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Genetic variants of homocysteine metabolism and Multiple Sclerosis: A case-control study

Benjamin V Ineichen¹, Salla Keskitalo¹, Melinda Farkas¹, Nadja Bain¹, Ulf Kallweit¹, Michael Weller¹, Luisa Klotz²*, Michael Linnebank¹*

¹ Dept. of Neurology, University Hospital Zurich (Zurich, CH); ² Dept. of Inflammatory Disorders of the Nervous System, and Neurooncology, University Hospital Münster (Münster, DE)

*LK and ML contributed equally.

Correspondence
PD Dr. Michael Linnebank, Department of Neurology, University Hospital Zurich, Frauenklinikstrasse 26, CH-8091 Zurich, Switzerland. Phone: 0041 44 2551111. Fax: 0041 44 2554507. E-mail: michael.linnebank@usz.ch

Running title: Homocysteine metabolism and Multiple Sclerosis

Abstract
Methylenetetrahydrofolate reductase (MTHFR) is necessary for the synthesis of methionine and S-adenosylmethionine, which is necessary for CNS (re-)myelination. The MTHFR variant c.1298A>C was associated with the development of Relapsing Remitting Multiple Sclerosis (RRMS) in a German population. This study aimed at analyzing whether further genetic variants of methionine metabolism are associated with the development or the clinical course of RRMS. Therefore, genomic DNA of 147 serial German RRMS patients and 147 matched healthy controls was genotyped for five polymorphic variants of methionine metabolism. Statistical analyses were performed using multivariate binary and linear regression analyses. We show that the insertion allele of cystathionine beta-synthase (CBS) c.844_855ins68bp and the G-allele of reduced folate carrier 1 (RFC1) c.80G>A were associated with an
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Conflict of Interest Statement

- ML is member of the D.A.CH Liga homocysteine which is an expert board of homocysteine metabolism sponsored by pharmaceutical companies that are involved in vitamin supplement production, and works as expert consultant for Desitin within a project on vitamin supplementation.
- The other authors declare no conflict of interest.
- This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Zurich, October 13th, 2013

Keywords
Homocysteine, multiple sclerosis, cystathionine beta-synthase, reduced folate carrier 1, S-adenosylmethionine, folate
Introduction

Multiple sclerosis (MS) is a complex neurological disease that affects the central nervous system (CNS) and is characterized by inflammation, demyelination, and neurodegeneration. Despite intensive research, both the etiology and the pathogenesis of MS are not completely understood. The current concepts suggest a multifactorial etiology with an interplay between immunological, environmental, and genetic factors.

Furthermore, MS has been explored with regard to methionine-homocysteine metabolism as an alliance of genetics and environment. For example, homocysteine plasma levels are increased in relapsing remitting MS (RRMS) patients [1]. In addition, vitamin B12 deficiency may be associated with RRMS, and severe vitamin B12 deficiency shares some neurodegenerative and inflammatory pathophysiological characteristics with RRMS including central demyelination [2]. Vitamin B12 is necessary for the remethylation of neurotoxic homocysteine to methionine. Methionine is a semi-essential amino acid that can be activated to S-adenosylmethionine (SAM) by methionine-adenosyltransferase (MAT, EC 2.5.1.6; figure 1). S-adenosylmethionine serves as ubiquitous methyl donor and is essential for several components of the myelin sheet, such as myelin basic protein or phospholipids. In experimental and clinical studies, anti-inflammatory and analgesic effects of SAM have been shown [3]. Transmethylation of S-adenosylmethionine results in S-adenosylhomocysteine, which becomes hydrolyzed to homocysteine, a reactive neuro- and vasculotoxic amino acid [4,5]. Homocysteine can be metabolized in two different pathways: First, cystathionine beta-synthase (CBS, EC 4.2.1.22), which depends on pyridoxal-phosphate (vitamin B6), can convert homocysteine to cystathionine. In the other pathway, methionine can be regenerated from homocysteine in the CNS via methyltetrahydrofolate-homocysteine S-methyltransferase (MTR, EC 2.1.1.13), which depends on cobalamin (vitamin B12) and 5-methyltetrahydrofolate.

Transcobalamin 2 (Tc2) acts as a transporter protein of cobalamin. Reduced folate carrier 1 (RFC1) is a transmembrane transporter protein mainly expressed in the brain [6]. Dihydrofolate reductase (DHFR, EC 1.5.1.3) reduces the precursor dihydrofolic acid (DHF) to the active component tetrahydrofolate and is thereby essential for the availability of folates in the CNS [7].

Interestingly, this metabolism shows high inter-individual variety depending on renal function, individual
vitamin and methionine uptake and on the genetic profile of methionine-homocysteine metabolism, i.e. on the expression of allelic variants with functional consequences for the encoded enzymes, transporter proteins or carriers. Severe mutations affecting the enzymes of methionine-homocysteine metabolism can lead to CNS demyelination [8]. Thus, we hypothesize that variants with functional consequences impact chronic inflammatory demyelinating diseases. Previously we observed that the allelic variant 5,10-methylenetetrahydrofolate reductase (MTHFR) c.1298A>C was associated with the incidence of RRMS in a German cohort [9]. In the present study we extended the analysis of that cohort by testing whether five further functionally relevant variants of methionine-homocysteine metabolism are associated with the development or the age of onset of RRMS (table 1).

Materials and methods

Study population

The study population consisted of 147 serial German patients of Caucasian origin with RRMS according to McDonald criteria [10] (106 female; mean age ± standard deviation (SD): 31.5±8.7 years). The patients were recruited from the Department of Neurology, University of Bonn, Germany. In addition, we analyzed 147 German age- and gender-matched healthy local controls of Caucasian origin without apparent signs or a history of neurological or immunological disease (106 female; 30.5±7.3 years). All individuals of the present study were unrelated. The local ethics committee approved the study and informed written consent was received from all subjects.

Genotyping

DNA was extracted from peripheral leukocytes using QIAquick DNA extracting Kit (Qiagen, Hilden, Germany). Genomic DNA was amplified by PCR (Thermocycler T Professional, Biometra, Göttingen, Germany; Taq Polymerase and Polymerase buffer, Roche Diagnostics, Basel, Switzerland; Primers, Microsynth, Balgach, Switzerland) followed by restriction enzyme digestion (New England Biolabs, Ipswich, USA) and agarose gel electrophoresis. The PCR and restriction analysis conditions were
described previously [11-15].

Statistical analysis
Data were analysed with the Statistical Package for the Social Sciences (SPSS statistics, Version 16). To test the independent association of the genetic variants on development of RRMS, all variants were simultaneously analysed together with age and gender as covariables in multivariate binary regression analysis. Accordingly, the association with age of onset was analysed using multivariate linear regression. Statistical significance was defined with $p < 0.05$.

Results
There were no significant differences concerning gender ($\chi^2 = 0.263, p = 0.627$) or age ($F = 0.88, p = 0.350$) between patients and controls. The distribution of genotypes did not deviate from the Hardy-Weinberg equilibrium. Allele frequencies of patients and controls were similar (table 2). However, the wildtype G allele of RFC1 c.80G>A was associated with an earlier age of RRMS onset, suggesting a gene dose effect (AA-AG-GG: 35-31-29; standardized regression coefficient Beta: 0.282; $p=0.005$, table 3).

Additionally, the mutant CBS c.844_855ins68bp allele carrying the insertion (“ins”-allele) was associated with an earlier age of RRMS onset, also suggesting a gene dose effect (del/del-del/ins-ins/ins, median age of onset in years: 32-26-25; standardized regression coefficient Beta: 0.216; $p=0.030$). The other allelic variants did not reveal a significant difference in the age of MS onset.

Discussion
This study indicates an association of variants of CBS and RFC1 with the age of RRMS onset. The corresponding proteins are involved in methionine metabolism that is necessary for CNS myelination. In the CNS of mice, CBS is highly expressed in the hippocampus and cerebellum, whereas expression levels are unknown for human CNS [12,16]. The incomplete tandem repeat CBS c.844_855ins68bp leads to splice variants of CBS mRNA associated with higher CBS expression and lower homocysteine plasma.
levels (figure 1) [13,14]. As a likely consequence, reduced amounts of homocysteine are metabolized via
the alternative pathway, i.e. remethylation to methionine and S-adenosylmethionine. By reducing the (re-)methylation capacity of the CNS, this may lead to an earlier clinical manifestation of demyelinating
diseases in predisposed individuals.

RFC1 acts as a transmembrane transporter protein for 5-methyltetrahydrofolate and is thereby essential for
folate distribution within the CNS. The RFC1 missense variant c.80G>A (p.R27H) might impact the CNS
folate levels [17] and since folate is a cofactor for methionine synthesis, this might result in lower (re-)methylation capacity, analogue to CBS c.844_855ins68bp.

Vitamin B12 has two functions in human metabolism: it functions as a cofactor for methylmalonyl CoA-
mutase for the synthesis of succinyl-CoA and as a cofactor for methionine synthesis. Previous studies
report vitamin B12 deficiency in patients with RRMS [18-20] and a specific association between the age
of onset of first neurological symptoms of MS and vitamin B12 metabolism [21]. Our data suggest that
also genetic factors of methionine metabolism may be relevant for RRMS etiology or pathology.
Methionine metabolism can easily be manipulated by the supplementation of vitamins and amino acids.
Therefore, the re-evaluation of our results in independent studies may be reasonable to search for
nutritional strategies to influence demyelinating diseases.
References


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al. The reduced folate carrier (SLC19A1) c.80G>A polymorphism is associated with red cell

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Tables

Table 1 – Genetic variants analyzed in this study

<table>
<thead>
<tr>
<th>Allelic variant</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cystathionine beta-synthase c.844_855ins68bp</td>
<td>splice alteration increasing the transcript and expression level</td>
<td>Linnebank et al., 2001</td>
</tr>
<tr>
<td>dihydrofolate reductase c.594+59del19bp</td>
<td>intronic deletion supposedly affecting the transcript level</td>
<td>Johnson et al., 2004</td>
</tr>
<tr>
<td>reduced folate carrier 1 c.80G&gt;A</td>
<td>missense mutation p.R27H</td>
<td>Yates et al., 2005</td>
</tr>
<tr>
<td>transcobalamin 2 c.776C&gt;G</td>
<td>missense mutation p.P259R</td>
<td>Afman et al., 2002</td>
</tr>
</tbody>
</table>
Table 2 – Frequencies of the allelic variants in patients and controls. There is no statistical significant difference in allelic frequencies between patients and control.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genotype</th>
<th>Patients</th>
<th>Controls</th>
<th>Wald; p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBS c.844_845ins68bp</td>
<td>del/del</td>
<td>0.79</td>
<td>0.83</td>
<td>0.817; 0.366</td>
</tr>
<tr>
<td></td>
<td>del/ins</td>
<td>0.20</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ins/ins</td>
<td>0.01</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DHFR c.594+59del19bp</td>
<td>del/del</td>
<td>0.10</td>
<td>0.20</td>
<td>0.010; 0.921</td>
</tr>
<tr>
<td></td>
<td>del/ins</td>
<td>0.61</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ins/ins</td>
<td>0.29</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>MTR c.2756 A&gt;G</td>
<td>AA</td>
<td>0.62</td>
<td>0.70</td>
<td>0.357; 0.550</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>0.33</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>0.05</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>RFC1 c.80 G&gt;A</td>
<td>GG</td>
<td>0.33</td>
<td>0.44</td>
<td>0.842; 0.359</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>0.48</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>0.19</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>Tc2 c.776 C&gt;G</td>
<td>CC</td>
<td>0.29</td>
<td>0.37</td>
<td>2.69; 0.101</td>
</tr>
<tr>
<td></td>
<td>CG</td>
<td>0.45</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>0.26</td>
<td>0.17</td>
<td></td>
</tr>
</tbody>
</table>
Table 3 – RFC1 and CBS genotypes and age of onset. The wildtype G allele of RFC1 c.80G>A was associated with an earlier age of RRMS onset, suggesting a gene dose effect (AA-AG-GG: 35-31-29; standardized regression coefficient Beta: 0.282; p=0.005, table 3). Additionally, the mutant CBS c.844_845ins68bp allele carrying the insertion (“ins”-allele) was associated with an earlier age of RRMS onset, also suggesting a gene dose effect (del/del-del/ins/ins/ins, median age of onset in years: 32-26-25; standardized regression coefficient Beta: 0.216; p=0.030). The other allelic variants did not reveal a significant difference in the age of MS onset. Median age of first symptoms of MS ± 1 standard deviation.

<table>
<thead>
<tr>
<th>RFC1 c.80G&gt;A</th>
<th>AA</th>
<th>GA</th>
<th>GG</th>
<th>Beta; p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age of MS onset</td>
<td>35 ± 14</td>
<td>31 ± 9</td>
<td>29 ± 8</td>
<td>0.282; 0.005</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CBS c.844_845ins68bp</th>
<th>del/del</th>
<th>del/ins</th>
<th>ins/ins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age of MS onset</td>
<td>32 ± 9</td>
<td>26 ± 6</td>
<td>25 ± 8</td>
</tr>
</tbody>
</table>
**Figure 1** - The sulfur-containing amino acid methionine can be activated by methionine-adenosyltransferase (MAT) to S-adenosylmethionine (AdoMet), which is an ubiquitous methyl-group donor e.g. for myelin sheet components like phospholipids or myelin basic protein. The degradation product of AdoMet is S-adenosylhomocysteine (AdoHcy), which becomes hydrolyzed to homocysteine in a reversible reaction. Homocysteine can either be transsulfurated by vitamin B6-dependent CBS or remethylated to methionine and AdoMet via methyltetrahydrofolate-homocysteine S-methyltransferase (MTR), which depends on vitamin B12 and 5-methyltetrahydrofolate (5-mTHF) as cofactors. The reduced folate carrier 1 (RFC1) acts as a transmembrane transport protein for 5-mTHF and is essential for folate uptake into the CNS. Dihydrofolate reductase (DHFR) reduces the precursor dihydrofolic acid (DHF) to the active tetrahydrofolate (THF), which is further converted into 5-methyltetrahydrofolate by 5,10-methylenetetrahydrofolate reductase (MTHFR). Tc2 is as a transport protein for vitamin B12.