regression analysis. Odds ratios and 95% confidence intervals were calculated to estimate the relative risk of recurrent venous thrombosis in individuals that carry the mutant allele using logistic regression analysis. A two-tailed $p<0.05$ was accepted as statistically significant. For all analyses SPSS 12.0 for Windows was used.

Results

Baseline characteristics of study populations

The control group consisted of 431 individuals (average age 50.7 ± 13.3 y) from which 41.1% was male (n=177). The case group consisted of 173 recurrent venous thrombosis patients (average age 61.5 ± 14.2 y) from which 50.9% was male (n=88). Fasting tHcy was 10.4 (95% CI 10.1 to 10.8) μmol/L and 12.5 (95% CI 11.8 to 13.3) μmol/L in controls and cases, respectively ($p<0.01$). Serum creatinine was 74.1 (95% CI 72.5 to 75.7) μmol/L and 83.3 (95% CI 78.9 to 87.7) μmol/L in controls and cases, respectively ($p<0.01$). Mean serum folate (95% CI) was 12.9 (12.2 to 13.7) nmol/L and 13.8 (12.5 to 15.1) in controls and cases, respectively ($p=0.21$), and the corresponding RBC folate level (95% CI) was 399 (378 to 420) and 404 (340 to 468) nmol/L ($p=0.23$).

Association between genetic variants and metabolites in population-based controls

The effects of the genetic variants on tHcy, folate and RBC folate levels in the control group are shown in Table 11. The crude analyses show that subjects with the TYMS 28-bp 3/3 repeat have the lowest RBC folate levels when compared to 2/2 repeat subjects (p trend=0.05), while plasma folate was the lowest in 3/3 subjects too (p trend=0.06). In addition, we observed a decrease in tHcy of about 12% in RFC1 80AA compared to 80GG subjects ($p=0.03$) in the crude analyses. However, after adjustment for age, sex, serum creatinine and MTHFR 677C>T genotype these associations disappeared ($p>0.05$)(Table 11). The ATIC 346C>G and TYMS 6-bp deletion variant had no effect on any of the metabolites.

Compound genotypes and tHcy, folate and RBC folate levels in controls

We tested whether the combinations of genotypes, defined by the variants under study, were associated with tHcy, serum folate or RBC folate levels. In subjects with the TYMS 28-bp 3/3 repeat genotype, the RFC1 80 G>A variant was associated with tHcy levels (95% CI), that is 12.1 (10.2 to 14.2), 10.7 (9.2 to 12.4) and 9.2 (7.3 to 11.5) μmol/L for 80GG (n=46), GA (n=56) and AA (n=15) individuals, respectively (p=0.02). In subjects with the TYMS 6-bp no del/del genotype, the ATIC 346 C>G variant was significantly associated with serum but not RBC folate levels. Serum folate (95% CI) was 14.1 (12.1 to 16.5), 12.3 (10.6 to 14.2) and 10.8 (8.6 to 13.5) nmol/L for 346CC (n=20), 346CG (n=82) and 346GG (n=87) individuals, respectively (p=0.02). Adjustment for confounders like age, sex, creatinine and MTHFR 677C>T genotype did not affect these associations. None of the other genotype combinations were associated with tHcy, serum folate or RBC folate levels (data not shown).

Table 12. Association between variants in folate genes and venous thrombosis risk

Variant	Genotype	Controls, n (%)	MAF[a] controls	Cases, n (%)	MAF[a] cases	Odds ratio (95% CI)
TYMS 28-bp rpt	2/2	85 (20.2)	0.46 (2x rpt)	33 (20.2)	0.44 (2x rpt)	1.0[b]
	2/3	214 (51.0)		78 (47.9)		0.94 (0.58 to 1.51)
	3/3	119 (28.3)		50 (30.7)		1.08 (0.64 to 1.82)
TYMS 6-bp del	no del/no del	176 (40.8)	0.36 (del)	66 (40.2)	0.34 (del)	1.0[b]
	no del/del	196 (45.5)		83 (50.6)		1.13 (0.77 to 1.66)
	del/del	59 (13.7)		15 (9.1)		0.68 (0.36 to 1.28)
RFC1 80 G>A	GG	158 (36.8)	0.39 (A)	65 (37.6)	0.39 (A)	1.0[b]
	GA	208 (48.5)		82 (47.4)		0.96 (0.65 to 1.41)
	AA	63 (14.7)		26 (15.0)		1.00 (0.58 to 1.72)
ATIC 346 C>G	CC	183 (44.3)	0.33 (G)	57 (43.8)	0.37 (G)	1.0[b]
	GC	184 (44.6)		51 (39.2)		0.89 (0.58 to 1.37)
	GG	46 (11.1)		22 (16.9)		1.54 (0.85 to 2.77)

[a] MAF, minor allelefrequency, [b] reference category

Genotype distribution and recurrent venous thrombosis risk

The genotype distributions of the variants in the control population were all in Hardy-Weinberg equilibrium (p>0.50). Details on genotype frequencies and associated recurrent venous thrombosis risk are shown in Table 12. The minor allele frequencies for RFC1 80G>A, ATIC 346C>G, TYMS 28-bp repeat and 6-bp deletion were 0.39/0.39 (A allele), 0.37/0.33 (G allele), 0.44/0.46 (2x repeat allele) and 0.34/0.36 (6-bp deletion allele) for cases and controls, respectively. The corresponding odds ratios indicate that none of the analysed variants were separately associated with venous thrombosis risk (Table 12). In addition, none of the genotype combinations were significantly associated with recurrent venous thrombosis risk (data not shown).

Discussion

In this study we assessed four common variants, that is TYMS 28-bp repeat and 6-bp deletion, the ATIC 346C>G and RFC1 80G>A polymorphisms, for their effect on serum and

RBC folate and tHcy. In addition, we investigated the prevalence of these polymorphisms in a case-control study on venous thrombosis.

We found no indications that the RFC1 80G>A, TYMS 28-bp repeat and TYMS 6-bp deletion were singularly associated with tHcy, serum or folate levels in the general population, which corresponds with the observations of other groups for the RFC1 and TYMS variants [110,210,212]. Likewise, the ATIC 346C>G variant was not associated with these metabolites as well.

There is much evidence that multiple genetic variants contribute to phenotypic changes, like hyperhomocysteinemia. For example, Trinh *et al.* observed an effect for the 3/3 repeat genotype on tHcy and plasma folate but only in MTHFR 677TT subjects [142]. A recent study by Yates and Lucock suggests that common folate polymorphisms and B-vitamin status modulate tHcy levels [184]. Lastly, Devlin *et al.* observed a gene-gene interaction between the MTHFR 677TT and RFC1 80GG genotypes on tHcy in a large group of elderly controls [210]. However, we found only slight indications that some genotype combinations may affect tHcy or serum folate levels, such as the RFC1 80G>A polymorphism in combination with the TYMS 28-bp 3/3 repeat. The TYMS 28-bp repeat was shown to increase translation efficiency with higher repeat sizes [141]. Although no evident functional effect was found for the 80G>A polymorphism on reduced folate cofactor transport *in vitro* [108], the combination may disturb folate metabolism and, hence, affect tHcy levels. The finding that no effect for this genotype combination on serum and RBC folate was observed, may be related to the fact that total folate is measured, leaving small changes in folate vitamer distribution unnoticed.

Regarding the genetic variants being a risk factor for venous thrombosis, only one group reported a protective effect of the RFC1 80A allele in a small study on thrombosis (OR 0.56 [95% CI 0.34 to 0.92]) [209]. Despite the fact that our study populations were larger we could not confirm their findings, as none of the genotypes were singularly associated with recurrent venous thrombosis risk. The genotype combinations did not affect disease risk as well, but our study populations may have been too small to detect slight changes, if any, in disease risk.

Given the complex etiology of hyperhomocysteinemia and thrombosis, studying the effect of genotype combinations in relation to multifactorial traits and diseases is of great interest. This will require large study populations in order to detect small phenotypic changes that may, consequently, modulate disease risk.

In conclusion, our data suggests that the TYMS 28-bp repeat and 6-bp deletion, RFC1 80G>A and ATIC 346G>C polymorphisms are not associated with tHcy, serum or RBC folate in the general population, nor did any of the polymorphisms affect venous thrombosis risk.

Acknowledgments

This study was supported by grant 2002B68 from the Netherlands Heart Foundation. Martin den Heijer, MD, PhD, is supported by a VENI-grant from the Dutch Organization for Scientific Research (NWO).

Chapter 5

Effect of Genetic Variation in the Human S-adenosylhomocysteine Hydrolase Gene on Total Homocysteine Concentrations and Risk of Recurrent Venous Thrombosis

Henkjan Gellekink, Martin den Heijer, Leo A.J. Kluijtmans, Henk J. Blom

Laboratory of Pediatrics and Neurology, Department of Endocrinology and Department of Epidemiology and Biostatistics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

European Journal Of Human Genetics 2004; 12: 942-8

Abstract

Hyperhomocysteinemia is an independent and graded risk factor for arterial vascular disease and venous thrombosis. It is still debated via which mechanism homocysteine (Hcy) causes vascular disease. S-adenosylhomocysteine hydrolase (AHCY) catalyzes the reversible hydrolysis of S-adenosylhomocysteine (AdoHcy) to Hcy. As an increase in AdoHcy, a strong inhibitor of many methyltransferases, is observed in hyperhomocysteinemic individuals, AdoHcy may play a role in the development of cardiovascular diseases by inhibiting transmethylation reactions. We sequenced the entire coding region and parts of the untranslated regions (UTRs) of the AHCY gene of 20 patients with recurrent venous thrombosis in order to identify genetic variation within this gene. We identified three sequence variants in the AHCY gene: a C>T transition in the 5' UTR (-34 C>T), a missense mutation in exon 2, which mandates an amino acid conversion at codon 38 (112 C>T; Arg38Trp), and a silent mutation in exon 4 (390 C>T; Asp130Asp). We studied the effect of the first two variants on total plasma homocysteine and venous thrombosis risk in a case-control study on recurrent venous thrombosis. The two polymorphisms under study seem to have no evident effect on tHcy. The adjusted relative risk of venous thrombosis associated with the 112CT genotype compared with 112CC individuals was 1.27 (95% CI [0.55 to 2.94]), whereas the -34CT genotype confers a risk of 1.25 (95% CI [0.44 to 3.52]) compared with the wild type genotype at this locus. However, the wide confidence intervals do not allow firm conclusions to be drawn.

Introduction

An elevated total plasma homocysteine (tHcy) concentration, also referred to as hyperhomocysteinemia, is an independent and graded risk factor for cardiovascular disease, including venous thrombosis, peripheral, cerebral and coronary artery disease [6,8,17,40,213,214]. Homocysteine is formed following S-adenosylmethionine (AdoMet)-dependent methylation reactions and subsequent hydrolysis of S-adenosylhomocysteine (AdoHcy) by AdoHcy hydrolase (AHCY; EC 3.3.1.1). Removal of homocysteine is essential as the equilibrium of the reaction catalyzed by AHCY strongly favours the formation of AdoHcy. Under normal conditions, the turnover rate of homocysteine is sufficient enough to favour the hydrolysis of AdoHcy. This is important as AdoHcy is a potent inhibitor of most methyltransferases thereby affecting DNA, RNA, protein and lipid methylation [59,60]. For example, *in vivo* studies have demonstrated that an elevated tHcy is associated with increased plasma and intracellular AdoHcy levels, which correlates well with DNA hypomethylation in different tissues including lymphocytes, brain and liver [34,55,57,60]. Therefore, it has been postulated that the increase in AdoHcy and associated inhibition of transmethylation may, in part, explain the increased risk of cardiovascular disease in hyperhomocysteinemic individuals.

The AHCY gene has been assigned to chromosome 20cen-q13.1 (Ensembl locus 20q11.22) and consists of 10 exons spanning about 23 Kb. Native human AHCY is a cytosolic protein composed of four identical subunits and requires NAD^+ as a cofactor [215]. In the past, three electrophoretic isoforms of AHCY have been identified [216-218], but the molecular basis underlying these isoforms is still obscure. Coulter-Karis and Hershfield were the first to report a full-length cDNA of AHCY from a human placental cDNA library [219]. They identified a transcript of 1299 bp encoding a 432 amino acid protein of approximately 48 kDa and reported a G>A transition at nucleotide 256 of this transcript. However, no AHCY sequence data or frequency data of this 256 G>A transition in a larger group of individuals is available yet.

Very recently, Baric and colleagues reported an AHCY-deficient patient with strongly reduced activity in liver (10%) and fibroblasts (3%) compared with controls. This 8-month-old boy suffered from severe myopathy and slow psychomotor development but showed no signs of cardiovascular disease, yet. In addition, an increased tHcy of 15.9 μmol/L (normal <8.9 μmol/L) was observed, while AdoMet and AdoHcy levels were increased ~30-fold and ~150-fold, respectively. Surprisingly, this condition was accompanied by DNA hypermethylation in leucocytes [220].

It is still debated via which mechanism homocysteine induces arterial vascular diseases and venous thrombosis. Because AHCY is thought to play a pivotal role in the control of transmethylation reactions via regulation of intracellular Hcy and AdoHcy levels we sequenced the AHCY gene of 20 patients with a history of venous thrombosis. We aimed to identify genetic variation in order to assess a possible effect on tHcy and disease risk in a group of recurrent venous thrombosis patients and population-based controls. We hypothesized that if a disturbed methylation increases the risk of venous thrombosis, functionally relevant polymorphisms within the AHCY gene may increase disease risk even without concomitant hyperhomocysteinemia.

Materials and Methods

Patients and controls used in the case-control study

Patients were selected from the files of the anticoagulant clinic in Leyenburg Hospital in The Hague [6,176] and a population-based control group was recruited via a general practice in The Hague [6]. Each study participant was subjected to a standardised methionine-loading test. This test included a basal homocysteine measurement (after overnight fasting) and a second homocysteine assessment 6 h after an oral methionine loading (100 mg L-methionine per kg body weight dissolved in 200 mL orange juice). During these 6 h, the subjects obtained a protein-restricted diet. For the current study, DNA was available of 173 recurrent venous thrombosis patients and 438 controls.

Patient selection for sequence analysis of the AHCY gene

We hypothesised that functional polymorphisms in the AHCY gene would decrease the conversion of methionine to homocysteine, which could be visualised by a diminished increase in tHcy upon methionine-loading; This so called delta (Δ) tHcy can be calculated by subtracting fasting tHcy from post-methionine load (PML) tHcy. A total of 10 recurrent venous thrombosis patients were selected for sequence analysis of the AHCY gene based on their capacity to convert methionine to homocysteine after a methionine load (Δ tHcy 15.1 ± 4.3 μmol/L; range 4 to 18 μmol/L). Another ten venous thrombosis patients were selected randomly (Δ tHcy 29.7 ± 10.0; range 20 to 53 μmol/L). Mean Δ tHcy in the entire case group was 33.1 ± 14.0 μmol/L (range 4 to 98 μmol/L).

Biochemical parameters

Blood samples were drawn from the antecubital vein for determination of total plasma homocysteine and for DNA extraction. EDTA samples for homocysteine measurement were placed on ice immediately and centrifuged at 3500 g for 5 minutes with minimal delay. The plasma was separated and stored at -20 ºC. Total plasma homocysteine concentrations were measured in our laboratory 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). The detection limit was less than 0.5 μmol/L with inter- and intra run variation was less than 6% [178]. A comparative study in which plasma total homocysteine was determined batch-wise in the laboratory of Pediatrics and Neurology of the University Medical Centre Nijmegen (The Netherlands) and at the Department of Pharmacology at the University of Bergen (Norway) showed a systematic overestimation in tHcy concentration in the Nijmegen laboratory of 2.4 μmol/L but the ranking of the subjects was found not to be different between the two tests (precision 93%). Therefore it was decided to subtract 2.4 μmol/L from all tHcy measurements (fasting and post-methionine load) conducted in Nijmegen [178,221]. DNA extraction was performed as described previously [179] and the DNA was stored at 4 ºC.

Sequence analysis of the AHCY gene

The molecular analysis of the AHCY gene was performed by genomic DNA sequencing of the entire coding region, including 75 nucleotides of the 5' untranslated region (UTR) and the

entire 3' UTR. Intron-based primers were developed to avoid amplification of pseudogenes (GenBank accession number NT_028392.4) and to reveal potential splice site variants. PCR amplifications were performed in a total volume of 50 μL on an iCycler (Bio-Rad, the Netherlands); each mixture contained 50 ng of both the forward and reverse primer (Biolegio BV, The Netherlands), 200 μM dNTPs, 10 mM Tris-HCl buffer (pH 8.2), 50 mM KCl, 1.0 to 2.0 mM $MgCl_2$ (see Table 13), 0.5 U of recombinant Taq polymerase, 5% DMSO (all from Invitrogen, The Netherlands) and 75 ng DNA. PCR conditions were as follows: initial denaturation of 4 minutes at 94°C, 35 cycles of 94°C/30s (denaturation), 50-60°C/30s (annealing, see Table 13), and 72°C/60s (elongation), and a final extension of 7 minutes at 72°C. The PCR products obtained were analysed on a 2% agarose gel and subsequently sequenced on an ABI Prism 377 automated sequencer using the ABI Prism Big Dye Terminator cycle sequence kit according to the instructions of the manufacturer (PE Biosystems, The Netherlands). Sequence variants identified during the experiment were screened for in our study populations using PCR-RFLP analyses.

Table 13. Primer sequences and PCR conditions used for amplification of the AHCY gene

Primer	Sequence (5'→ 3')	[$MgCl_2$] (mM)	T_{ann} (°C)	Product size (bp)
5' UTR+exon1 F	cgccacgcgcatatccctg			
5' UTR+exon1 R	cccccgccacgaacaagc	2.0	60	195
Exon 2 F	gtgaccgcccctcttggttgg			
Exon 2 R	ccaccctggcacagtcgtcttc	1.5	51	338
Exon 3 F	caccctttcccttccacacag			
Exon 3 R	gctaggattttgtggcggtgac	1.5	51	442
Exon 4 F	gttgggaaggaggtagttttggc			
Exon 4 R	gctgcttgaggtgatgggagtc	1.5	51	350
Exon 5 F	gctttagggggtaaagatgagg			
Exon 5 R	gcctgtctataaccgcttttgcc	1.0	55	330
Exon 6 F	ggccttgcctgcccctattg			
Exon 6 R	ctctggccccagtggctgac	1.5	51	295
Exon 7/8 F	ggcaagggttggttgtcagc			
Exon 7/8 R	ccatctcctcagctctcctccc	1.0	60	476
Exon 9 F	ggcactgttggaggcagagag			
Exon 9 R	ctctggcaagccctgtgtgg	1.0	60	363
Exon 10 F	cagacgcgtgtaggggtcc			
Exon 10 R	gaggagaggtggggcctg	1.5	60	254
3' UTR1 F	cctccagctgctgtccttgc			
3' UTR1 R	gggctgaagaaccactggacc	1.5	59	383
3' UTR2 F	ggacttatacctgtgtgcttgg			
3' UTR2 R	gcagagtaccccgtgaatgc	1.0	59	513

T_{ann} = annealing temperature

Mutation analysis of AHCY 112 C>T variant

A C>T transition in exon 2 at nucleotide position 112 (relative to the adenosine in the translation initiation codon; AHCY mRNA reference sequence NM_000678) of the AHCY gene was found. This transition abolishes a *Bsr*B1 restriction site, allowing RFLP analysis.

PCR amplification, using primerset 2 (see Table 13), resulted in a fragment of 338 bp and was digested by 5 units of the restriction enzyme *Bsr*B1 (New England Biolabs, Inc.) for 2 hours at 37°C, followed by gel electrophoresis analysis on a 2% agarose gel. Fragments of 159 bp and 179 bp identify the 112C allele, while the 112T allele shows only one fragment of 338 bp. Genotype data was not available from one recurrent venous thrombosis patient (n=172) and one control individual (n=437).

Mutation analysis of AHCY -34 C>T variant

We found a C to T transition (nucleotide -34 relative to the adenosine in the translation initiation codon; AHCY mRNA reference sequence NM_000678) in the 5' untranslated region (UTR) of the AHCY gene. This transition creates an additional *Mnl*I restriction site, allowing RFLP analysis. PCR amplification, using primerset 1 (see Table 13), resulted in a fragment of 195 bp, which was digested by 5 units of the restriction enzyme *Mnl*I for 2 hours at 37°C, followed by gel electrophoresis analysis on a 4% agarose gel. Fragments of 148 and 47 bp identify the -34C allele, while the -34T allele shows fragments of 121, 47 and 27 bp. The additional *Mnl*I restriction site forms an internal control of the assay. Genotype data was not available for three recurrent venous thrombosis patients (n=170) and thirteen control individuals (n=425).

Statistics

All the analyses were performed using SPSS software. Total homocysteine concentrations were logarithmically transformed prior to all analyses, and are expressed as a geometric mean with a 95% confidence interval (CI). Differences in tHcy concentration were determined by linear regression analysis and expressed as changes relative to the wild type. Odds ratios and 95% confidence intervals were calculated to estimate the relative risk of venous thrombosis in individuals that carry the mutant allele using logistic regression analysis.

Table 14. Sequence variants in the S-adenosylhomocysteine hydrolase gene identified in this study

DNA variants	Location	Amino acid (substitution)	Screening method
-34 C>T	5' UTR	Untranslated region	RFLP; -BsrB1
112 C>T	Exon 2	Arg38Trp	RFLP; +MnlI
390 C>T	Exon 4	Asp130Asp	Not screened

Results

AHCY sequence analysis

In order to identify genetic determinants of homocysteine we sequenced the AHCY gene of 20 recurrent venous thrombosis patients. Sequence analysis of all 10 exons, part of the 5' UTR (from nucleotide -75 to the translation initiation codon) and the entire 3' UTR (nucleotide +22353 to +23369) of the AHCY gene revealed 3 sequence variants (Table 14 and Figure 6). The first was located in the 5' UTR 34 bp upstream of the translation initiation codon (-34 C>T). In one patient a C>T transition was found in exon 2 of the AHCY gene at cDNA position 112 (112 C>T). This mutation mandates an amino acid substitution of a basic arginine to a neutral tryptophan at codon 38 (R38W). Finally, a synonymous C>T variant was

detected in exon 4 (nucleotide position 390, amino acid D130D). The variants were present in three separate patients, all in a heterozygous state. Because the -34 C>T variant may affect gene expression and the 112 C>T variant mandates an amino acid change and potentially affects protein function, we screened our population of recurrent venous thrombosis patients and population-based controls for these variants to assess their effects on tHcy levels and recurrent venous thrombosis risk. The synonymous 390 C>T variant was not studied in further detail, although an effect on mRNA stability or the splicing process cannot be ruled out.

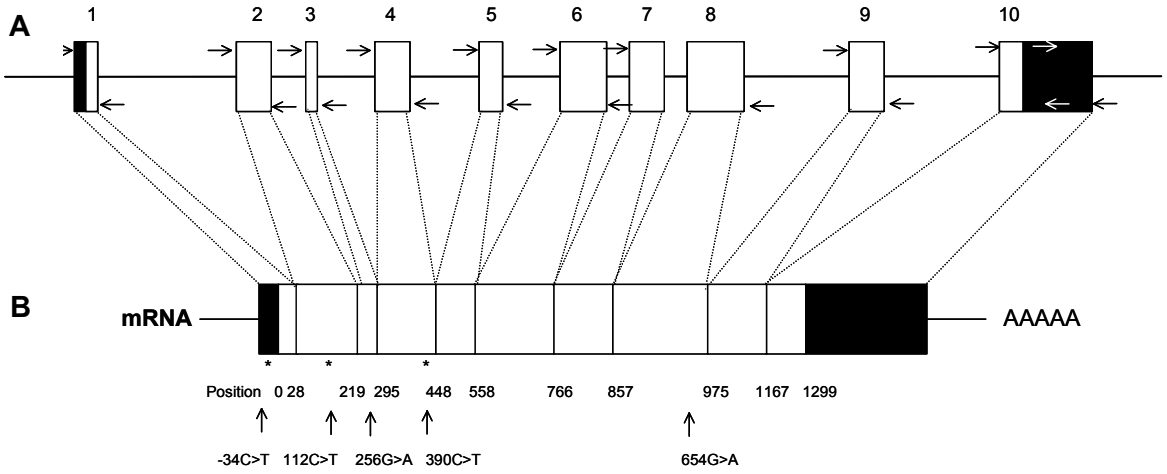

Figure 6. Schematic representation of the AHCY gene

A) The coding regions are depicted as white boxes and the intronic regions as a horizontal line. The black boxes represent the untranslated regions. The arrows indicate the location of the (intronic) primers used for sequence analysis. B) In this mRNA representation sequence variants are indicated by vertical arrows (identified in this study denoted by *) with their cDNA position and corresponding codon.

Baseline characteristics of study population

The control group consisted of 438 individuals (average age 50.7 ± 13.3 y) from which 41.1% was male (n=180). Fasting and post-load tHcy were 10.4 (95% CI 10.1 to 10.8) μmol/L and 38.3 (95% CI 37.2 to 39.5) μmol/L, respectively. The case group consisted of 180 recurrent venous thrombosis patients (average age 61.5 ± 14.2 y) from which 50.9% was male (n=88). Fasting and post-load tHcy were 12.5 (95% CI 11.8 to 13.3) μmol/L and 44.4 (95% CI 42.3 to 46.6) μmol/L, respectively.

Association of AHCY sequence variants and total homocysteine

Fasting tHcy concentrations did not differ between the AHCY 112 C>T genotypes in recurrent venous thrombosis patients and controls (Table 15). However, in heterozygous subjects tHcy concentrations seemed to be increased after a methionine load compared with 112CC individuals but only in the case group. With respect to the -34 C>T variant, no differences in tHcy (fasting or PML) were observed between the genotypes (Table 16).

Table 15. Association between AHCY genotypes defined by the 112 C>T variant and fasting and post-methionine load (PML) tHcy concentrations in venous thrombosis patients versus control individuals

		AHCY genotype	n	Geometric mean tHcy [95% CI] (μmol/l)	Crude increase % [95% CI]	Adjusted increase % [95% CI][a]
Fasting tHcy	Control	112CC	415	10.4 [10.0 to 10.8]	1.0[b]	1.0[b]
		112CT	21	11.3 [9.5 to 13.3]	8.3 [-9.0 to 28.0]	14.4 [-2.1 to 33.6]
		112TT	1	13.3	28.1	13.0
	Case	112CC	162	12.5 [11.8 to 13.3]	1.0[b]	1.0[b]
		112CT	10	12.5 [9.8 to 16.0]	0 [-23.0 to 29.0]	3.8 [-16.6 to 29.3]
PML tHcy	Control	112CC	415	38.2 [37.1 to 39.5]	1.0[b]	1.0[b]
		112CT	21	39.3 [34.2 to 45.1]	2.7 [-11.0 to 18.0]	6.1 [-7.7 to 22.1]
		112TT	1	36.5	-4.5	-4.5
	Case	112CC	162	43.8 [41.7 to 46.1]	1.0[b]	1.0[b]
		112CT	10	54.8 [44.8 to 67.2]	25.1 [1.5 to 54.3]	27.7 [3.9 to 56.8]

[a] adjusted for age, sex and serum creatinine, [b] reference category

Table 16. Association between AHCY genotypes defined by the -34 C>T variant and fasting and post-methionine-load (PML) tHcy concentrations in recurrent venous thrombosis patients versus control individuals

		AHCY genotype	n	Geometric mean tHcy [95% CI] (μmol/l)	Crude increase % [95% CI]	Adjusted increase % [95% CI][a]
Fasting tHcy	Control	-34CC	411	10.4 [10.1 to 10.8]	1[b]	1[b]
		-34CT	14	11.5 [9.5 to 14.1]	10.5 [-9.9 to 35.4]	11.1 [-7.8 to 33.7]
	Case	-34CC	164	12.7 [11.9 to 13.5]	1[b]	1[b]
		-34CT	6	11.0 [8.0 to 16.0]	-12.9 [-37.1 to 20.6]	-5.7 [-29.0 to 25.3]
PML tHcy	Control	-34CC	411	38.3 [37.1 to 39.5]	1[b]	1[b]
		-34CT	14	39.3 [33.0 to 46.9]	2.8 [-14.1 to 22.9]	6.1 [-7.7 to 22.1]
	Case	-34CC	164	44.4 [42.3 to 46.6]	1[b]	1[b]
		-34CT	6	48.4 [37.4 to 62.6]	9.0 [-16.2 to 41.7]	11.7 [-13.6 to 44.5]

[a] adjusted for age, sex and serum creatinine, [b] reference category

AHCY genotype analyses and associated RVT risk

In the control group, the distribution of the genotypes defined by the 112 C>T variant was in Hardy-Weinberg equilibrium (p=0.19) with a 112T allele frequency of 3.0% among recurrent venous thrombosis patients and 2.7% among the controls. The crude odds ratio as an estimation of the relative risk of recurrent venous thrombosis for the 112CT genotype compared with the 112CC genotype was 1.22 (95% CI [0.56 to 2.65]). Adjustment for age and sex did not change this risk estimate (Table 17).

The genotype distribution defined by the -34 C>T transition in the control group was in Hardy-Weinberg equilibrium (p=0.73). The frequency of the -34T allele was 1.8% among the recurrent venous thrombosis patients and 1.6% among the controls. The -34TT genotype was not observed in our study populations. The relative risk of recurrent venous thrombosis due to the -34CT genotype compared with the wild type was 1.07 (95% CI [0.41 to 2.84]). After adjustment for age and sex this odds ratio increased to 1.25 (95% CI [0.44 to 3.52]) (Table 17).

Table 17. Distribution of AHCY 112 C>T and -34 C>T genotypes among recurrent venous thrombosis patients and control individuals and risk for recurrent venous thrombosis

Genotype	RVT Patients, n (%) (N=172)	Controls, n (%) (N=437)	Crude odds ratio [95% CI]	Adjusted odds ratio [95% CI][a]
112 CC	162 (94.2)	415 (95.0)	1.0[b]	1.0[b]
112 CT	10 (5.8)	21 (4.8)	1.22 [0.56 to 2.65]	1.27 [0.55 to 2.94]
112 TT	n.o.	1 (0.2)	-	-
	RVT patients, n (%) (N=170)	Controls, n (%) (N=425)	Crude odds ratio [95% CI]	Adjusted odds ratio [95% CI][a]
-34 CC	164 (96.5)	411 (96.7)	1.0[b]	1.0[b]
-34 CT	6 (3.5)	14 (3.3)	1.07 [0.41 to 2.84]	1.25 [0.44 to 3.52]
-34 TT	n.o.	n.o.	-	-

[a] adjusted for age and gender, [b] reference category, n.o. not observed

Other sequence variants in AHCY coding region

Coulter-Karis and Hershfield [219] were the first to report a sequence of a full-length human placental cDNA of the AHCY gene. They reported a G to A transition in exon 3 at nucleotide 256, which results in an aspartic acid to asparagine conversion and abrogates a *Sau*96I restriction site. We screened 50 randomly selected controls for this polymorphism but did not detect this variant in the 100 alleles screened, nor did we find this mutation in 40 recurrent venous thrombosis patients (including the 20 patients used for the sequence analysis).

From these findings we conclude that this transition may either represent a sequence artifact or is present only at a frequency <0.5%. In addition, a synonymous mutation in exon 8 (954 G>A, K318K) reported in the NCBI SNP Database was not found by sequencing analysis of the AHCY gene in the 20 recurrent venous thrombosis patients, and was not studied in further detail.

Discussion

It is still debated via which mechanism hyperhomocysteinemia induces arterial vascular disease and venous thrombosis. In addition to the direct effects of homocysteine, the parallel increase in AdoHcy observed in hyperhomocysteinemic individuals [55,56] and associated effects on adenosine [222] and transmethylation [59,60] have emerged as alternative mechanisms for hyperhomocysteinemia-related cardiovascular disease. Laukkanen and colleagues found that local CpG-methylation in the superoxide dismutase gene in rabbit atherosclerotic aortas was reduced [223], while others observed global DNA hypomethylation in atherosclerotic lesions and leucocytes of cardiovascular disease patients [57,224].

This is the first report of a systematic sequence analysis of the human AHCY gene in a group of recurrent venous thrombosis patients. We found three sequence variants of which two, that is 112 C>T and -34 C>T, were studied in a group of recurrent venous thrombosis cases and population-based controls to assess their effect on tHcy and recurrent venous thrombosis risk.

No effect of both sequence variants on fasting tHcy was observed in our case and control group. A higher tHcy after a methionine-load was observed but only in 112CT cases. This is in contrast to our expectations but may be explained by an effect of the polymorphism on the

synthesis of AdoHcy out of homocysteine resulting in elevation of tHcy, especially after increased methionine-intake (i.e. methionine-load). However, this effect was not observed in our control population making it questionable whether the effect on tHcy is due to the polymorphism or simply a chance finding (due to low number of individuals carrying the T allele). Expression studies are needed to study the impact of these polymorphisms at the protein level that may reveal if and how the reversible AHCY-catalyzed reaction is affected. The effect of these polymorphisms on electrophoretic mobility may also be investigated since differently migrating isoforms have been described in the past [216-218]. However, no tissue samples were available to assess whether a mobility shift of the AHCY enzyme was correlated with the polymorphisms.

Very recently, Baric and colleagues reported an AHCY deficient patient with only a slightly elevated tHcy (15.9 μmol/L) while AdoHcy levels were increased ~150-fold. This case shows that inhibition of AHCY, and associated changes in AdoHcy, does not necessarily lead to a dramatic change in tHcy [220]. It would be interesting to measure AdoMet and AdoHcy levels, the ratio of which is an important predictor of cellular methylation capacity, to test the hypothesis that the polymorphisms described in this study affect AdoHcy and AdoMet levels. Unfortunately, AdoMet is very unstable in untreated blood samples and is partly degraded into AdoHcy [225] making it impossible to measure these metabolites in the stored plasma samples of our study populations. This also prompted us to use tHcy levels as a selection criterion although AdoHcy would have been a better candidate.

We assessed the relative risk conferred by these variants on developing two or more venous thrombosis events. The odds ratio for recurrent venous thrombosis was 1.22 (95% CI [0.56 to2.65]) for the 112 CT genotype compared with the 112CC genotype. Similar results were obtained for the -34bp C>T variant; an odds ratio of 1.07 (95% CI [0.41 to 2.84] was observed for the -34CT genotype, which increased to 1.25 (95% CI [0.44 to 3.52]) after adjustment for age and sex. Although these risk estimates are in the range of venous thrombosis risk associated with the MTHFR 677 TT genotype [18], the low frequency of the polymorphisms described above underpowers this study to draw firm conclusions on disease risk conferred by these variants.

In conclusion, the AHCY gene harbors only little variation in our Dutch population of recurrent venous thrombosis patients. This underlines the importance of AHCY in methionine metabolism. The detected polymorphisms seem to have no important effect on tHcy, but an effect on AdoHcy and recurrent venous thrombosis risk cannot be ruled out and needs additional studies in larger populations. These studies, in which AdoMet and AdoHcy concentrations are measured as well, may contribute to our understanding via which mechanism a disturbed homocysteine metabolism is associated with cardiovascular disease.

Acknowledgments

This study was in part supported by grant 2002B68 from the Netherlands Heart Foundation. Martin den Heijer, MD, PhD, is supported by a VENI-grant from the Dutch Organization for Scientific Research (NWO). Leo A.J. Kluijtmans, PhD, is a post-doctoral fellow of the Netherlands Heart Foundation (1999T023). Henk J. Blom, PhD, is an Established Investigator of the Netherlands Heart Foundation (D97.021).

Chapter 6

Catechol-O-methyltransferase Genotype is Associated With Plasma Total Homocysteine Levels and May Increase Recurrent Venous Thrombosis Risk

Henkjan Gellekink*, Jan-Willem Muntjewerff*, Sita H.H.M. Vermeulen, Ad R.M.M. Hermus, Henk J. Blom, Martin den Heijer

Laboratory of Pediatrics and Neurology, Department of Endocrinology and Department of Epidemiology and Biostatistics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; GGz Nijmegen, Mental Health Institute, Nijmegen, The Netherlands.
* these authors contributed equally to the manuscript

Submitted for publication

Abstract

Background: 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-adenosylmethionine-dependent methylation of catecholamines and catecholestrogens and in this way contributes to homocysteine synthesis. COMT dysfunction has been related to schizophrenia and breast cancer. Objectives: We hypothesized that COMT dysfunction affects plasma total homocysteine (tHcy). Because we did find an association between COMT genotype and tHcy we also evaluate this genotype as a risk factor for recurrent venous thrombosis. Methods: 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). Results: 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. Conclusion: 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 [17-19]. In addition, high plasma total homocysteine (tHcy) increases the risk of spina bifida, and mental disorders like schizophrenia and Alzheimer's disease [23,36,39]. The mechanism of how homocysteine is related to disease is still obscure, but there are strong indications that a disturbed transmethylation may partly explain this association [60,226,227]. Several studies show that plasma total homocysteine levels (tHcy) correlate well with plasma S-adenosylhomocysteine (AdoHcy) [55,57], a strong inhibitor of S-adenosylmethionine (AdoMet)-dependent methylation. Given the importance of methylation of nucleic acids, proteins, lipids but also hormones and neurotransmitters, 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 [228]. 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 [34,160,229,230]. COMT enzyme exists as a membrane-bound (MB) and soluble (S) isoform, the expression of which is regulated by two different promotors. 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 nonconsistent [231,232].

The metabolic pathways of COMT and homocysteine are interconnected as the O-methylation of catecholamines and catecholestrogens catalyzed by COMT produces homocysteine (Figure 1). Therefore, functional variants within the COMT gene may influence tHcy levels and possibly reflect disturbed transmethylation capacity implicated in COMT-related diseases.

The aim of our study was to investigate the effect of COMT variants on tHcy. Considering the controversy whether the 324G>A variant is causally related to decreased enzyme activity or expression, we included three other variants (rs2097603 [231], rs4633 and rs174699) in or directly adjacent to the COMT gene and performed haplotype analysis. Because we did find an association between COMT genotype and plasma homocysteine levels we also evaluate this genotype as a risk factor for recurrent venous thrombosis, which is also related to homocysteine.

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 [6]. Recurrent venous thrombosis cases were selected from the files of the anticoagulant clinic in Leyenburg Hospital in The Hague, The Netherlands [6]. For the association study, DNA was available from 438 subjects. For the case-control study, COMT 324 G>A genotyping was performed in 169 recurrent venous thrombosis patients.

Biochemical parameters

Blood samples were drawn 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 3500 g for 5 minutes with minimal delay. The plasma was separated and then stored at -20 ºC. 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) [178]. DNA extraction was performed as described previously [179] and the DNA was stored at 4 ºC.

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 18. PCR conditions: 4 minutes at 94°C, 35 cycles of 94°C/60s, 52-60°C/60s, and 72°C/30s, and a final extension of 7 minutes 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 analysed 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). In addition, data of four other reported variants (i.e. rs6267 [Ala22Ser] [233], 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, additional screening (rs 4680) was performed in 169 cases with a history of venous thrombosis [6].

Statistical analyses

Haploview [234] 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 were evaluated by linear regression analysis (SPSS 12.0). In the haplotype analysis, changes in homocysteine levels are expressed relative to the most frequent haplotype group. Odds ratios were estimated using logistic regression analysis (SPSS 12.0). A p-value <0.05 was considered statistically significant.

Table 18. Relevant data of analysed polymorphisms and method of screening

Variant	Reported MAF[a]	DNA	Protein[b]	HW P value	Primers (5'→ 3')	Validation method	$[Mg^{2+}]$ (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 tactgtggctactcagctgtgc R gtgaacgtggtgtgaacacc	*+NlaIII*	2.0	59
rs174699	0.19 (C)	Intron 3 T>C	-	1.0	F cctctgtgaggctccaactc R gaaagaaggcagcacctgtc	*-NcoI*	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

Results

SNP genotyping and linkage disequilibrium

For details about these SNPs regarding location, function and validation method, see Table 18 and Figure 7. 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 18). Linkage disequilibrium (D') and squared correlation coefficients (r^2) between each of the genotyped variants are shown in Table 19. The SNP pair rs4680 (324G>A) and rs4633 (36T>C) was in almost complete LD, with a D' of 0.96 and r^2 of 0.89. No strong LD was observed for the other SNP pairs.

Table 19. Linkage disequilibrium coefficient (D') between SNPs across the COMT gene in the general population
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*	-

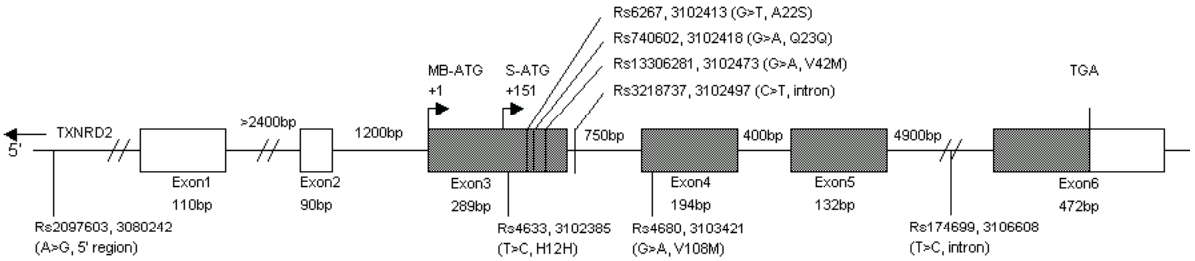

Figure 7. Gene structure of the COMT gene

The boxes represent exons and the thin lines in between represent introns. Gray boxes indicate protein-coding regions. The length (bp) of each region is indicated, as are the translation initiation codons for the soluble (S-ATG) and membrane-bound (MB-ATG) COMT isoform. In the lower part of the figure the polymorphic variants assessed in this study are shown (rs number, relative position, type of polymorphism), while four other variants that appeared non-polymorphic are depicted in the upper part. TXNDR2=thioredoxin reductase 2. Adapted from Zhu *et al.*, 2002 [59].

Haplotype associations in the general population

Genotype data for all four polymorphisms was obtained from 401 individuals (mean age 50.6 ± 13.3 y), from which 41.0% was male (n=164). Mean fasting tHcy was 10.4 (95% CI 10.0 to 10.8) μmol/L, serum creatinin was 74.3 (9% CI 72.7 to 75.9) μmol/L, vitamin B12 was 221 (95% CI 208 to 234) pmol/L and folate was 13.1 (95% CI 12.3 to 13.9) nmol/L.

The haplotype frequencies and their effects on tHcy relative to the most frequent haplotype (G-T-A-T) are presented in Table 20. 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 21). Furthermore, by comparing a model in which the effects of the 3 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.

Table 20. Haplotype frequencies and relative change in tHcy in population-based controls 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 21. COMT single-locus genotype effects on tHcy concentrations in population-based controls

Variant	Genotype	n (%)	MAF[b]	Crude mean tHcy [95% CI] (μmol/l)	Crude change % [95% CI]	P
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)	6.8 (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

The 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 en blood parameters were similar as those of the individuals included in the haplotype analysis. For the recurrent venous thrombosis cases (mean age 61.5 ± 14.3 y) geometric mean (95% CI) tHcy was 12.6 (11.6 to 13.6) μmol/L, 83.1 (78.6 to 87.6) μmol/L for serum creatinin, 237 (207 to 267) pmol/L for vitamin B12 and 13.7 (12.4 to 15.0) nmol/L 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, 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 odds ratio for individuals with the 324GA genotype was 1.21 (95% CI 0.76 to 1.94, p=0.18).

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) [229,235]. 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 [231,236] although the Val-allele was expressed at a slightly lower level in human brain [232]. In the past, Goodman *et al.* [160] showed that the COMT 324G>A variant affected tHcy in controls, while Geisel *et al.* did not find such an effect in elderly subjects [129]. In addition, it has been suggested that other variants might explain the observed associations [229]. Our results show that 324AA genotype is significantly associated with increased tHcy levels, which may support the observation of higher expression of the Met-allele by Bray *et al.* [232]. 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 [237], although negative effects have also been reported [59]. 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 [238-240], indicate that a higher COMT flux is reflected in plasma tHcy levels. In addition, a recent genome-wide linkage scan performed by Souto and colleagues identified another methyltransferase, Nicotinamide N-methyltransferase (NNMT), as a possible major determinant of tHcy [241]. This shows that not only methyltransferases with a high flux-rate, like guanidinoacetate- and phosphatidylethanolamine methyltransferase [242], 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 venous 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.

Acknowledgments

This study was in part supported by grant 2002B68 from the Netherlands Heart Foundation. Martin den Heijer, MD, PhD, is supported by a VENI-grant from the Dutch Organization for Scientific Research (NWO).

Part *II*

Pathophysiology - Homocysteine and Disturbed Transmethylation

Chapter 7

Stable-isotope Dilution Liquid Chromatography-electrospray Injection Tandem Mass Spectrometry Method for Fast, Selective Measurement of S-adenosylmethionine and S-adenosylhomocysteine in Plasma

Henkjan Gellekink, Dinny van Oppenraay-Emmerzaal, Arno van Rooij,
Eduard A. Struys, Martin Den Heijer, and Henk J. Blom

Laboratory of Pediatrics and Neurology, Department of Endocrinology, and Department of Epidemiology and Biostatistics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; Metabolic Unit, Department of Clinical Chemistry, VU University Medical Centre Amsterdam, Amsterdam, The Netherlands

Clinical Chemistry 2005; 51: 1487-1492

Abstract

It has been postulated that changes in S-adenosylhomocysteine (AdoHcy), a potent inhibitor of transmethylation, provide a mechanism via which elevated homocysteine causes its detrimental effects. We aimed to develop a rapid and sensitive method to measure AdoHcy and its precursor S-adenosylmethionine (AdoMet). We used stable-isotope dilution electron spray injection liquid chromatography tandem mass spectrometry (LC-ESI-MS/MS) to measure AdoMet and AdoHcy in plasma. Acetic acid was added to prevent AdoMet degradation. Phenylboronic acid containing solid-phase extraction (SPE) columns were used to bind AdoMet, AdoHcy and their internal standards and for sample cleanup. An HPLC C-18 column directly coupled to the LC-MS/MS was used for separation and detection. In plasma samples, the interassay CVs for AdoMet and AdoHcy were 3.9% and 8.3%, while the intraassay CV were 4.2% and 6.7%, respectively. Mean recovery for AdoMet was 94.5% and 96.8% for AdoHcy. The quantification limits were 2.0 and 1.0 nmol/L for AdoMet and AdoHcy, respectively. Immediate acidification of the plasma samples with acetic acid prevented the observed AdoMet degradation. In a group of controls (mean tHcy 11.2 μmol/L) plasma AdoMet and AdoHcy were 94.5 nmol/L and 12.3 nmol/L, respectively. Stable-isotope dilution LC-ESI-MS/MS allows a sensitive and rapid measurement of AdoMet and AdoHcy. The SPE columns enable a simple cleanup step and no metabolite derivatisation is needed. The instability of AdoMet is a serious problem and can be prevented easily by immediate acidification of the samples.

Introduction

Increased plasma total homocysteine (tHcy) is a risk factor for many pathological conditions including cardiovascular disease, congenital abnormalities, certain malignancies and neurological disorders [59,243]. However, whether increased homocysteine itself is causally related to these disease states or is a marker of impaired one-carbon metabolism remains subject of debate.

Homocysteine is a sulphur-containing amino acid derived from demethylation of the essential amino acid methionine. After condensation of methionine and ATP, S-adenosylmethionine (AdoMet), the principle methyldonor in the human body, is formed. The methyl group can be donated to a variety of macromolecules, such as DNA, RNA, proteins and lipids, as well as to (small) precursor molecules such as guanidinoacetate and catechol(amine)s. The demethylated product of AdoMet is S-adenosylhomocysteine (AdoHcy) which is hydrolyzed to homocysteine and adenosine in a reversible reaction catalyzed by AdoHcy hydrolase.

Efficient removal of adenosine and homocysteine is essential for cellular function as the equilibrium of the reaction catalyzed by AdoHcy hydrolase strongly favors the formation of AdoHcy, a strong inhibitor of most cellular methylation reactions. *In vivo* studies have demonstrated that increased tHcy is associated with increased plasma AdoHcy levels and a reduced AdoMet / AdoHcy ratio [244], also called methylation index, which correlates well with global DNA hypomethylation in cardiovascular disease patients [57] as well as different tissues including lymphocytes, brain and liver [55,60]. It has been suggested that AdoHcy-mediated hypomethylation provides an alternative mechanism for the pathogenesis of diseases related to hyperhomocysteinemia. Moreover, several studies have shown that AdoHcy is a stronger risk factor for cardiovascular disease than homocysteine [56,57], a finding that makes the determination of AdoMet and AdoHcy an important tool to evaluate the clinical conditions associated with hyperhomocysteinemia.

Because the concentratioins of AdoMet and AdoHcy in body fluids are low (about 10-100 nmol/l), their measurement is time-consuming and difficult. In addition, AdoMet is unstable and partially degrades into AdoHcy in untreated samples (this study). We therefore aimed to develop a sensitive and rapid high-throughput method for simultaneous measurement of AdoMet and AdoHcy in biological samples using liquid chromatography-electron spray injection tandem mass spectrometry (LC-ESI-MS/MS).

Materials and Methods

Sample collection and storage

Blood samples were drawn from the antecubital vein into 4.5 mL evacuated glass tubes containing EDTA (BD Vacutainer Systems, Plymouth, UK), placed on ice immediately and centrifuged at 3500 g for 5 minutes with minimal delay. The plasma was separated and stored at -20ºC until analysis. For AdoMet and AdoHcy measurements, 500 μL of plasma was directly acidified with 50μL 1 mol/L acetic acid to a final acetic acid concentration of 0.091 M, mixed thoroughly and then stored at -20°C. All study participants gave informed consent.

Homocysteine measurements

Plasma total homocysteine concentrations were measured in our laboratory by an automated HPLC method with reverse-phase separation and fluorescent detection. The HPLC system consisted of a Gilson 232-401 sample processor, Spectra Physics 8800 solvent delivery system and LC 304 fluorometer [178].

Plasma sample preparation for AdoMet and AdoHcy measurements

Sample cleanup was performed with solid-phase extraction (SPE) columns (Varian Inc.), containing phenylboronic acid, which at pH of 7 to 8 selectively bind cis diol groups. The SPE columns were preconditioned by addition of five 1-mL volumes of 0.1 M formic acid and five 1-mL volumes of 20 mM ammonium acetate (pH 7.4). Before SPE, the acidified samples were neutralised with 55 μL 1 mol/L ammonia to a pH of 7.4 to 7.5, and 110 μl internal standard [1.5 μM for $^{2}H_{3}$-AdoMet (CDN Isotopes) and 0.41 μM for $^{13}C_{5}$-AdoHcy [245]] was added. The mixture was then applied to the SPE column for binding of AdoMet, AdoHcy and their internal standards $^{2}H_{3}$-AdoMet and $^{13}C_{5}$-AdoHcy. Water-soluble impurities were removed by washing the column twice with one mL 20 mmol/L ammoniumacetate (pH 7.4) [246] and AdoHcy and AdoMet were eluted in one mL 0.1 mol/L formic acid. After SPE, AdoMet and AdoHcy were stable for at least 6 months (at -20°C) because elution from the SPE column by formic acid (pH 2-3) stabilises AdoMet and AdoHcy. The samples (20 μl) were loaded on an equilibrated (0.2 mL/L acetic acid) Symmetry-Shield HPLC C-18 column [100 mm x 2.1 mm i.d.; Waters Corporate] and eluted in a gradient (0%-0.3%) of methanol in 0.2 mL/L aqueous acetic acid delivered by a HP 1100 binary pump (Agilent Technologies) with the splitter (Acurate; LC Packings) in the 1:4 mode allowing the injection of 4 μL sample into the electrospray injection chamber. The retention times were 2.40 and 2.80 minutes for AdoMet and AdoHcy, respectively. AdoMet and AdoHcy levels were measured by LC-ESI-MS/MS with the Micromass Quattro LC (Waters) in the positive-ion (ESI+) mode. Optimal multiple reaction monitoring conditions were obtained for four channels: AdoMet (m/z 399>250), $^{2}H_{3}$-AdoMet (m/z 402>250), AdoHcy (m/z 385>136) and $^{13}C_{5}$-AdoHcy (m/z 390>136). Data were acquired and processed by Quanlynx for Windows NT software (Micromass).

AdoMet and AdoHcy quantification and ion suppression

Calibrators (AdoMet and AdoHcy) and internal standards ($^{2}H_{3}$-AdoMet and $^{13}C_{5}$-AdoHcy) were included in each analytical run for calibration. Briefly, stock solutions of AdoMet and AdoHcy in deionized water were diluted in ammonium acetate (pH 7.4) to concentrations of 10, 20, 50, 100, 200, 400 and 800 nmol/L for AdoMet and 5, 10, 20, 50, 100, 200 and 400 nmol/L for AdoHcy. We added 110 μl of internal standard to 500 μl of calibration solution and then processed as described above for the samples. Calibration curves were obtained by plotting ratios of the peak area (calibrator/internal standard) against the concentration of the calibrator. We quantified AdoMet and AdoHcy by interpolating the observed peak area ratio (m/z 399 and 385 peaks for endogenous AdoMet and AdoHcy vs m/z 402 and m/z 390 peaks for the $^{2}H_{3}$-AdoMet and $^{13}C_{5}$-AdoHcy internal standards) on the linear regression line

for the calibration curve. When AdoMet or AdoHcy concentrations were low, the samples were measured again, and an additional low-range calibration curve was prepared (2, 5, 10, 20 and 50 nmol/L or 1, 2.5, 5, 10 and 20 nmol/L for AdoMet and AdoHcy, respectively) as described above.

Ion suppression was calculated from the peak areas of the internal standards added to the calibrator solutions and compared with the peak areas of the internal standard that was added to each plasma sample. The relative change in peak area of the internal standard was attributed to matrix effects.

Statistics

Linear regression analysis (Excel) was used to verify linearity of the calibration curves and one-way ANOVA (SPSS, version 12.0) was used to assess differences in AdoMet and AdoHcy concentrations in pooled plasma samples.

Results

Chromatography and mass spectra

Shown in Figure 8 are typical chromatograms of a control plasma prepared and subjected to LC-ESI-MS/MS analysis as described in the *Materials and Methods* section. Elution time were 2.4 min for AdoMet and $^{2}H_3$-AdoMet and 2.8 minutes for AdoHcy and $^{13}C_5$-AdoHcy. Decomposition MS/MS mass spectra of AdoMet and AdoHcy are shown in Figure 9. Optimal multiple reaction monitoring conditions were obtained in the positive-ion mode: AdoMet, m/z 399>250 (adenosine) and AdoHcy, m/z 385>136 (adenine).

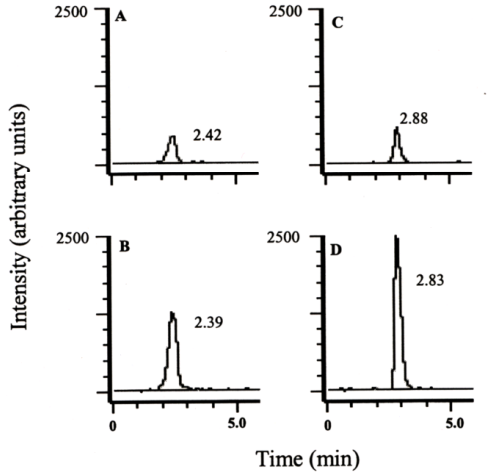

Figure 8. Typical MRM chromatograms of control serum
The left panels show the peak of endogenous AdoMet monitored at *m/z* 399>250 (A) and the internal standard peak of $^{2}H_3$-AdoMet monitored at *m/z* 402>250 (B) eluting at 2.4 minutes. The right panels show the peak of endogenous AdoHcy monitored at *m/z* 385>136 (C) and the internal standard peak $^{13}C_5$-AdoHcy monitored at *m/z* 390>136 (D) eluting at 2.8 minutes

Linearity of AdoMet and AdoHcy

The calibration curve was linear over the ranges 10-800 nmol/L for AdoMet and 5-400 nmol/L for AdoHcy, as determined by 3 separate measurements. The coefficient of linear correlation (r^2) was >0.999 for the calibration curves of both AdoMet (▪) and AdoHcy (♦) (see Figure 10). For the lower-range calibration curves (2-50 nmol/L for AdoMet and 1-20 nmol/L for AdoHcy), the coefficient of linear correlation was also >0.999 for both curves. The quantification limits,

derived from the lower-range calibration curve, were 2.0 nM for AdoMet and 1.0 nM for AdoHcy (mean signal-to-noise ratio>10).

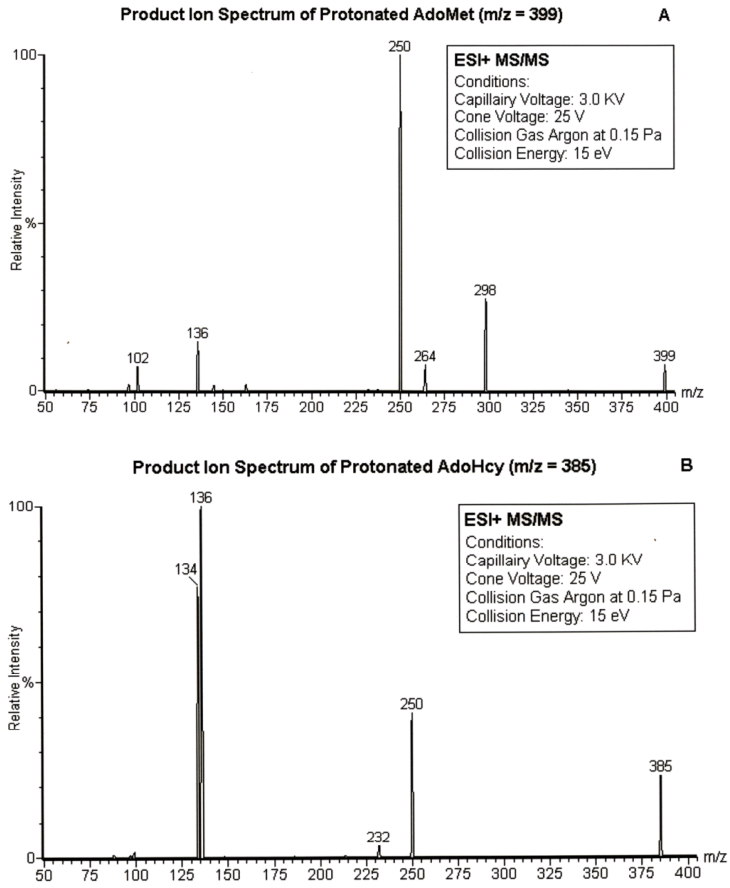

Figure 9. Mass fragmentogram of AdoMet and AdoHcy generated in the positive-ion mode

A) AdoMet (*m/z* 399) and products (*m/z* 250, adenosine);

B) AdoHcy (*m/z* 385) and products (*m/z* 136, adenine)

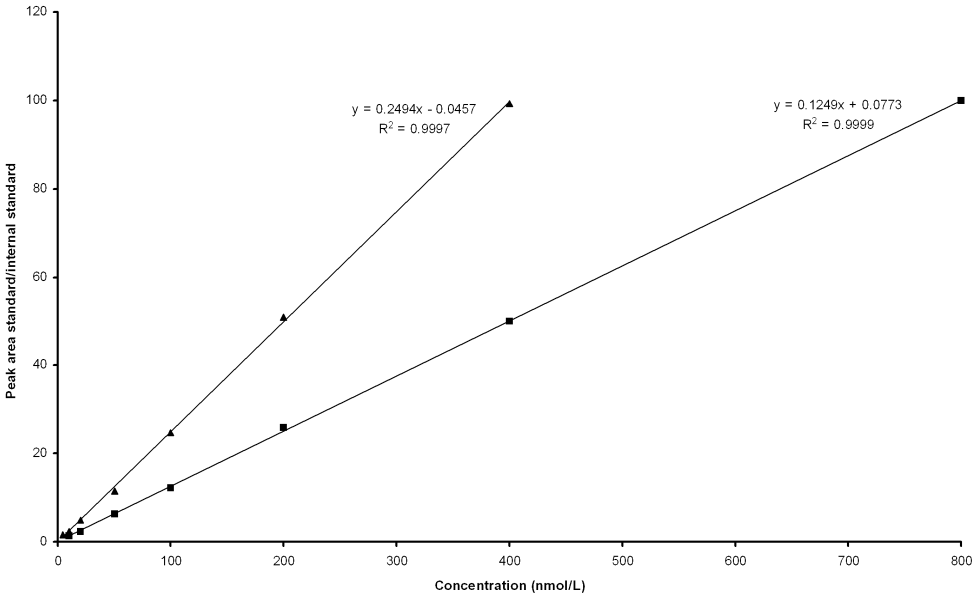

Figure 10. Calibration curves for AdoMet and AdoHcy determination by LC-MS/MS

Standard calibration curves were linear over a range of 10 to 800 nmol/L for AdoMet (▪) and 5 to 400 nmol/L for AdoHcy (♦) with a coefficient of linear correlation >0.999 for both metabolites

Quality control, recovery, and precision

Recovery experiments were performed within the physiological ranges of AdoMet and AdoHcy, as determined in healthy controls (see below), by use of nonacidified pooled plasma samples. The AdoMet concentration of the test pool was 77.3 nmol/L, and for AdoHcy 17.6 nmol/L. Mean recoveries were 94.5% for AdoMet (100 nmol/L added to the test pool) and 96.8% for AdoHcy (20 nmol/L added to the testpool) with CVs of 5.0% and 6.1% respectively (see Table 25A). The precision data for the method are presented in Table 25B. For this purpose, a large test pool of plasma was collected and treated according to the standard procedure used by our laboratory to assure metabolite stability over time (see also next section). The intraassay CVs (n=9) for AdoMet and AdoHcy were 4.2% and 6.7%, respectively, and the interassay CVs (n=12) for AdoMet and AdoHcy were 3.9% and 8.3%, respectively (Table 25B). Ion suppression in plasma was 30% and 20% for AdoMet and AdoHcy, respectively. This assay comprises a fast sample preparation step (10 samples in 30 minutes) and a measurement / column regeneration time of 8.5 minutes, which enables us to handle 100 samples/day.

Table 25. Recovery (A) and precision (B) of AdoMet and AdoHcy assay in plasma by LC-MS/MS

A) Recovery of the assay	Testpool[a]	Added	Mean ± SD	CV, %	Mean recovery, %
AdoMet (nmol/L, n=6)	77.3	100	171.8±8.6	5.0	94.5
AdoHcy (nmol/L, n=3)	17.6	20	37.0±2.3	6.1	96.8

B) Precision of the assay	Intra assay (n=9)			Inter assay (n=12)		
	Mean[b]	SD	CV	Mean	SD	CV
AdoMet (nmol/L)	126.1	5.4	4.2	131.2	5.1	3.9
AdoHcy (nmol/L)	19.7	1.3	6.7	16.9	1.4	8.3

Testpool of nonacidified [a] or acidified [b] plasma

Stability of AdoMet and AdoHcy

We observed a decrease in AdoMet over time in nonacidified plasma samples and a simultaneous increase of AdoHcy, suggesting a partial degradation of AdoMet into AdoHcy in our plasma samples. We therefore evaluated the AdoMet degradation rate during storage in treated and nontreated EDTA plasma samples. After only 3 h at room temperature, a marked decrease in AdoMet (~10%) and an increase in AdoHcy (~24%) were observed in the nonacidified plasma samples. This degradation process was not prevented by sample storage at -20°C, and after 1 month, the AdoMet concentrations had decreased to 43% of the initial value (p=0.009), and AdoHcy had increased to 150% of the initial value (p=0.067). Acidification of aliquots of the same plasma samples (with 1 mol/L acetic acid) stabilised both AdoMet and AdoHcy (Table 26). A decrease in AdoMet and a parallel increase in AdoHcy were also observed in nontreated plasma samples when the samples were collected in sodium citrate (pH 5.5) or heparin Vacutainer Tubes (BD Vacutainer Systems; data not shown). These observations are in line with earlier results of Stabler and Allen, who observed the same phenomenon in plasma and urine samples that were stored at room temperature and in samples stored at 4°C and below [247]. Even the use of acidic citrate (pH

Table 26. AdoMet degradation over time is observed in non-acidified but not in acidified EDTA plasma samples

	AdoMet (nmol/L)		AdoHcy (nmol/L)	
Incubation time	Non-acidified plasma (n=2)	Acidified plasma (n=4)	Non-acidified plasma (n=2)	Acidified plasma (n=4)
0 hrs	96.9 ± 7.8	96.5 ± 11.7	16.1 ± 0.4	15.6 ± 1.3
3 hrs rT[a]	88.4 ± 4.0	102.8 ± 8.6	21.2 ± 1.6	15.8 ± 0.9
1 day -20 °C	90.2 ± 3.4	-	20.3 ± 2.8	-
1 week -20 °C	65.0 ± 10.9	92.5 ± 6.8	24.3 ± 2.6	15.3 ± 0.9
1 month -20 °C	61.4 ± 2.7	98.3 ± 2.8	24.1 ± 2.8	17.2 ± 0.7
ANOVA	P=0.009	P=0.891	p=0.067	P=0.673

[a] room temperature

Table 27. AdoMet, AdoHcy and tHcy concentrations in plasma samples of healthy controls

N	AdoMet, nmol/L Mean ± SD	AdoHcy, nmol/L Mean ± SD	AdoMet/AdoHcy ratio Mean ± SD	Method of detection	tHcy, μmol/L Mean ± SD	Reference
26	94.5 ± 15.2	12.3 ± 3.7	8.5 ± 3.0	Stable-isotope dilution LC-MS/MS	11.2 ± 4.8	This study
15	74.7 ± 14.5 (range 49.5 to 90.7)	26.2 ± 6.1 (range 18.6 to 40.1)	2.9	Stable-isotope dilution LC-MS/MS	normal	[245]
48	109 (95% CI 71 to 168)	15.0 (95% CI 8 to 26)	7.4 (95% CI 4.4 to 12.4)	Stable-isotope dilution LC-MS	7.7 (95% CI 4.3 to 13.8)	[247]
7	102.7 ± 9.9	22.7 ± 3.1	4.5	HPLC-fluorescence	-	[250]
40	60.0 ± 19.0	24.4 ± 7.0	2.7 ± 1.3	HPLC-fluorescence	6.8 ± 2.5	[251,252]
28	155.9 ± 14.1	20.0 ± 5.6	8.5 ± 2.9	Coulorometric electrochemical detection	7.3 ± 1.1	[253]
30	154.8 ± 16.9	40.1 ± 12.5	4.5 ± 2.7	Coulorometric electrochemical detection	12.3 ± 1.8	[253]

4.3) may not prevent degradation as the pH increases to ~6.0 after blood sampling [248]. We therefore acidified plasma samples for AdoMet and AdoHcy measurements with acetic acid (final concentration, 0.091 mol/L acetic acid; final pH 4.5-5.0) with minimal delay after blood sampling to prevent AdoMet degradation. At this pH, no protein precipitation was observed.

AdoMet and AdoHcy concentrations in control individuals

As controls, 26 apparently healthy individuals from the Radboud University Nijmegen Medical Centre [mean (SD) age, 28.3 (8.2) years; 69% women) participated in this study to verify our method. Plasma samples were prepared for AdoMet, AdoHcy and tHcy determination as described. Mean (SD) concentrations were 11.2 (4.8) μmol/L for tHcy (range, 7.0-29.7 μmol/L), 94.5 (15.2) nmol/L for AdoMet (range, 69.4-121.8 nmol/L), and 12.3 (3.7) nmol/L for AdoHcy (range, 6.2-21.9 nmol/L). The resulting mean AdoMet / AdoHcy ratio was 8.5 (3.0). The AdoMet and AdoHcy values we obtained from control individuals are summarized in Table 27, along with data reported by other groups.

Discussion

Interest in AdoMet and AdoHcy measurement has increased over the last few years, particularly since increased AdoHcy and decreased cellular methylation capacity have emerged as a mechanism explaining the association between hyperhomocysteinemic individuals and increased risk for cardiovascular and neurological diseases [56,57,59,249].

In this report we present a highly selective and sensitive high-throughput method for the simultaneous measurement of AdoMet and AdoHcy in plasma samples by means of stable-isotope dilution LC-ESI-MS/MS. Phenylboronic acid-containing SPE columns were used for AdoMet and AdoHcy extraction, and no metabolite derivatisation was needed. Our method meets the criteria of minimal time required for sample preparation (10 samples in 30 min) and measurement / column regeneration (8.5 min), enabling us to process 100 samples per day. The method was linear over a broad range for both AdoMet and AdoHcy (r^2 >0.999). Recoveries >94% were obtained at physiological concentrations, and the inter- and intraassay CVs were <10%. Quantification limits of the assay were 2.0 and 1.0 nmol/L for AdoMet and AdoHcy, respectively, enabling measurement of AdoMet and AdoHcy within the (patho)physiological range.

Several methods to measure AdoMet and AdoHcy have been described, including HPLC with fluorescence detection [250-252], coulometric electrochemical detection [253], and stable-isotope dilution LC-MS/MS [245,247](Table 3). The disadvantages of the published methods include long sample preparation times attributable to the use of inorganic chemicals (e.g., phosphate buffers [245] or perchloric acid for deproteinisation [247]), which are less compatible with MS. Other time-consuming steps include derivatisation [251], sample analysis, and column regeneration [247,250,253]. Our method used MS-compatible buffers and solutions, thereby reducing potential ion suppression effects. The use of stable isotopes enables the adjustment for ion suppression due to matrix effects or early eluting compounds.

The observation that AdoMet is unstable in untreated samples and partially degrades into AdoHcy, as observed by us (this study) and others [247], may confound the results obtained in previous studies that report relatively low AdoMet and high AdoHcy concentrations. The

sensitivity of the assay may be compromised, and the AdoMet / AdoHcy ratio may change because the quantitative decrease in AdoMet is not reflected by the increase in AdoHcy. To prevent this degradation process, we acidified plasma samples with acetic acid to a pH<5.0 immediately after blood sampling and centrifugation. Interassay CVs calculated over a 4-month period indicated that acidification of plasma samples with acetic acid stabilises AdoMet and AdoHcy for at least 4 months.

We measured AdoMet and AdoHcy in 26 healthy individuals and found AdoMet and AdoHcy concentrations similar to those reported by others [247,250], and our calculated methylation index approached values observed by Stabler and Allen, and Melnyk *et al.* [247,253]. We found only a slight correlation between tHcy and AdoHcy (data not shown). This may be attributable to the fact that most of our observations were within the physiological range for Hcy; we may have observed a stronger correlation if hyperhomocysteinemic individuals had been included in the study.

In conclusion, the presented method provides a fast, reliable way for the routine measurement of AdoMet and AdoHcy in plasma, enabling investigation of disturbed 1-carbon metabolism in various disease states associated with hyperhomocysteinemia.

Acknowledgments

This study was supported in part by The Netherlands Heart Foundation (grant 2002B68). Martin den Heijer is supported by a VENI-grant from the Dutch Organization for Scientific Research (NWO). Henk J. Blom is an Established Investigator of the Netherlands Heart Foundation (D97.021). We greatly acknowledge S.G. Heil for critical evaluation of the manuscript.

Chapter 8

Investigation of the Human Umbilical Vein Endothelial Cell Proteome and Protein Methylation by Nano-liquid Chromatography Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

Henkjan Gellekink, Martin den Heijer, Wendy J.G. Pluk, Bert van den Heuvel and Henk J. Blom

Laboratory of Pediatrics and Neurology, Department of Endocrinology and
Department of Epidemiology and Biostatistics, Radboud University Nijmegen Medical Centre,
Nijmegen, The Netherlands

In preparation

Abstract

Hyperhomocysteinemia is a risk factor for arterial vascular disease and venous thrombosis. High homocysteine causes an increase in S-adenosylhomocysteine, a strong inhibitor of S-adenosylmethionine-dependent transmethylation, which is thought to explain the association between high homocysteine and cardiovascular disease. Methylation is a covalent modification involved in key processes like gene expression, genomic stability, protein-protein interactions and protein maturation. In contrast to DNA methylation, protein methylation is poorly studied due to technical difficulties. The introduction of high-throughput and sensitive methods for mass determination is of great benefit to proteomic applications, including the identification of posttranslational modifications. We aimed to study the proteome of cultured human umbilical vein endothelial cells (HUVECs) and protein methylation by means of SDS-PAGE fractionation, followed by in-gel trypsin digestion, peptide extraction and nano-liquid chromatography Fourier transform ion cyclotron resonance mass spectrometry (nano-LC FTICR MS).

We identified about 1500 proteins in cultured HUVECs, which could, after orthologous clustering, be assigned to the following main eukaryotic orthologous group (KOG)-divisions: Cellular processes and signaling (22.0%), Information storage and processing (10.3%), and Metabolism (9.2%). Based on peptide counts, we enlisted the most abundant proteins and provided additional data, like GI/IPI entry, mass and KOG (sub)divisions for each of those proteins. In order to illustrate the potential for identifying methylation sites we examined the Von Willebrand Factor precursor protein in more detail. A sequence coverage of 53.3% (1500 amino acids out of 2813) was obtained, and fourteen methylation sites were identified at arginine or lysine residues. The ions scores (mean 53) and expectation values (mean 0.027) indicate that these sites are likely to represent true methylation sites.

Our study shows that we are able to identify proteins in complex samples and assign methylation sites within specific proteins. Future studies should focus on nano-LC FTICR MS in combination with stable-isotope labeling, allowing the quantification of global and specific protein methylation for studying differential protein methylation *in vitro*.

Introduction

High levels of plasma total homocysteine (tHcy) are associated with an increased risk of cardiovascular disease. Endothelial dysfunction, an early marker of atherosclerosis, is one of the proposed mechanisms via which high tHcy compromises the vascular system [49,254,255]. The endothelium makes up the inner lining of the blood vessel, and is involved in important processes like regulation of blood flow, production of bioactive molecules (hormones, growth factors), production of (anti)coagulation factors (such as Von Willebrand factor and nitric oxide), and immune responses. Because the endothelium is directly exposed to the blood, toxic metabolites including reactive oxygen species may disturb endothelial function and ultimately lead or contribute to vascular damage.

The exact mechanism how high tHcy causes endothelial dysfunction and subsequent vascular disease is still obscure, but some studies suggest that a disturbed methylation may be involved [55,60]. Methylation of macromolecules, like proteins, represents an important modification involved in numerous processes. Because the addition of a methyl group to proteins may cause steric hindrance or affect protein properties (such as hydrophobicity), methylation may be important for protein-protein interactions or interactions between proteins and other molecules (such as DNA and RNA). As such, protein methylation is thought to be involved in protein repair and turnover, gene expression, maturation of RNA-binding proteins and cellular signaling [256-258].

Several studies have shown that high tHcy is associated with an increase in S-adenosylhomocysteine (AdoHcy), a strong inhibitor of S-adenosylmethionine (AdoMet)-dependent transmethylation [55-57,60]. The inhibitory effect of AdoHcy is thought to affect many methyltransferases [228] and, hence, disturbed methylation may represent a general mechanism via which homocysteine causes disease. Recently, Bjorklund suggested that disturbed protein methylation may be involved in the etiology of neural tube defects [227].

The study of protein methylation has been hindered because it is laborious, requires large amounts of sample and the techniques are not applicable for studying complex samples [259]. The increasing accuracy in mass determination by mass spectrometry has greatly facilitated the study of proteomes, and posttranslational modifications, including methylation. In general, these methods use the mass shift, introduced by the addition of a methyl group (14.0157 Da), to find peptides that contain modified amino acids. Recently, Rappsilber and co-workers used precursor ion scanning mass spectrometry to study arginine dimethylated peptides [260]. They focused on the FUS/TLS and Sam68 proteins and were able to identify several arginine dimethylated sites within these proteins.

We aimed to study the proteome of cultured human umbilical vein endothelial cells (HUVECs). Because of the potential role of disturbed protein methylation in disease, we also studied protein specific methylation in HUVECs. After SDS-PAGE of crude HUVEC extracts, we performed in-gel digestion followed by nano-LC FTICR MS analysis for the separation and sequencing of peptides. We used Mascot software for the identification of peptides and their methylation sites.

Materials and Methods

Cell culture

Culture medium was prepared by suppletion of M199 medium (Sigma-Aldrich) with 0.1% heparin, 10% newborn calf serum (Gibco), 10% human serum (Biowhittaker), 1% glutamine (ICN), 1% penicillin/streptomycin and 7.5 mg / 50 ml growthfactor (home-made calf brain extract). The medium was sterilized using a 0.2 μm filter. HUVECs were harvested from fresh umbilical veins by collagenase treatment (0.06%, Worthington/Aristofarma) and grown to confluence (cobblestone) on 1% gelatin-coated wells in complete medium at 37°C (5% CO_2). Cells were diluted (1:3) after every passage and grown in 175 cm^2 culture flasks. Cells were harvested at the fourth passage by trypsin treatment, washed in phosphate-buffered saline and collected at 3000 rpm for 5 minutes (4°C). Cells were snap-frozen in liquid nitrogen and stored at –80°C until further analysis.

Sample preparation

Cells were resuspended in lysis buffer (7M ureum, 2M thioureum, 4% CHAPS, 5% glycerol and 1 mM protease inhibitors [Complete, Roche]) and sonicated on ice (3 x 5 seconds) to facilitate cell lysis. The membrane and cytosolic fractions were separated by centrifugation (Sorvall RC5B plus DuPont, 20.000 g, 1 hour at 4°C). The membrane fraction was solubilized in membrane buffer (5M urea, 2M thiourea, 10% glycerol, 50mM Tris-HCl pH 8.0, 2% SBS-10, 5mM TCEP and 1 mM protease inhibitors) and diluted in tricine buffer (+2% β-mercaptoethanol, both from Bio-Rad). The protein concentration of the cytoplasmic fraction was measured by the method of Lowry and samples were diluted in tricine buffer (+2% β-mercaptoethanol). Thirty microgram of both fractions was separated on a 12% SDS-polyacrylamide gel (90 minutes, 100V). Each lane was cut into 10 slices, which were then prepared for FT-MS analysis.

Gel slices were cut into pieces with a scalpel and proteins were in-gel reduced (10 mM DTT) and alkylated (50 mM iodoacetamide in 50 mM NH_4HCO_3). Proteins were in-gel digested overnight at 37°C in a solution containing 12.5 ng/μl trypsin (Promega). Peptides were extracted from the gel by 2% trifluoro acetic acid (TFA) and the extract was desalted and concentrated using C_{18}-stage tips. Peptides were eluted in 2-4 μl buffer containing 0.5% acetic acid and 1% TFA (buffer A), and then diluted ten times in a buffer containing 80% acetonitrile, 0.5% acetic acid and 1% TFA (buffer B). Extracts were stored at -20°C until analysis.

FT-MS analysis

Digested proteins were separated using an Agilent 1100 LC system (Agilent, Palo Alto, CA, USA). The peptides were loaded onto 100 μm columns packed by bomb loading with 3 μm C18 beads (Reprosil-Pur C18 AQ, Dr Maisch GMBH, Ammerbuch-Entringen, Germany). Peptides were eluted in a gradient of solution B (80% acetonitrile, 0.5% acetic acid) in solution A (0.5% acetic acid). First a gradient of 3% solution B (97% solution A) to 10% (90% solution A) was built up in 5 minutes. In the next 55 minutes, the gradient increased to 30% solution B (70% solution A), and then in seven minutes from 30% solution B (70% solution A) to equivalent amounts of solution A and B to elute the residual peptides. A flow of 600 nl/min

during loading and 300 nl/min during elution was used. Peptides were eluted directly into the mass spectrometer (LTQ FTICR MS, Thermo Electron Corporation, Bremen, Germany) by nano-electrospray ionization. In each cycle, full scan MS spectra were recorded (400-1500 m/z) in the Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FTICR MS). The four most abundant ions were selected data-dependent and fragmented in the linear ion trap by collision-induced dissociation (using helium as a collision gas) to determine their amino acid sequence.

Data analysis

The mass spectrometry data were converted to a Mascot Generic data format with a Perl script and searched against the International Protein Index (IPI) database (version 3.03, 02-14-2005, http://www.ebi.ac.uk/IPI/IPIhelp.html) using Mascot (Matrix Science Inc., Boston, MA USA). The search algorithm was set to perform a tryptic search allowing 4 miscleavages and searched for carbamidomethyl modifications on cysteine residues, mono- di- and trimethylation of both arginine and lysine residues and possible oxidation of methionine. A mass accuracy of 20 ppm for the FTICR MS and 0.8 Da for the Linear Ion Trap was used. Protein hits were extracted from the Mascot search file using MSQuant (http://msquant.sourceforge.net/) and a Mascot (ions) score of 30 and peptide delta score >10 were used for peptide selection and protein identification. Using these cut-off values, the false positive rate for identifying proteins based on a single peptide is negligible (E. Lasonder, personal communication). The ions score and related expectation value (e-value), two statistical measures allowing a better estimation of significance in order to reduce the possibility of false positives, were used for the identification of methylated peptides.

Results

HUVEC proteome

The obtained mass data from both the MS and MS/MS spectra were compared to a protein database and enable peptide and protein identification. We were able to identify 1517 proteins in our HUVEC extracts. The mean protein sequence coverage in the whole set of proteins was about 25%, which allows protein identification with high confidence. In order to cluster these proteins according to their function, we performed orthologous (functional) clustering. This way the proteins are assigned to one of the three “eukaryotic orthologous group” (KOG) divisions. The percentage of proteins assigned to each KOG division is given in parenthesis: “1) Cellular processes and signaling” (22.0%), “2) Information storage and processing” (10.3%) and “3) Metabolism” (9.2%), while 52.1% was not annotated or poorly characterized (6.4%) using this cluster algorithm. Within each KOG division, the proteins can be assigned to subgroups. From the proteins assigned to KOG division 1, most were involved in posttranslational modification, protein turnover and chaperones (28.4%), signal transduction (19.8%), trafficking (18.1%) or were cytoskeletal proteins (17.2%). In KOG division 2, most proteins were involved in translation, ribosomal structure and biogenesis (47.4%), RNA processing and modification (26.6%) and transcription (9.5%) and chromatin structure (11.8%). Finally, within KOG 3 most proteins were involved in energy production

Table 28. The most abundant proteins observed in cultured HUVECs with their corresponding KOG annotation

IPI entry	Gi Entry	Protein description	Mass (Da)	KOG Division	Description Subdivision
IPI00000816	gi|5803225	14-3-3 protein epsilon	29326	CELLULAR PROCESSES AND SIGNALING	PTM, protein turnover, chaperones
IPI00000874	gi|4505591	Peroxiredoxin 1	22324	CELLULAR PROCESSES AND SIGNALING	PTM, protein turnover, chaperones
IPI00003362	gi|16507237	78 kDa glucose-regulated protein precursor	72402	CELLULAR PROCESSES AND SIGNALING	PTM, protein turnover, chaperones
IPI00003865	gi|5729877	Splice Isoform 1 Of Heat shock cognate 71 kDa protein	71082	CELLULAR PROCESSES AND SIGNALING	PTM, protein turnover, chaperones
IPI00003918	gi|16579885	60S ribosomal protein L4	51482	INFORMATION STORAGE AND PROCESSING	RNA processing and modification
IPI00005614	gi|None	Splice Isoform 1 Of Spectrin beta chain, brain 1	275259	Not annotated	Not annotated
IPI00008274	gi|5453595	adenylate cyclase-associated protein 1	52325	CELLULAR PROCESSES AND SIGNALING	Signal transduction mechanisms / Cytoskeleton
IPI00009342	gi|4506787	Ras GTPase-activating-like protein IQGAP1	189761	CELLULAR PROCESSES AND SIGNALING	Signal transduction mechanisms
IPI00009867	gi|4557890	Keratin, type II cytoskeletal 5	62637	CELLULAR PROCESSES AND SIGNALING	Cytoskeleton
IPI00009904	gi|4758304	Protein disulfide-isomerase A4 precursor	73229	CELLULAR PROCESSES AND SIGNALING	PTM, protein turnover, chaperones
IPI00010796	gi|20070125	Protein disulfide-isomerase precursor	57480	CELLULAR PROCESSES AND SIGNALING	PTM, protein turnover, chaperones
IPI00011229	gi|4503143	Cathepsin D precursor	45037	CELLULAR PROCESSES AND SIGNALING	PTM, protein turnover, chaperones
IPI00011253	gi|15718687	40S ribosomal protein S3	26842	INFORMATION STORAGE AND PROCESSING	Translation, ribosomal structure and biogenesis
IPI00011654	gi|29788785	Tubulin beta-2 chain	50095	Not annotated	Not annotated
IPI00012011	gi|5031635	Cofilin 1 (non-musCle)	18719	CELLULAR PROCESSES AND SIGNALING	Cytoskeleton
IPI00013508	gi|4501891	Alpha-actinin 1	103563	CELLULAR PROCESSES AND SIGNALING	Cytoskeleton
IPI00013808	gi|12025678	Alpha-actinin 4	105245	CELLULAR PROCESSES AND SIGNALING	Cytoskeleton
IPI00016610	gi|5453854	Poly	37987	INFORMATION STORAGE AND PROCESSING	RNA processing and modification
IPI00019502	gi|12667788	Myosin heavy chain, nonmuscle type A	227646	CELLULAR PROCESSES AND SIGNALING	Cytoskeleton
IPI00020599	gi|4757900	Calreticulin precursor	48283	CELLULAR PROCESSES AND SIGNALING	PTM, protein turnover, chaperones
IPI00020984	gi|10716563	Calnexin precursor	67982	CELLULAR PROCESSES AND SIGNALING	PTM, protein turnover, chaperones
IPI00021263	gi|4507953	Hypothetical protein	31996	CELLULAR PROCESSES AND SIGNALING	PTM, protein turnover, chaperones
IPI00021405	gi|27436946	Lamin A/C	81487	Not annotated	Not annotated
IPI00021440	gi|4501887	Actin, cytoplasmic 2	42108	CELLULAR PROCESSES AND SIGNALING	Cytoskeleton
IPI00021716	gi|4507521	Transketolase	68519	METABOLISM	Carbohydrate transport and metabolism
IPI00022434	gi|None	Serum albumin precursor	71317	Not annotated	Not annotated
IPI00022774	gi|6005942	Valosin-containing protein	89950	CELLULAR PROCESSES AND SIGNALING	PTM, protein turnover, chaperones
IPI00023014	**gi|4507907**	**Von Willebrand factor precursor**	**322435**	**CELLULAR PROCESSES AND SIGNALING**	**Defense mechanisms / Extracellular structures**
IPI00024057	gi|None	Transgelin-2	22548	Not annotated	Not annotated
IPI00024067	gi|4758012	Clathrin heavy Chain 1	193260	CELLULAR PROCESSES AND SIGNALING	Intracellular trafficking, secretion, and vesicular transport
IPI00024284	gi|62859979	Basement membrane-specific HSPG core protein precursor	479248	Not annotated	Not annotated
IPI00025054	gi|None	Splice Isoform 1 Of Heterogenous nuclear ribonucleoprotein U	91164	Not annotated	Not annotated
IPI00025091	gi|4506681	40S ribosomal protein S11	18590	INFORMATION STORAGE AND PROCESSING	Translation, ribosomal structure and biogenesis
IPI00025252	gi|21361657	Protein disulfide-isomerase A3 precursor	57146	Not annotated	Not annotated
IPI00025447	gi|4503471	Elongation factor 1-alpha 1	50451	INFORMATION STORAGE AND PROCESSING	Translation, ribosomal structure and biogenesis
IPI00025491	gi|4503529	Eukaryotic initiation factor 4A-I	46353	INFORMATION STORAGE AND PROCESSING	Translation, ribosomal structure and biogenesis
IPI00025512	gi|4504517	Heat-shock protein beta-1	22826	CELLULAR PROCESSES AND SIGNALING	PTM, protein turnover, chaperones
IPI00026119	gi|None	Ubiquitin-activating enzyme E1	122537	Not annotated	Not annotated
IPI00027230	gi|4507677	Endoplasmin precursor	97170	CELLULAR PROCESSES AND SIGNALING	PTM, protein turnover, chaperones
IPI00032140	gi|32454741	Collagen-binding protein 2 precursor	46525	Not annotated	Not annotated
IPI00141318	gi|19920317	P63 protein	66097	Not annotated	Not annotated
IPI00163187	gi|4507115	Fascin	59068	CELLULAR PROCESSES AND SIGNALING	Cell motility / Cytoskeleton
IPI00169383	gi|4505763	PhosPhoglycerate kinase 1	44985	METABOLISM	Carbohydrate transport and metabolism
IPI00171438	gi|42794771	Thioredoxin domain containing protein 5 precursor	48283	Not annotated	Not annotated
IPI00176903	gi|None	Splice Isoform 1 Of Polymerase I and transcript release factor	43450	Not annotated	Not annotated
IPI00180675	gi|17986283	Tubulin alpha-3 chain	50788	Not annotated	Not annotated
IPI00186290	gi|4503483	Eukaryotic translation Elongation factor 2	96246	INFORMATION STORAGE AND PROCESSING	Translation, ribosomal structure and biogenesis

Table 28. Continued

IPI entry	Gi Entry	Protein description	Mass (Da)	KOG Division	Description Subdivision
IPI00215719	gi|4506607	60S ribosomal protein L18	22901	INFORMATION STORAGE AND PROCESSING	Translation, ribosomal structure and biogenesis
IPI00216587	gi|4506743	40S ribosomal protein S8	24503	INFORMATION STORAGE AND PROCESSING	Translation, ribosomal structure and biogenesis
IPI00216691	gi|4826898	Profilin I	19553	CELLULAR PROCESSES AND SIGNALING	Cytoskeleton
IPI00216694	gi|7549809	Plastin 3	71279	CELLULAR PROCESSES AND SIGNALING	Cytoskeleton
IPI00216746	gi|14165437	Splice Isoform 2 Of Heterogeneous nuclear ribonucleoprotein K	51281	INFORMATION STORAGE AND PROCESSING	RNA processing and modification
IPI00217030	gi|4506725	Ribosomal pRotein S4, X-linked X isofoRm	29807	INFORMATION STORAGE AND PROCESSING	Translation, ribosomal structure and biogenesis
IPI00218319	gi|24119203	Splice Isoform 2 Of Tropomyosin alpha 3 chain	29243	Not annotated	Not annotated
IPI00218914	gi|21361176	Aldehyde dehydrogenase 1A1	56836	Not annotated	Not annotated
IPI00219018	gi|7669492	Similar to Glyceraldehyde 3-phosphate dehydrogenase, liver	44332	METABOLISM	Carbohydrate transport and metabolism
IPI00219365	gi|4505257	Moesin	67892	POORLY CHARACTERIZED	General function prediction only
IPI00220644	gi|None	Pyruvate kinase 3 isoform 2	58538	Not annotated	Not annotated
IPI00221088	gi|14141193	40S ribosomal protein S9	24242	INFORMATION STORAGE AND PROCESSING	Translation, ribosomal structure and biogenesis
IPI00221224	gi|4502095	MeMbrane alanine aMinopeptidase precursor	109842	CELLULAR PROCESSES AND SIGNALING / METABOLISM	PTM, protein turnover, chaperones / Amino acid transpo
IPI00221226	gi|71773329	Annexin VI	77986	Not annotated	Not annotated
IPI00289334	gi|None	Splice Isoform 1 Of Filamin B	280188	Not annotated	Not annotated
IPI00291175	gi|None	Vinculin isoform VCL	117220	Not annotated	Not annotated
IPI00294578	gi|39777597	Splice Isoform 1 Of Protein-glutamine gamma-glutamyltransferase	78420	Not annotated	Not annotated
IPI00295741	gi|4503139	Cathepsin B precursor	38752	CELLULAR PROCESSES AND SIGNALING	PTM, protein turnover, chaperones
IPI00296337	gi|None	Splice Isoform 1 Of DNA-dependent protein kinase catalytic subunit	473749	Not annotated	Not annotated
IPI00298994	gi|16753233	Talin 1	273479	CELLULAR PROCESSES AND SIGNALING	Cytoskeleton
IPI00303476	gi|32189394	ATP synthase beta chain, mitochondrial precursor	56525	Not annotated	Not annotated
IPI00329801	gi|4502107	Annexin 5	35971	CELLULAR PROCESSES AND SIGNALING	Intracellular trafficking, secretion, and vesicular transpo
IPI00333541	gi|4503745	Filamin A	283323	CELLULAR PROCESSES AND SIGNALING	Cytoskeleton
IPI00334775	gi|None	Heat shock protein HSP 90-beta	86248	Not annotated	Not annotated
IPI00374260	gi|41151097	Ribosomal pRotein L10	25017	Not annotated	Not annotated
IPI00383581	gi|38202257	Splice Isoform 1 Of Neutral alpha-glucosidase AB precursor	107263	Not annotated	Not annotated
IPI00384444	gi|15431310	Keratin, type I cytoskeletal 14	54092	CELLULAR PROCESSES AND SIGNALING	Cytoskeleton
IPI00387144	gi|57013276	Tubulin alpha-ubiquitous chain	50804	Not annotated	Not annotated
IPI00395646	gi|42794775	Thioredoxin domain conTaining 5 isoform 2	44440	Not annotated	Not annotated
IPI00397526	gi|41406064	Myosin heavy chain, nonmuscle type B	229824	Not annotated	Not annotated
IPI00398699	gi|None	29 kDa protein	29250	Not annotated	Not annotated
IPI00398776	gi|41322910	Plectin 7	514231	Not annotated	Not annotated
IPI00418471	gi|62414289	Vimentin	58351	Not annotated	Not annotated
IPI00419262	gi|None	Peptidyl-prolyl cis-trans isomerase	23771	Not annotated	Not annotated
IPI00419585	gi|10863927	Peptidyl-prolyl cis-trans isomerase A	19943	CELLULAR PROCESSES AND SIGNALING	PTM, protein turnover, chaperones
IPI00440493	gi|4757810	ATP synthase alpha chain, mitochondrial precursor	59828	METABOLISM	Energy production and conversion
IPI00455315	gi|4757756	Annexin A2 isoform 2	38808	CELLULAR PROCESSES AND SIGNALING	Intracellular trafficking, secretion, and vesicular transpo
IPI00456969	gi|33350932	Dynein heavy chain, cytosolic	534809	Not annotated	Not annotated
IPI00465028	gi|None	Triosephosphate isomerase	28209	Not annotated	Not annotated
IPI00465070	gi|4504281	PREDICTED: similar to CG31613-PA	15624	INFORMATION STORAGE AND PROCESSING	Chromatin structure and dynamics
IPI00465248	gi|4503571	Enolase 1	47481	METABOLISM	Carbohydrate transport and metabolism
IPI00465361	gi|15431295	60S ribosomal protein L13	24318	INFORMATION STORAGE AND PROCESSING	Translation, ribosomal structure and biogenesis
IPI00472102	gi|41399285	60 kDa heat shock protein, mitochondrial precursor	61346	Not annotated	Not annotated
IPI00477536	gi|None	Filamin B	278875	Not annotated	Not annotated
IPI00478790	gi|None	Ribosomal pRotein L3	46365	Not annotated	Not annotated
IPI00549682	gi|None	Fructose-bisphosphate aldolase A	52762	Not annotated	Not annotated
IPI00550458	gi|None	40S ribosomal protein S6	30491	Not annotated	Not annotated
IPI00551050	gi|None	Similar to H4 histone	12496	Not annotated	Not annotated

(27.1%), lipid (17.2%), carbohydrate (16.5%) or amino acid (15.9%) transport and metabolism.

Abundant proteins in HUVECs

All proteins that were identified by 30 or more peptide hits are listed in Table 28 (n=96). For these most abundant proteins, a total 13072 of peptides were identified with a mean Mascot score of 52 (SD 17). These proteins could be assigned into KOG divisions "1) Cellular processes and signaling" (35.9%), "2) Information storage and processing" (13.6%) and "3) Metabolism" (5.8%), while 41.8% was not annotated or poorly characterized (2.9%). Among the most abundant proteins were cytoskeletal proteins like α-actinin, lamin A/C, actin and myosin heavy chain. In addition, proteins involved in posttranslational modification, protein turnover and chaperones, like 14-3-3 protein, peroxiredoxin, calnexin and heat-shock protein β-1 were abundantly present. Lastly, proteins involved in translation, like 60S and 40S ribosomal proteins, elongation factor α and eukaryotic initiation factor were also identified in HUVEC extracts. See Table 28 for details on IPI/GI entry, description, mass and KOG (sub)division for these proteins.

Protein methylation sites on Von Willebrand Factor precursor protein

In order to identify protein specific methylation sites, all peptides with an ions score >20 were selected from the data set. Because the addition of a methyl group results in a mass-shift of 14.02 Da, we were able to detect methylated peptides. Figure 11 shows two MS/MS spectra from the same peptide that is either methylated or non-methylated. The differences in masses of the whole peptide and the corresponding b-ions show that this peptide contains a methylation site. We studied Von Willebrand factor precursor protein (VWFp, IPI00023014) in more detail and show the methylation sites within that protein (see Table 29 and Figure 12). The sequence coverage was 53.3% (1500 out of 2813 amino acids) for VWFp. We identified fourteen (mono) methylation sites dispersed throughout the protein: five methylgroups were located at lysine residues and nine at arginine residues. For all peptides specific to VWFp and with a presumed methylation site, the mean ions score was 45 (SD 14) and the mean e-value was 0.0616 (SD 0.1289) (Table 29), meaning that the annotation of methyl groups at these amino acid residues most likely represent true methylation sites. The mean ions score and e-value of the non-methylated peptides was 58 (SD 17.6) and 0.0093 (SD 0.0029), respectively, showing that non-modified peptides can be identified with higher confidence (Table 29). The percentage of peptides, for which both methylated and non-methylated peptides were identified, that was methylated was 37.4%. Theoretically, based on the mass shift ($[14.02]_n$) we should be able to identify dimethylation sites, but the ions scores and expectation values (<20 or >1, respectively) did not allow definite annotation of these sites.

Figure 11. MS/MS spectra derived from the same peptide (YAGSQVASTSEVLK) that is either non-methylated (A) or methylated (B). The observed masses, b- and y-ions are shown for each peptide

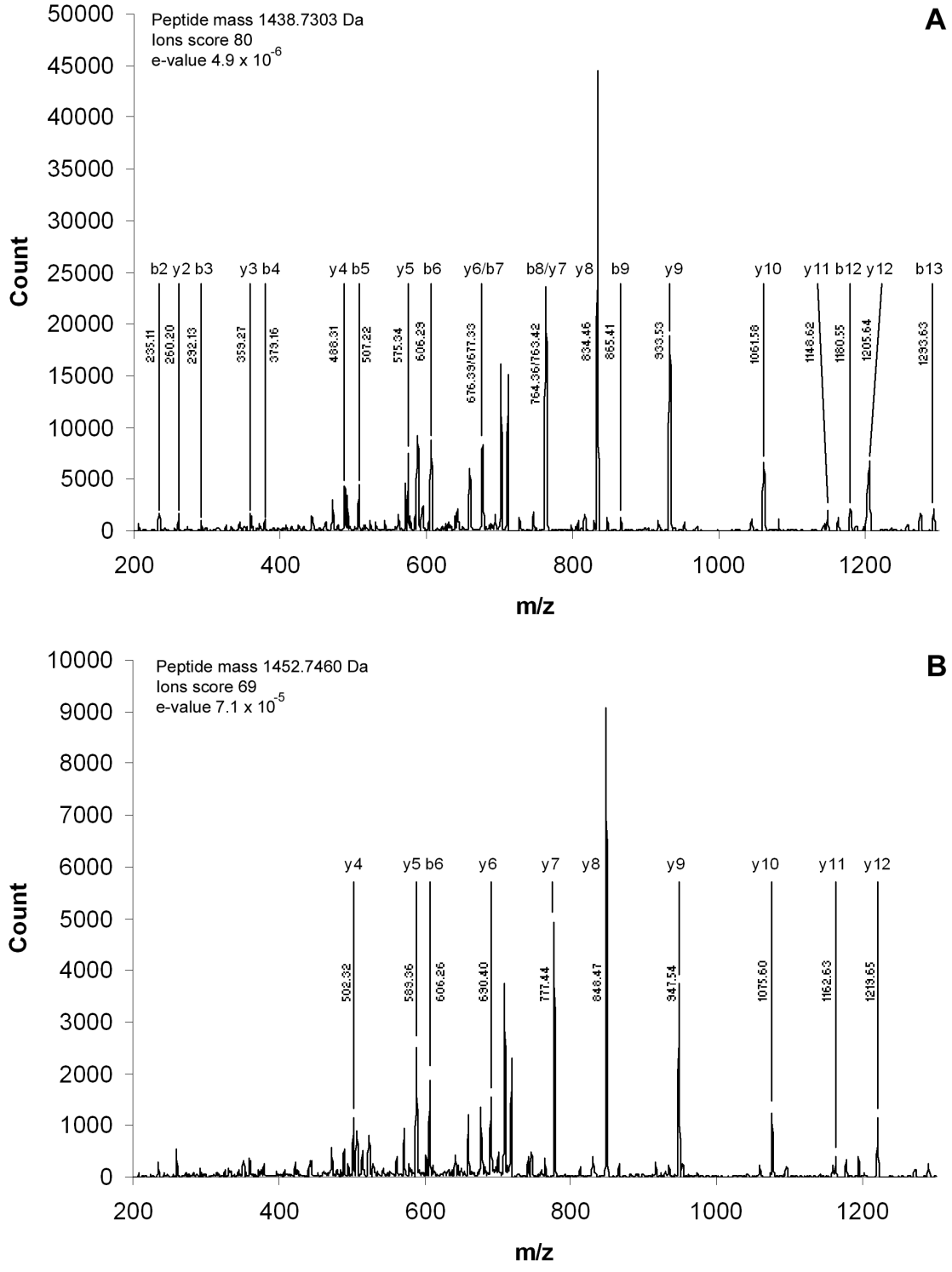

Table 29. Identified methylation sites (underlined) for Von Willebrand factor precursor protein

Peptide sequence	Position	Characteristcis of non-methylated peptides				Characteristcis of methylated peptides			
		Mass (Da)	Peptides (n)	Mean ions score (SD)	Mean e-value (SD)	Mass (Da)	Peptides (n)	Mean ions score (SD)	Mean e-value (SD)
TCGLCGNYNGNQGDDFLTPSGLAEPR	520-545	2812.2658	3	64 ± 11	$2.2x10^{-4}$ ($2.1x10^{-4}$)	2826.2337	2	40 ± 6	0.061 (0.070)
FSEEACAVLTSPTFEACHR	579-597	2210.9724	4	54 ± 6	0.0013 (0.0018)	2224.9881	2	52 ± 18	0.014 (0.019)
ILTSDVFQDCNK	1062-1073	1438.6762	15	64 ± 12	$2.5x10^{-4}$ ($5.6x10^{-4}$)	1452.6918	9	47 ± 7	0.0072 (0.011)
TATLCPQSCEER	1122-1133	1450.6180	9	47 ± 7	0.0023 (0.0039)	1464.6337	7	41 ± 12	0.025 (0.038)
LSEAEFEVLK	1288-1297	1163.6073	11	61 ± 8	0.0013 (0.002)	1177.6230	3	36 ± 1	0.16 (0.044)
YAGSQVASTSEVLK	1349-1362	1438.7303	9	89 ± 7	$1.4x10^{-6}$ ($1.6x10^{-6}$)	1452.7460	7	58 ± 12	0.0090 (0.016)
AFVLSSVDELEQQR	1437-1450	1619.8198	8	58 ± 7	0.0066 (0.014)	1633.8311	2	33 ± 1	0.6 (0.3)
EQAPNLVYMVTGNPASDEIK	1598-1618	2175.0469	2	42 ± 11	0.047 (0.030)	2189.0674	1	42	0.073
LPGDIQVVPIVGVPNANVQELER	1619-1641	2413.3110	7	71 ± 16	$8.9x10^{-4}$ (0.0014)	2427.3121	5	61 ± 14	0.023 (0.032)
ICMDEDGNEK	1878-1887	1209.4692	5	38 ± 9	0.0029 (0.0051)	1223.4798	3	30 ± 4	0.0087 (0.0059)
GLRPSCPNSQSPVK	1922-1935	1525.7671	5	43 ± 7	0.049 (0.041)	1539.7827	1	36	0.16
TPDFCAMSCPPSLVYNHCEHGCPR	1995-2219	2891.1894	1	46	$6.1x10^{-4}$	2905.1863	1	24	0.098
APTCGLCEVAR	2301-2311	1232.5641	2	51 ± 9	0.0019 (0.0023)	1246.5798	1	38	0.046
VAQCSQKPCEDSCR	2465-2478	1723.7129	8	35 ± 8	0.017 (0.026)	1737.7233	3	27 ± 2	0.061 (0.029)

Figure 12. Sequence coverage Von Willebrand factor precursor and identified methylation sites

Matched peptides are shown in **bold** (53.3%), methylation sites in **bold and underlined**

1 MIPARFAGVL LALALILPGT LCAEGTRGRS STARCSLFGS DFVNTFDGSM
101 TVTQGDQR**VS MPYASKGLYL ETEAGYYKLS GEAYGFVARI DGSGNFQVLL**
201 ERASPPSSSC NISSGEMQK**G LWEQCQLLKS TSVFARCHPL VDPEPFVALC**
301 YR**QCVSPCAR TCQSLHINEM CQER**CVDGCS CPEGQLLDEG LCVESTECPC
401 NRYFTFSGIC QYLLARDCQD HSFSIVIETV QCADDRDAVC TR**SVTVRLPG**
501 **DWDGRGR**LLV K**LSPVYAGKT CGLCGNYNGN QGDDFLTPSG LAEPRVEDFG**
601 **PLPYLR**NCR**Y DVCSCSDGRE CLCGALASYA AACAGR**GVRV AWREPGR**CEL**
701 CVPKAQCPCY YDGEIFQPED IFSDHHTMCY CEDGFMHCTM SGVPGSLLPD
801 SMGCVSGCLC PPGMVRHENR **CVALERCPCF HQGKEYAPGE TVKIGCNTCV**
901 **NPGTFRILVG NKGCSHPSVK** CKKRVTILVE GGEIELFDGE VNVK**RPMKDE**
1001 **GIQNNDLTSS NLQVEEDPVD FGNSWKVSSQ CADTRKVPLD SSPATCHNNI**
1101 CDTIAAYAHV CAQHGKVVTW R**TATLCPQSC EERNLRENGY ECEWR**YNSCA
1201 **VAGR**RFASGK KVTLNPSDPE HCQICHCDVV NLTCEACQEP GGLVVPPTDA
1301 **VDMMER**LRIS QKWVR**VAVVE YHDGSHAYIG LK**DR**KRPSEL RRIASQVKYA**
1401 **VQGLKK**K**KVI VIPVGIGPHA NLK**QIRLIEK QAPENK**AFVL SSVDELEQQR**
1501 FVLEGSDK**IG EADFNRSKEF MEEVIQR**MDV GQDSIHVTVL QYSYMVTVEY
1601 **PNLVYMVTGN PASDEIKRLP GDIQVVPIGV GPNANVQELE R**IGWPNAPIL
1701 SFPASYFDEM KSFAK**AFISK** ANIGPRLTQV SVLQYGSITT IDVPWNVVPE
1801 TDVSVDSVDA AADAARSNR**V TVFPIGIGDR YDAAQLRILA GPAGDSNVVK**
1901 TVTCQPDGQT LLKSHRVNCD R**GLRPSCPNS QSPVKVEETC GCRWTCPCVC**
2001 CMK**SIEVK**HS ALSVELHSDM EVTVNGRLVS VPYVGGNMEV NVYGAIMHEV
2101 **GTVTTDWK**TL VQEWTVQRPG QTCQPILEEQ CLVPDSSHCQ VLLLPLFAEC
2201 **MSCPPSLVYN HCEHGCPR**HC DGNVSSCGDH PSEGCFCPPD KVMLEGSCVP
2301 **APTCGLCEVA R**LR**QNADQCC PEYECVCDPV SCDLPPVPHC ERGLQPTLTN**
2401 STVSCPLGYL ASTATNDCGC TTTTCLPDKV CVHRSTIYPV GQFWEEGCDV
2501 **VVTGSPR**GDS QSS**WKSVGSQ WASPENPCLI NECVRVKEEV FIQQR**NVSCP
2601 **TTCRCMVQVG VISGFK**LECR **KTTCNPCPLG YKEENNTGEC CGRCLPTACT**
2701 **CLAEGGK**IMK **IPGTCCDTCE EPECNDITAR LQYVK**VGSCK **SEVEVDIHYC**
2801 AMECKCSPRK CSK

51 YSFAGYCSYL LAGGCQKR**SF SIIGDFQNGK** RVSLSVYLGE FFDIHLFVNG
151 **SDR**YFNKTCG LCGNFNIFAE DDFMTQEGTL TSDPYDFANS WALSSGEQWC
251 **EKTLCECAGG LECACPALLE YAR**TCAQEGM VLYGWTDHSA CSPVCPAGME
351 VHSGK**RYPPG TSLSRDCNTC ICRNSQWICS NEECPGECLV TGQSHFK**SFD
451 **LHNSLVKLK**H GAGVAMDGQD IQLPLLKGDL R**IQHTVTASV RLSYGEDLQM**
551 **NAWKLHGDCQ DLQKQHSDPC ALNPR**MTR**FS EEACAVLTSP TFEACHRAVS**
651 **NCPKGQVYLQ CGTPCNLTCR** SLSYPDEECN EACLEGCFCP PGLYMDERGD
751 AVLSSPLSHR SKR**SLSCRPP MVKLVCPADN LRAEGLECTK** TCQNYDLECM
851 **CR**DRKWNCTD HVCDATCSTI GMAHYLTFDG LK**YLFPGECQ YVLVQDYCGS**
951 **THFEVVESGR YIILLLGKAL SVVWDR**HLSI SVVLKQTYQE K**VCGLCGNFD**
1051 **MKQTMVDSSC RILTSDVFQD CNK**LVDPEPY LDVCIYDTCS CESIGDCACF
1151 PACQVTCQHP EPLACPVQCV EGCHAHCPPG K**ILDELLQTC VDPEDCPVCE**
1251 PVSPTTLYVE DISEPPLHDF YCSRLLDLVF LLDGSSR**LSE AEFEVLKAFV**
1351 **GSQVASTSEV LKYTLFQIFS KIDRPEASRI ALLLMASQEP QR**MSR**NFVRY**
1451 DEIVSYLCDL APEAPPPTLP PHMAQVTVGP GLLGVSTLGP KRNSMVLDVA
1551 PFSEAQSK**GD ILQR**VREIRY QGGNR**TNTGL ALRYLSDHSF LVSQGDREQA**
1651 IQDFETLPR**E APDLVLQR**CC SGEGLQIPTL SPAPDCSQPL DVILLLDGSS
1751 K**AHLLSLVDV MQREGGPSQI GDALGFAVRY LTSEMHGARP GASK**AVVILV
1851 LQR**IEDLPTM VTLGNSFLHK LCSGFVRICM DEDGNEK**RPG DVWTLPDQCH
1951 **TGSSTRHIVT FDGQNFKLTG SCSYVLFQNK EQDLEVILHN GACSPGAR**QG
2051 RFNHLGHIFT FTPQNNEFQL QLSPK**TFASK TYGLCGICDE NGANDFMLRD**
2151 HKVLAPATFY AICQQDSCHQ EQVCEVIASY AHLCR**TNGVC VDWRTPDFCA**
2251 EEACTQCIGE DGVQHQFLEA WVPDHQPCQI CTCLSGRK**VN CTTQPCPTAK**
2351 **PGECRPNFTC ACRKEECKRV SPPSCPPHRL PTLR**KTQCCD EYECACNCVN
2451 CTCTDMEDAV MGLR**VAQCSQ KPCEDSCRSG FTYVLHEGEC CGRCLPSACE**
2551 QLEVPVCPSG FQLSCK**TSAC CPSCRCERME ACMLNGTVIG PGKTVMIDVC**
2651 **IQLRGGQIMT LKRDETLQDG CDTHFCKVNE RGEYFWEKRV TGCPPFDEHK**
2751 **QGK**CASK**AMY SIDINDVQDQ CSCCSPTR**TE PMQVALHCTN GSVVYHEVLN

Discussion

We aimed to study the proteome and protein methylation in HUVECs by means of nano-LC FTICR MS analysis. We identified about 1500 proteins, from which the most abundant represented cytoskeletal proteins, proteins involved in posttranslational modifications, protein turnover, chaperones and translation. Recently, Bruneel and co-workers studied the proteome of HUVECs by means of 2D gel electrophoresis and matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) analysis and were able to identify 160 proteins (http://www.huvec.com) [261,262]. We were able to identify a nearly ten-fold higher number of proteins, illustrating the power of our FTICR-MS method. Because the databases used for orthologous clustering include only few eukaryotic genomes, the annotation within the clustering was rather low (50%).

We aimed to identify methyl groups, which correspond to a mass shift of 14.02 Da relative to the non-methylated peptide, and their position within the protein. We used Von Willebrand factor precursor protein (VWFp) as an example, because of its size, abundancy and well-known role as a platelet-vessel wall mediator in the blood coagulation system. VWF is synthesized as an inactive precursor protein in endothelial cells and becomes activated, for example, upon damage of the vascular endothelial cells. Indeed, we identified the inactive form of VWF in our extracts, for which a sequence coverage of 53.3% was obtained and fourteen methylation sites were identified on either an arginine or lysine residue with high confidence. The mean ions score and expectation values indicate that these residues most likely represent methylation sites. Several domains within VWFp have been identified, such as the D'/D3 domain (residues 1-272) for factor VIII binding and C1 domain (residues 1744-1746) for integrin binding on platelets [263]. We did not observe methylation sites within these domains, however.

There are a few limitations of our study to consider. The sequence coverage is not 100%, which is necessary to identify all methylation sites. This could be accomplished by further fractionation of the protein mixture or enrichment of specific proteins, i.e. by immunoprecipitation with antibodies directed against specific proteins or methylated arginine residues. Furthermore, longer recording time of peptide spectra will increase protein coverage. Another point of consideration is the potential effect of protein methylation on trypsin cleavage. Trypsin is known to cut at the same (arginine and lysine) residues, which are potentially methylated. It has been suggested that trypsin may not cleave methylated sites as efficiently as non-methylated sites [264]. An alternative would be to use chymotrypsin (which cleaves at Trp, Phe and Tyr residues) or GluC (which cleaves primarily at Gln residues) and, hence, its activity is unlikely to be affected by methylation.

The identification of protein methylation sites, and the quantitation of this modification, has gained increasing interest because of its potential role in cell function. Recently, Bulau and co-workers described a method to study global arginine methylation by means of hydrolysis of enriched protein samples followed by high-performance liquid chromatography analysis [265]. This may give an indication of overall (arginine) methylation between two (experimental) states, but specific proteins or methylation sites will not be identified this way. Ong and colleagues [264] described a method, called "stable isotope labelling of amino acids in cell culture" (SILAC), for the identification of methylation sites and their relative or (when using

internal standards) absolute quantification. In this technique, cells are cultured in the presence of $^{13}CD_3$-methionine ("heavy" methionine), which is then metabolically converted to $^{13}CD_3$-AdoMet. These "heavy" methylgroups are directly incorporated into *in vivo* methylation sites and cause a mass shift relative to the non-methylated peptide. The lysates form "heavy" and "light" labelled cells are mixed in known ratios. Quantification is based on individual peptides, allowing site-specific quantitation of protein methylation by determining intensity ratios of methylated/unmethylated peptide pairs [264].

A disturbed homocysteine metabolism is known to affect methylation status *in vivo* [55,57,60]. An increase in AdoHcy, a strong inhibitor of AdoMet-dependent transmethylation, is postulated to be the underlying cause. These conditions can be imitated *in vitro* by manipulating homocysteine metabolism. For example, the use of periodate oxidized adenosine (Adox), a well-known inhibitor of S-adenosylhomocysteine hydrolase, increases intracellular AdoHcy and decreases global DNA methylation in HUVECs [266]. In a pilot experiment using Adox-treated cultured HUVECs, we were able to establish an accumulation of intracellular AdoHcy, resulting in a strong decrease in AdoMet/AdoHcy ratio (an indicator of cellular methylation capacity). At a concentration of 50 μmol/L Adox (Sigma-Aldrich), the AdoMet/AdoHcy ratio decreased from 5.63 (standard deviation=0.73) in control cells (0 μmol/L Adox), to 0.82 (standard deviation=0.08) in treated cells (unpublished results). This will allow us to study protein methylation under "hypomethylating" conditions. In addition, the investigation of protein methylation in experimental models [267] or patients assumed to have a reduced methylation capacity [268] may confirm that this modification also occurs *in vivo.* However, the quantification of protein methylation in primary tissues is not compatible with the SILAC method, and would require chemical labeling of proteins, like the isotope-coded affinity tag (ICAT) method [269]. Ultimately, protein function needs to be studied in order to reveal the consequences of differential methylation of specific proteins and potential contribution to disease initiation or progression.

Our study shows that we are able to identify proteins in complex samples and methyl groups on peptides derived from specific proteins, as described for Von Willebrand factor precursor protein. Future studies should focus on nano-LC FTICR MS in combination with stable-isotope labeling, allowing the quantification of global and specific protein methylation for studying differential protein methylation *in vitro*. This may contribute to our understanding of how a disturbed one-carbon metabolism may contribute to disease development.

Acknowledgments

This study was supported by grant 2002B68 from the Netherlands Heart Foundation. The authors greatfully acknowledge Edwin Lasonder for technical assistance in FTICR MS and data analysis, and Ralf Weber for developing the Clue-p script for orthologous cluster analysis.

Chapter 9

Summary,

General discussion

and

Future perspectives

Part of the General discussion is published in modified form as
Genetic Determinants of Plasma Total Homocysteine
Henkjan Gellekink, Martin den Heijer, Sandra G. Heil, Henk J. Blom
Seminars in Vascular Medicine 2005; 5: 98-109

Summary

A high plasma total homocysteine (tHcy) is a risk factor for cardiovascular diseases, congenital malformations and neurological disorders. Besides environmental factors and lifestyle, also genetic factors contribute to the variation in tHcy concentrations. The genetic contribution to the variation in tHcy, however, is only partly explained by known genetic factors. The aim of the studies described in this thesis was to identify novel genetic determinants of tHcy, especially those that are also associated with disease risk. In this thesis we focused on recurrent venous thrombosis. A second goal was to develop methods to study the role of disturbed transmethylation as a mechanism for homocysteine-related pathology.

In Chapter 1, a general introduction on homocysteine metabolism, a description of homocysteine-related diseases, and an overview of the genetic variants that have been assessed for their effect on tHcy is given.

The studies presented in this thesis are divided into two parts: **Part I** describes the search for novel determinants of tHcy, focusing on homocysteine remethylation *(Chapters 2-4)* and on genes that are involved in transmethylation *(Chapters 5 and 6)*. **Part II** of this thesis describes the studies that were performed in order to facilitate the study of transmethylation as a mechanism for homocysteine-related pathology *(Chapter 7 and 8).*

In *Chapter 2*, we studied common variants in genes encoding homocysteine remethylation enzymes (MTR 2756A>G, MTRR 66A>G) and their associations with tHcy, folate and venous thrombosis risk. Given the importance of vitamin B12 in homocysteine remethylation we also assessed vitamin B12 status in relation to venous thrombosis, by assessing plasma vitamin B12 and methylmalonic acid (MMA). Interestingly, we found that high MMA, indicative of low intracellular vitamin B12 status, increased the risk of venous thrombosis. The fact that low plasma vitamin B12 levels did not confer a risk for venous thrombosis, suggests that plasma vitamin B12 is not a sensitive marker for vitamin B12 status. Both genetic variants had no effect on tHcy, but we observed an interaction between the MTRR 66GG genotype and high MMA, increasing venous thrombosis risk more than 5-fold.

In *Chapter 3*, the molecular genetic analysis of the human DHFR gene is described. This gene is essential for the continuous reduction of dihydrofolate to tetrahydrofolate required for homocysteine remethylation. We identified a 9-bp repeat in the 5' upstream region of DHFR, but observed no effect on tHcy in the general population. The recently identified 19-bp deletion in intron 1 was also assessed for its effect on tHcy. Subjects with the 19-bp del/del genotype had lower tHcy levels compared to those with the wild type genotype, suggesting that DHFR expression may be increased. No effect on serum and RBC folate levels was observed. Since this was the first study to explore the effects of this variant at the metabolic level, future studies are awaited to verify our findings on tHcy, as well as gene expression.

In *Chapter 4*, we focused on common variants in folate metabolizing enzymes (TYMS 5' UTR 28-bp repeat and 3' UTR 6-bp deletion, RFC1 80G>A and ATIC 346G>C) for their effect on tHcy, folate and venous thrombosis risk. In the crude analysis, the RFC1 80AA genotype was associated with a lower tHcy, while a higher number of TYMS 28-bp repeats decreased

serum and RBC folate levels in the general population. These effects disappeared, however, when age, sex and serum creatinin were taken into account. Some of the genotype combinations, like TYMS 28 bp 3/3 repeat and RFC1 66AA or TYMS 6 bp no del/del and ATIC 346 GG, were associated with lower tHcy and serum folate, respectively, which may point to gene-gene interaction. However, the size of our study population does not allow definite conclusions to be drawn. No effect for any of the genetic variants on venous thrombosis risk, alone or in combination, was observed.

Because of the possible role for high S-adenosylhomocysteine (AdoHcy), secondary to high tHcy, in cardiovascular disease, we sequenced the human AHCY gene *(Chapter 5)*. We identified several variants and screened two of them (-34C>T and 112C>T) for their effect on tHcy levels and venous thrombosis risk. We did not find an evident effect on tHcy levels or venous thrombosis risk but the low frequency of these variants requires larger populations to draw firm conclusions.

Because of its role in transmethylation and homocysteine synthesis, variants within the COMT gene may modulate homocysteine levels and methylation of catecholamines. We performed haplotype analysis to assess the role of COMT variants as determinants of homocysteine *(Chapter 6)*. We show that the functional 324G>A (Val108/158Met) variant was associated with tHcy, which explained the observed haplotype effect. COMT 324AA individuals had a 10.4% higher tHcy (p=0.036) when compared with those carrying the 324GG genotype. Interestingly, the odds ratio for subjects with the 324AA genotype for recurrent venous thrombosis was 1.61 (95% CI 0.97 to 2.65, p=0.06). This may point to a role for COMT as a determinant of tHcy and a possible modulator of recurrent venous thrombosis risk.

In *Chapter 7*, we describe a method for the measurement of AdoMet and AdoHcy in body fluids, the ratio of which is an important indicator of methylation capacity. Our method comprises sample concentration and clean up using solid-phase extraction columns, HPLC separation and detection by tandem mass spectrometry. The interassay CVs for AdoMet and AdoHcy were 3.9% and 8.3%, while the intraassay CV were 4.2% and 6.7%, respectively. Mean recovery for AdoMet was 94.5% and 96.8% for AdoHcy. The quantification limits were 2.0 and 1.0 nmol/L for AdoMet and AdoHcy, respectively. In a group of controls (mean tHcy 11.2 μmol/L), mean plasma AdoMet and AdoHcy were 94.5 nmol/L and 12.3 nmol/L, respectively. We observed that AdoMet degraded at neutral pH, which could be easily prevented by addition of acetic acid.

Hyperhomocysteinemia is associated with DNA hypomethylation, which is likely due to an increase in AdoHcy, a strong inhibitor of AdoMet-dependent transmethylation. Besides DNA methyltransferases, AdoHcy may also inhibit protein methyltransferases. The study of large-scale protein methylation is in its infancy. We performed nano-LC Fourier transform ion cyclotron resonance mass spectrometry (nano-LC FTICR MS) in order to study the proteome of HUVECs and protein specific methylation *(Chapter 8)*. We identified about 1500 proteins that, by means of orthologous clustering, could be assigned to the following categories: cellular processes and signalling (22.0%), information storage and processing (10.3%), and metabolism (9.2%). Because of the high mass accuracy of our FTICR-MS method, we were

able to identify protein methylation sites and used Von Willebrand factor precursor (VWFp) protein as an example. Although only 53% of the sequence of VWFp was covered, we were able to identify 14 methylation sites at either arginine or lysine residues. This study is a first attempt to assess protein methylation in crude extracts. Future studies should focus on nano-LC FTICR MS in combination with stable-isotope labeling, allowing the quantification of global and specific protein methylation for studying differential protein methylation *in vitro*.

In conclusion, our results show that low intracellular vitamin B12 status (high MMA) is (partly) responsible for the high homocysteine levels in our population, which is in agreement with the proven role of remethylation in the regulation of tHcy. The effect of the COMT 324G>A polymorphism on tHcy suggests that (a disturbed) transmethylation also plays a role in this regulation process and possibly in the risk of recurrent venous thrombosis as well. The central role of these so-called methyltransferases in homocysteine synthesis makes them obvious candidate genes in the search of genetic determinants of tHcy. To study a disturbed transmethylation, as a mechanism via which homocysteine exerts its detrimental effects, and the role of genetic variants in associated genes in this process, will require methods to assess transmethylation. In this thesis we describe a method to measure AdoMet and AdoHcy, the ratio of which is called the methylation index, in biological samples. In addition, we performed a pilot experiment with the ultimate goal to assess the effect of a disturbed homocysteine metabolism on protein methylation. Future genetic and *in vitro* studies will provide evidence to what extent a disturbed transmethylation, as one of the possible mechanisms, explains how homocysteine exerts its negative effect and results in diseases like cardiovascular diseases, congenital malformations and neurological disorders.

General discussion

After the identification of MTHFR 677C>T variant as an important determinant of tHcy, many groups have searched for other polymorphisms that affect tHcy and its related diseases. Although a large number of polymorphisms in genes involved in homocysteine and folate/cobalamin metabolism have been identified, the MTHFR 677C>T polymorphism is by far the strongest genetic determinant of tHcy in the general population. The research described in Part I of this thesis aimed to identify novel genetic determinants of tHcy and to investigate their potential role in homocysteine-related disease. In the past decade, our group studied the effect of about 37 polymorphisms in genes involved in homocysteine metabolism for their effect on tHcy in population-based controls. Figure 13 shows the obtained results for those genetic variants with the strongest effect on fasting tHcy in the general Dutch population identified so far. Some polymorphisms may be regarded as genetic determinants of tHcy, although to a lesser extent when compared with the MTHFR 677C>T variant. These include the CBS 31bp VNTR (especially after a methionine-load) and the polymorphisms in COMT (324G>A) and DHFR (19-bp deletion), the latter two being described in this thesis.

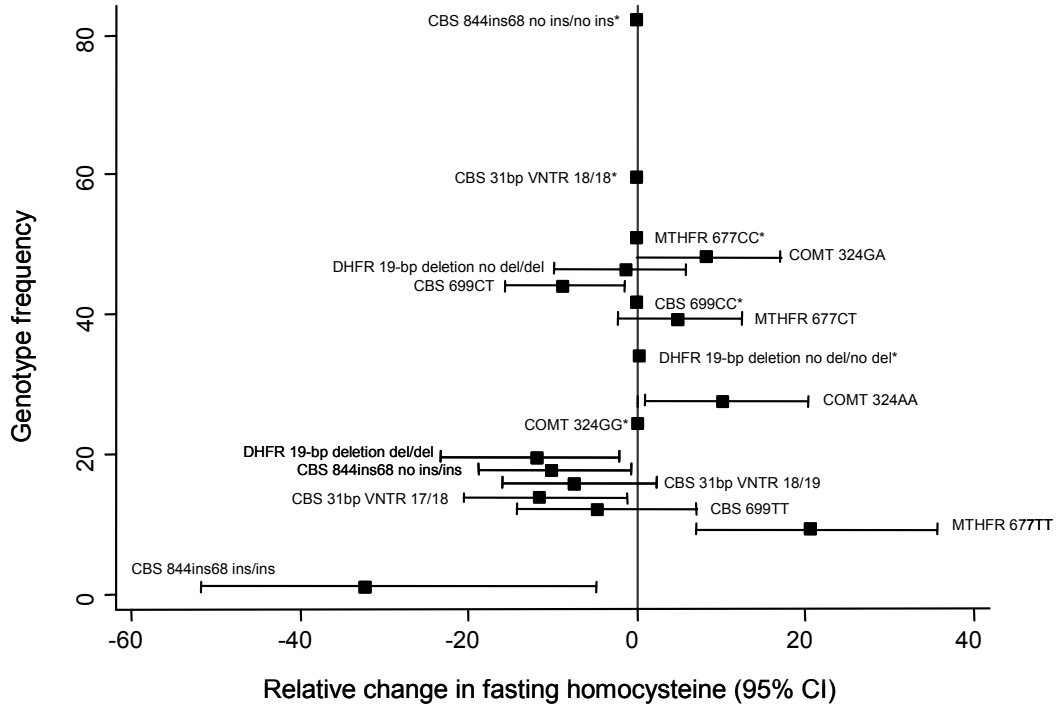

Figure 13. Overview of genetic variants that are associated with altered tHcy in the general Dutch population.
The frequency of the corresponding genotype is plotted (Y-axis) against the relative change in tHcy (X-axis) compared to the most frequent homozygous genotype (indicated with an asterisk).

Folate cycle and homocysteine remethylation

The impact of MTHFR enzyme dysfunction on folate and homocysteine in health and disease has been confirmed in many studies. Because these data point to a remethylation defect in hyperhomocysteinemia, much research has focused on variants in folate-metabolizing genes other than MTHFR. We investigated the effect of variants in genes that are related to folate metabolism and homocysteine remethylation for their effect on tHcy, folate levels and venous thrombosis risk, including DHFR, RFC1, TYMS, ATIC, MTR and MTRR.

The DHFR gene plays an essential role in folate metabolism and, hence, in homocysteine remethylation and DNA synthesis. We were the first to study the effect of the DHFR 19-bp deletion on tHcy, and observed a decrease in tHcy for 19-bp del/del subjects. Johnson and co-workers identified this del/del genotype as a risk factor for neural tube defects [137], and suggested that the deletion removed a potential (Sp1) transcription factor binding site, hence leading to a decreased DHFR expression. However, this family of transcription factors (Sp1–8) comprises both repressive and activating members [270], so the removal of this Sp1 site may also lead to an increased DHFR expression thereby facilitating homocysteine remethylation.

Our observation of a lower tHcy in 19-bp del/del subjects, suggests that DHFR expression is increased, and facilitates homocysteine remethylation, with possible repercussions on DNA synthesis and, hence, cell proliferation. This presumed effect should be confirmed in expression studies.

In another study, we observed small effects of genotype combinations at the metabolite level tHcy, suggesting gene-gene interaction between these specific genetic variants (Chapter 4). For example, in individuals with the TYMS 6 bp no del/del genotype, the ATIC 346 C>G variant was associated with serum, but not RBC, folate levels (-23% for 346GG relative to 346AA individuals, p=0.02). Of note, serum folate reflects daily intake (short-term), while RBC folate is thought to reflect long-term folate intake. However, this effect was not paralleled by an increase in tHcy levels. One possible explanation might be that homocysteine remethylation via BHMT in the liver or kidney compensates for the "lack" or redistribution of folate in order to maintain tHcy at a constant level. It is questionable what the clinical relevance of our observation is, also considering the idea that especially chronically low folate levels will lead to disease.

Also B-vitamins are essential in homocysteine remethylation and, in subjects with normal folate status, vitamin B12 deficiency may be an important cause of hyperhomocysteinemia in the general population [271]. An interesting finding in the study described in Chapter 2 is that high plasma MMA, but not low plasma vitamin B12, increases the risk of venous thrombosis. This shows that the higher tHcy in our venous thrombosis cases is, at least partly, explained by lower intracellular vitamin B12 availability (i.e. high MMA). Some studies suggest that low levels of B-vitamins *per se* increase thrombosis risk [161,163,165-167], in addition to the effects of high homocysteine [272,273]. However, it is difficult to disentangle the effects of two components that are part of the same pathway. We also confirmed that plasma vitamin B12 is not the best functional marker of vitamin B12 status [169,272]. The use of plasma vitamin B12 for assessing vitamin B12 status is questionable, considering the fact that up to 50% of subjects with low intracellular vitamin B12 status have normal plasma vitamin B12 levels [274]. Given the relation between suboptimal vitamin B12 status and cognitive function [183], and its presumed effect on transmethylation [186,275], this observation may be of interest for assessing vitamin B12 status in the elderly as well.

The effect of a single polymorphism on metabolites or disease risk is often small and difficult to reveal. There is increasing evidence that gene-gene or gene environment interactions may modulate tHcy or disease risk [47,184,210,276]. Our results suggest that genotype combinations of folate-metabolizing enzymes may lower folate or tHcy levels in the general population (Chapter 4). In addition, we show that high MMA and the MTRR 66GG genotype may increase venous thrombosis risk (Chapter 2). These results should be replicated in large, well-documented, populations in order to confirm the importance of interactions as contributors to a disturbed folate and homocysteine metabolism or disease.

Homocysteine and transmethylation

Several studies have shown that high homocysteine is related to disturbed transmethylation. Irrespective of the underlying cause, hyperhomocysteinemia leads to high AdoHcy levels and, as a consequence, to a lower AdoMet/AdoHcy ratio and global DNA hypomethylation [55-57,60,268]. Transmethylation is important in many aspects of cell function, like genomic imprinting, RNA stability, protein function and degradation. Therefore, genetic variation within methyltransferases that catalyze these reactions have become of great interest, especially since these transmethylation reactions contribute to homocysteine synthesis. Up till now,

most genetic variants studied were located in genes involved in homocysteine transsulfuration or remethylation. Because of their direct involvement in homocysteine synthesis, functional variants in methyltransferases may have effects on tHcy levels, in addition to the intrinsic effect on the reaction they catalyze. Interestingly, we showed that a common haplotype (with a prevalence of about 12% in the general population) in the COMT gene was associated with tHcy levels (Chapter 6). This was mainly due to the functional 324G>A polymorphism, and a 10.4% increase in tHcy for 324AA compared to 324GG individuals was observed. We also showed that the COMT 324AA genotype was more common in venous thrombosis patients (Chapter 6). One may hypothesize that COMT dysfunction itself, i.e. disturbed catecholamine or catecholestrogen metabolism [277], and the concomitant effect on tHcy [59], both modulate disease susceptibility. Assessing the effect of COMT genotypes on AdoMet and AdoHcy would be a first step to confirm that the transmethylation pathway may be affected.

Pathophysiology – homocysteine and disturbed transmethylation

Plasma AdoHcy represents a sensitive indicator of cardiovascular disease [55-57,223,224] and potentially also for the increased risk of cardiovascular disease secondary to end-stage renal disease [278,279]. Several studies indicate that high AdoHcy leads to DNA hypomethylation [55] in cardiovascular disease [57] and renal insufficiency [280]. DNA methylation contributes to the epigenetic regulation of gene expression, and constitutes an important mechanism for transcriptional repression or activation [281]. In order to investigate the association between disturbed homocysteine metabolism and a disturbed methylation capacity, represented by the AdoMet/AdoHcy ratio (also called the methylation index), we developed an assay to measure these two metabolites in body fluids (Chapter 7) [225]. This mass spectrometry-based approach together with the use of stable isotopes enables us to selectively measure AdoMet and AdoHcy in a reproducible and sensitive way. An important finding was that AdoMet is not stable at pH >5, and partially degrades into AdoHcy. Hence, studies in which nonacidified samples were used are likely to be confounded and should be interpreted with caution. We found that acidification of plasma with acetic acid could prevent this degradation process. The recruitment of new study populations will enable us to study the associations between high homocysteine, AdoMet and AdoHcy in the future.

The inhibition by AdoHcy is not restricted to DNA methyltransferases and also other methyltransferases, such as protein methyltransferases, are likely to be inhibited [228]. Protein methylation is thought to be involved in RNA processing, transcriptional regulation, DNA repair and signal transduction [282]. It has been postulated that a disturbed protein methylation is involved in the pathogenesis of neural tube defects [227]. Given the importance of protein methylation for proper cell functioning we studied the proteome of human umbilical vein endothelial cells (HUVEC) and methylation status of specific proteins. We show in Chapter 8 that we are able to identify proteins in complex samples and to detect methyl groups on specific proteins. The use of stable-isotope labeling of proteins in combination with immunoprecipitation should allow the quantification of protein-specific methylation [264]. By quantitating of methyl groups in complex protein samples, the effect of high AdoHcy on

protein-specific or global protein methylation can be studied, which may contribute to our understanding of the mechanisms involved in homocysteine-related diseases.

Homocysteine-lowering intervention trials

Meta-analyses, including both retrospective and prospective studies, have shown that high homocysteine is associated with an increased risk of cardiovascular disease [17-19]. In addition, the genetic association studies investigating the MTHFR 677C>T variant as a risk factor for arterial vascular disease and thrombosis further support the finding that a disturbed homocysteine metabolism causes cardiovascular disease [19,283]. The main question to be answered is whether homocysteine lowering prevents disease [284]. Several intervention studies have been published but they did not show a beneficial effect of B-vitamin supplementation on (recurrent) vascular events, such as recurrent venous thrombosis, stable coronary artery disease, myocardial infarction or stroke [285-287]. In two other studies, folic acid and vitamin B12 supplementation had a marginal effect on markers of atherosclerosis [288] or restenosis rate after coronary angioplasty [289]. Very recently, two large clinical trials were published, the Norwegian vitamin (NORVIT) trial and the Canadian Heart Outcome Prevention Evaluation (HOPE-2) trial. These studies were designed to study the effect of vitamin supplementation (folic acid, vitamin B12 and/or B6) on secondary prevention of acute myocardial infarction and stroke. Vitamin supplementation in the NORVIT trial decreased tHcy by 27% (3.6 μmol/L) in the treated group (n=937) but had no beneficial effect on vascular outcome [290]. In the HOPE-2 study, tHcy decreased by 2.4 μmol/L in the treated group (n=2758), but did not reduce the risk for major cardiovascular events in patients with vascular disease [291]. This may point to homocysteine as an innocent bystander, not causally linked to disease. Indeed, an increasing number of studies suggest that homocysteine is a marker and that AdoHcy or AdoMet are causally related to disease [56,244,292]. Unfortunately, these parameters have not been assessed in the large intervention trials described above, but it is possible that homocysteine-lowering is not paralleled by a normalization of the AdoMet/AdoHcy ratio [293], which is an important predictor of cellular methylation capacity. In addition, we showed that low methionine is a risk factor for venous thrombosis, suggesting that low substrate for AdoMet synthesis may affect transmethylation processes and predispose to disease [294]. However, there are some other points to consider when interpreting the recent intervention trials. First, the effect of folic acid supplementation is not completely understood and may not be limited to a decrease in homocysteine alone. Because of its role in DNA synthesis and transmethylation, it is possible that folate might accelerate several processes (such as cell proliferation in the vessel wall or increased arginine methylation), which may overrule or masque the beneficial effects of homocysteine-lowering [295]. This may be especially true for those subjects that had normal folate status at baseline. Secondly, most observational studies focused on homocysteine as a risk factor for primary events whereas most trials are designed to study recurrent events. When only the primary event is sensitive to treatment, no strong effect from vitamin intervention is to be expected. Finally, when considering intervention trials in homocystinuric patients, the treatment with B-vitamins prevented cardiovascular disease by 90% [20]. This shows that cardiovascular disease can be prevented by B-vitamin intervention. Additional intervention

studies with lower amounts of vitamin or alternative treatment regimens, such as betaine, are warranted.

Future perspectives

Searching for novel genetic determinants of tHcy

Heritability studies suggest that the variation in plasma total homocysteine levels is under genetic control. For example, Souto *et al.* reported a strong genetic correlation for homocysteine (r=0.652) in twenty-one families from the Genetic Analysis of Idiopathic Thrombophilia (GAIT) Study. From their data a heritability for homocysteine of approximately 23% can be derived [44]. Another group calculated a heritability for fasting homocysteine levels of 47% in coronary artery disease cases, and suggested the presence of a co-dominantly expressed major gene, in addition to the effects of the MTHFR 677C>T polymorphism [45]. Finally, in hyperhomocysteinemic cardiovascular disease cases included in the HOmocysteine in FAMilies (HOFAM) study we calculated a crude genetic correlation of 22% and 67% for fasting and post-load tHcy, respectively [46]. Kluijtmans *et al.* reported that five common genetic variants (MTHFR 677C>T & 1298A>C, MTR 2756A>G, MTRR 66A>G and CBS 844ins68) account for only a small fraction (9%) of the variability in fasting tHcy in young adults which was mainly explained by the MTHFR 677C>T polymorphism [47]. Although the clinical relevance of post-load hyperhomocysteinemia is not fully understood, the high heritability observed in the HOFAM study warrants further exploration of genetic determinants of post-load homocysteine levels and their potential (predictive) role in disease. Collectively, these data suggest that other genetic determinants of fasting or post-load tHcy may emerge as we continue our search.

If we consider homocysteine metabolism, not all candidate genes have been fully explored. In the past cDNA's or exons of genes selected on *a priori* assumptions were sequenced to identify variation within those regions, leaving intronic and promoter regions more or less aside of the screening process. Because these sequences may have important functions in gene expression or transcription regulation, we should also look for genetic variation within these areas and assess their effect on tHcy. In addition, functional enzyme studies may help to identify aberrant proteins as was successfully demonstrated by Kang *et al.* for thermolabile MTHFR, enabling the identification of the 677C>T polymorphism [43,296].

The individual testing of variants in candidate genes has been a great effort albeit with limited success. Small effects are often difficult to reveal because these effects may not rise above the background level. The concept of gene-gene and gene-environment interaction has gained increasing attention. It is thought that several polymorphisms may act in concert thereby increasing the risk of a certain phenotype or disease in an additive or synergistic fashion. Interactions between common CBS genotypes and MTHFR and MTR genotypes or folate polymorphisms and B-vitamin status on tHcy and disease risk have been reported [184,210,276]. Another well-known example of gene-environment interaction is illustrated by the fact that low folate status may increase tHcy by 25-100% among MTHFR 677TT individuals compared to 677CC subjects [47]. Technological improvements will facilitate the screening of

(multiple) genetic variants in larger study populations, which will be required to perform proper analyses.

What strategies are available to identify new genetic determinants of tHcy? First, haplotype analysis is used to assess the impact of several variants within one locus, so-called haplotypes, on a phenotypic trait [297,298]. This approach may provide more information about the impact of that locus, potentially harboring a causative allele, than individual polymorphisms do [153,299]. The size of the study groups needed for haplotype analysis and the time-consuming process to screen for several variants per gene may be limiting factors. The use of mass spectrometry was demonstrated to be a valuable tool to screen for twelve (and potentially more) important polymorphisms related to homocysteine metabolism in a rapid and reproducible way [300]. In addition, the increasing use of SNP arrays may overcome these problems as the arrays enable the analysis of thousands of SNP's simultaneously. This development also facilitated the introduction of another approach, i.e. genome wide association studies, in which SNPs dispersed throughout the genome can be analysed on a single array and assessed for their effect on a certain trait [301]. In addition, the data generated by the HapMap consortium [302] may also provide information as to which variants must be genotyped in order to cover all the "haplotype blocks" that exist within a certain gene. Another, albeit more time-consuming, approach to identify new determinants of tHcy is positional cloning. The first step may be a linkage analysis, which involves the identification of a correlation between the inheritance pattern of a phenotypic trait (e.g. homocysteine levels) and genetic markers. Once a chromosomal region has been identified, candidate genes within this region can be cloned and studied in more detail. Many Mendelian disease genes have been successfully identified using this technique, which is also applicable to multifactorial diseases [303]. The combination of the candidate-gene approach and positional cloning may help us to identify genes that have not been previously related to homocysteine metabolism but potentially contain variation that predisposes to Hhcy and diseases associated with disturbed homocysteine metabolism.

Candidate genes in hyperhomocysteinemia and disease

Vitamin B12 is an important cofactor in homocysteine remethylation. We have shown that high MMA, indicative of low intracellular vitamin B12 availability, may increase venous thrombosis risk. The complex uptake and transport mechanisms of vitamin B12 involve several transporter and carrier proteins. Hence, functional genetic variants in vitamin B12 transport genes like intrinsic factor, cubulin (the intrinsic factor-B12 receptor) or haptocorrin may disturb vitamin B12 metabolism and affect tHcy and MMA levels, and predispose to venous thrombosis.

The effect of common variants in the COMT gene on tHcy described in Chapter 6 shows that methyltransferases may represent an important group of candidate genes in the search for genetic determinants of tHcy, especially considering their direct involvement in homocysteine synthesis. In addition, functional variants in these genes may pose a negative effect on the reaction they catalyze, and potentially contribute to disease risk as well. Besides the COMT gene, other methyltransferases have been linked to tHcy levels, such as nicotinamide N-methyltransferase (NNMT) [241] and glycine N-methyltransferase (GNMT) [304]. Other candidates

include guanidinoacetate methyltransferase (GAMT) and phosphatidylethanolamine methyltransferase (PEMT), both being main contributors to homocysteine synthesis [242]. In order to study the importance of disturbed methylation capacity in hyperhomocysteinemia, promising genetic variants should be related not only to tHcy, but also to AdoMet and AdoHcy and ultimately to the methylation status of various substrates, including DNA and proteins. An effect of the MTHFR 677C>T polymorphism on DNA methylation has already been successfully demonstrated [305]. Another candidate is adenosine deaminase (ADA), the enzyme responsible for adenosine removal produced during AdoHcy hydrolysis. It has been hypothesized that the AdoHcy accumulation in hyperhomocysteinemic individuals reduces adenosine levels resulting in a reduction of its vasoprotective actions [222].

Many polymorphisms have been identified in genes involved in homocysteine metabolism. The majority of these genes are part of folate metabolism, and in this way involved in homocysteine remethylation. In most studies, total serum or red blood cell folate have been measured in order to assess the effect of a genetic variant on folate levels. It has been demonstrated that the MTHFR 677C>T polymorphism causes an accumulation of formylated tetrahydrofolates in erythrocytes [207,306]. One may postulate that other functional genetic variants also cause a redistribution of folate derivatives, which is not detected when measuring total folate levels. The application of methods that discriminate between different folate derivatives [307] may reveal shifts in folate distribution that contribute to the development of folate-related diseases, in the absence of altered homocysteine levels.

Pathophysiology of hyperhomocysteinemia

The pathophysiology of hyperhomocysteinemia remains to be elucidated. Our results suggest that low vitamin B12 status impairs homocysteine remethylation and in this way contributes to hyperhomocysteinemia in our group of recurrent venous thrombosis cases. It has been suggested that low vitamin B12 status may affect transmethylation and explain the symptoms in neurological disease [186,275]. An effect of high MMA on transmethylation may be postulated, in a similar way that folate status affects AdoHcy levels and DNA methylation in cardiovascular disease and cancer patients [57,305,308]. The measurement of AdoMet, AdoHcy and global and gene-specific DNA methylation may provide support for the involvement of disturbed transmethylation when vitamin B12 levels are low, in this way contributing to diseases like venous thrombosis and neurological disorders.

In addition, there is accumulating evidence that high homocysteine is associated with disturbed transmethylation [55,60]. The observation that hyperhomocysteinemia is associated with global DNA hypomethylation is not easily interpreted, however, and gene-specific methylation, and associated effects on gene-expression, would provide more information on the functional effects of unbalanced methylation. DNA methylation occurs at cytosine nucleotides of repetitive (viral) elements, CpG dinucleotides throughout the genes/genome or CpG islands present in promoter regions. Methylation of promoter regions is associated with transcriptional repression, hence affecting the activity of the corresponding gene. For example, the COMT gene is known to have CpG islands in its promoter region, the methylation of which is said to affect its expression [309]. Studies of COMT promoter methylation in subjects/animal models with high tHcy or AdoHcy and the possible effects on

gene expression are warranted. Methods for studying gene-specific DNA methylation, like array-based bisulfite sequencing, will be of great help in identifying other genes that are susceptible to differential methylation in different experimental conditions [310,311]. This way, the effects of hyperhomocysteinemia on gene-specific methylation and gene expression can be studied, that may potentially contribute to the development of disease.

If a disturbed methylation in hyperhomocysteinemia is a universal process, than it is likely that other molecules such as proteins, lipids and neurotransmitters are also affected. Bulau *et al.* described a method to quantify arginine methylation by high-performance liquid chromatography, which allows the assessment of global arginine methylation between two samples [265]. This approach may be used a prognostic tool, although protein-specific data is required to assess which proteins are affected. In order to obtain protein specific data, mass spectrometric techniques are of great value, as has been shown by several groups [260,312]. For example, Ong *et al.* used stable-isotope labeling of amino acids in cultured cells (SILAC) to study protein methylation in a quantitative fashion [264]. Once specific proteins have been identified that are differentially methylated, the proteome from subjects in which a disturbed transmethylation is thought to be involved, or the proteome from animal models, may be analysed in order to investigate whether these proteins are altered *in vivo* too [269]. The investigation of protein methylation status in health and disease may provide more insight into the pathological mechanisms in hyperhomocysteinemia.

In addition to unbalanced transmethylation, high homocysteine may also reflect a disturbed folate metabolism. Because of the importance of folates in purine and thymidylate synthesis, high homocysteine may mirror a decreased capacity of DNA synthesis and, hence, cell proliferation. This may be of critical importance during “normal” cell turnover or vessel repair in response to vascular damage and thus contribute to cardiovascular disease. In addition, low folate is associated with uracil misincorporation and chromosomal breakage, which is an important feature of cancer development [29]. These aspects should be taken into account too, when studying the complex mechanisms of homocysteine-related diseases.

Concluding remarks

Although the first studies indicated the involvement of a disturbed *transsulfuration* in hyperhomocysteinemia [11,40], the identification of the MTHFR 677C>T polymorphism as a determinant of tHcy in the general population, pointed to the involvement of a disturbed *remethylation* instead [43,296,313]. Many genes involved in folate and vitamin B12 metabolism have been assessed since then, but the effects observed in single populations appear difficult to replicate in other populations. The evidence that in most cases multiple genetic variants (i.e. genetic background), in combination with lifestyle factors, contribute to phenotypic changes, may explain these discrepancies. It will require large well-documented populations to reveal small changes in phenotype that may contribute to disease, in addition to high-throughput SNP genotyping technology and proper statistical data analysis and interpretation. The involvement of a disturbed *transmethylation* in hyperhomocysteinemic subjects is now subject of interest. The observations that an increased flux through the transmethylation pathway increases tHcy levels and that hyperhomocysteinemia may cause

an inhibition of these processes, warrants the study of methylation parameters, methyltransferases and methylation status of their corresponding substrates (such as nucleic acids and proteins). This will provide evidence whether unbalanced methylation occurs in diseases associated with a disturbed homocysteine metabolism.

Chapter *10*

Samenvatting

Een hoog plasma totaal homocysteine (tHcy) is een risicofactor voor onder andere hart- en vaatziekten, congenitale afwijkingen en neurologische aandoeningen. Naast het effect van omgevingsfactoren en leefstijl is de variatie in tHcy erfelijk bepaald. De genetische bijdrage aan de variatie van tHcy kan slechts gedeeltelijk verklaard worden door bekende genetische varianten. Het doel van de in dit proefschrift beschreven studies was om onbekende genetische determinanten van tHcy te identificeren, en met name die varianten die ook geassocieerd zijn met een hoger ziekterisico, waarbij in dit proefschrift recidief veneuze trombose centraal staat. Een tweede doel was om methodes te ontwikkelen waarmee de rol van een verstoorde transmethylering, als mechanisme voor homocysteine-gerelateerde pathologie, bestudeerd kan worden.

Hoofdstuk 1 van dit proefschrift bestaat uit een algemene inleiding over het homocysteine metabolisme, een korte beschrijving van ziekten die geassocieerd zijn met een verstoord homocysteine metabolisme en een overzicht van genetische varianten die reeds onderzocht zijn in relatie tot tHcy.
De studies die in dit proefschrift worden beschreven, zijn ondergebracht in twee delen: **Deel I** omvat de studies die tot doel hadden om nieuwe determinanten van tHcy te identificeren, waarbij wij ons richtten op genen betrokken bij de remethylering (Hoofdstuk 2-4) en transmethylering (Hoofdstuk 5 en 6). **Deel II** van dit proefschrift beschrijft de studies die tot doel hadden het bestuderen van transmethylering, als een mechanisme voor homocysteine-gerelateerde pathologie, beter mogelijk te maken.
In *Hoofdstuk 2* hebben we varianten in genen betrokken bij homocysteine remethylering (de methionine synthase [MTR] 2756A>G en methionine synthase reductase [MTRR] 66A>G polymorfismen) bestudeerd als mogelijke determinanten van tHcy en serum folaat en als risicofactor voor recidief veneuze trombose. Gezien het belang van vitamine B12 (cobalamine) voor homocysteine remethylering, hebben we ook een lage vitamine B12 status als risicofactor bestudeerd. Daarvoor hebben we gebruik gemaakt van zowel plasma vitamine B12 als plasma methylmalonzuur (MMA), een betrouwbare maat voor de intracellulaire beschikbaarheid van vitamine B12. Uit onze studie bleek dat een hoog plasma MMA tot een hoger risico voor recidief veneuze trombose leidt. Een laag plasma vitamine B12 was géén risicofactor, wat verklaart kan worden met het gegeven dat plasma vitamine B12 geen gevoelige maat is voor de intracellulaire vitamine B12 status. De MTR 2756A>G en MTRR 66A>G varianten hadden beide géén effect op tHcy of folaat, maar de combinatie van MTRR 66GG genotype én hoog MMA (>80^{e} percentiel van de controle populatie) was geassocieerd met een 5 maal verhoogde kans op het risico op recidief trombose.
In *Hoofdstuk 3* hebben we de moleculair genetische analyse van het dihydrofolaat reductase (DHFR) gen beschreven. Dit gen is essentiëel voor de reductie van dihydrofolaat tot tetrahydrofolaat en is vereist voor homocysteine remethylering. Door sequencen van het DHFR gen hebben we een 9-bp repeat geïdentificeerd in het gebied voorafgaand aan het DHFR gen (aansluitend op het 5' untranslated region [UTR]). Echter, de repeat had géén effect op tHcy of folaat concentraties. Onlangs is een 19-bp deletie beschreven in intron 1 van dit gen die, gezien het belnag van intron 1 als transcriptiefactor binding site, een effect zou kunnen hebben op de genexpressie. Uit onze studie bleek dat mensen die homozygoot

zijn voor de deletie een lager tHcy hadden dan mensen met het wild type genotype. Dit zou kunnen betekenen dat het DHFR gen sterker tot expressie komt, waardoor homocysteine beter omgezet kan worden. Er werd geen effect van deze variant op serum en erytrocyten folaat gevonden. Dit is de eerste studie naar het effect van DHFR varianten op tHcy en folaat, en toekomstige studies zijn nodig om onze bevindingen, en de effecten op genexpressie, verder te bestuderen.

In *Hoofdstuk 4* hebben we vier varianten in genen van het folaat metabolisme (thymidylaat synthase [TYMS] 5' UTR 28-bp repeat and 3' UTR 6-bp deletie, reduced folate carrier [RFC1] 80G>A en AICAR transformylase/IMP cyclohydrolase [ATIC] 346G>C) bestudeerd als mogelijke determinanten van folaat en tHcy en als risicofactor voor recidief trombose. In de algemene populatie lijkt het RFC1 80AA genotype geassocieerd met een lager tHcy, terwijl de TYMS 28-bp repeat een lager serum en erytrocyten folaat tot gevolg had bij een groter aantal repeats. Deze effecten verdwenen na correctie voor leeftijd, geslacht en serum kreatinine. Sommige genotype combinaties, zoals de TYMS 28 bp 3/3 repeat met RFC1 66AA of TYMS 6 bp no del/del met ATIC 346 GG, leken een verlagend effect te hebben op, respectievelijk, tHcy en serum folate, wat een aanwijzing zou kunnen zijn voor gen-gen interacties. Deze observaties moeten in grotere populaties bevestigd worden. Géén van de varianten (alléén of in combinatie) had een effect op het risico op recidief trombose.

Omdat bij mensen met hyperhomocysteinemie ook een verhoogd S-adenosylhomocysteine (AdoHcy, de precursor van homocysteine) wordt gevonden, hebben wij het AHCY gen (verantwoordelijk voor de hydrolyse van AdoHcy naar homocysteine en adenosine) gesequenced (*Hoofdstuk 5*). We hebben enkele polymorfismen geïdentificeerd (waaronder -34C>T en 112C>T) en we hebben onderzocht of deze een effect hadden op tHcy of de kans op recidief trombose. We vonden geen duidelijke aanwijzingen dat deze varianten een effect hadden op tHcy of het ziekterisico, maar door de lage frequenties van deze polymorfismen kan een mogelijk effect ook niet uitgesloten worden.

Door de rol van catechol-O-methyltransferase (COMT) bij de transmethylering van catecholamines en bij homocysteine synthese, kunnen genetische varianten in dit gen een invloed hebben op tHcy en transmethylering. Om het effect van de methyltransferase COMT op tHcy en recidief trombose te bestuderen hebben we haplotype (genotype op basis van meerdere genetische varianten) analyse uitgevoerd (*Hoofdstuk 6*). We laten zien dat er een haplotype bestaat binnen het COMT gen dat geassocieerd is met tHcy; dit effect wordt bijna geheel verklaard door de functionele 324G>A variant (Val108/158Met). Mensen met het 324AA genotype hadden een 10.4% hoger tHcy dan mensen met het 324GG genotype. Bovendien kwam het 324AA genotype vaker voor bij recidief trombose patiënten (relatief risico 1.61 [95% betrouwbaarheidsinterval 0.97 tot 2.65], p=0.06). Dit wijst op COMT als determinant van tHcy en een mogelijk effect van deze variant op het recidief veneuze trombose risico.

In *Hoofdstuk 7* beschrijven we een methode voor het meten van S-adenosylmethionine (AdoMet) en S-adenosylhomocysteine (AdoHcy) in lichaamsvloeistoffen. De ratio van deze metabolieten is een belangrijke maat voor de methyleringscapaciteit. Onze methode bevat een eenvoudige verrijkings- en zuiveringsstap door het gebruik van solid-phase extraction kolommetjes, HPLC scheiding en tandem massa spectrometrie detectie. De interassay

variatie coëfficiënten (VC) voor AdoMet en AdoHcy zijn respectievelijk 3.9% en 8.3%, en de intraassay VC's zijn 4.2% en 6.7%. De gemiddelde recovery van AdoMet was 94.5% en die van AdoHcy 96.8%. De kwantificatie limiet was 2.0 en 1.0 nmol/L voor respectievelijk AdoMet en AdoHcy. In een groep controles (n=29, gemiddelde tHcy concentratie 11.2 μmol/L) was de gemiddelde AdoMet concentratie 94.5 nmol/L en de gemiddelde AdoHcy concentratie 12.3 nmol/L. De bij neutrale pH waargenomen AdoMet degradatie kan eenvoudig voorkomen worden door aanzuring van het te meten plasma monster met azijnzuur direct na bloedafname.
Hyperhomocysteinemie is geassocieerd met hypomethylering, waarschijnlijk door een stijging van de methyleringsremmer AdoHcy. Omdat niet alleen DNA methyltransferases worden geremd door AdoHcy, is het aannemelijk dat óók methyltransferases worden geremd die betrokken zijn bij de eiwitmethylering. Het op grote schaal bestuderen van eiwitmethylering staat nog in de kinderschoenen. Wij hebben van een techniek gebruik gemaakt, die nano-LC Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) wordt genoemd, om het proteoom van veneuze endotheelcellen uit humane navelstrengen (HUVECs) te bestuderen (*Hoofdstuk 8)*. We hebben ongeveer 1500 eiwitten geïdentificeerd die na orthologe clustering toegewezen konden worden aan de volgende categorieën van eiwitten: cellulaire processen en signaaltransductie (22.0%), informatie opslag en verwerking (10.3%) en metabolisme (9.2%). Door de hoge nauwkeurigheid in massabepaling, zijn we in staat om methyleringssites (+/-14.0157 Da) in eiwitten te identificeren. We hebben het Von Willebrand Factor precursor (VWFp) eiwit als voorbeeld uitgewerkt en 14 (arginine/lysine) methyleringssites kunnen identificeren. Deze studie is een eerste poging tot het bestuderen van specifieke eiwitmethylering in complexe eiwitlysaten. Het gebruik van stabiele isotopen maakt de kwantificering van differentiële eiwitmethylering in verschillende experimentele settings mogelijk.

Samenvattend blijkt uit de resultaten van dit proefschrift dat een lage intracellulaire vitamine B12 status (hoog MMA) in onze populatie (deels) verantwoordelijk is voor verhoogde tHcy concentraties, wat aansluit op de bewezen rol van remethylering bij de regulatie van tHcy. Het effect van de COMT 324G>A variant op tHcy suggereert dat (een verstoring van) de transmethylering óók een rol speelt bij de regulatie van tHcy en mogelijk ook bij recidief veneuze trombose. De centrale rol van deze zogenaamde methyltransferases in homocysteine synthese maakt hen voor de hand liggende kandidaatgenen in de zoektocht naar genetische determinanten van tHcy. Een verstoorde transmethylering, als één van de mechanismen voor homocysteine-gerelateerde pathologie, en de rol van genetische variatie in de betrokken genen, vereist methodes om die transmethylering te bestuderen. In dit proefschrift is een methode beschreven om de methyleringsparameters AdoMet en AdoHcy te meten in biologische materialen. Daarnaast is een pilot experiment gedaan met als uiteindelijke doel om het effect van een verstoord homocysteine metabolisme op eiwitmethylering te bestuderen. Toekomstige genetische en *in vitro* studies zullen moeten uitwijzen in hoeverre een verstoorde transmethylering kan verklaren hoe een verstoord homocysteine metabolisme leidt tot hart- en vaatziekten, congenitale afwijkingen en neurologische aandoeningen.

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Dankwoord

Ik heb onderzoek mogen doen in een omgeving met mensen die enthousiast, behulpzaam en geïnteresseerd zijn. Dat heeft mijn promotietijd tot een aangename tijd gemaakt en heeft zeker ook zijn weerslag gehad op het proefschrift zoals het nu verschenen is. Bij deze wil ik dan ook mijn collega's van Lab Kindergeneeskunde en Neurologie en afdeling Endocrinologie, die op welke manier dan ook betrokken zijn geweest bij mijn promotie, bedanken voor hun inzet en gezelligheid de afgelopen jaren.

Henk Blom en Martin den Heijer
Jullie gezamenlijke begeleiding heb ik als zeer postief ervaren. Door de biochemische kennis en brede wetenschappelijke interesse van Henk gecombineerd met de "artsenvisie", veelzijdigheid en statistische hoogstandjes van Martin heb ik van jullie beider kennis kunnen profiteren. Ook in het nakijken van manuscripten vulden jullie elkaar aan, waardoor ik uit het commentaar maximaal rendement kon halen. Jullie gaven me de vrijheid om na twee jaar "iets anders te doen", waardoor er niet alléén SNP-werk in mijn proefschrift staat. Daardoor heb ik mijzelf ook op andere vlakken kunnen ontwikkelen. Bedankt voor jullie inzet en, op zijn tijd, gezelligheid. Henk, succes in Amsterdam en ik hoop dat alles daar volgens plan verloopt. Martin, succes met je vele bezigheden als arts/onderzoeker.

Prof. Hermus
Als promotor hebt u het onderzoek van een afstand gevolgd. Eens in de zoveel tijd hadden we samen een overleg om de vorderingen binnen het onderzoek te bespreken. Daaruit bleek betrokkenheid en interesse, waardoor ik altijd met een goed gevoel wegging. Bedankt!

Mijn paranimfen Sandra Heil en Ivon van der Linden
Als collega junior-onderzoekers (en Sandra later als post-doc) hebben we samen een kamer (kantoortuin) gedeeld en derhalve altijd discussies kunnen voeren als we iets niet begrepen of juist wél, zowel op wetenschappelijk gebied als daarbuiten. Ik denk dat dat een essentiëel onderdeel is in iemands (wetenschappelijke) ontwikkeling. Ik heb het enorm naar mijn zin gehad en wil jullie daarvoor bedanken, evenals voor jullie inzet voor/tijdens mijn promotie. Sandra, succes met je carrière in Wageningen. Ivon, veel succes met de afronding van je promotie en verdere carrière.

Homocysteine research groep (en geadopteerde leden)
Ik wil alle collega's van de homocysteine groep (inclusief Maria, Anita en Henriëtte) bedanken voor de gezelligheid, de borrels, barbecues en congressen. Brenda, Alexandra, Suzanne en Gerry: op het onderzoekslab hing een goede sfeer en ik heb prettig met jullie samengewerkt. Altijd goedlachs en bereid om te helpen. Ik noem met name Gerry's onuitputtelijke energie om het lab toonbaar, en de stagiaires in het gareel, te houden. Sterkte! Voor allen, bedankt en succes voor de toekomst!

Dinny van Oppenraaij-Emmerzaal, Addy de Graaf-Hess en Gwendolyn Beckmann:
Dinny, bedankt voor de leuke samenwerking en je enthousiasme bij het opzetten en valideren van de AdoMet/AdoHcy meting. Hopelijk kun je de toepassing van de meting in de toekomst uitbreiden. Addy, en later ook Gwendolyn, jullie hebben me geholpen als er weer eens een tHcy of totaal eiwit gemeten moest worden, of dat nu voor het prakticum was, voor een artikel of eigen gebruik. Ik wil ook Leo Kluijtmans bedanken voor zijn bijdrage aan het onderzoek en de publicaties in mijn "beginjaren".

Collega's van LKN en Afdeling Endocrinologie
Omdat ik bij twee afdelingen een aanstelling had heb ik (af en toe) bij meerdere dagjes-uit of borrels aan kunnen schuiven. Ik heb het "aan beide kanten" altijd naar mijn zin gehad. Daarnaast zal ik de niet altijd ongevaarlijke mountainbiketochten van LKN zeker onthouden. In dat kader hebben Herman en Richard terecht een fiets-licentie voor de deelnemers in het leven geroepen.
Naast het genetische werk heb ik endotheelcellen gekweekt om vervolgens het proteoom te bestuderen. Ik wil Joyce en Thea bedanken voor de aanvoer van de navelstrengen en het aanleren hoe de endotheelcellen te isoleren en te kweken. Ik ben dank verschuldigd aan Wendy, die veel werk heeft verzet om de aangeleverde HUVEC-fracties om te toveren in interpreteerbare eiwitlijsten. Edwin en Lambert, bedankt voor jullie bijdrage bij respectievelijk de MS-data analyse en 2D-electroforese. Bert, bedankt voor je begeleiding bij dit project.
Hanneke wil ik bedanken voor de aminozuur-analyses van de HUVEC-extracten. Jammer genoeg hadden we niet meer tijd, wellicht hadden we er dan meer uit kunnen halen.

Daarnaast wil ik mijn collega's(-promovendi) bedanken en succes wensen: Udo (óók voor de last-minute figuurbewerkingen), Suzan W., Nienke, Miranda, Murtada, Rutger, Joyce, Rolf, Cindy, Saskia, Paula, Ingrid, Marije en Marloes. De EpiHomLip-club: Sita, Gerly en Jacqueline. Ook Hans en Anja wil ik bedanken voor de leuke tijd als "kantoortuincommissie".
Maarten van den Hurk, Geert Corstens en Ruud Clarijs: in het begin veel hardgelopen, nu iets minder. Misschien toch nog weer eens de 7-heuvelenloop lopen?

Studenten
Tijdens mijn promotie heb ik vier studenten mogen begeleiden, en met veel plezier. Ik wil Femke Philips, Karin von Winckelmann, Daniëlle Groen en Els(je) Cornelissen bedanken voor hun inzet. Jullie waren zeer verschillende persoonlijkheden, maar allen even enthousiast. Karin en Daniëlle, jullie hebben een vervolgstudie opgepakt waarmee ik jullie veel succes wens. Femke en Els, ik vraag me af of jullie in het onderzoek blijven, de tijd zal het uitwijzen. Succes!

Samenwerkingen
Met Jan-Willem Muntjewerff (GGz, Nijmegen) hebben we gekeken naar genetische variatie in relatie tot schizofrenie. Jan-Willem, bedankt voor de leuke samenwerking en gefeliciteerd met je proefschrift. Ook wil ik Eduard Struys (VU Medisch Centrum, Amsterdam) bedanken voor zijn bijdrage aan het artikel beschreven in hoofdstuk 7.

Prof. dr. Per Ueland, Halvard Bergesen and Tove Følid
Per, I would like to thank you for letting me visit your lab to learn the vitamin B12 and folate assays. I appreciate your presence on January 11th. Halvard, thanks for teaching me the ins and outs of the assays in a humoristic way. Tove, thanks for your help in running the many samples that I brought with me. Of course, I'd like to thank Øivind, Klaus and Amrei for taking me out to see Bergen and for inviting me to the seventies party. It was great fun!

Ouders
Ik heb een omweggetje gemaakt om te komen waar ik nu ben. Jullie hebben me altijd gesteund in mijn keuzes en vertrouwen in mij gehad. Dat had ik nodig om tot dit punt te komen. Pa, je sprak tijdens de bruiloft van Nathalie en mij de volledige titel van mijn proefschrift moeiteloos in één adem uit. Deze woorden zullen ongetwijfeld meer betekenis krijgen tijdens de verdediging. Nu op weg naar je eigen promotie!

Schoonouders
Ook jullie hebben altijd interesse getoond in het onderzoek, hoewel dat laatste betekende dat Nathalie uiteindelijk zou verhuizen naar Nijmegen. Bedankt voor jullie steun.

Nathalie
Lieve Nathalie, we kennen elkaar al een hele tijd en sinds kort zijn we een "echt paar". Je hebt me in 1998 zien vertrekken naar Wageningen en later naar Nijmegen. In 2003, je werk, familie en vrienden achterlatend, ben je me gevolgd naar Nijmegen, waar je het nu goed naar je zin hebt. Wellicht kunnen we onze toekomstplannen verwezenlijken in Nijmegen, de tijd zal het leren. Dank je voor je onvoorwaardelijke steun en vertrouwen.

Henkjan

Curriculum Vitae

Henkjan Gellekink was born on March 10, 1975 in Enschede, The Netherlands. In 1995 he passed pre-university education (VWO) at the Stedelijk Lyceum in Enschede. In 1999 he received his bachelor degree for Hoger Laboratorium Onderwijs at Hogeschool Enschede. In the same year he started a study Biology at the Radboud University Nijmegen. He performed research internships at the Dept. of Pathology and Dept. of Neurology (Prof. dr. H.P.H. Kremer), Radboud University Nijmegen Medical Centre and the Dept. of Cellular Animal Physiology (Prof. dr. E.W. Roubos), Radboud University Nijmegen. In May 2002 he received his master degree in Medical Biology. After that he started a PhD project at the Dept. of Endocrinology (Promotor Prof. dr. A.R.M.M. Hermus, Radboud University Nijmegen Medical Centre) and the Laboratory of Pediatrics and Neurology (Prof. Dr. R.A. Wevers, Radboud University Nijmegen Medical Centre) under supervision of Dr. H.J. Blom and Dr. M. den Heijer (co-promotors). During this period he focused on molecular genetics of hyperhomocysteinemia, but also explored the field of mass spectrometry in order to study the effect of a disturbed homocysteine metabolism at the metabolic and protein level. The research that was performed during this period has been described in this thesis. Henkjan is married to Nathalie Stol.

Henkjan Gellekink is geboren op 10 maart 1975 te Enschede, Nederland. In 1995 behaalde hij zijn VWO diploma aan het Stedelijk Lyceum in Enschede. Daarna volgde hij het Hoger Laboratorium Onderwijs (specialisatie biochemie) aan de Hogeschool Enschede dat in 1999 met een diploma werd afgesloten. In datzelfde jaar startte hij een studie Biologie aan de Radboud Universiteit Nijmegen. Hij liep stage bij de afdeling Pathologie / afdeling Neurologie (Prof. dr. H.P.H. Kremer), Radboud Universiteit Nijmegen Medisch Centrum en bij de afdeling Cellulaire Dierfysiologie (Prof. dr. E.W. Roubos), Radboud Universiteit Nijmegen. In mei 2002 behaalde hij zijn master titel in de Medische Biologie. Aansluitend begon hij een promotie onderzoek bij de afdeling Endocrinologie (Promotor Prof. dr. A.R.M.M. Hermus, Radboud Universiteit Nijmegen Medisch Centrum) en het Laboratorium voor Kindergeneeskunde en Neurologie (Prof. dr. R.A. Wevers, Radboud Universiteit Nijmegen Medisch Centrum) onder directe supervisie van dr. H.J. Blom en dr. M. den Heijer (beiden co-promotor). Tijdens zijn promotie deed hij onderzoek naar genetische oorzaken van hyperhomocysteinemie, maar ook verkende hij het veld van massa spectrometrie om het effect van een verstoord homocysteine metabolisme op andere metabolieten en het proteoom van endotheelcellen te bestuderen. Het onderzoek dat hij als junior onderzoeker uitvoerde is beschreven in dit proefschrift. Henkjan is getrouwd met Nathalie Stol.

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