

## EXTENDED REPORT

# Methotrexate related adverse effects in patients with rheumatoid arthritis are associated with the A1298C polymorphism of the MTHFR gene

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**Background:** There is an association between C677T polymorphism of the methylenetetrahydrofolate reductase (MTHFR) gene and methotrexate related toxicity.

**Objective:** To examine the relations between the recently described A1298C polymorphism of the MTHFR gene, plasma homocysteine, methotrexate toxicity, and disease activity in patients with rheumatoid arthritis.

**Design:** A cross sectional study on 93 methotrexate treated patients with rheumatoid arthritis, comprising a clinical interview and physical examination to determine disease activity and methotrexate related adverse reactions. Genotype analysis of the MTHFR gene was carried out and fasting plasma homocysteine and serum folate concentrations were measured. The data were analysed using univariate analysis. Allele and genotype distributions were compared with those of a healthy control group.

**Results:** The frequency of the 1298CC genotype (24.7%) in the rheumatoid study group was greater than expected in the general population (12.8%,  $p < 0.001$ ). This genotype was associated with a significantly low rate of methotrexate related side effects. The odds ratio for side effects in patients with wild type 1298AA genotype v 1298CC genotype was 5.24 (95% confidence interval, 1.38 to 20). No correlation of disease activity variables or plasma homocysteine with MTHFR A1298C and C677T polymorphisms was observed.

**Conclusions:** 1298CC polymorphism was more common in methotrexate treated rheumatoid patients than expected in the population, and was associated with a reduction in methotrexate related adverse effects. The A1298C polymorphism of the MTHFR gene may indicate a need to adjust the dose of methotrexate given to patients with rheumatoid arthritis.

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Methotrexate is the most commonly used disease modifying antirheumatic drug (DMARD) for rheumatoid arthritis and many other immune diseases.<sup>1, 2</sup> Methotrexate is a structural analogue of folic acid which inhibits dihydrofolate reductase, an enzyme responsible for tetrahydrofolate regeneration. Methotrexate may influence several other steps in folate metabolism and causes cellular folate depletion and possibly inhibition of methylenetetrahydrofolate reductase (MTHFR). MTHFR synthesises 5-methyltetrahydrofolate, which acts as the methyl donor for remethylation of homocysteine to methionine.<sup>3, 4</sup>

Several polymorphisms of the MTHFR gene have been described. The most studied—C677T polymorphism—results in decreased enzyme activity and hyperhomocysteinaemia in the general population.<sup>5</sup> Studies of homocysteine metabolism in patients with rheumatoid arthritis treated with methotrexate have produced controversial results,<sup>6–12</sup> and contradictory effects of C677T polymorphism on plasma homocysteine concentrations have been found.<sup>9, 11</sup> An association between the 677TT polymorphism and methotrexate toxicity has been demonstrated in patients suffering from various malignant diseases,<sup>13–15</sup> and two studies have shown such an association in patients with rheumatoid arthritis.<sup>16, 17</sup> This toxicity is correlated with raised plasma homocysteine concentrations.<sup>15</sup>

The recently described A1298C polymorphism is associated with MTHFR activity and may affect plasma homocysteine level.<sup>18–20</sup>

Our aims in the present study were, first, to investigate the distribution of A1298C and C677T polymorphism in our methotrexate treated rheumatoid patients compared with a healthy control group; second, to determine the relation between A1298C polymorphism and rheumatoid arthritis activity, methotrexate efficacy, and adverse effects; and third, to explore the influence of A1298C polymorphisms on plasma homocysteine levels in methotrexate treated patients with rheumatoid arthritis.

## METHODS

### Patients

Ninety three consecutive patients with rheumatoid arthritis who were treated with methotrexate were recruited from the rheumatology outpatient clinics of three university hospitals. All patients met the American College of Rheumatology revised criteria for rheumatoid arthritis.<sup>21</sup> One patient with compromised renal function was not included.

All participants underwent a clinical interview and physical examination to determine methotrexate toxicity, disease activity, and dietetic history. Methotrexate related adverse effects were defined as one or a combination of gastrointestinal symptoms (nausea, abdominal pain, diarrhoea) appearing repeatedly in association with methotrexate

**Abbreviations:** DMARD, disease modifying antirheumatic drug; EULAR, European League Against Rheumatism; MTHFR, methylenetetrahydrofolate reductase

consumption, oral ulcers, disturbed liver functions tests (alanine aminotransferase more than twice the upper limit of normal values), methotrexate induced or aggravated skin nodules, and haematological adverse reactions (leucocyte count below 3500/mm<sup>3</sup>). Side effects were evaluated during patient recruitment. Disease activity was evaluated by the EULAR criteria for rheumatoid arthritis activity, which include the number of tender and swollen joints, pain score, and physician's and patient's global score on a visual analogue scale.<sup>22</sup>

Blood was drawn after 10 hours of fasting on the third to the sixth day after the weekly methotrexate dose. Plasma and serum were separated promptly and stored until assayed at -80°C. Both the rheumatoid group and the control group for genotype frequency analysis were Jewish. The population control sample was drawn from the national population registry.<sup>20</sup> In both groups, the proportion of Ashkenazi to non-Ashkenazi Jews was 1:1.

The study was approved by the ethics committee of the Israeli Ministry of Health and informed consent was obtained from all participants.

**Biochemical measurements and genetic analysis**

Serum folate concentrations were determined by commercial sets (Vitamin B12 Elecsys reagent kit, catalogue No 1820753, and Folate Elecsys reagent kit, catalogue No 1820761), using an automated electrochemiluminescence immunoassay (ELICIA). The assays were done on a Roche Elecsys 2010 immunoassay analyser.

Plasma total homocysteine—the sum of protein bound and free homocysteine—was determined by a procedure modified from Araki and Sako.<sup>23</sup> In our procedure a 100 µl plasma sample was treated with tributylphosphine to reduce disulphide bonds, resulting in free homocysteine. After protein precipitation, the supernatant fraction was alkalinised and treated with a fluorescent probe (fluorobenzo-2-oxa-1,3-diazole-4-sulfonate, SDBF). Total homocysteine was determined after reverse phase high performance liquid chromatography using isocratic elution and fluorimetric detection.

Genomic DNA was prepared from peripheral blood. The A1298C and C677T polymorphisms of the MTHFR gene were analysed by polymerase chain reaction amplification, restriction enzyme digestion, and DNA fragment separation by electrophoresis, as described previously.<sup>20</sup>

**Statistical methods**

For the comparison of quantitative variables between the two groups (with and without mutation), two-sample *t* tests and

the non-parametric Mann-Whitney tests were applied. The  $\chi^2$  test and Fisher's exact test were used to assess the association between the mutation groups and the different side effects, or any other qualitative variables. In order to test whether the distributions of the genotypes or the alleles in the patient group differed from the distribution in the control group, the one-sample  $\chi^2$  test was applied.

All statistical tests were two tailed and a probability (*p*) value of 5% or less was considered statistically significant.

**RESULTS**

Table 1 compares various characteristics between patients carrying the 1298 AA genotype and those carrying the 1298 AC and CC genotypes. Patients in all three groups received similar doses of methotrexate (mean (SD), 11.9 (3.8) mg/week) for a similar duration of time (3.7 (3.4) years). The cumulative methotrexate dose did not differ significantly between the three groups. No difference between groups was observed for the number of patients using additional DMARDs or prednisone and individual indices of disease activity. None of the 1298 genotypes (AA, AC, and CC) showed an association with disease activity. The only significant difference between the genotypes was the amount of folate supplementation: patients with the 1298 AA genotype received 7.45 (10.26) mg/week of folic acid, while those carrying the 1298AC and CC received 2.61 (3.48) and 3.73 (3.34) mg/week, respectively.

**Genotype and allele distribution**

We examined allele and genotype distribution among our rheumatoid patients and compared them with the allele and genotype distribution among a control population of 377 healthy subjects previously characterised by our group.<sup>20</sup> Using a sample  $\chi^2$  analysis, the genotype distribution among the rheumatoid patients was significantly different from the genotype distribution among the control population: 24.7% of the rheumatoid population carried the 1298CC genotype, compared with only 12.8% of the control population (*p*<0.001) (table 2). When allele frequency was compared between the two groups, no significant difference was found, and the distribution of the A and C alleles was similar (data not shown).

**MTHFR A1298C genotype polymorphism, clinical factors, and methotrexate related side effects**

Previous reports have found an association between the MTHFR 677 TT genotype and methotrexate induced side effects.<sup>16 17</sup> We showed that the genotype 1298CC is associated with a low rate of methotrexate related side effects

**Table 1** Patient characteristics

	Total (n = 93)	Genotype		
		1298CC (n = 23)	1298AC (n = 20)	1298AA (n = 50)
Age (years)	58.74 (13.69)	54.78 (14.17)	59.79 (10.92)	60.16 (14.50)
Female (%)	82.8	78.3	90	82
Duration of methotrexate treatment (years)	3.70 (3.43)	2.92 (3.18)	4.05 (3.44)	3.92 (3.48)
Methotrexate dose (mg/week)	11.93 (3.81)	11.41 (3.76)	11.58 (3.84)	12.40 (3.88)
Treated with folate (%)	58.8	56.5	40.0	67.3
Folate supplementation (mg/week)	5.42 (8.03)	3.73 (3.34)	2.61 (3.48)	7.45 (10.26)*
C reactive protein (mg/100 ml)	2.14 (3.15)	2.87 (3.86)	1.41 (1.06)	2.09 (2.85)
No of tender joints	6.68 (1.3)	7.30 (1.50)	4.74 (1.20)	7.18 (1.30)
No of swollen joints	2.47 (1.20)	3.30 (1.30)	2.21 (1.10)	2.20 (1.20)
Pain score	3.59 (2.91)	3.65 (2.93)	4.00 (3.00)	3.40 (2.85)
Patient assessment	3.55 (2.81)	3.54 (2.74)	2.93 (2.18)	3.80 (2.71)
Physician assessment	2.94 (2.26)	2.77 (1.97)	2.83 (2.31)	3.06 (2.38)
Morning stiffness (hours)	0.53 (0.84)	0.58 (0.70)	0.43 (0.93)	0.54 (0.79)

Values are mean (SD).

No significant differences between 1298 AA AC and CC genotype carriers for various clinical determinants except for weekly folate supplementation (\**p*<0.005).

**Table 2** C677T and A1298C polymorphism distribution in patients with rheumatoid arthritis and controls

	C677T polymorphism			A1298C polymorphism				
	n	CC	CT	TT	n	AA	AC	CC*
Study group	93	48.4%	40.9%	10.8%	93	53.8%	21.5%	24.7%
Control group	377	40.6%	44.1%	15.2%	377	44.8%	42.3%	12.8%

\*1298CC genotype distribution significantly different between rheumatoid patients and controls (one sample  $\chi^2$  analysis,  $p < 0.001$ ).

(see Methods for details) among our rheumatoid patients (table 3). Thirty three patients experienced any side effect, of whom only 9.1% carried the 1298CC genotype, while among the 60 patients who were free of side effects 33% were 1298CC carriers ( $p = 0.035$ ). The odds ratio for developing side effects in patients with wild type 1298AA versus 1298CC genotype was 5.24 (95% confidence interval, 1.38 to 20). In contrast to A1298C polymorphism, we did not find any association between C677T polymorphism and methotrexate related adverse effects among our patients.

It is expected that various clinical factors may contribute to methotrexate induced side effects. Previous studies reported a significant association between plasma homocysteine levels and methotrexate related adverse reactions.<sup>15</sup> Using univariate analysis, methotrexate dosage, the use of other DMARDs, folic acid supplementation, and plasma homocysteine concentrations were not significantly different between rheumatoid patients with and without methotrexate related side effects (table 4). In conclusion, the 1298CC genotype was the only significant predictor of lack of methotrexate related side effects.

#### MTHFR A1298C genotype polymorphism, serum folate, and plasma homocysteine

It has been reported previously that homozygosity for the 677T and 1298C alleles does not coexist in a particular individual.<sup>17-20</sup> We examined whether the "protective" effect of the 1298CC genotype is simply the result of an association between the 677TT genotype and the presence of methotrexate related side effects in our rheumatoid patients. We found no association between 677 genotype polymorphism distribution and side effects (table 3). Rheumatoid patients carrying the 1298CC genotype had higher plasma concentrations of homocysteine (mean 13.63 (4.03)  $\mu\text{mol/l}$ ), which were unaffected by serum folic acid levels, compared with patients who carried AA (13.00 (4.47)  $\mu\text{mol/l}$ ) and AC (13.18 (2.88)  $\mu\text{mol/l}$ ) genotypes, in whom higher serum folic acid was associated with decreased concentrations of homocysteine (fig 1; differences did not reach statistical significance). Considered together, these results suggest that there is no association between the 677TT genotype, homocysteine concentrations, and the occurrence of side effects.

#### DISCUSSION

The main findings of our study are a high rate of 1298CC homozygosity among our rheumatoid patients treated with

methotrexate and a significant decrease in methotrexate related side effects in patients carrying the 1298CC genotype of MTHFR gene A1298C polymorphism. We also found no relation between plasma homocysteine level and the various A1298C polymorphisms.

The rate of 1298CC homozygosity among our methotrexate treated rheumatoid patients was 24.7%, significantly higher than that found in an unselected population (12.8%).<sup>20</sup> This may indicate that rheumatoid arthritis is more common among 1298CC homozygotes in our population. However, it could also result from the cross sectional type of study that we conducted. Such a design may have led to a selection bias towards patients carrying a genotype that was protective against methotrexate related side effects or towards patients in whom methotrexate was effective. Both possibilities should be tested in further studies and in various populations.

An association between the MTHFR gene C677T polymorphism and methotrexate related toxicity has been reported in patients with malignant conditions.<sup>13-15</sup> Patients carrying the 677TT genotype experienced increased prevalence of toxicity, evidenced by a higher incidence of oral mucositis and a trend toward delayed platelet recovery when treated with methotrexate for bone marrow transplantation.<sup>13</sup> Toffoli *et al* reported that neutropenia following methotrexate chemotherapy was more common in patients with the 677TT polymorphism of the MTHFR gene.<sup>14</sup> Patients with rheumatoid arthritis are treated with much lower methotrexate doses. Recently, van Ede *et al* reported that in rheumatoid patients receiving low dose methotrexate the presence of the 677T allele was associated with an increased risk for drug withdrawal because of adverse events, mainly hepatotoxicity.<sup>16</sup>

Our results indicate no association between C677T polymorphisms and methotrexate related side effects. This may be the result of a selection bias against hepatotoxicity in our population because of the cross sectional design of our study. Indeed, the major adverse effects among our rheumatoid patients were methotrexate induced nodules and gastrointestinal toxicity, and not hepatotoxicity.

Supplementation with folic acid reduces the incidence of methotrexate related side effects.<sup>24-25</sup> Although our patients with the wild type 1298AA genotype received a significantly higher folic acid dose than patients with the 1298CC genotype, they had more methotrexate related side effects, suggesting an important role for the A1298C polymorphism

**Table 3** A1298C and C677T genotypes and methotrexate related side effects

	A1298C genotype			C677T genotype				
	n	AA	AC	CC*	n	CC	CT	TT
No with any side effect	33	22 (66.7%)	8 (24.2%)	3 (9.1%)	33	15 (45.5%)	14 (42.4%)	4 (12.1%)
No with no side effects	60	28 (4.07%)	12 (20.0%)	20 (33.0%)	60	30 (50.0%)	24 (40.0%)	6 (10.0%)

\*Number of 1298CC carriers significantly higher among rheumatoid patients with no methotrexate related side effects than among those with side effects ( $\chi^2$  test,  $p = 0.035$ ).

**Table 4** Clinical and laboratory factors and methotrexate related side effects

	Side effects	No of patients	Mean (SD)	p Value
Folate supplementation (mg/week)	No	60	5.30 (8.44)	0.733
	Yes	33	5.93 (9.19)	
Serum folate (ng/ml)*	No	45	9.11 (5.41)	0.965
	Yes	27	9.05 (6.54)	
Plasma homocysteine ( $\mu\text{mol/l}$ )†	No	49	13.31 (4.27)	0.697
	Yes	28	12.88 (5.07)	
Methotrexate dose (mg/week)	No	60	11.91 (3.77)	0.800
	Yes	33	11.99 (3.42)	

No significant difference between patients with or without side effects in folic acid consumption, serum folic acid, plasma homocysteine, or methotrexate dose (*t* test).

\*Data available from only 72 participants.

†Data available from only 77 participants.

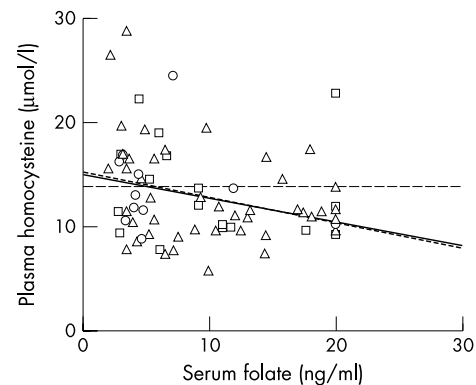
in determining such side effects, a role that may override folic acid supplementation.

Using haplotype analysis, a previous study indicated that rheumatoid patients with the 677C-1298C haplotype were receiving lower doses of methotrexate because of better efficacy than were those without it, while subjects with the 677T-1298A haplotype had a higher frequency of side effects than those without the haplotype.<sup>17</sup> Looking at polymorphisms, however, the investigators concluded that methotrexate related toxicity was more common in patients with the 677T allele than in those without it. For the 1298C polymorphism, the frequencies of patients who experienced toxicity did not differ between the different genotypes.<sup>17</sup> The number of patients with rheumatoid arthritis carrying the 1298CC genotype in their study was too low (3/106) to evaluate the association of this genotype with methotrexate induced side effects. The protection from adverse effects by the 1298CC genotype could result from the lack of the 677T allele in 1298CC carriers. This is unlikely, as we did not find any association between C677T polymorphisms and side effects. In addition, one would expect that patients suffering from methotrexate induced side effects and carrying the 677T allele will have higher plasma homocysteine concentrations; however, we found that there was no correlation between adverse side effects and plasma homocysteine. Figure 1 shows that patients carrying the 1298CC genotype have higher plasma homocysteine despite a significantly lower prevalence of methotrexate induced side effects. These results suggest that the 1298CC genotype predicts immunity from methotrexate induced side effects irrespective of either the C677T polymorphism or the levels of plasma homocysteine. A similar protective effect of this polymorphism has been demonstrated in type 2 diabetic patients with a reduced rate of diabetic nephropathy.<sup>26</sup>

We studied the relation between plasma homocysteine level and the various MTHFR gene polymorphisms in our population. Low serum folate was the only factor associated with hyperhomocysteinaemia in the present study. This association was restricted to the 1298AA and the 1298AC genotypes but was not statistically significant. The plasma homocysteine concentration in 1298CC carriers was independent of serum folate level (fig 1). In our study, no association emerged between homocysteine concentrations and methotrexate dose or duration of treatment. Various previous reports have been contradictory concerning the influence of methotrexate on homocysteine levels. Hyperhomocysteinaemia was found to be independent of methotrexate use in several studies.<sup>6-8, 12</sup> Roubenoff *et al*<sup>6</sup> found higher plasma homocysteine concentrations in rheumatoid patients than in healthy controls, with the highest

levels in patients not receiving methotrexate compared with those treated with low dose methotrexate. Morgan *et al* detected no change in plasma homocysteine after six months of methotrexate treatment.<sup>8</sup> Hernanz *et al* also found increased plasma homocysteine in rheumatoid patients in comparison with healthy controls; the values were similar in both untreated and methotrexate treated patients.<sup>7</sup> Jensen *et al* recently showed that patients receiving methotrexate supplemented with folic acid had plasma homocysteine concentrations similar to rheumatoid patients treated with other DMARD.<sup>12</sup> However, in other studies, methotrexate was found to cause hyperhomocysteinaemia in rheumatoid patients.<sup>9-11</sup> Morgan *et al* and van Ede *et al* prospectively evaluated the effect of methotrexate and folate supplementation on homocysteine concentrations in patients with rheumatoid arthritis. Plasma homocysteine was decreased in the folate supplemented group and increased during methotrexate treatment without folate supplements. It was concluded that folic acid supplementation prevents the increase in plasma homocysteine induced by methotrexate treatment.<sup>10, 11</sup>

The principal MTHFR gene polymorphism evaluated in rheumatoid arthritis is the C677T. Recently Haagsma *et al* reported the influence of this polymorphism on the impact of methotrexate on plasma homocysteine concentration.<sup>9</sup> Heterozygous patients had a higher plasma homocysteine during methotrexate treatment than those carrying the wild type 677CC. No increase in homocysteine was demonstrated in the 677TT carriers. In another study the fluctuation in



**Figure 1** Relation between plasma homocysteine and serum folate concentrations for various A1298C polymorphisms. Regression lines represent the relation between plasma homocysteine concentration ( $\mu\text{mol/l}$ ) and serum folate (ng/ml) for various A1298C polymorphisms. Triangles, 1298AA; circles, 1298AC; squares, 1298CC.

plasma homocysteine during methotrexate treatment was not influenced by C677T polymorphism.<sup>11</sup> In our study we found no significant association between plasma homocysteine and C677T and A1298C polymorphisms. We assume that this outcome is attributable to the relatively high doses of methotrexate given to our patients.

Although one possible mechanism of methotrexate effect is anti-folate action, we found no evidence that MTHFR gene polymorphisms (C677T or A1298C) were associated with a therapeutic response to methotrexate. In concordance with other studies, none of the disease activity factors correlated with homocysteine level.<sup>11</sup>

In summary, in this observational study we investigated the A1298C polymorphism of the MTHFR gene in patients with rheumatoid arthritis treated with methotrexate. Our findings suggest that the A1298C polymorphism has no significant influence on homocysteine levels in these patients; however, our study shows that the 1298CC polymorphism may protect against methotrexate related side effects, while the 1298AA genotype is associated with such adverse effects despite higher doses of folic acid supplementation. Additional studies are required to establish the importance of the A1298C polymorphism in rheumatoid arthritis in other populations, and its role in adjusting methotrexate dosage. A futuristic look at pharmacogenetics implies that gene polymorphism for one gene or another may predict not only disease susceptibility, but also the response to a particular drug treatment and the potential side effects.

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