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Genetic and Environmental Determinants of Plasma Total Homocysteine Levels: Impact of Population-wide Folate Fortification

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Abstract

Objectives—Folate metabolism is an important target for drug therapy. Drug-induced inhibition of folate metabolism often causes an elevation of plasma total homocysteine (tHcy). Plasma tHcy levels are influenced by several non-genetic (e.g., folate intake, age, smoking) as well as genetic factors. Over the last decade, several countries have implemented a nation-wide folate fortification program of all grain products. This investigation sought to determine the impact of folate fortification on the relative contribution of environmental and genetic factors to the variability of plasma tHcy.

Methods—Two cohorts were compared in this study, one from the U.S. (with folate fortification, n=281), and one from Austria (without folate fortification, n=139). Several environmental factors as well as previously identified gene variants important for tHcy levels (*MTHFR* C677T, *MTHFR* A1298C, *MTRR* A66G) were examined for their ability to predict plasma tHcy in a multiple linear regression model.

Results—Non-genetic, environmental factors had a comparable influence on plasma tHcy between the two cohorts ($R^2 \sim 0.19$). However, after adjusting for other covariates, the tested gene variants had a substantially smaller impact among patients from the folate fortified cohort ($R^2 = 0.021$) compared to the non-folate fortified cohort ($R^2 = 0.095$). The *MTHFR* C677T polymorphism was the single most important genetic factor. Male gender, smoking and folate levels were important predictors for non-folate fortified patients; age for folate fortified.

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Conclusions—Population-wide folate fortification had a significant effect on the variability of plasma tHcy and reduced the influence of genetic factors, most importantly the *MTHFR* 677TT genotype, and may be an important confounder for a personalized drug therapy.

Keywords

Homocysteine; folate fortification; folic acid

Introduction

Folate metabolism is an important target for drug therapy.^{1, 2} Several classes of drugs either directly or indirectly affect folate metabolism. Anticancer drugs, such as pyrimidine analogues and methotrexate,³ or nitrous oxide (laughing gas)⁴ which inactivates cobalamin (vitamin B₁₂) are well-known examples. On the other hand, folic acid itself, as well as other B-vitamins, is commonly used for dietary supplementation. Drug-induced inhibition of the folate cycle often leads to an increase in blood homocysteine levels, as the critical one-carbon (methyl) groups from the folate cycle are missing and methionine cannot be produced from its precursor homocysteine.^{5–7} Thus, elevated plasma total homocysteine (tHcy) is often an indicator of folate cycle inhibition, either inborn – for example due to the *MTHFR* 677TT genotype –, drug-induced or due to low folate intake. Plasma tHcy is influenced by non-genetic and environmental factors such as smoking, age, intake of B-vitamins (most importantly folic acid), but also genetic factors.⁸ Among the most important genetic factors are variants in genes involved in folate metabolism (e.g., *MTHFR*, *MTRR*), with the *MTHFR* C677T variant (rs1801133) considered the most important genetic determinant of plasma tHcy.^{9–11}

Several countries, including the U.S., have implemented a mandatory folic acid fortification program of grain products to reduce the incidence of neural tube defects,¹² since evidence indicated a strong association with low folate levels. While folate levels rose on a population level after the implementation of the folate fortification program¹³ and the incidence of neural tube defects declined steeply, the effects on the variability of tHcy levels and the relative impact of genetic and environmental factors are largely unknown.^{13–15} Thus, we hypothesized that folate fortification would lead to a reduced variability of plasma tHcy levels and a reduced relative contribution of *MTHFR* C677T, *MTHFR* A1298C, *MTRR* A66G to plasma tHcy levels. To test this hypothesis, we compared two patient cohorts, one from the U.S. (with folate fortification program) and one from Austria (no folate fortification).

Materials and Methods

Patient Cohorts

In this study, we compared two separate patient cohorts previously enrolled in two clinical trials (NCT00482456 and NCT00655980) that tested the effects of nitrous oxide, a general anesthetic and vitamin B₁₂ antagonist, on plasma tHcy. Both studies were approved by the local IRB (Medical University in Vienna, Austria, and Washington University, St. Louis, MO) and all patients gave informed consent. The Austrian cohort consisted of 140 patients scheduled for surgery at the Vienna General Hospital and the U.S. cohort of 281 patients from Barnes-Jewish-Hospital, St. Louis, MO.

Study Protocol

Patients could be enrolled in the study if they met the following inclusion criteria: age >18 yrs and scheduled for major surgery. Patients in the U.S. cohort also needed to be diagnosed

with or at risk for coronary artery disease. Patients were excluded if they met any of the following criteria: patients with significant pulmonary disease or requiring supplemental oxygen, vitamin B₁₂ or folate deficiency, patients taking supplemental vitamin B₁₂ or folic acid, contraindication against nitrous oxide, hypersensitivity to cobalamins, Leber's disease (hereditary optic nerve atrophy), and seizure disorder.

Per study protocol, patients had their morning fasting plasma tHcy, serum folate and vitamin B₁₂ concentrations measured and no intervention occurred before the blood draw. Additionally, venous blood was drawn to obtain DNA for determination of *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *MTRR* A66G (rs1801394) polymorphisms. DNA isolation was done using standard methods (Qiagen PureGene™ System, Qiagen, Valencia, CA). Genotyping was performed by PCR-RFLP as previously described.^{9, 16, 17}

Statistical Analysis

Continuous variables are described as mean ($\pm$ SD) for normally distributed data and as median (minimum-maximum or interquartile range) for skewed data. Binary variables are described as absolute frequencies and percentages. Two-sided t-tests were used for group comparisons of normally distributed continuous variables and chi-square test for categorical and binary data. Non-normally distributed continuous variables (most blood levels) were log-transformed to achieve a normal distribution.

To test each variable's ability to predict plasma tHcy levels, we used a simple linear regression with log tHcy as dependent variable without adjustment for covariates. Genotypes were dummy-coded for the regression. We computed separate regression models for each cohort to reduce confounding.

All variables that were significant at a <0.10 level were included in a multiple linear regression analysis. We tested several models to determine the incremental improvement in model fit and explained variance as measured by r^2 . The final regression model underwent several tests to rule out violations of linear regression model assumptions (multicollinearity, homoscedasticity). A chi-square statistic was used to determine violations of the Hardy-Weinberg equilibrium in both cohorts separately. A p value < 0.05 was considered statistically significant.

Results

Because the two trials targeted different study populations, the Austrian cohort generally younger and healthier than the U.S. cohort, the comparison in baseline variables showed substantial differences (Table 1). The U.S. cohort had more smokers and older patients and lower serum folate and higher plasma total homocysteine (tHcy) levels, despite living in a folate fortified environment.

The genotype frequencies within all three single nucleotide polymorphisms (SNPs), *MTHFR* C677T (rs1801133), *MTHFR* A1298C (rs1801131), *MTRR* A66G (rs1801394), were in Hardy-Weinberg equilibrium. To determine if the C677T and A1298C variant in the *MTHFR* gene were in linkage disequilibrium, we calculated D' and r^2 for each cohort. The analysis showed that despite a high D' of 1.0 and 0.96 in the Austrian and U.S. cohort, respectively, both SNPs had only a marginal r^2 of 0.22 and 0.14.

Next, we analyzed how environmental and genetic factors were individually able to predict plasma tHcy levels in a simple linear regression analysis in both cohorts, one with a national folate fortification program, the other one without (Table 2 and Figure 1). In the cohort without folate fortification (Austria), the genetic factors had the largest impact on plasma

tHcy levels with the *MTHFR* C677T polymorphism making the largest contribution ($R^2 = 0.107$). Environmental factors, most importantly male gender, smoking and plasma folate levels explained each between 4–7% of the variance. In the cohort with a national folate fortification program (U.S.), the influence of the three gene variants on plasma tHcy levels was substantially less, again with the *MTHFR* C677T polymorphism making the largest contribution ($R^2 = 0.021$). Environmental factors had a substantially larger impact on plasma tHcy with age ($R^2 = 0.106$) and smoking ($R^2 = 0.065$) having the largest impact.

In the multiple linear regression analyses (Table 3), a clear difference could be observed between both cohorts. Although the relative contribution of environmental and non-genetic factors was nearly identical ($R^2 \sim 0.19$), genetic factors (*MTHFR* C677T, A1298C) explained an additional 9.2% of the observed variance in the non-folate fortified cohort, but only 2.1% in the U.S. cohort.

Of the significant environmental and non-genetic predictors (age, gender, smoking, folate and vitamin B₁₂ levels), male gender, smoking and baseline folate levels had the largest influence on plasma tHcy in the cohort without folate fortification program; in the U.S. cohort with national folate fortification, age was by far the most important predictor for plasma tHcy.

Among the genetic predictors, the homozygous *MTHFR* 677TT genotype was the most important predictor for plasma tHcy. Neither of the other two genetic polymorphisms, *MTHFR* A1298C or *MTRR* A66G, were significant predictors of plasma tHcy in the adjusted multiple regression model.

Finally, we pooled the data from both cohorts ($n = 420$) and fit a multiple regression model which included folate fortification as covariate (Table 4). The model explained 34% of the observed variance. Folate fortification was the strongest predictor, followed by age and baseline folate levels and the C677T genotype. A gene x environment interaction between *MTHFR* C677T and folate fortification was not significant ($p = 0.086$).

Discussion

The results of this investigation show that a population-wide folate fortification program has an impact on the relative influence of genetic and environmental factors on plasma total homocysteine. Gene variants that have long been regarded and replicated as important predictors for plasma tHcy, such as the *MTHFR* C677T polymorphism,^{9, 11, 18} were reduced to marginal relevance and explained cumulatively less than 3% of the observed variance among patients with a national folate fortification program (*versus* 9.5% in patients without national folate fortification). The second *MTHFR* polymorphism, A1298C,^{17, 19} was a strong and significant predictor in patients without folate fortification program, yet became non-significant among the cohort with folate fortification. On the other hand, non-genetic factors such as age and smoking became substantially more relevant in patients living in an environment with a folate fortification program.²⁰

In the United States, the FDA has instituted a mandatory nation-wide fortification of breads, cereals, and other grain products with folic acid (140 µg of folic acid into each 100 g of grain product) since 1998. The main goal was to reduce the incidence of neural tube defects (spina bifida, anencephaly). The program is considered a success: it consistently raised folate levels^{14, 21} and reduced the incidence of neural tube defects by about 25%.²² Besides raising folate levels, population-wide folate fortification programs also reduce plasma tHcy levels, on average by about 10%.¹³

In a recent, large-scale genome-wide association study from a U.S. sample whose goal was to identify novel genetic predictors for plasma tHcy,²³ the significance of the *MTHFR* C677T variant was again confirmed, but accounted for only 1% of the observed variance. Clinical and environmental covariates explained about 9% of the observed variance. In a family-based segregation analysis of patients with coronary artery disease in Korea (no folate fortification program),¹⁰ the final regression model including environmental covariates and the *MTHFR* C677T variant explained between 16–20% of the observed variance with the genetic variant contributing between 4–5%, a result similar to ours. While a large-scale study of more than 10,000 individuals from Norway showed that, among other variants, both *MTHFR* polymorphisms (C677T and A1298C) were significant predictors of plasma tHcy,²⁴ our results indicate that the additional effects of the *MTHFR* A1298C variant on top of the C677T variant are likely small and clinically probably negligible. A large study from the U.S. before the implementation of the nationwide folate fortification program corroborates these findings: the *MTHFR* C677T polymorphism was the single most important genetic determinant of plasma tHcy and interestingly they also found that moderate folate intake attenuated the effects of this gene variant on plasma tHcy levels,²⁵ a finding that was corroborated in a large meta-analysis.²⁶

What could our findings mean for genetics-based, personalized drug therapy? It appears that folate fortification may be an important confounder that ameliorates several of the distinct metabolic features of gene variants in the folate cycle, such as the *MTHFR* 677TT variant.^{11, 15} Folate fortification may be viewed as another type of phenocopying, where the resultant phenotype has a low correlation to the original phenotype. For patients living in a folate fortified environment, carrying a heterozygous or even homozygous gene variant within the folate metabolism may be less important for a rational, personalized drug therapy.

Our study had several limitations. First, the two cohorts that we used for comparisons were not analogous at baseline since both were recruited for separate clinical trials and the U.S. cohort targeted sicker patients with or at risk for coronary artery disease. Patients in the U.S. cohort had lower plasma folate and higher tHcy levels, and were significantly older than patients from the Austrian cohort. It is a well-known effect of advanced age to lead to an increase in median plasma tHcy levels and also lower folate levels, which may account for the apparent paradox that patients in the U.S. cohort had lower plasma folate levels despite living in a folate-fortified environment. However, to address this dissimilarity we performed two separate regression analyses within each cohort with the goal to limit confounding. Since the focus of this study was not on absolute plasma tHcy levels, but on the relative contribution of genetic and environmental factors to plasma tHcy, we believe the dissimilarity of the two cohorts does not detract from our conclusions. Second, for budgetary reasons we could genotype only three common variants, well aware that other gene variants relevant for folate and homocysteine metabolism may exist, such as the *844ins68* allele of cystathionine- β -synthase or the *MTR* A2756G polymorphism. Third, while our analyses demonstrate that the variance explained by the genetic variants is substantially reduced in the folate-fortified cohort compared to the non-folate-fortified cohort, a test of environmental interaction with C677T (the polymorphism of strongest effect in the pooled analysis) was suggestive but not significant ($p = 0.1$). Larger samples would allow more powerful tests of specific gene-environment interactions. Finally, we were unable to obtain detailed nutritional and dietary information from the patients, which would have given us a better understanding of baseline folate, vitamin B₁₂ and B₆ intake, among other important dietary determinants of plasma tHcy levels.

In conclusion, our study shows that a population-wide folate fortification program reduced the relative influence of genetic factors to plasma total homocysteine levels and should be

considered an important confounder for personalized drug therapy targeting folate metabolism.

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Figure 1. Plasma total homocysteine concentrations are shown as geometric means plus SD stratified by cohort and *MTHFR* C677T (1A) and A1298C (1B), and *MTRR* A66G (1C) genotype.

Table 1

Baseline Comparison of the Two Study Cohorts.

Variable	No Folate Fortification (n=139)	Folate Fortification (n=281)	P-value
Age (median, range)	40 (18–78)	60 (19–92)	<0.001
Gender, %males	58%	57%	0.849
Smoking	40.3%	72.0%	<0.001
<i>MTHFR</i> C677T			0.197
677CC	62 (45%)	150 (53%)	
677CT	61 (44%)	99 (35%)	
677TT	16 (12%)	32 (11%)	
<i>MTHFR</i> A1298C			0.04
1298AA	60 (43%)	149 (53%)	
1298AC	71 (51%)	108 (38%)	
1298CC	8 (6%)	24 (9%)	
<i>MTRR</i> A66G			0.205
66AA	27 (19%)	61 (28%)	
66AG	72 (52%)	102 (46%)	
66GG	40 (29%)	57 (26%)	
Folate (median, range)	20.2 (1.5 – 45.0)	15.8 (3.3 – 110.6)	0.005
B₁₂ (median, range)	274 (50 – 885)	410 (13.2 – 2990)	<0.001
tHcy (mean, SD)	9.4 (4.8)	13.6 (5.8)	<0.001

Table 2
Unadjusted Predictors for Plasma Total Homocysteine from Simple Linear Regression.

Variable	No Folate Fortification (n=139)				Folate Fortification (n=281)			
	β	SE	P-value	R ²	β	SE	P-value	R ²
Age	0.001	0.002	0.664	0.001	0.009	0.001	<0.001	0.106
Gender	0.224	0.070	0.002	0.07	0.072	0.047	0.126	0.008
Smoking	0.173	0.071	0.016	0.041	0.222	0.051	<0.001	0.065
Folate (log)	-0.168	0.054	0.006	0.054	-0.144	0.044	0.001	0.037
B ₁₂ (log)	0.093	0.077	0.228	0.011	-0.114	0.045	0.011	0.023
<i>MTHFR</i> C677T			<0.001	0.092			0.019	0.016
<i>MTHFR</i> A1298C			0.013	0.062			0.224	0.011
<i>MTRR</i> A66G			0.944	0.001			0.316	0.011

Table 3

Multiple Linear Regression Models for Plasma Total Homocysteine.

Models	R ²	SE	R ² change	F	Sig.
No Folate Fortification					
1. All covariates, no gene variants	0.197	0.38		6.359	<0.001
2. All covariates, <i>MTHFR</i> C677T	0.275	0.37	0.077	6.987	0.001
3. All covariates, <i>MTHFR</i> C677T, A1298C	0.289	0.37	0.015	1.319	0.27
Folate Fortification					
1. All covariates, no gene variants	0.188	0.36		12.558	<0.001
2. All covariates, <i>MTHFR</i> C677T	0.205	0.35	0.018	3.020	0.05
3. All covariates, <i>MTHFR</i> C677T, A1298C	0.209	0.35	0.003	0.538	0.59

Table 4

Multiple Regression Analysis from pooled cohort (n= 420)

Variable	Pooled model (both cohorts)			
	β	SE	Stand. β	P-value
Age	0.005	0.002	0.183	0.002
Gender	0.072	0.040	0.80	0.073
Smoking	0.131	0.046	0.141	0.004
Folate Fortification	0.272	0.064	0.298	<0.001
Folate (log)	-0.141	0.039	-0.174	<0.001
B ₁₂ (log)	-0.014	0.044	-0.017	0.742
<i>MTHFR</i> C677T	0.096	0.031	0.149	0.002
<i>MTHFR</i> A1298C	-0.027	0.034	-0.038	0.426
<i>MTRR</i> A66G	-0.009	0.027	-0.015	0.733
Model R ² = 0.338				