Chapter 7

The influence of the number of haplotypes of \textit{MTHFR} 1298A-677C alleles on the predicted probability to respond to methotrexate in early RA patients

Wouter M. Kooloos\textsuperscript{1}, Judith A.M Wessels\textsuperscript{1}, S.M. van der Kooij\textsuperscript{2}, Cornelia F. Allaart\textsuperscript{2}, Tom W.J. Huizinga\textsuperscript{2} and Henk-Jan Guchelaar\textsuperscript{1}

\textsuperscript{1}Clinical Pharmacy & Toxicology, Leiden University Medical Center, Leiden, The Netherlands.
\textsuperscript{2}Rheumatology, Leiden University Medical Center, Leiden, The Netherlands.

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**Introduction**

Two nonsynonymous genetic variants within the methylenetetrahydrofolate reductase (*MTHFR*) gene, *MTHFR* 677C>T (rs1801133) and *MTHFR* 1298A>C (rs1801131), have been extensively studied over the last decade. It has been demonstrated that these single nucleotide polymorphisms (SNPs) cause an amino acid replacement at codon 222 (Ala222Val) and at codon 429 (Glu429Ala), respectively (1;2). Additional haplotype analyses have revealed that these polymorphisms are in linkage disequilibrium (LD), meaning that a combination of C and A alleles of the two SNPs occur more frequently in a population than would be expected on a random basis (3).

*MTHFR* is a key enzyme in the folate pathway, which catalyzes the conversion of homocysteine to methionine for a variety of metabolic reactions (4). In vitro studies have demonstrated that *MTHFR* 677C>T and *MTHFR* 1298A>C, are associated with diminished enzyme activity of MTHFR leading to homocysteinemia and a disturbed folate balance (1;2;5). Moreover, a functional interaction between these two SNPs have been observed, which resulted in a synergistic effect on MTHFR enzyme activity (6).

Alteration of enzymatic function related to these SNPs might modulate disease susceptibility, but studies also have proposed *MTHFR* 677C>T and *MTHFR* 1298A>C as genetic determinants of clinical therapy outcome. These studies have mainly been performed with MTX (7-10). Notably, MTX functions as a folate antagonist, which inhibits indirectly MTHFR. Recently, a meta-analysis reported that the *MTHFR* 677C>T polymorphism was significantly associated with increased toxicity in rheumatoid arthritis (RA) patients on MTX treatment (11). Regarding efficacy, opposing results have been found for *MTHFR* 677C>T and *MTHFR* 1298A>C, whereas most of the performed studies did not find any association (12-17). Notably, a substantial part of the studies did not take linkage disequilibrium between these two *MTHFR* SNPs into account. Only a few studies have examined associations between haplotypes of *MTHFR* 1298A>C and *MTHFR* 677C>T and MTX efficacy in RA patients. In the reports of Urano et al and Taniguchi et al no significant associations of response defined as lower MTX dosages and the haplotype *MTHFR* 1298A-677C were found (16;18). In addition, in the study of Urano et al, carriers of the *MTHFR* 1298C-677C haplotype did show a relation with lower MTX dosage (18). Furthermore, Hughes et al reported no significant associations with MTX treatment outcome with any of the haplotypes comprising *MTHFR* 1298A>C and *MTHFR* 677C>T (14). In the study of Kurzawski et al a lower probability of remission was seen in patients genotyped for *MTHFR* 1298AA and 677CC in comparison with patients with 1 or 0 copies of 677C-1298A. Moreover, the presence of both 677T and 1298C alleles was associated with an increased frequency of remission (8).

We have reported that MTX treated RA patients genotyped for *MTHFR* 1298AA and 677CC were associated with efficacy (19). Additional analyses demonstrated that the number copies of the haplotype as determined by the 1298A-677C SNPs importantly strengthened the association with good clinical improvement (achieving an improvement of DAS >1.2) at 6 months.

Previously, we developed a pharmacogenetic model in combination with clinical factors to predict MTX efficacy in recent-onset RA. In this study it was reported that the clinical factors gender, rheumatoid factor combined with smoking status and disease activity at baseline together with genetic factors were predictive for MTX response. The included genetic factors were the SNPs *AMPD1* 34C>T, *ATIC* 347C>G, *ITPA* 94 A>C and *MTHFD1* 1958G>A. The prediction resulted in the classification of 60% of the RA patients into MTX responders and nonresponders (defined as achieving DAS >2.4 or DAS ≤2.4, respectively), with a 95% and 86% true positive and negative response rate, respectively. Thus 40% of the patients (n=74) could not be classified resulting in a group with a predicted intermediate probability of response to MTX (a score between 3.5 and 6 points). For these patients an evaluation was added using good clinical improvement at 3 months as an intermediate
The influence of the number of haplotypes of MTHFR 1298A-677C alleles on the predicted probability to respond to methotrexate in early RA patients

endpoint. This would enable clinicians to decide on continuation or alteration of MTX therapy at an early stage of treatment and could possibly prevent treatment delay of three months (unnecessary MTX exposure due to inefficacy). Still, with the addition of this interim evaluation, categorization into responders and nonresponders at 6 months remained suboptimal. Regarding the influence of MTHFR 1298A>C and MTHFR 677C>T and particularly their haplotypes on achieving good clinical improvement, addition of these genotypes to the pharmacogenetic model to improve categorization of patients in the predicted intermediate response group could be beneficial.

In this paper, it is aimed to assess the discriminative performance of the pharmacogenetic predictive model by the addition of the number of copies of the MTHFR 1298A-677C haplotype. Also, it is aimed to increase the percentage of patients for which the pharmacogenetic model predicts response or nonresponse by incorporating the number of copies of this haplotype.

Methods

Patients
Characteristics of the patients enrolled in this study are similar to characteristics earlier described by our group (19;20). Briefly, the 205 patients enrolled in this study comprised a subcohort of the patients participating in the BeSt study. Main inclusion criteria were a diagnosis of early RA as defined by the American College of Rheumatology (ACR) 1987 criteria for RA (21), age ≥18 years, symptom duration of <2 years and active RA according to the BeSt study protocol (22). Main exclusion criteria were previous treatment with DMARDs other than antimalarials and concomitant treatment with an experimental drug. Further details have been published elsewhere (22). The local ethics committee at each participating hospital approved the study protocol. All patients gave informed consent before enrolment into the study.

Study design and evaluation of clinical efficacy
The discriminative performance of the pharmacogenetic model was defined as the difference in area under the curves (AUCs), obtained by plotting receiver operating curves (ROCs), between curves with and without inclusion of two copies of the haplotype into the model. Notably, AMPD1 34C>T, ITPA 94C>A, ATIC 347C>G and MTHFD1 1958G>A have been related with good response at 6 months (defined as obtaining a DAS ≤2.4) (23). Therefore, the discriminative performance of carrying 1 or 2 and 2 copies alone of the MTHFR haplotype in comparison with these four good response-related SNPs were presented according to the endpoint good response at 6 months. For this analysis 205 patients were included in the study. Of these, 74 patients were predicted having an intermediate probability of response to MTX (20) and this cohort was analysed for increasing the percentage of patients for which the pharmacogenetic model predicts response or nonresponse by incorporating the number of copies of the haplotype. Hereby, the primary goal was good clinical improvement (achieving an improvement of DAS >1.2) at 3 months.

Genotyping
DNA isolation, genotyping techniques and success rates of the SNPs MTHFR 1298A>C, MTHFR 677C>T, AMPD1 34C>T, ATIC 347C>G, ITPA 94 A>C and MTHFD1 1958G>A were as previously described (19;23).

Statistical analysis
Frequencies of the number of copies of the MTHFR haplotypes were calculated using chi-square tests. Additionally, the estimated probability for achieving good response was calculated for each
individual patient by adding the number of *MTHFR* haplotype to the prediction model. Next, receiver operating characteristic (ROCs) were derived to demonstrate the discriminative performance of the model with and without 1 or 2 and 2 copies alone of the *MTHFR* haplotype. In the predicted intermediate responders, associations of genotypes and haplotypes with treatment outcome were analyzed using chi-square tests and/or Fisher exact tests. All statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

**Results**

**Assessment of the discriminative performance of the pharmacogenetic predictive model by the addition of the number of copies of the *MTHFR* 1298A-677C haplotype.**

In the cohort (N=205) the following haplotype distributions were present: for 2 copies 677C-1298A 13% (n=27); for 1 copy 677C-1298A 40% (n=82); for 0 copies 677C-1298A 46% (n=94) (Table 1). No differences in predicted probability with the addition of number of copies of the haplotypes as predictors for good response were found (p>0.05). Specifically, similar AUCs were observed for all three ROC curves, as depicted in figure 1 (AUC=86% and 95% C.I.= 81-91%). Furthermore, comparison of ROC curves showed that the AUCs of the *MTHFR* 1 or 2 and 2 copies genotypes were smaller compared to the AUCs of other SNPs included in the model (table 2- ROCs not displayed). Specifically, the discriminative ability (AUC) of both number carriers of the *MTHFR* haplotype was 50% (95%C.I. 40-59%) in comparison with an AUC of 61% of the single SNP *ATIC* 347 C>G. Also, when the AUC of the *MTHFR* 2 copies genotype was compared with the AUC of *AMPD1* 34C>T, *ITPA* 94C>A, *ATIC* 347C>G and *MTHFD1* 1958G>A combined a significant difference was observed (50% vs. 69%, respectively: p=0.009- not displayed in table 2).

<table>
<thead>
<tr>
<th>MTHFR 1298 A&gt;C</th>
<th>AA</th>
<th>AC</th>
<th>CC</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td><strong>MTHFR 677C&gt;T</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>27</td>
<td>43</td>
<td>22</td>
<td>92</td>
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<td>CT</td>
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<tr>
<td>TT</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>84</td>
<td>96</td>
<td>23</td>
<td>203</td>
</tr>
</tbody>
</table>

Table 1. *MTHFR* 1298A>C and *MTHFR* 677C>T genotype distribution to demonstrate the number of copies of the *MTHFR* 1298A-677C haplotype
The influence of the number of haplotypes of MTHFR 1298A-677C alleles on the predicted probability to respond to methotrexate in early RA patients

Figure 1. Receiver operating characteristic curve for predicting the response to methotrexate with and without the number of copies of the MTHFR 1298A-677C haplotype

Abbreviation(s): MTHFR= methylenetetrahydrofolate reductase.
Optimalization of the clinical pharmacogenetic model to predict methotrexate treatment response: the influence of the number of haplotypes of MTHFR 1298A-677C alleles on probability to respond.

In the group of patients with a predicted intermediate response to MTX (n=74), the number of haplotypes of the MTHFR 1298A>C and 677C>T SNPs was assessed. The genotypes of the two single SNPs were not significantly associated with good clinical improvement at 3 months (data not shown). Subsequently, diplotype analyses demonstrated that none of these diplotypes in this specific group of patients appeared able to significantly enhance prediction of achieving good clinical improvement at 3 months (data not shown). Additionally, trend analyses were performed to explore differences in carrying the number of alleles of the 1298A and 677C haplotype (0, 1 and 2). The data revealed no significant differences between the number of MTHFR haplotype and good clinical improvement at 3 months (table 3).

<table>
<thead>
<tr>
<th>SNPs</th>
<th>AUC in % (95% C.I.)</th>
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<tbody>
<tr>
<td>MTHFR 2 copies vs. 0 or 1</td>
<td>50% (42-59%)</td>
</tr>
<tr>
<td>MTHFR 1 or 2 copies vs. 0</td>
<td>50% (42-59%)</td>
</tr>
<tr>
<td>ITPA 94 CC vs. CA or AA</td>
<td>56% (47-64%)</td>
</tr>
<tr>
<td>MTHFD1 1958 GG or GA vs. AA</td>
<td>56% (47-64%)</td>
</tr>
<tr>
<td>AMPD1 34 CC vs. CT or TT</td>
<td>57% (49-66%)</td>
</tr>
<tr>
<td>ATIC 347 CC vs. CG or GG</td>
<td>61% (53-70%)</td>
</tr>
</tbody>
</table>

Table 2. AUCs (95% C.I.) for predicting the response to methotrexate of the number of copies of the haplotype MTHFR 1298A-677C, ITPA 94C>A, MTHFD1 1958G>A, AMPD1 34C>T, ATIC 347C>G

Abbreviation(s): AUC= area under the curve; MTHFR= methylenetetrahydrofolate reductase, AMPD1=adenosine monophosphate deaminase, ATIC= aminomimidazole, carboxamide ribonucleotide transformylase, ITPA= inosine triphosphate pyrophosphatase; MTHFD1= methylenetetrahydrofolate dehydrogenase and SNP= single nucleotide polymorphism
The influence of the number of haplotypes of MTHFR 1298A-677C alleles on the predicted probability to respond to methotrexate in early RA patients

Discussion

In this study, it is concluded that the predictive performance of the pharmacogenetic model to predict the efficacy of MTX therapy in this group of early RA patients is not improved when the MTHFR haplotype is included in the model. Moreover, the discriminative effect for the prediction of MTX efficacy including 1 or 2 or (solely) 2 copies of the 1298A and 677C haplotype was significantly smaller compared with the four SNPs AMPD1 34C>T, ITPA 94C>A, ATIC 347C>G and MTHFD1 1958G>A.

Furthermore, it is observed that incorporation of the number of alleles of the favorable MTHFR 1298A en 677C haplotype (0, 1 or 2) into the predictive model does not lead to improvement of the number of classifiable patients in those with an intermediate probability of response to MTX. However, some remarks have to be made concerning the analysis of the MTHFR haplotype.

Notably, current analyses were performed in Caucasian patients. In this way, our results can not easily be compared with studies representing other ethnicities. This is demonstrated in the study of Hughes et al (14), which revealed significant differences in haplotype distribution between Caucasians and African-Americans. Namely, alternative distributions in haplotypes between ethnic groups are caused by differences in allele frequencies of the two SNPs in these groups resulting in different values for D’ (D-prime), a value ascribing LD. Specifically, Hughes et al (14) reported that the D’ value for the two SNPs was 0.955, indicating strong LD. However, in African-Americans the D’ value is much lower (0.408), indicating less linkage disequilibrium (www.hapmap.org). Alternative values of linkage disequilibrium could therefore explain the different results seen in the reports of Urano et al (18) and Taniguchi et al (16), which studied the influence of the haplotype on response in patients with Asian backgrounds. Furthermore, the degree of LD could be biased by the number of patients under study, since small numbers of included patients may lead to differences in haplotype distribution.

Also, to elucidate an additive effect of the number of risk haplotypes on treatment outcome, a gene dose effect is informative. Hereby, a gene dose effect compromises a linear relationship between the number of haplotypes and clinical response. In a part of above described studies, this specific trend is not seen. For example in study of Kurzawski et al (8), patients carrying 1 haplotype MTHFR 1298A-677C showed increased response when compared with patients carrying 0 or 2 haplotypes meaning that a gene dose effect was lacking. In this way, a clear biological explanation for involvement in MTX efficacy remains difficult to acquire.

Finally, it has been demonstrated that the pathophysiological consequences of MTHFR genetic variants (especially the C677T polymorphism) are significantly affected by demographic and environmental factors such as nutritional (folate) status, age, smoking and alcohol intake, parameters that may bias genetic associations with therapy outcome to MTX (24-26). Therefore, multivariate analysis including these confounding factors is inevitable. Regarding our analyses, no significant changes in results are expected.

In conclusion, incorporation of the number of alleles of the MTHFR 1298A and 677C haplotype (0, 1 or 2) into the pharmacogenetic model did not lead to improvement of the model, since no associations with achieving good clinical improvement at 3 months (ΔDAS>1.2) were seen in patients with an intermediate probability of response to MTX. Moreover, the results presented in this paper suggest that a (leading) role for the MTHFR 1298A and 677C haplotype with regard to predicting efficacy of MTX monotherapy in early RA patients seems unlikely. Future research is necessary to elucidate the exact pharmacogenetic and biological role of MTHFR 1298A>C and MTHFR 677C>T and their haplotypes in the efficacy of MTX in RA.
The influence of the number of haplotypes of MTHFR 1298A-677C alleles on the predicted probability to respond to methotrexate in early RA patients

References


(20) Wessels JA, van der Kooij SM, le CS, Kievit W, Barerra P, Allaart CF et al. A clinical pharmacogenetic model to predict the efficacy of methotrexate monotherapy in recent-
The influence of the number of haplotypes of MTHFR 1298A-677C alleles on the predicted probability to respond to methotrexate in early RA patients.


