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RESEARCH ARTICLE

Large-Scale Population-Based Metabolic Phenotyping of Thirteen Genetic Polymorphisms Related to One-Carbon Metabolism

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Several polymorphisms of genes involved in one-carbon metabolism have been identified. The reported metabolic phenotypes are often based on small studies providing inconsistent results. This large-scale study of 10,601 population-based samples was carried out to investigate the association between a panel of biochemical parameters and genetics variants related to one-carbon metabolism. Concentrations of total homocysteine (tHcy), folate, vitamin B_{12} (cobalamin), methylmalonic acid (MMA), vitamin B_2 (riboflavin), vitamin B_6 (PLP), choline, betaine, dimethylglycine (DMG), cystathionine, cysteine, methionine, and creatinine were determined in serum/plasma. All subjects were genotyped for 13 common polymorphisms: methylenetetrahydrofolate reductase (*MTHFR*) c.665C>T (known as 677C>T; p.Ala222Val) and c.1286A>C (known as 1298A>C; p.Glu429Ala); methionine synthase (*MTR*) c.2756A>G (p.Asp919Gly); methionine synthase reductase (*MTRR*) c.66A>G (p.Ile22Met); methylenetetrahydrofolate dehydrogenase (*MTHFD1*) c.1958G>A (p.Arg653Gln); betaine homocysteine methyltransferase (*BHMT*) c.716G>A (known as 742G>A; p.Arg239Gln); cystathionine β-synthase (*CBS*) c.844_845ins68 and c.699C>T (p.Tyr233Tyr); transcobalamin-II (*TCN2*) c.67A>G (p.Ile23Val) and c.776C>G (p.Pro259Arg); reduced folate carrier-1 (*SLC19A1*) c.80G>A (p.Arg27His); and paraoxonase-1 (*PON1*) c.163T>A (p.Leu55Met) and c.575A>G (p.Gln192Arg). The metabolic profile in terms of the measured vitamins and metabolites were investigated for these 13 polymorphisms. We confirmed the strong associations of *MTHFR* c.665C>T with tHcy and folate, but also observed significant ($P<0.01$) changes in metabolite concentrations according to other gene polymorphisms. These include *MTHFR* c.1286A>C (associations with tHcy, folate and betaine), *MTR* c.2756A>G (tHcy), *BHMT* c.716G>A (DMG), *CBS* c.844_845ins68 (tHcy, betaine), *CBS* c.699C>T (tHcy, betaine, cystathionine) and *TCN2* c.776C>G (MMA). No associations were observed for the other polymorphisms investigated. Hum Mutat 28(9), 856–865, 2007.

KEY WORDS: *MTHFR*; homocysteine; one-carbon metabolism; metabolic phenotyping; NORCCAP

INTRODUCTION

Homocysteine and folate are key components of one-carbon metabolism. Elevated concentrations of total homocysteine (tHcy) in plasma or inadequate folate status have been associated with many pathological states, including cardiovascular disease, cognitive dysfunction, adverse pregnancy outcomes, osteoporosis, and cancer. Plasma tHcy serves as a marker of impaired folate and cobalamin status [Fowler, 2005], but is also influenced by a variety of lifestyle and nutritional factors. The numerous disease associations have also stimulated the investigation of polymorphic variants of genes involved in one-carbon metabolism, their metabolic effects, and their relation to risk of chronic diseases [Gellekink et al., 2005; Molloy, 2004].

The flavoenzyme, methylenetetrahydrofolate reductase (*MTHFR*; MIM# 607093), has a major impact on folate distribution by reducing 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the primary methyl donor for remethylation of homocysteine to methionine. A SNP of *MTHFR*, c.665C>T (p.Ala222Val; known as 677C>T), was described by Frosst et al. [1995]. Numerous studies have since consistently demonstrated that the *MTHFR* c.665T-allele is associated with elevated tHcy under conditions of impaired folate status, and the *MTHFR* c.665C>T polymorphism has been linked to most pathologies associated with hyperhomocysteinemia [Ueland et al., 2001]. The *MTHFR* c.665C>T polymorphism is in linkage disequilibrium with a second nearby SNP; i.e., *MTHFR* c.1286A>C (p.Glu429Ala; known as 1298A>C). The effects of

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this SNP on tHcy and folate in blood are weaker [Kim, 2005], and could not always be detected.

Numerous other polymorphisms of genes involved in one-carbon metabolism have been identified. These include: methionine synthase (*MTR*; MIM# 156570) c.2756A>G (p.Asp919Gly) [Leclerc et al., 1996]; methionine synthase reductase (*MTRR*; MIM# 602568) c.66A>G (p.Ile22Met) [Wilson et al., 1999]; methylenetetrahydrofolate dehydrogenase (*MTHFD1*; MIM# 172460) c.1958G>A (p.Arg653Gln) [Hol et al., 1998]; betaine homocysteine methyltransferase (*BHMT*; MIM# 602888) c.716A>G (p.Arg239Gln, known as 742G>A) [Heil et al., 2000]; cystathionine β-synthase (*CBS*; MIM# 236200) c.844_845ins68 [Sebastio et al., 1995] and c.699C>T (p.Tyr233Tyr) [Kraus et al., 1998]; transcobalamin-II (*TCN2*; MIM# 275350) c.67A>G (p.Ile23Val) [Li et al., 1995] and c.776C>G (p.Pro259Arg) [Li et al., 1993]; reduced folate carrier-1 (*SLC19A1*; MIM# 600424) c.80G>A (p.Arg27His) [Chango et al., 2000]; and paraoxonase-1 (*PON1*; MIM# 168820) 163T>A (p.Leu55Met) [Adkins et al., 1993] and c.575A>G (p.Gln192Arg) [Humbert et al., 1993]. The enzymatic reactions involved and their role in one-carbon metabolism are summarized in Fig. 1. The metabolic effects of these polymorphisms, often in terms of concentrations of folate and cobalamin, and their metabolic markers, tHcy and methylmalonic acid (MMA), have been assessed, and variable and inconsistent results have been obtained. This is probably explained by insufficient statistical power to detect moderate metabolic effects in studies involving a small number of subjects (often less than 500 subjects). The fact that the frequencies of the least common variants are often less than 10% adds to these methodological difficulties.

The aim of the present work was to carry out metabolic profiling of genetic polymorphisms related to one-carbon metabolism in a large-scale population-based study to obtain sufficient statistical power to detect moderate associations in small subgroups. We measured a panel of 13 metabolites and 13 polymorphisms of nine genes in a large population of 10,601 subjects.

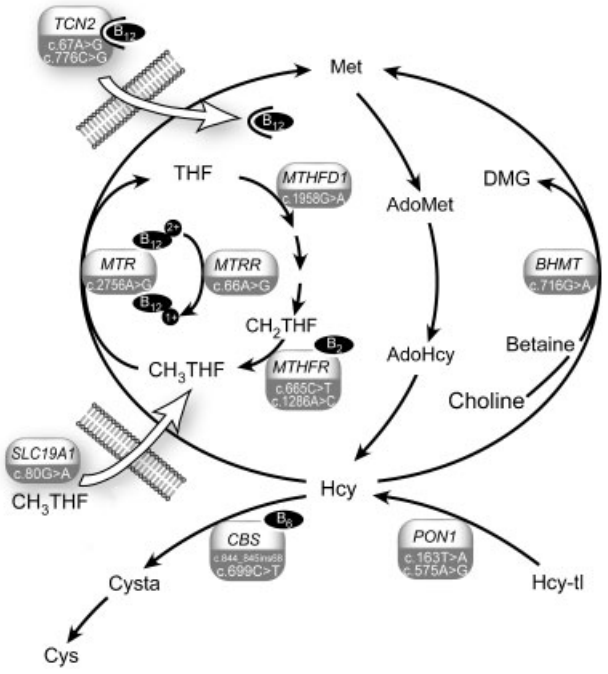

FIGURE 1. **One-carbon metabolism and related gene polymorphisms. AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; *BHMT*, betaine homocysteine methyltransferase; Cysta, cystathionine; Cys, cysteine; *CBS*, cystathionine β-synthase; CH_2THF, methylenetetrahydrofolate; CH_3THF, methyltetrahydrofolate; DMG, dimethylglycine; Hcy, homocysteine; Hcy-tl, homocysteine thiolactone; Met, methionine; *MTHFD1*, trifunctional enzyme methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase; *MTHFR*, methylenetetrahydrofolate reductase; *MTR*, methionine synthase; *MTRR*, methionine synthase reductase; *PON1*, paraoxonase-1; *SLC19A1*, reduced folate carrier-1; *TCN2*, transcobalamin-II; THF, tetrahydrofolate.**

MATERIALS AND METHODS

Subjects

A total of 13,823 subjects were drawn randomly from the population registries in Oslo and Telemark County between 1999 and 2001 for participation in the Norwegian Colorectal Cancer Prevention (NORCCAP) study [Bretthauer et al., 2002]. Participants were of both genders, homogeneous with respect to ethnicity, and their age ranged from 50 to 64 years. Exclusion criteria were previous open colorectal surgery, need of long-lasting attention and nursing services, on-going cytotoxic or radiotherapy for malignant disease, severe chronic cardiopulmonary disease, lifelong anticoagulant therapy, a coronary episode requiring hospital admission during the last 3 months, cerebrovascular accident during the last 3 months, and resident abroad or postal return of unopened mail. Using these criteria, 535 individuals were excluded and for 10,601 eligible subjects, we obtained blood samples where metabolites and genotypes were determined for the present work. The study was approved by the Regional Ethics Committee and The Data Inspectorate. Written informed consent was obtained from all participants.

Collection of Blood

Blood samples were collected from nonfasting subjects into EDTA Vacutainer tubes and tubes without additive. EDTA samples were immediately put on ice, whereas serum was allowed to clot for 1 hr at room temperature. Samples were centrifuged at 1100 *g* for 10 minutes. Whole blood, the plasma and serum fraction were stored at −20°C for 6 to 12 months, and then transferred to the research laboratory, where they were stored at −80°C until analysis.

Vitamin and Metabolite Determination

Folate and vitamin B_{12} (cobalamin) were determined in serum, and tHcy, MMA, vitamin B_2 (riboflavin), vitamin B_6 (pyridoxal 5′-phosphate, PLP), choline, betaine, dimethylglycine (DMG), cystathionine, cysteine, methionine, and creatinine were determined in plasma, using published methods [Holm et al., 2003; Midttun et al., 2005; Molloy and Scott, 1997; Windelberg et al., 2005].

DNA Extraction and Genotyping

DNA was extracted from the 25 μL cell pellet obtained after removal of the plasma fraction, using a Geno M-96 workstation (Geno Vision, Qiagen, Oslo, Norway). *MTHFR* c.665C>T genotyping was performed by real-time PCR with 5′ exonuclease (Taqman®, Applied Biosystems, Foster City, CA) probes [Ulvik and Ueland, 2001]. The other polymorphisms were determined by a matrix-assisted laser-desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry (MS) assay as described [Meyer et al., 2004]. The amount of DNA was variable and small in this sample collection, and in order to increase accuracy of the genotyping, samples were analyzed twice by the MALDI-TOF MS assay and

TABLE 1. Characteristics of the Study Population

	Total[a]	Men[a]	Women[a]	P[b]
Age (years)	55.00 (51.00–63.00)	55.00 (51.00–63.00)	55.00 (51.00–63.00)	0.189
tHcy (μM)	10.23 (6.78–16.43)	10.87 (7.51–17.08)	9.57 (6.40–15.70)	<0.001
Folate (nM)	13.70 (6.59–39.44)	12.84 (6.39–34.86)	14.75 (6.83–43.34)	<0.001
Cobalamin (pM)	307.20 (171.95–535.90)	304.60 (171.70–518.40)	309.70 (172.70–555.62)	0.001
MMA (μM)	0.169 (0.110–0.288)	0.169 (0.109–0.289)	0.170 (0.111–0.287)	0.331
Riboflavin (nM)	10.50 (4.11–55.92)	9.92 (4.00–46.31)	11.00 (4.23–65.81)	<0.001
PLP (nM)	48.00 (18.70–152.37)	48.70 (19.62–134.00)	47.20 (17.60–170.05)	0.082
Choline (μM)	8.62 (5.79–12.90)	9.04 (6.12–13.45)	8.26 (5.56–12.10)	<0.001
Betaine (μM)	35.40 (19.12–58.30)	39.66 (25.44–63.04)	31.00 (16.80–52.00)	<0.001
DMG (μM)	3.73 (2.44–5.93)	3.95 (2.70–6.27)	3.50 (2.30–5.59)	<0.001
Cystathionine (μM)	0.190 (0.091–0.525)	0.202 (0.099–0.579)	0.177 (0.086–0.471)	<0.001
Cysteine (μM)	283.70 (237.13–338.17)	285.79 (240.08–338.63)	281.49 (234.47–337.58)	<0.001
Methionine (μM)	21.41 (15.26–33.31)	22.37 (16.03–34.34)	20.36 (14.82–32.33)	<0.001
Creatinine (μM)	68.94 (50.60–92.17)	75.55 (58.26–96.78)	62.60 (48.00–81.30)	<0.001

[a]Values are median (fifth–95th percentiles) of individuals.
[b]By Mann-Whitney *U* test.

results were compared. Conflicting genotypes were inspected manually, corrected or defined as undetermined. The *MTHFD1* c.1958G>A polymorphism was included in the assay using the sense primer, 5′-CTGGTTTCCACAGGGCACTC-3′, and antisense primer, 5′-ACAAACCCTTCTGGGCCAAC-3′ for PCR, and the primer 5′-TCCTCCATCATTGCAGACC-3′ as extension probe. The polymorphism numbering is based on the cDNA sequences with GenBank accession numbers NM_005957.3 for *MTHFR*, NM_000254.1 for *MTR*, NM_002454.1 for *MTRR*, NM_005956.2 for *MTHFD1*, NM_001713.1 for *BHMT*, NM_000071.1 for *CBS*, M60396.1 for *TCN2*, U19720.1 for *SLC19A1*, and NM_000446.3 for *PON1*, with+1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Statistical Methods

Descriptive measures include medians with fifth and 95th percentiles, and means. Adjusted mean values were corrected for age, sex, creatinine, and center and were computed by analysis of covariance. Differences between women and men were assessed with the Mann-Whitney *U* test. Because of skewed distributions, logarithmic transformation was applied to all analyte concentrations. Three samples with extreme MMA values (>2 μM) were excluded from the analysis. A test for linear trend across genotypes (wild types, heterozygotes, homozygotes; coded 0, 1, 2) was estimated by linear regression analysis with adjustment for age, sex, creatinine, and center. P-values<0.01 were considered statistically significant. Hardy-Weinberg equilibrium (HWE) was measured by the chi-squared test and equilibrium was defined as P>0.05. In addition, the disequilibrium coefficient D was determined [Weir, 1996]. The linkage disequilibrium between two loci was described by the normalized disequilibrium coefficient D′ [Lewontin, 1964]. The sign of the D′ coefficient indicates whether the rare alleles are associated (positive) or whether the rare allele at one locus is associated with the most common allele at the other locus (negative). SPSS 12 for Windows (SPSS, Chicago, IL) was used for calculation of descriptive statistics and for the regression analysis. Haploview (www.broad.mit.edu/mpg/haploview/index.php) was used for determination of the linkage coefficient D′ with 95% confidence interval (CI).

RESULTS

Characteristics of the Study Population

The study population consisted of 10,601 subjects, 49.2% males. The median (5th–95th percentiles) age was 55 (51–63) years. The median (5th–95th percentiles) concentrations of tHcy, folate, cobalamin, MMA, riboflavin, PLP, choline, betaine, DMG, cystathionine, cysteine, creatinine, and methionine are listed for the total study group and separately for men and women in Table 1. The concentrations of tHcy, betaine, choline, DMG, cystathionine, cysteine, methionine and creatinine were higher and folate, cobalamin, and riboflavin lower in men than in women. MMA and PLP were not significantly different according to gender.

Genotype Distributions

DNA samples from all subjects were genotyped for 13 polymorphisms related to one-carbon metabolism. Their success rates (ratio of successfully genotyped samples to total number of samples), genotypes, and allele frequencies are summarized in Table 2. The success rates vary between 1.00 for the *MTHFR* c.665C>T and 0.85 for the *SLC19A1* c.80G>A, respectively. Allele frequencies are compared with those in the literature [Brophy et al., 2001; Janosikova et al., 2005; Pepe et al., 1999] and are in accordance with published data.

The observed genotype frequencies are shown in Fig. 2. The distributions were in HWE (0.0001<D<0.0021) for the genotypes of *MTHFR* c.665C>T, *MTR* c.2756A>G, and *MTHFD1* c.1958A>G. Deviations from HWE were observed for the other polymorphisms ranging from the lowest D=0.0041 for *TCN2* c.67A>G to the highest D=0.0675 for *SLC19A1* c.80G>A.

Linkage disequilibrium was detected for all polymorphisms located in the same gene (Table 3), and the following coefficients D′ (95% CI) were calculated:−0.95 (−0.93,−0.97) for *MTHFR* c.665C>T and c.1286A>C,−0.89 (−0.85,−0.93) for *CBS* c.844_845ins68 and c.699C>T,−0.41 (−0.35,−0.46) for *TCN2* c.67A>G and c.776C>G, and −0.84 (−0.81,−0.87) for *PON1* c.163T>A and c.575A>G.

Associations Between Genotypes and Biochemical Variables

The concentrations of 12 biochemical parameters according to 13 polymorphisms were calculated as mean values for each genotype, and the changes according to the number of the rare allele for each polymorphism were assessed by analysis for trend (Supplementary Table S1; available online at http://www.interscience.wiley.com/jpages/1059–7794/suppmat). Due to linkage disequilibrium between the polymorphisms located in the same genes, the variants of *MTHFR* c.665C>T and c.1286A>C, *CBS*

TABLE 2. Characteristics of the Study Population*

			Current study					Literature	
Gene	DNA change	Protein change	Success rate	Number of wild-types (%)	Number of heterozygotes (%)	Number of homozygotes (%)	Frequency of rare allele	Frequency of rare allele	References
MTHFR	c.665C>T	p.Ala222Val	1.00	5452 (51.5)	4299 (40.6)	847 (8.0)	0.28	0.30–0.40	Janosikova et al. [2005]
MTHFR	c.1286A>C	p.Glu429Ala	0.96	4506 (44.5)	4336 (42.8)	1292 (12.8)	0.34	0.33	Janosikova et al. [2005]
MTR	c.2756A>G	p.Asp919Gly	0.96	6707 (66.1)	3059 (30.1)	381 (3.8)	0.19	0.19–0.22	Janosikova et al. [2005]
MTRR	c.66A>G	p.Ile22Met	0.93	1883 (19.1)	4218 (42.8)	3765 (38.2)	0.60	0.35–0.59	Janosikova et al. [2005]
MTHFD1	c.1958G>A	p.Arg653Gln	0.95	3060 (30.2)	4994 (49.4)	2065 (20.4)	0.45	0.44–0.45	Janosikova et al. [2005]
BHMT	c.716G>A	p.Arg239Gln	0.95	5236 (51.8)	3992 (39.5)	871 (8.6)	0.28	0.23–0.32	Janosikova et al. [2005]
CBS	c.844_845ins68	–	0.95	8364 (82.9)	1575 (15.6)	152 (1.5)	0.09	0.05–0.40	Pepe et al. [1999]
CBS	c.699C>T	p.Tyr233Tyr	0.93	4596 (46.7)	3922 (39.8)	1332 (13.5)	0.33	0.29–0.42	Janosikova et al. [2005]
TCN2	c.67A>G	p.Ile23Val	0.95	7885 (78.3)	2000 (19.9)	179 (1.8)	0.12	0.13	Janosikova et al. [2005]
TCN2	c.776C>G	p.Pro259Arg	0.95	3278 (32.5)	4783 (47.4)	2020 (20.0)	0.44	0.45–0.47	Janosikova et al. [2005]
SLC19A1	c.80G>A	p.Arg27His	0.85	3693 (40.7)	3183 (35.1)	2187 (24.1)	0.42	0.13–0.47	Janosikova et al. [2005]
PON1	c.163T>A	p.Leu55Met	0.94	4647 (46.8)	4009 (40.3)	1282 (12.9)	0.33	0.06–0.40	Brophy et al. [2001]
PON1	c.575A>G	p.Gln192Arg	0.95	5356 (53.0)	3891 (38.5)	858 (8.5)	0.28	0.27–0.60	Brophy et al. [2001]

*Reference sequences: *MTHFR* (GenBank: NM_005957.3), *MTR* (GenBank: NM_000254.1), *MTRR* (GenBank: NM_002454.1), *MTHFD1* (GenBank: NM_005956.2), *BHMT* (GenBank: NM_001713.1), *CBS* (GenBank: NM_000071.1), *TCN2* (GenBank: M60396.1), *SLC19A1* (GenBank: U19720.1), and *PON1* (GenBank: NM_000446.3) with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

c.844_845ins68 and c.699C>T, *TCN2* c.67A>G and c.776C>G, and *PON1* c.163T>A and c.575A>G polymorphisms were evaluated in the stratum of the wild-type genotype of the linked polymorphism.

The strongest associations were observed for the *MTHFR* c.665C>T SNP. tHcy increased ($P<0.001$), whereas folate ($P<0.001$), betaine ($P<0.001$), DMG ($P<0.001$), and PLP ($P<0.001$) decreased according to the number of T-alleles. Also *MTHFR* c.1286A>C affected tHcy, folate and betaine as tHcy increased ($P<0.001$), while folate ($P<0.001$) and betaine ($P<0.008$) decreased with the number of *MTHFR* c.1286C alleles.

The *MTR* c.2756A>G transition was inversely associated with tHcy ($P<0.001$).

BHMT c.716G>A had a significant effect on the product of the *BHMT* reaction, DMG, which decreased according to the number of *BHMT* c.716A alleles ($P=0.001$).

The *CBS* c.844_845ins68 was negatively associated with tHcy ($P<0.001$) and positively associated with betaine ($P<0.001$). Subjects carrying the T-allele of the silent *CBS* c.699C>T polymorphism had decreased tHcy ($P=0.002$) and increased betaine ($P=0.003$) and slightly increased cystathionine ($P=0.001$). These small effects from the *CBS* c.699C>T polymorphism motivated us to investigate the relation between the substrate (homocysteine) and product (cystathionine) of the *CBS* reaction according to *CBS* c.699C>T genotype. Fig. 3 demonstrates that the tHcy/cystathionine ratio declined according to the number of *CBS* c.699T alleles ($P<0.001$).

Both *TCN2* c.776C>G ($P<0.001$) and c.67A>G ($P=0.011$) were positively associated with MMA (Supplementary Table S1).

NULL EFFECTS

MTRR c.66A>G, *MTHFD1* c.1958G>A, *SLC19A1* c.80G>A, and *PON1* c.163T>A and c.575A>G were not significantly associated with changes in any of the biochemical parameters investigated. However *MTHFD1* c.1958G>A and *SLC19A1* c.80G>A had borderline significant effects on folate. Cobalamin, riboflavin, choline, cysteine, and methionine were not significantly influenced by any of the investigated polymorphisms.

Effect Size

For all significant associations, the relative changes of the mean concentrations between homozygous rare and wild types were illustrated in Fig. 4. The strongest effects were found for the *MTHFR* c.665C>T polymorphism, with 32% difference in tHcy, 29% in folate, 11% in DMG, 10% in betaine, and 8% in PLP. For the *MTHFR* c.1286A>C polymorphism, the differences were 5% in tHcy, 9% in folate, and 3% in betaine. The effects of *BHMT* c.716G>A, *CBS* c.844_845ins68, *CBS* c.699C>T, and *TCN2* c.776C>G were in the range of 2 to 7%.

DISCUSSION

This study demonstrates that the polymorphisms *MTHFR* c.665C>T and c.1286A>C, *MTR* c.2756A>G, *BHMT* c.716G>A, *CBS* c.844_845ins68 and c.699C>T, and *TCN2* c.776C>G were associated with the concentrations of biochemical parameters related to one-carbon metabolism in a population of 10,601 healthy adults. The strongest associations were observed for the *MTHFR* c.665C>T polymorphism, which had profound effects on homocysteine and folate status, whereas *MTRR* c.66A>G, *MTHFD1* c.1958G>A, *SLC19A1* c.80G>A, and

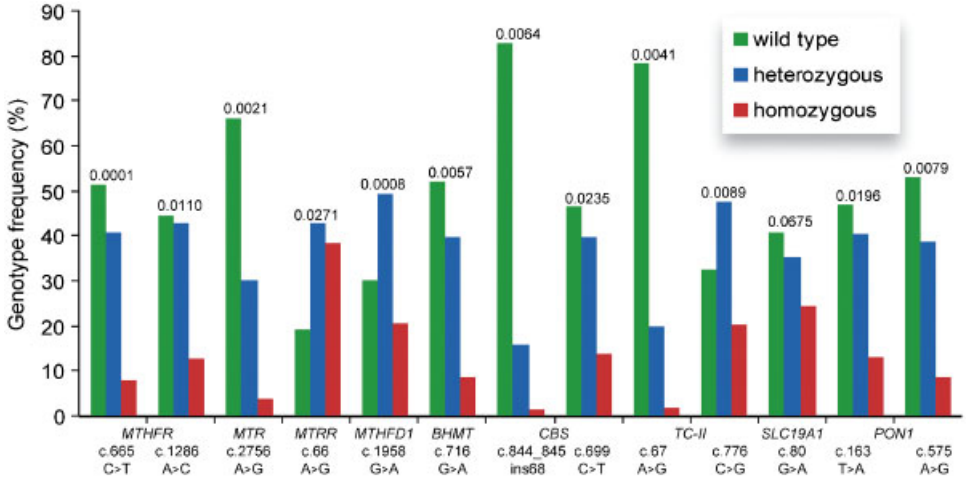

FIGURE 2. **Genotype frequencies. The observed genotype distributions are given as colored bars and the departure from HWE is given as disequilibrium coefficient D.**

TABLE 3. **Number of Genotype Combinations of Polymorphisms in the *MTHFR*, *CBS*, *TCN2*, and *PON1* Genes**

	CC	CT	TT		wt wt	wt ins	ins ins
***MTHFR*, c.665**				***CBS*, c.844_845ins68**			
c.1286AA	1492	2217	783	c.699CC	3448	985	134
c.1286AC	2469	1848	16	c.699CT	3366	544	9
c.1286CC	1246	43	0	c.699TT	1298	24	0
	CC	**CG**	**GG**		**AA**	**AG**	**GG**
***TCN2*, c.776**				***PON1*, c.575**			
c.67AA	2322	3745	1711	c.163TT	1728	2111	768
c.67AG	805	952	235	c.163TA	2326	1608	57
c.67GG	103	52	21	c.163AA	1150	107	6

PON1 c.163T>A and c.575A>G did not significantly affect the investigated parameters.

Study Design, Strength, and Weakness

The strength of this study is the large population, which allows the detection of moderate associations, also in subgroups. Another advantage is the number of parameters used for metabolite profiling, which includes 13 markers and metabolites related to one-carbon metabolism. This contrasts to most published studies [Molloy, 2004], which measure tHcy and folate. Finally, the study population is homogeneous with respect to age and ethnicity, and was recruited from an area with no mandatory folate fortification.

Since the age of the female subjects ranged from 50 to 64 years, the presented results are restricted to postmenopausal women.

Among the 13 polymorphisms, three were in HWE, whereas 10 SNPs showed deviation from equilibrium. Deviation becomes significant at a small D of 0.0041 (*TCN2* c.67A>G) due to the large size of the study population. The positive D [Leal, 2005] demonstrates the underestimation of heterozygous genotypes. This is explained by genotyping errors due to low amounts of DNA in some blood samples, resulting in poor amplification of some PCR products and misinterpretation of some heterozygous genotypes as homozygous genotypes. Notably, the success rate was lowest for polymorphisms, which suffered from the highest deviations from HWE. Such reading errors may lead to underestimation of "true" associations, especially for metabolites with highest assay imprecision (high coefficient of variation). Such attenuation of the metabolite-genotypes relation may become substantial for the polymorphisms estimated with the highest error rate, in particular *SLC19A1* c.80G>A. Conceivably, decrease in folate according to the number of A-alleles, which was of borderline significance, may actually reflect a true metabolic effect from this polymorphism.

Metabolic Phenotyping

This work confirmed the strong effect of *MTHFR* c.665C>T on homocysteine and folate status that has been consistently demonstrated in numerous studies [Jacques et al., 1996; Ma et al., 1996; Rozen, 2000]. In addition, we observed a 10% reduction of betaine in subjects with c.665TT compared with the c.665CC genotype, which has not been previously reported. This reduction may be linked to a decrease in serum folate, because betaine has previously been observed to be positively related to folate [Holm et al., 2005] and increases after supplementation with folic acid [Melse-Boonstra et al., 2005].

We observed a simultaneous increase in tHcy and decrease in folate by the *MTHFR* c.1286AC and c.1286CC genotypes, which has not been reported before. An increase of tHcy in c.665CT/c.1286AC compared to c.665CT/c.1286AA has been demonstrated in two studies [van der Put et al., 1998; Weisberg et al., 2001], while others reported an increase [Castro et al., 2003] or decrease [Friedman et al., 1999] of tHcy in c.665CC/c.1286CC compared to c.665CC/c.1286AA. Associations with folate were often not reported [Castro et al., 2003; Dekou et al.,

2001; Gueant-Rodriguez et al., 2005; Weisberg et al., 2001]. A reduction of betaine was also observed for the *MTHFR* c.1286A>C variant, but was only one-third of the decrease found for the c.665C>T. In addition, we have investigated in detail the changes in folate and tHcy levels according to *MTHFR* c.665/c.1286 genotype combinations, and the results have been published in a separate work [Ulvik et al., 2007] (http://www.springerlink.com/content/051213l4t2254328/).

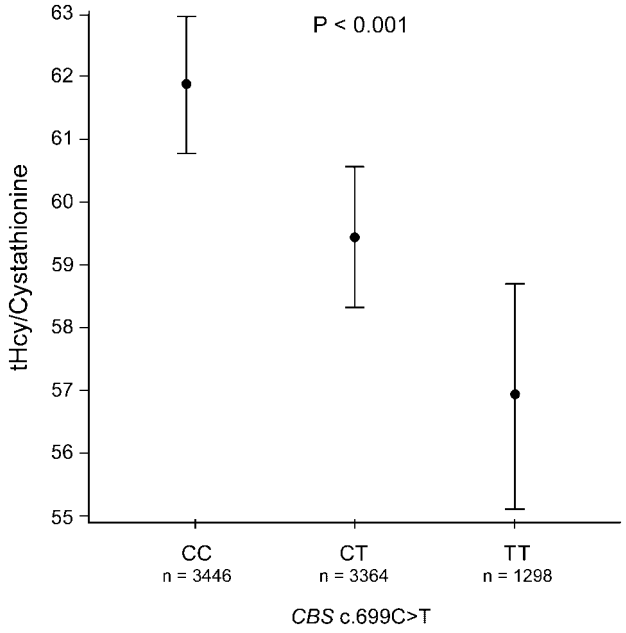

FIGURE 3. **The relation of the homocysteine/cystathionine ratio to the *CBS* c.699C>T genotypes. The ratio between the substrate (homocysteine) and product (cystathionine) is plotted according to genotype as means with 95% CI after adjustment for age, sex, creatinine, and center. P-value for trend is given.**

MTR and *MTRR* are both involved in homocysteine remethylation [Finkelstein, 1990]. The decrease in tHcy according to the number of *MTR* c.2756G alleles observed by us, confirms the results from many previous studies [Chen et al., 2001; Harmon et al., 1999; Tsai et al., 2000; Yates and Lucock, 2002], but this is not a consistent finding [Hyndman et al., 2000; Klerk et al., 2003; Ma et al., 1999; Morita et al., 1999; Ulvik et al., 2004; van der Put et al., 1997]. In contrast to published results [Chen et al., 2001; Hyndman et al., 2000; Klerk et al., 2003], we and others [Harmon et al., 1999; Ma et al., 1999] found no relation of *MTR* c.2756A>G genotypes to the concentrations of folate or cobalamin. Furthermore, there are reports on association of *MTRR* c.66A>G polymorphism with tHcy [Gaughan et al., 2001; Gueant-Rodriguez et al., 2005], which could not be confirmed by us or others [Brilakis et al., 2003; Jacques et al., 2003].

The relation of the *BHMT* c.716G>A polymorphism to tHcy has so far been investigated in three studies [Heil et al., 2000; Morin et al., 2003b; Weisberg et al., 2003] and no associations have been found. We did not find any significant effect of this SNP on tHcy, but a significant decrease of DMG according to the number of c.716A alleles. This is the first indication that *BHMT* c.716G>A might have metabolic effects.

Cystathionine β-synthase is the rate limiting step in the transsulfuration pathway that degrades superfluous homocysteine [Finkelstein, 1990]. A few studies [Janosikova et al., 2003; Tsai et al., 1999; Tsai et al., 2000] have reported lower levels of postmethionine load (PML) tHcy in subjects with the *CBS* c.844_845ins68. In accordance with the role of cystathionine β-synthase in homocysteine homeostasis, they suggested that the 68-bp insertion may enhance transsulfuration efficiency by

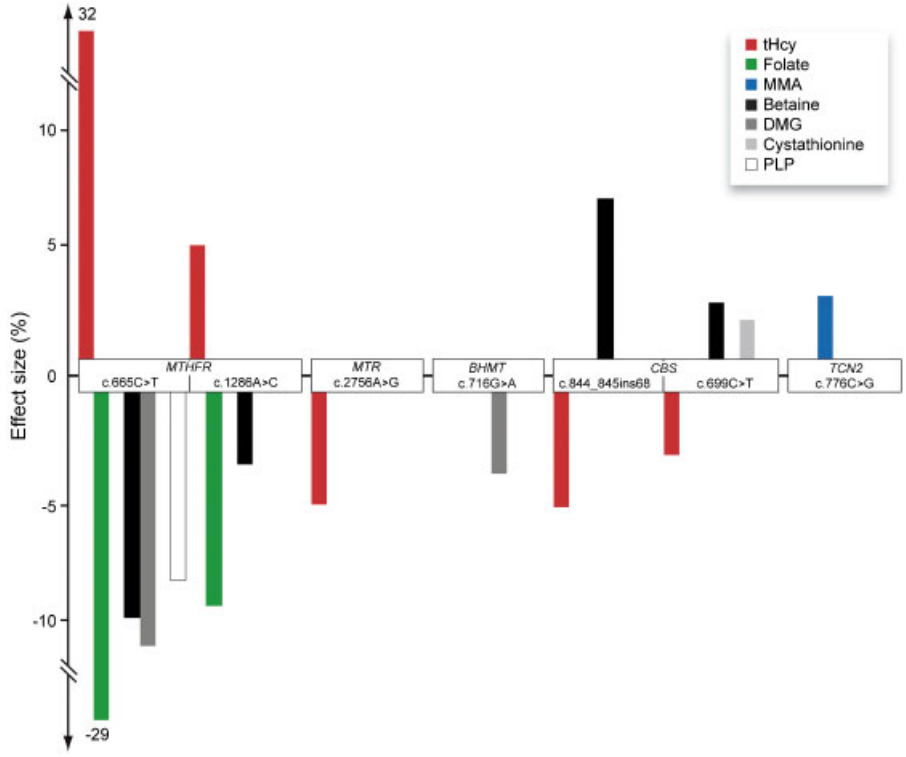

FIGURE 4. **Effect sizes for the associations of gene polymorphisms with concentrations of metabolites. The effect sizes are given as relative difference in means between the two homozygous genotypes. Mean values of the wild types were used as reference.**

increasing the *CBS* activity or amount of the *CBS* mRNA. tHcy in subjects not loaded with methionine or in the fasting state has shown no relation to the c.844_845ins68 [Bowron et al., 2005; Janosikova et al., 2003; Kluijtmans et al., 1997, 2003], which is in agreement with homocysteine remethylation rather than transsulfuration as the main determinant of this tHcy modality [Refsum et al., 2004]. However, we observed a minor but significant reduction of tHcy in subjects with the insertion. This probably reflects the power of the present study to detect small changes in tHcy according to genotype. Notably, the 68-bp insertion was associated with a significant increase in betaine, which adds to the general observation [Holm et al., 2005] that conditions that lower tHcy increase betaine.

Our observation that the 68-bp insertion is in strong linkage disequilibrium with the *CBS* c.699C>T ($D' = -0.89$) is in accordance with earlier reported data [De Stefano et al., 1998]. Published results [Aras et al., 2000; Kruger et al., 2000] demonstrate a tHcy lowering effect of the *CBS* c.699T alleles in subjects after PML or folate supplementation. Likewise, we found a decrease in tHcy and increase in betaine with increasing number of *CBS* c.699 T alleles. Thus, the metabolic phenotype of the *CBS* c.699C>T polymorphism is similar to that of the 68-bp insertion, except for the decline of the tHcy/cystathionine ratio, which was confined to the *CBS* c.699C>T. Since the c.699C>T transition is silent, it has been speculated that this SNP might be in linkage disequilibrium with regulatory elements that control *CBS* gene transcription [Aras et al., 2000].

Two SNPs in the gene encoding for the cobalamin transporter, transcobalamin II, *TCN2* c.776C>G and c.67A>G, have been reported to have metabolic effects. The c.776C>G transversion has been associated with low plasma holo-transcobalamin (holoTC) [McCaddon et al., 2004; von Castel-Dunwoody et al., 2005], increased [Miller et al., 2002] or no change [Wans et al., 2003] in MMA, and the reported effects on tHcy have been minor and inconsistent [Afman et al., 2002; Lievers et al., 2002; Miller et al., 2002; Namour et al., 2001; Wans et al., 2003]. Homozygosity for the low frequency SNP, c.67A>G has been associated with reduced tHcy in one study [Lievers et al., 2002]. We found that the c.776C>G transversion had a minor nonsignificant influence on tHcy, whereas this SNP was associated with increased MMA, indicating reduced availability of intracellular cobalamin. The *TCN2* c.67A>G polymorphism also increased MMA, but this effect was of borderline significance.

NULL EFFECTS

The *MTHFD1* c.1958G>A, *SLC19A1* c.80G>A, and *PON1* c.163T>A and c.575A>G showed no associations with the investigated vitamins and metabolites, except for *MTHFD1* c.1958G>A and *SLC19A1* c.80G>A transitions, which were related to a minor decrease in folate of borderline significance. These null findings actually confirm most published results for these SNPs. Among the studies evaluating the metabolic phenotype of *MTHFD1* c.1958G>A [Chen et al., 2004; Cheng et al., 2005; Konrad et al., 2004] and *SLC19A1* c.80G>A [Chango et al., 2000; Morin et al., 2003a; Vesela et al., 2005; Yates and Lucock, 2005], four suggest a possible effect on tHcy or folate [Chango et al., 2000; Cheng et al., 2005; Morin et al., 2003a; Yates and Lucock, 2005]. The *PON1* c.163T>A and c.575A>G have both been associated with an influence on homocysteine thiolactonase activity [Jakubowski et al., 2001; Lacinski et al., 2004], but there are no data suggesting effects of these SNPs on plasma tHcy.

Implications and Conclusion

The *MTHFR* c.665C>T polymorphism has a well-established and marked effect on folate and homocysteine status [Ueland et al., 2001]. The reports on metabolic phenotypes of most other genetic polymorphisms related to one-carbon metabolism have been inconsistent. The present study has sufficient power to demonstrate that *MTHFR* c.1286A>C, *MTR* c.2756A>G, *BHMT* c.716G>A, *CBS* c.844_845ins68, *CBS* c.699C>T, and *TCN2* c.776C>G have significant associations with concentrations of vitamins or metabolites in plasma/serum. However, one can not infer from the small effect size measured for some genetic variants that their biological importance is minor, because small changes in extracellular markers may reflect substantial perturbation of intracellular metabolism. Therefore, the metabolic profile demonstrated for several polymorphisms in the present study should stimulate further studies on their biological effects as well are their relations to chronic diseases.

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