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Alcohol abuse and cigarette smoking are associated with global DNA hypermethylation: results from the German Investigation on Neurobiology in Alcoholism (GINA)

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Running title: Alcohol abuse and smoking are associated with DNA hypermethylation

Abstract

Recent studies have shown that smoking and alcoholism may be associated with altered DNA methylation and that alcohol consumption might induce changes in DNA methylation by altering homocysteine metabolism.

In this monocenter study, we included 363 consecutive patients referred to hospital for alcohol detoxification treatment. Blood samples were obtained on treatment days 1, 3 and 7 for measurement of global DNA methylation in leucocytes by liquid chromatography tandem mass spectrometry. Genomic DNA was used for genotyping the following seven genetic variants of homocysteine metabolism: cystathionine beta-synthase (CBS) c.844_855ins68, dihydrofolate-reductase (DHFR) c.594+59del19bp, methylenetetrahydrofolate-reductase (MTHFR) c.677C>T and c.1298A>C, methyltetrahydrofolate-transferase (MTR) c.2756A>G, reduced folate carrier 1 (RFC1) c.80G>A, and transcobalamin 2 c.776C>G.

Multivariate linear regression showed a positive correlation of global DNA methylation with alcohol consumption and smoking on day 1 of hospitalization. DNA methylation was not correlated with homocysteine or vitamin plasma levels nor with the tested genetic variants of homocysteine metabolism.

This suggests a direct effect of alcohol consumption and smoking on DNA methylation, which is not mediated by effects of alcohol on homocysteine metabolism.

Keywords: alcohol; alcohol abuse; alcohol dependence; smoking; DNA methylation; homocysteine; folate; vitamin B1, vitamin B2; vitamin B6, vitamin B12; single nucleotide polymorphism

Introduction

Tobacco and alcohol are the most commonly used noxious compounds worldwide. Cigarette smoking and alcoholism significantly increase the risk for a variety of medical and psychiatric conditions and different forms of cancer (Thun et al., 1997; Zaridze et al., 2009). Because of the high prevalence of alcoholism and tobacco abuse and their negative health consequences, it is important to understand the mechanisms involved in alcohol and tobacco dependence and toxicity. A growing number of studies have shown that alcoholism and chronic alcohol intake in non-addicted subjects may be associated with altered DNA methylation (Bonsch, Lenz, Reulbach, Kornhuber, & Bleich, 2004; Harlaar & Hutchison, 2013; Starkman, Sakharkar, & Pandey, 2012). Some studies have demonstrated an additive effect of alcohol consumption and smoking on changes in DNA methylation (Thapar, Covault, Hesselbrock, & Bonkovsky, 2012). Alcohol might induce changes in DNA methylation by altering homocysteine metabolism (Bleich et al., 2000; Bleich & Hillemacher, 2009; Bonsch et al., 2004; Varela-Rey, Woodhoo, Martinez-Chantar, Mato, & Lu, 2013).

DNA methylation depends on S-adenosylmethionine (SAM) as a methyl group donor. The demethylated residue of SAM is S-adenosylhomocysteine (SAH), which is reversibly hydrolyzed to homocysteine. In a vitamin B6-dependent pathway, homocysteine can be transsulfurated to cystathionine and cysteine (Figure 1). Alternatively, homocysteine can be remethylated, depending on the essential co-factors folate, vitamin B2 and vitamin B12. As vitamin deficiency commonly occurs in alcohol-dependent patients, alcohol-induced changes in DNA methylation and homocysteine might be explained by vitamin deficiencies (Cravo & Camilo, 2000; Heese et al., 2012). Not only the vitamin status, but also genetic variants may modify folate, vitamin B12 and homocysteine metabolism (Stover, 2011). Alcohol-induced changes of DNA

methylation are possibly influenced by these genetic variants which are common in the general population.

In a cohort of 363 patients with alcohol dependency, we analyzed changes of DNA methylation and attempted to identify parameters related to homocysteine metabolism which may mediate or modify the association of alcohol and DNA methylation, i.e. plasma levels of homocysteine and vitamins involved in homocysteine metabolism as well as genetic variants of homocysteine metabolism.

Materials and Methods

Patients

The present study is part of the German Investigation on Neurobiology in Alcoholism (GINA)(Heese et al., 2012). Consecutive patients were recruited from the Department of Addiction and Psychotherapy of the LVR-Clinic in Bonn, Germany (Heese et al., 2012). All participants were diagnosed with alcohol dependency according to ICD-10 and were included in the study on admission for alcohol detoxification. Patients were mainly detoxified with clomethiazole following a symptom-triggered regime using the Banger-Score (Banger, Philipp, Herth, Hebenstreit, & Aldenhoff, 1992). If, for clinical reasons, clomethiazole could not be used, benzodiazepines were administered. Patients diagnosed with dependence from other substances were excluded. Daily alcohol consumption was calculated per day according to patients' self-reported alcohol consumption within the last week before admission to the hospital. Fasting blood samples were obtained on days 1 (admission), 3 and 7 of the detoxification treatment. Blood samples were centrifuged and consecutive serum and lithium heparin plasma samples were stored at −80°C directly after collection. Homocysteine and global DNA

methylation were assessed at all three time points, while vitamin serum levels were obtained at admission.

This study was approved by the local ethics committee. All patients gave their informed written consent.

Biochemical measurements

Serum alcohol concentrations were measured by an enzymatic test (alcohol dehydrogenase method) with a Dimension Vista™ system (Siemens Healthcare Diagnostics, Eschborn, Germany).

Serum alanine aminotransferase (ALT) activity, aspartate aminotransferase (AST) activity and gamma glutamyl transferase (GGT) activity were measured by means of an enzymatic test (ALTI method, AST method, GGT method) with a Dimension Vista™ system (Siemens Healthcare Diagnostics). Reference intervals for ALT ranged up to 45 U/l for men and 34 U/l for women, reference intervals for AST ranged from up to 35 U/l for men and 31 U/l for women, and reference intervals for GGT ranged up to 55 U/l for men and 38 U/l for women.

Serum carbohydrate-deficient transferrin (CDT) and serum transferrin were measured by means of particle-enhanced immunonephelometry using a BN Prospec™ System (Siemens Healthcare Diagnostics). Reference intervals given by Siemens Healthcare Diagnostics ranged from 28.1 - 76.0 mg/l (1^{st} to 99^{th} percentile), CDT values with reference to the results obtained with the N antiserum to human transferrin ranged from 1.19 – 2.47 %.

Blood cell count was measured using a Sysmex XE 5000™ System (Sysmex Corporation, Kobe, Japan). Erythrocyte count was performed in a separate measuring channel according to the principle of impedance measurement with hydrodynamic

focusing. Measurement of hematocrit was based on the precise erythrocyte count. The impulse of each cell is proportional to its cell volume. Cumulative impulse height summation adds the analyzed impulses and corresponding cell volumes in a defined sample volume to reach the hematocrit value. Mean corpuscular volume (MCV, [fl]) was calculated from hematocrit [%] x 10 divided by erythrocyte count [Mio/µl].

Homocysteine was determined by fully automated particle-enhanced immunonephelometry with a BN II System (Siemens Healthcare Diagnostics, Eschborn, Germany) by enzymatic conversion to S-adenosyl-homocysteine (SAH). The reference range for homocysteine is 5.8 - 11.9 µmol/L. The intra-assay coefficient of variation of the homocysteine assay was 3.4% (mean: 11 µmol/L, n=20), the inter-assay coefficient was 5.6% (mean: 11 µmol/L l, n=20).

Plasma concentrations of vitamin B1, vitamin B2 and vitamin B6 were analyzed using commercially available HPLC assays (Chromsystems Instruments & Chemicals GmbH, Munich, Germany) on an HPLC system with a fluorescence detector (Agilent.Series 1200, Agilent, Waldbronn, Germany). Reference intervals given by Chromsystems were 66.5 – 200 nmol/L for vitamin B1, 174 – 471 nmol/L for vitamin B2 and 35.2 – 110.5 nmol/L for vitamin B6. The intra-assay coefficient of variation of the vitamin B6 assay was 5.3% (mean: 10.4 µg/l; n=20), while the inter-assay coefficient of variation was 6.5% (mean: 10.5 µg/l; n=20).

Plasma concentrations of vitamin B12 and folate were measured by means of a competitive chemiluminescence immunoassay with an Access™ Immunoassay System (Beckman Coulter, Krefeld, Germany) according to the manufacturer’s instructions. The reference range of vitamin B12 is 130-680 pmol/L and the reference range of folate is 6.8-30 nmol/L. The intra-assay coefficient of variation of the vitamin B12 assay was 3.8% (mean: 487 pmol/L; n=20), the inter-assay coefficient was 4.2% (mean: 492

pmol/L; n=20). The intra-assay coefficient of variation of the folate assay was 3.1% (mean: 14.1 nmol/L; n=20), while the inter-assay coefficient of variation was 3.6% (mean: 14.3 nmol/L; n=20).

Genotyping

Genomic DNA prepared from leukocytes was used for genotyping by PCR amplification and subsequent restriction analysis of seven genetic variants of homocysteine metabolism. CBS c.844_855ins68 (change of transcript levels, Genbank Number S78267.1, no restriction digest necessary)(Tsai, Bignell, Schwichtenberg, & Hanson, 1996), DHFR c.594+59del19bp (change of transcript levels, no restriction digest necessary, rs70991108)(Johnson et al., 2004), MTHFR c.677C>T (A222V, restriction enzyme Hinf, rs1801133)(Frosst et al., 1995) and c.1298A>C (E429A, restriction enzyme MboII, rs1801131)(van der Put et al., 1998), MTR c.2756A>G (D919G, restriction enzyme HaeIII, rs1805087)(Leclerc et al., 1996), reduced folate carrier 1 (RFC1) c.80G>A (R27H, restriction enzyme Cfo1, rs1051266)(Chango et al., 2000; Linnebank et al., 2006; Moskau et al., 2005), and transcobalamin 2 c.776C>G (P259R, restriction enzyme ScrF1, rs 1801198)(Afman, Lievers, van der Put, Trijbels, & Blom, 2002).

Determination of global DNA methylation

Global DNA methylation was measured by ultra-high performance liquid chromatography (UHPLC) tandem mass spectrometry with a method adapted from the work done by Kok and coworkers (Kok et al., 2007). In short, 2 μg of DNA were hydrolyzed using formic acid. Cytosine (cyt) and 5-methylcytosine (mcyt) were separated using an Ultimate 3000 LC system (Dionex, Sunnyval, CA, USA) and an

Acquity BEH Amide column (1.7 µm, 2.1x100 mm, Water Corporation, Milford, MA, USA). The mobile phase was consisting of (A) MeCN/H_2O 1:1 and (B) MeCN/H_2O 95:5, both buffered with 10 mM NH_4HCO_2 and 0.125% HCOOH (v/v). The compounds were eluted with a linear gradient from 95 to 85% in 4 min at 0.5 mL•min^{-1} flow rate. The column was then washed with 50% of solvent B during 2 min and reconditioned during 2 min until the next injection. The UHPLC system was coupled to a 3200 QTRAP electospray ionization tandem mass spectrometer (AB Sciex, Foster City, CA, USA) operating in the multiple reaction monitoring mode and quantified using labeled internal standards. MS/MS transitions with 150 ms dwell time from *m/*z 112.0 to 95.0, 117.0 to 99.0, 126.0 to 109.0, and 130.1 to 113.0 were chosen for the detection of cyt, mcyt, 2,4-[$^{13}C_2$,$^{15}N_3$]-cyt, and 5-methyl-d_3-cytosine-6-d_1, respectively. The level of DNA methylation is expressed as the mcyt/total-cytosine ratio (mcyt/tcyt).

The intra- and inter-assay coefficient of variation (CV) for the mcyt/tcyt was 1.7% (*n* = 9) and 3.5% (*n* = 9) for calf thymus DNA (mean mCyt/tCyt ratio 6.5%), respectively.

Statistics

Deviations from Hardy-Weinberg equilibrium were separately analyzed using the χ^2 goodness of fit test, comparing observed and expected numbers for each genetic variant (α = 0.05). We used multivariate linear regression with the global leukocyte DNA methylation on the day of admission as dependent variable and age, gender, body mass index, cigarette consumption per day, daily alcohol consumption, vitamin plasma levels and the seven included single nucleotide polymorphisms as independent variables. α = 0.05 was defined as significance level.

Results

We included 363 serial patients (250 men, 113 women). Demographic and laboratory data determined at admission are shown in Table 1.

From the day of admission (day 1) 344 samples were available. A significant number of patients left the hospital before alcohol detoxification was completed or refused participation on some days of the study. Therefore, on day 3, n=59 samples and on day 7, n=75 samples were available. Self-reported alcohol consumption before admission was 218 g/d (± 119), Figure 2. 23% (n=83) of the study population were non-smokers. The rest of the study population reported tobacco consumption with a mean consumption of 18.4 (± 13.6 1SD) cigarettes per day.

Multivariate linear regression showed that on the day of admission (day 1), global leucocyte DNA methylation correlated with alcohol consumption per day and cigarette consumption per day. There was no correlation of global leukocyte DNA methylation with age, gender, vitamin plasma levels or the seven single nucleotide polymorphisms (table 3).

Samples drawn on day 3 of the study showed a positive correlation of global DNA methylation with cigarette consumption, but not with alcohol consumption. On day 7, neither alcohol consumption nor cigarette consumption were significantly associated with global DNA methylation in leucocytes. When analysis was confined to patients who remained in hospital until days 3 or 7, no significant change in DNA methylation was detectable over time.

When the small group of non-smokers was analyzed separately, there was no significant association between the global DNA methylation and daily alcohol consumption (Beta 0.162; p=0.27). When only smokers were included analysis showed an association between global DNA methylation and daily alcohol consumption on the day of admission (Beta 0.154; p=0.04).

As homocysteine plasma levels strongly depend on serum levels of folate, vitamin B1, B6 and B12 (Heese et al., 2012; Mudd, Levy, & Kraus, 2001), homocysteine was not included in multivariate analyses. Bivariate Pearson analyses revealed no significant correlation between homocysteine plasma levels and DNA methylation on day 1 ($p=0.626$), day 3 ($p=0.871$) or day 7 ($p=0.624$).

Discussion

This study shows that global DNA methylation in leukocytes correlates with the amount of daily alcohol consumption and cigarette smoking in alcohol dependent patients suggesting that both alcohol and tobacco provide an increase in global DNA methylation. We found no positive correlation with alcohol consumption on day 3, and no correlation with alcohol consumption or smoking on day 7. However, a large number of patients left the hospital before detoxification was completed on day 7. When analysis was confined to patients who remained in hospital until days 3 or 7, no significant change in DNA methylation was detectable over time. Thus, the lack of a correlation between alcohol consumption and DNA methylation on days 3 and 7 might be explained by drop-outs rather than by a change of methylation during detoxification. When smokers and non-smokers were analyzed separately, the association of global DNA methylation and daily alcohol consumption could only be found in smokers. The data of this study does not allow to decide whether this finding is due to separate analysis of a small subgroup (non-smokers), or if any impact of alcohol on DNA methylation only becomes effective in smokers.

No association of DNA methylation with vitamin or homocysteine plasma levels was found. Therefore, the increase in global DNA methylation cannot be explained by

vitamin deficiency or metabolic consequences of chronic alcohol consumption, such as liver dysfunction. This is surprising as dietary and metabolic factors are known to influence DNA methylation (Davis & Uthus, 2004; Ulrey, Liu, Andrews, & Tollefsbol, 2005), and malnutrition and liver dysfunction are common in alcohol-dependent patients. Possibly, DNA hypermethylation in alcohol-dependent patients is a direct, dose-related consequence of alcohol consumption rather than a dietary and metabolic consequence.

Our findings also show that genetic variants of key enzymes of methylation metabolism are not associated with DNA methylation in alcohol dependent patients, suggesting that the tested variants do not contribute to hypermethylation in alcohol dependent patients. According to our findings, changes in global DNA methylation in alcohol dependent patients cannot be explained by changes in homocysteine metabolism. Direct inhibition of DNA methyltransferases by alcohol has previously been described as a potential mechanism of DNA hypermethylation in alcohol dependence, which could also be the case in our patient sample (Bonsch et al., 2006; Varela-Rey et al., 2013).

Cigarette smoking and alcoholism significantly contribute to a great variety of medical conditions, especially to different forms of cancer (Varela-Rey et al., 2013; Zaridze et al., 2009). Several factors potentially promote alcohol-induced cancer development, such as toxic effects of ethanol metabolites and oxidative stress. This study provides additional evidence that aberrant patterns of DNA methylation could also contribute to alcohol-tobacco induced disease development.

Other authors have described different results. Thapar et al. found decreased DNA methylation when alcohol dependence was combined with smoking (Thapar et al., 2012). The difference between this these findings and the present study may be explained by a smaller sample size and a lower amount of alcohol consumption in the

alcohol dependent subjects included in the study of Thapar et al. (n=25 alcoholics). It has also been reported that occasional alcohol consumption in healthy subjects leads to DNA hypomethylation (Zhu et al., 2012). However, studies including healthy subjects with occasional alcohol consumption and studies including subjects with alcohol dependence and heavy consumption may not be comparable. The same argument holds true for studies with negative results of alcohol consumption and DNA methylation in healthy subjects (Ono et al., 2012; Zhang et al., 2011).

The strengths of the present study are its sample size, the characteristics of the study population including only alcohol dependent patients with heavy alcohol consumption, and the inclusion of important dietary and genetic factors, such as homocysteine and vitamin plasma levels and the polymorphisms of seven important genetic variations of methylation metabolism. One important limitation is that a significant number of study participants dropped out of the study after day 1, considerably limiting the number of available samples from day 3 and day 7 after alcohol cessation. Unfortunately, this study does not contain data which relate the differences in DNA methylation with a functional effect. Neither can it provide data on the molecular mechanisms underlying the effects of alcohol and smoking on global DNA methylation. Also, DNA methylation patterns are frequently tissue- and cell-specific, reflecting tissue-specific regulation of DNA methylation (Khavari, Sen, & Rinn, 2010). Therefore, methylation patterns should ideally be examined in the tissue that is directly related to the outcome of interest. Thus, further tissues of interest, such as brain and liver, should be tested in animal studies.

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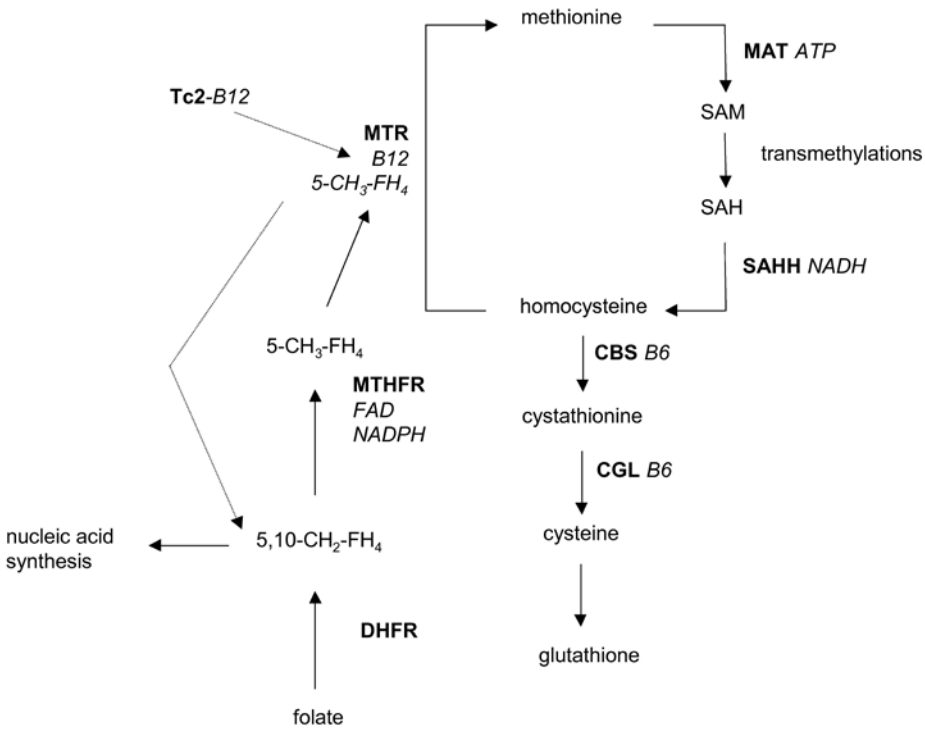

Figure 1. Homocysteine metabolism

The sulfur-containing amino acid methionine is activated to S-adenosylmethionine (SAM), which is a ubiquitous methyl group donor. The degradation product of SAM is S-adenosylhomocysteine (SAH), which is hydrolyzed to homocysteine. Homocysteine can be remethylated to methionine and SAM via methionine synthase (MTR), which depends on derivatives of folate and vitamin B12 as cofactors. Lack of these vitamins is a common cause of hyperhomocysteinemia (Mudd et al., 2001). The folate derivative is synthesized by methylenetetrahydrofolate reductase (MTHFR) and dihydrofolate reductase (DHFR), and the derivative of vitamin B12 is transported by transcobalamin 2 (Tc2). Alternatively, homocysteine can be transsulfurated by vitamin B6 dependent cystathionine β-synthase (CBS) and cystathionine gamma-lyase (CGL) to cysteine as a component of glutathione. Due to the existence of several functional variants in the genes involved in homocysteine metabolism, and to differences in dietary vitamin and amino acid uptake, disorders of homocysteine metabolism exhibit marked inter-individual differences.

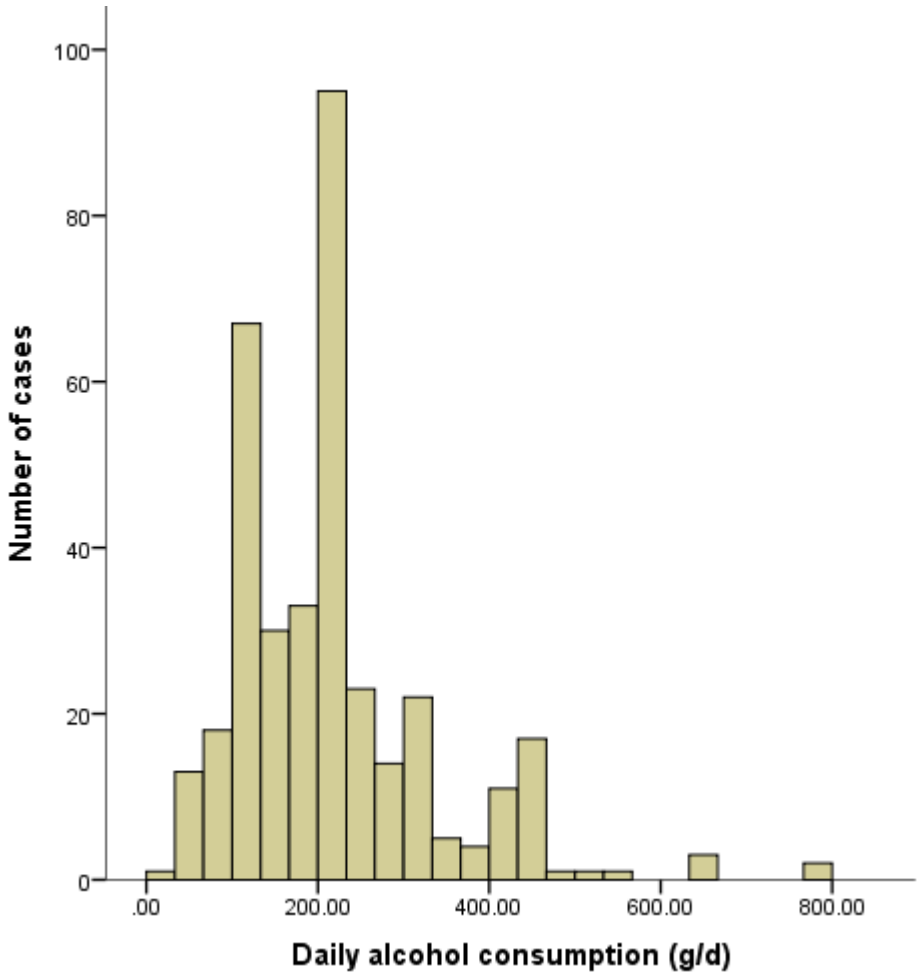

Figure 2. Histogram of the daily alcohol consumption within the study population

	minimum	maximum	mean	1 standard deviation
Age (yr)	35	87	61.8	9.4
Height (cm)	150	197	173.8	8.7
Weight (kg)	41	137	75.9	16.0
Body mass index	15.2	47.1	25.1	4.6
Daily alcohol (g)	30	840	218	119
CDT (%)	1.50	16.8	4.6	3.0
Pack years	0	76	22.4	17.3
Daily cigarettes	0	60	18.4	13.7
GGT (U/L)	3	5787	296	578
ALT (U/L)	9	311	66.6	59.3
AST (U/L)	4.9	566	83.5	87.5
MCV (fl)	34	219	95	9.7
Homocysteine (µmol/L)	5.5	131	20.8	15.2
Folate (ng/ml)	1.6	247.9	10.2	14.2
Vitamin B1 (nmol/L)	16.8	670.0	168.4	52.8
Vitamin B2 (nmol/L)	7.85	2551	308.6	136.1
Vitamin B6 (µmol/L)	3.74	522.60	30.9	38.1
Vitamin B12 (pg/L)	4.1	1500	685	295
DNA methylation (%)	1.50	5.50	3.72	0.66

Table 1. Demographic data and descriptive statistics of the study population.

The distribution of all genotypes was within the Hardy-Weinberg-equilibrium. Genotype analysis was not possible for 20 patients, either due to the quality of the sample or because patients did not agree with the genetic component of the study.

MTHFR c.1298A>C	**AA**	**AC**	**CC**
	0.48	0.44	0.08
MTHFR c.677C>T	**CC**	**CT**	**TT**
	0.46	0.44	0.10
Tc2 c.776C>G	**CC**	**CG**	**GG**
	0.26	0.50	0.24
MTR 2756A>G	**AA**	**AG**	**GG**
	0.69	0.26	0.24
RFC1 c.80G>A	**GG**	**AG**	**AA**
	0.32	0.45	0.23
CBS844ins68bp	**del/del**	**del/ins**	**ins/ins**
	0.83	0.16	0.02
DHFR c.594+59del19bp	**del/del**	**del/ins**	**ins/ins**
	0.30	0.47	0.23

Table 2. Results of genotyping for seven genetic variants of methylation metabolism.

	Beta	p
Age	-0.044	0.48
Gender	0.21	0.73
Cigarettes per day	**0.159**	**0.01**
Daily alcohol consumption g/d	**0.188**	**<0.001**
Vitamin B1 nmol/L	0.102	0.11
Vitamin B2 nmol/L	0.069	0.35
Vitamin B6 µg/L	0.057	0.356
Vitamin B12 pg/L	-0.051	0.42
Folate ng/ml	-0.072	0.33
MTHFR c.1298A>C	-0.013	0.85
MTHFR c.677C>T	0.006	0.92
Tc2 c.776C>G	0.104	0.09
MTR 2756A>G	0.019	0.76
RFC1 c.80G>A	0.063	0.31
CBS844ins68bp	0.022	0.72
DHFR c.594+59del19bp	0.010	0.87

Table 3. Multivariate linear regression of global leukocyte DNA methylation on the day of admission with age, gender, cigarette consumption per day, vitamins and seven single nucleotide polymorphisms as co-factors. $\alpha = 0.05$ was defined as significance level. n= 344 patients were included on the day of admission to the hospital.

	Beta	p
Cigarettes per day	0.374	0.04
Daily alcohol consumption g/d	0.109	0.41

Table 4. Multivariate linear regression of the global leukocyte DNA methylation on the third day after admission with age, gender, cigarette consumption per day, blood alcohol on admission, vitamins and seven single nucleotide polymorphisms as co-factors. α = 0.05 was defined as significance level. Results, which were not significant on day 1, are not shown. n = 59 patients were included.

	Beta	p
Cigarettes per day	0.084	0.57
Daily alcohol consumption g/d	-0.021	0.86

Table 5. Multivariate linear regression of the global leukocyte DNA methylation on day 7 after admission with age, gender, cigarette consumption per day, blood alcohol on admission, vitamins and the seven included single nucleotide polymorphisms as co-factors; α = 0.05 was defined as significance level. Results, which were not significant on day 1, are not shown. n= 75 patients were included.