Methylenetetrahydrofolate reductase C677T and A1298C gene variants in adult non-Hodgkin's lymphoma patients: association with toxicity and survival

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ABSTRACT

Background and Objectives

Common methylenetetrahydrofolate reductase gene variants (*MTHFR* C677T and A1298C) have been described to have opposite effects on cancer patients. They may reduce cancer susceptibility and increase drug-related toxicity when folate antagonists (e.g. methotrexate) are utilized. We analyzed 110 patients with high-grade non-Hodgkin's lymphoma (NHL), 68 of whom were eligible for a chemotherapy combination containing methotrexate (MACOP-B) and 42 for chemotherapy without methotrexate (CHOP).

Design and Methods

Patients were genotyped by polymerase chain reaction and stratified by *MTHFR* variants. These data were related to the toxicity (WHO grade G0-4) that the patients suffered and their survival. Overall 64 cases (58.2%) developed some form of toxicity and 23 (20.9%) had grade 3/4 toxicity.

Results

When considering toxicity of any grade (grade 1-4), the 677TT genotype was significantly over-represented among cases with mucositis (OR=4.85; 95%CI, 1.47-15.97; p=0.009) and those with hepatic toxicity (OR=3.43; 95%CI, 0.99-11.86; p=0.052). Sub-analyses in the group treated with MACOP-B showed a slight increase in the risk of developing mucositis (OR=5.22; 95%CI, 1.20-27.27; p=0.03), and a strong increase in the risk of hepatic toxicity (OR=7.08; 95%CI, 1.38-36.2; p=0.019) and thrombocytopenia (OR=7.69, 95%CI 1.0-58.94; p=0.05). Interestingly, compared to the risk of developing toxicity of any grade, the risk of developing severe (grade 3/4) mucositis was almost doubled in the whole group of cases with 677TT (OR=8.13; 95%CI 1.61-41.04; p=0.011) and dramatically increased in the MACOP-B-treated cases with this gene variant (OR=24.6; 95%CI 2.49-87.41; p=0.001). There were significant results for 1298CC cases exclusively for mucositis (any grade, OR=5.33; 95%CI, 1.25-22.70; p=0.023 and OR=9.15; 95%CI, 1.14-73.41; p=0.037; for the whole group and the MACOP-B-treated group, respectively). Similarly, the risk of 1298CC patients developing severe mucositis increased (OR=9.24; 95%CI, 1.47-58.0; p=0.017 and OR=11.53; 0.93-143.18; p=0.057; in the whole group and in the MACOP-B-treated group, respectively). Event-free survival analysis revealed a lower probability of event-free survival at 5 years for 677T-carriers (log-ranks, p=0.05 and p=0.07 in the whole group and in the MACOP-B-treated group, respectively). More significant results were obtained when 1298CC cases were excluded from the reference group (log-ranks, p=0.03 and p=0.04, respectively). No significant associations were found in the CHOP-treated group.

Interpretation and Conclusions

Our data suggest that *MTHFR* gene variants play a critical role in NHL outcome, possibly by interfering with the action of methotrexate with significant effects on toxicity and survival. Genotyping of folate pathway gene variants might be useful to enable reduction of chemotherapy toxicity and/or to improve survival by indicating when dose adjustments or alternative treatments are necessary.

Key words: non-Hodgkin's lymphoma, *MTHFR* SNP, toxicity, survival, pharmacogenetics.

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Methylenetetrahydrofolate reductase (MTHFR) converts methylenetetrahydrofolate to methyltetrahydrofolate, the major circulating form of folate, so providing methyl groups for methionine synthesis. Methylenetetrahydrofolate and its derivates are essential for purine and pyrimidine synthesis. Therefore, the activity of MTHFR plays an important role in both DNA synthesis and methylation, which are critical processes for rapidly growing malignant and non-malignant cells. Two common single nucleotide polymorphisms (SNP) have been described to affect the activity of the MTHFR enzyme, a C to T nucleotide transition at position 677 and an A to C nucleotide transversion at position 1298.[1,2] Reduced enzyme activity has been reported in 677TT and 1298CC homozygotes as well as in combined carriers and to a lesser extent in heterozygous individuals.[1-4] Several studies have investigated *MTHFR* gene variants and disorders involving folate metabolism,[5-9] and recently there has been growing interest in the pharmacogenetics of antifolate drugs.[10,11] In particular, it was reported that individuals carrying *MTHFR* polymorphisms may have a lower susceptibility to develop solid or hematologic cancers.[12-18] On the other hand, a few recent studies suggested that carrier patients may have exacerbated toxicity when treated with antifolate drugs[19-21] or reduced survival, possibly through interference with the action of methotrexate.[22,23] Such a dualism has also been reported for additional folate metabolizing gene polymorphisms.[14,23-25] Overall, relationships between clinical outcome and folate pathway gene variants in lymphomas have been poorly investigated.[26,27] Methotrexate, an antifolate chemotherapeutic agent, is widely used, alone or in combination with other drugs, in the treatment of a number of solid, hematologic malignancies[28-31] as well as non-malignant disease.[32,33] In particular, the MACOP-B combined scheme is a third-generation regimen that is very effective against high-grade non-Hodgkin's lymphomas (NHL).[34,35] Similarly to many other anticancer drugs, methotrexate has little selectivity for cancer cells, thus its effectiveness is limited by toxicity against normal tissues, particularly towards gastrointestinal epithelium, bone marrow and liver.[36,37] A less aggressive drug combination not containing methotrexate (i.e. CHOP) shows similar effectiveness in the treatment of high-grade NHL.[38,39] We previously reported that SNP in the genes of folate metabolizing enzymes play a greater role in acute lymphoblastic leukemia than in NHL.[18] In the present study we investigated the possible effects of two common *MTHFR* gene variants on toxicity and on clinical outcome in a group of 110 NHL patients treated with different chemotherapeutic regimens.

Design and Methods

Study design, selection of patients and their main characteristics

The aim of our study was to investigate any possible role of *MTHFR* genotypes on the clinical outcome of NHL patients and whether there were differences according to the chemotherapeutic regimens used. For this purpose, we analyzed therapy-related toxicity and survival data in the whole group and in the sub-groups of cases treated with MACOP-B or CHOP (*For details see online supplementary Appendix at www.haematologica.org/*).

Chemotherapeutic regimens and toxicity evaluation

After stratification according to age, Eastern Cooperative Oncology Group performance status,[42] and presence of mediastinal mass, the patients were assigned to the MACOP-B or CHOP chemotherapy regimen protocol. (*For details see online supplementary Appendix at www.haematologica.org/*).

Genotype analyses

DNA was isolated from peripheral whole blood by using proteinase-K treatment followed by phenol/chloroform extraction and ethanol precipitation. The genotyping protocol for detection of the *MTHFR* C677T polymorphism used the following primers: 5'-TGA AGG AGA AGG TGT CTG CGG GA-3' and 5'-AGG ACG GTG CGG TGA GAG TG-3'.[1] (*For details see online supplementary Appendix at www.haematologica.org/*). The genotyping protocol for detecting the *MTHFR* A1298C polymorphism used the following primers: 5'-GGG AGG AGC TGA CCA GTG CAG-3' and 5'-GGG GTC AGG CCA GGG GCA G-3'.[2] (*For details see online supplementary Appendix at www.haematologica.org/*).

Statistical analysis

Statistical differences between groups were assessed by the Student's *t*-test and the χ^2 test. When appropriate, Yates' correction or Fisher's exact test was applied. Odds ratio (OR) and 95% confidence intervals (95% CI) were used to estimate the risk of developing different grades of toxicity after chemotherapy. Adjusted OR were calculated with logistic regression models, with the dependent variable being the toxicity grade according to WHO criteria subdivided as grades 1-4 or grades 3-4 versus grade 0 (*see specifics in legends to the Tables*). (*For details see online supplementary Appendix at www.haematologica.org/*).

Results

Main clinical characteristics and genotype distributions in the groups of NHL patients

The main clinical characteristics of the groups of patients considered are listed in Table 1. The whole group consisted of 110 patients with high-grade NHL. Among these 110 patients, 68 received MACOP-B treatment and 42 received CHOP treatment. Stratification according to stage differed significantly between the two treatment groups (p=0.027), whereas there were no differences in performance status or in the presence of a mediastinal mass. Among the whole group of patients, 64 (58.2%)

developed toxicity of some grade (hematologic and non-hematologic) and 23 (20.9%) developed severe, grade 3/4 toxicity. The global treatment-related death rate was 3.6% (two cases in the MACOP-B group and two cases in the CHOP group). The global pattern of toxicities differed according to the chemotherapy regimen used, being more severe in the MACOP-B-treated group (p=0.05) probably due to the presence of methotrexate in this chemotherapeutic combination. Accordingly, mucositis was statistically overrepresented in the MACOP-B group with respect to the CHOP group (30.9% vs 7.1%; p=0.007). Conversely, the rate of the other toxicities considered did not differ statistically between the two groups, and neither did the *MTHFR* genotype.

Treatment was adapted in all patients developing severe (grade 3-4) toxicity (11 treated with MACOP-B, 16.2% and 12 treated with CHOP, 28.6%). In detail, these patients experienced lymphocytic toxicity (65.2%), mucositis (39.1%), anemia (26.1%), thrombocytopenia (21.7%) and hepatic toxicity (13.0%). Treatment was temporarily suspended in 40% of cases and the dose of chemotherapeutic agents reduced (by 20%) in 60% of the patients. When stratified by *MTHFR* polymorphisms, performance status revealed a slight 677 genotype dependence only in the MACOP-B subgroup. A worse performance status was observed as the number of 677T alleles increased (test-trend, p=0.051). Conversely, no significant associations were identified for disease stage or presence of a mediastinal mass with particular *MTHFR* genotypes in the whole group or in the two differently treated subgroups (*data not shown*).

Toxicity and MTHFR polymorphisms in the whole NHL group

Among all the patients with NHL who developed toxicity (n=64), the prevalence of hematologic and non-hematologic toxicities was as follows: 24 mucositis (37.5%), 26 hepatic toxicity (40.6%), 42 lymphocytopenia (65.6%), 21 anemia (32.8%) and 18 thrombocytopenia (28.1%). Table 2 shows the different kinds of toxicities stratified by *MTHFR* 677-genotypes in the whole group of NHL cases. Globally, mucositis was significantly overrepresented among 677TT-homozygotes when compared with both the 677CC reference group (OR=2.21; 95%CI, 1.08-8.75; p=0.045) and the group with the other genotypes (OR= 4.85; 95% CI, 1.47-15.97; p=0.009). Hepatic toxicity was slightly associated with the 677TT-genotype when compared with the other genotypes (OR=3.43; 95%CI; 0.99-11.86; p=0.052). It is worth noting that there was an unexpected low prevalence of 677CT cases (11.3%) among patients with mucositis compared to the prevalence of patients with the other 677-genotypes (23.7% and 47.4% for the CC and TT genotypes, respectively). This was responsible for a low, but not statistically significant, OR-value ascribable to the CT-genotype.

The risk of developing severe mucositis (grade 3/4) was double in 677TT-carriers compared to that in patients with the other genotypes (OR=8.13; 95% CI, 1.61-41.04; p=0.011) (Table 3). Conversely, no further increase in risk was observed when only severe hepatic toxicity was considered. No associations between particular 677-genotypes and thrombocytopenia, nor lymphocytic toxicity, or anemia were found (*data not shown*). As far as concerns the A1298C polymorphism, a statistical significance was found only for the occurrence of mucositis in 1298CC homozygotes when compared to patients with other genotypes, yielding an OR of 5.33 (95% CI, 1.25-22.70; p=0.023). When only severe mucositis was considered (Table 3) the risk increased further (OR=9.24; 95% CI, 1.47-58.01; p=0.017). In combined analyses to evaluate the effect of both *MTHFR* variants, the absence of double wild-type carriers with mucositis did not allow any relative risk evaluation. This supports the idea that wild-type alleles are underrepresented among patients developing toxicity.

Toxicity and MTHFR polymorphisms in the two treatment groups

Among NHL patients treated with MACOP-B who developed toxicity (n=45), the prevalence of hematologic and non-hematologic toxicities was as follows: 21 mucositis (46.6%), 18 hepatic toxicity (40.0%), 27 lymphocytopenia (60.0%), 9 anemia (20.0%) and 11 thrombocytopenia (24.4%). Table 4 shows the different kinds of toxicities stratified by MTHFR 677-genotypes in the MACOP-B-treated subgroup of patients. Among MACOP-B-treated patients with the 677TT genotype, the risk of developing any grade of mucositis, hepatic toxicity or thrombocytopenia was about 5 to 7-fold higher than that in patients with the other genotypes. When only grade 3/4 mucositis was considered (Table 3), the risk for 677TT-carriers increased dramatically (OR=24.60; 95% CI, 2.49-87.41; p=0.001). A further increase in the risk value related to the development of more severe hepatic toxicity or thrombocytopenia was not observed. No association between particular 677-genotypes and lymphocytic toxicity or anemia was found. As for the whole group, a low rate of 677CT cases among those with mucositis was responsible for an associated non-significant decreased OR value.

When the A1298C polymorphism was analyzed, only the occurrence of mucositis in the 1298CC homozygotes was statistically significant when compared with toxicity in patients with the other genotypes, yielding an OR of 9.15 (95% CI, 1.14-73.41; p=0.037). When severe mucositis was considered (Table 3) the risk further increased (OR=11.53; 95%CI, 0.93-143.18; p=0.057). Among NHL patients treated with CHOP who developed toxicity (n=19), the prevalence of hematologic and non-hematologic toxicities was as follows: 3 mucositis (15.8%), 8 hepatic toxicity (42.1%), 15 lymphocytopenia (79.0%), 12 anemia (63.1%) and 7 thrombocytopenia (36.8%). It should be noted that a very low percentage of cases of mucositis was observed in patients treated with CHOP in

Table 1. Main characteristics of the patients.

	Total cases n=110	*MACOP-B n=68*	*CHOP n=42*	*p**
Age (mean, SD), years	56.8±17.1	49.9±16.6	67.9±10.9	0.001
Range, years	18-80	18-80	32-80	--
Sex (male/female)	67/43 (60.9)	47/21 (69.1)	20/22 (47.6)	0.04
Stage				
I	16 (14.5)	6 (8.8)	10 (23.8)	0.027°
II	18 (16.4)	16 (23.5)	2 (4.8)	
III	37 (33.6)	22 (32.3)	15 (35.7)	
IV	39 (35.4)	24 (35.3)	15 (35.7)	
Performance status (ECOG)#				
Fully active or ambulatory (0-1)	69 (62.7)	42 (61.8)	27 (64.3)	NS
NS				
Bedridden (≥2)	33 (30.0)	21 (30.9)	12 (28.6)	NS
Mediastinal mass#				
Yes/No	33/69	22/41	11/28	NS
Toxicity				
Mucositis (n, %)	24 (21.8)	21 (30.9)	3 (7.1)	0.007
Hepatic (n, %)	26 (23.6)	18 (26.4)	8 (19.0)	NS
Lymphocytopenia (n, %)	42 (38.2)	27 (39.7)	15 (35.7)	NS
Anemia (n, %)	21 (19.1)	9 (13.2)	12 (28.6)	NS
Thrombocytopenia (n, %)	18 (16.4)	11 (16.2)	7 (16.7)	NS
Overall, grades 1-4 (n, %)	64 (58.2)	45 (66.2)	19 (45.2)	0.05
MTHFR genotype (n, %)				
677 CC	38 (34.5)	22 (32.3)	16 (38.1)	NS°
677 CT	53 (48.2)	36 (52.9)	17 (40.5)	
677 TT	19 (17.3)	10 (14.7)	9 (21.4)	
1298 AA	44 (40.0)	27 (39.7)	17 (40.5)	NS°
1298 AC	56 (50.9)	34 (50.0)	22 (52.4)	
1298 CC	10 (9.1)	7 (10.3)	3 (7.1)	

**p-values are referred to comparisons between the MACOP-B and the CHOP group; #Data are not available for five MACOP-B- and for three CHOP- treated-patients. °p-values are referred to data distribution.*

Table 2. ***MTHFR*** **C677T genotype and toxicity risk evaluation in the whole NHL group (n=110).**

MTHFR C677T (n)	*Toxicity grade 0 n (%)*	*Toxicity grade 1-4 n (%)*	*OR (95% CI)*	*P*
		Mucositis		
CC (38)	29 (76.3)	9 (23.7)	Reference	
CT (53)	47 (88.7)	6 (11.3)	0.50 (0.21-1.42)	NS
TT (19)	10 (52.6)	9 (47.4)	2.21 (1.08-8.75)	0.045
TT vs CC+CT*			4.85 (1.47-15.97)	0.009
		Hepatic toxicity		
CC (38)	31 (81.6)	7 (18.4)	Reference	
CT (53)	42 (79.2)	11 (20.7)	1.17 (0.41-3.39)	NS
TT (19)	11 (57.9)	8 (42.1)	2.94 (0.85-10.20)	0.089
TT vs CC+CT*			3.43 (0.99-11.86)	0.052
		Lymphocytic toxicity		
CC (38)	26 (68.4)	12 (31.6)	Reference	
CT (53)	31 (58.5)	22 (41.5)	1.49 (0.57-3.89)	NS
TT (19)	11 (57.9)	8 (42.1)	0.95 (0.25-3.71)	NS
TT vs CC+CT*			0.78 (0.24-2.50)	NS
		Anemia		
CC (38)	31 (81.6)	7 (18.4)	Reference	
CT (53)	42 (79.2)	11 (20.7)	0.98 (0.30-3.18)	NS
TT (19)	16 (84.2)	3 (15.8)	0.54 (0.10-2.98)	NS
TT vs CC+CT*			0.52 (0.12-2.32)	NS
		Thrombocytopenia		
CC (38)	32 (84.2)	6 (15.8)	Reference	
CT (53)	47 (88.7)	6 (11.3)	0.56 (0.14-2.22)	NS
TT (19)	13 (68.4)	6 (31.6)	1.92 (0.38-9.64)	NS
TT vs CC+CT*			2.31 (0.62-8.60)	NS

*OR-values were computed considering the number of MTHFR 677CC cases as the reference. *OR-values were computed comparing the number of MTHFR 677TT cases versus the number of cases with the remaining genotypes for each type of toxicity. p-values above 0.100 are not shown.*

comparison to the percentage among patients treated with MACOP-B (Table 1). This could be strongly associated with the presence/absence of methotrexate in the two different chemotherapy regimens. Finally, in the patients treated with CHOP, there were no significant associations between any type or grade of toxicity and specific *MTHFR* genotypes for either the C677T or A1298C polymorphism (*data not shown*).

Survival and MTHFR polymorphisms in NHL groups

Kaplan-Meier analysis comparing EFS curves at 5 years of follow-up for the two treatment groups (MACOP-B and CHOP) did not show significant difference (p=0.81; Figure 1). When the whole group of NHL cases was stratified according to 677-genotype, Kaplan-Meier analysis showed that 677T-carriers had a lower probability of EFS compared to cases with the 677CC-genotype (log-rank, p=0.05; Figure 2). Accordingly, 677T-carriers were at higher risk of adverse events compared to patients with the 677CC-genotype (HR=1.99; 95% CI, 1.05-3.55; p=0.046).

Although no significant differences in EFS rate were found between patients stratified according to 1298-variant, 1298CC-homozygotes had the lowest probability of survival at 5 years (*data not shown*). For this reason, and taking into account that only 1298CC homozygotes showed significant toxicity patterns, in an exploratory analysis we excluded these cases from the reference group in the subsequent survival analyses. In the whole group, excluding 1298CC-homozygotes, 677T-carriers had a further reduction in EFS probability (log-rank, p=0.03) and the associated risk of developing adverse events increased (HR=2.40; 95% CI, 1.10-5.10; p=0.024).

The pattern of EFS in the MACOP-B-treated subgroup was similar. Again, the significance was higher when 1298CC-homozygotes were excluded from the reference group and the associated log-rank values were p=0.07 and p=0.04 (when the 1298CC homozygotes were or were not included, respectively, in the reference group). Accordingly, the respective HR for adverse events reserved to the 677T-carriers were 2.21 (95% CI, 0.95-5.11; p=0.070) and 2.99 (95% CI; 1.19-9.50; p=0.030). Finally, EFS among the subgroup treated with CHOP did not differ significantly in relation to specific *MTHFR* geno-

Table 3. ***MTHFR*** **C677T and A1298C genotypes and risk of severe mucositis in the whole group of NHL patients (n=110) and in those treated with MACOP-B (n=68).**

MTHFR	*Whole group (n=110)*			*MACOP-B group (n=68)*		
C677T	WHO G0 n (%)	WHO G3-4 n (%)	OR (95% CI; P)	WHO G0 n (%)	WHO G3-4 n (%)	OR (95% CI; P)
CC	29 (90.6)	3 (9.4)	Reference	13 (81.2)	3 (18.7)	Reference
CT	47 (97.9)	1 (2.1)	0.22 (0.02-2.41; NS)	30 (96.8)	1 (3.2)	0.12 (0.01-1.71; NS)
TT	10 (66.7)	5 (33.3)	3.86 (0.84-23.15; 0.080)	4 (50.0)	4 (50.0)	9.40 (0.64-138.80; NS)
TT *vs* CC+CT*			8.13 (1.61-41.04; 0.011)			24.60 (2.49-87.41; 0.001)
A1298C						
AA	32 (86.5)	5 (13.5)	Reference	18 (81.8)	4 (18.2)	Reference
AC	49 (98.0)	1 (2.0)	0.12 (0.01-1.31; 0.082)	27 (96.4)	1 (3.6)	0.02 (0.0003-1.42;0.072)
CC	5 (62.5)	3 (37.5)	6.11 (0.66-56.25; NS)	2 (40.0)	3 (60.0)	24.36 (0.28-2119.33; NS)
CC *vs* AA+AC*			9.24 (1.47-58.01; 0.017)			11.53 (0.93-143.18;0.057)

*OR-values were computed considering the number of 677CC or 1298AA cases as the reference. *OR-values were computed comparing the number of MTHFR 677TT or 1298CC cases versus the number of the cases with respective remaining genotypes. p-values above 0.100 are not shown.*

Table 4. ***MTHFR*** **C677T genotype and risk of toxicity in the MACOP-B-treated group (n=68).**

MTHFR C677T (n)	*Toxicity grade 0 n (%)*	*Toxicity grade 1-4 n (%)*	*OR (95% CI)*	*p*
		Mucositis		
CC (22)	13 (59.1)	9 (40.9)	Reference	
CT (36)	30 (83.3)	6 (16.7)	0.62 (0.34-1.58)	NS
TT (10)	4 (40.0)	6 (60.0)	3.15 (1.05-9.43)	0.042
TT *vs* CC+CT*			5.22 (1.20-27.27)	0.030
		Hepatic toxicity		
CC (22)	17 (77.3)	5 (22.7)	Reference	
CT (36)	29 (80.5)	7 (19.4)	1.02 (0.24-4.30)	NS
TT (10)	4 (40.0)	6 (60.0)	5.34 (1.04-27.21)	0.044
TT *vs* CC+CT*			7.08 (1.38-36.21)	0.019
		Lymphocytic toxicity		
CC (22)	16 (72.7)	6 (27.3)	Reference	
CT (36)	20 (55.5)	16 (44.4)	2.04 (0.62-6.73)	NS
TT (10)	5 (50.5)	5 (50.5)	2.03 (0.29-14.08)	NS
TT *vs* CC+CT*			1.33 (0.27-6.51)	NS
		Anemia		
CC (22)	20 (90.9)	2 (9.1)	Reference	
CT (36)	30 (83.3)	6 (16.7)	1.90 (0.34-10.51)	NS
TT (10)	9 (90.0)	1 (10.0)	0.82 (0.04-16.43)	NS
TT *vs*CC+CT*			0.75 (0.08-7.40)	NS
		Thrombocytopenia		
CC (22)	20 (90.9)	2 (9.1)	Reference	
CT (36)	31 (86.1)	5 (13.9)	1.59 (0.26-9.59)	NS
TT (10)	6 (60.0)	4 (40.0)	4.87 (0.59-40.08)	NS
TT *vs* CC+CT*			7.69 (1.00-58.94)	0.050

*OR values were computed considering the number of MTHFR 677CC cases as the reference. *OR values were computed comparing the number of MTHFR 677TT cases versus the number of cases with the remaining genotypes for each toxicity. °p-values above 0.100 are not shown.*

types (log-ranks p=0.32 and p=0.26, when computing or not the 1298CC cases in the reference group). Likewise, the respective HRs were 1.50 (95% CI, 0.62-4.3; p=0.39) and 1.81 (95% CI, 0.62-5.69; p=0.30). The A1298C polymorphism by itself did not have a significant effect on survival.

Discussion

The great interindividual variability in drug effect and efficacy is one of the major issues in the clinical management of patients with cancer. Resistance and toxicity greatly affect the clinical outcome of treated patients.[10,36] SNP are emerging as important pharmacogenetic prognostic determinants of response to chemotherapy. Recent studies have investigated the toxic effects of antifolate drugs in relation to folate metabolizing SNP in both hematologic and solid cancers.

In the present study, we investigated whether specific *MTHFR* genotypes were associated with survival and toxicity in a cohort of 110 adults with high-grade NHL treated with different pharmacological regimens containing or not methotrexate (MACOP-B and CHOP, respectively). The first outcome of our survey was that patients with NHL who carry the 677TT-genotype had about a 3- to 7-fold increased risk of developing different kinds of toxicities when compared to patients with other genotypes in both the whole group and in the subgroup treated with MACOP-B. This effect of genotype was particularly evident when severe toxicity phenotypes were considered. Indeed, 677TT cases treated with MACOP-B had an approximately 24-fold increased risk of developing severe (grade 3-4) mucositis. Similarly, hepatic toxicity and thrombocytopenia were more strongly related to the 677TT genotype in the MACOP-B-treated group than in the whole group but the risks were quite similar consid-

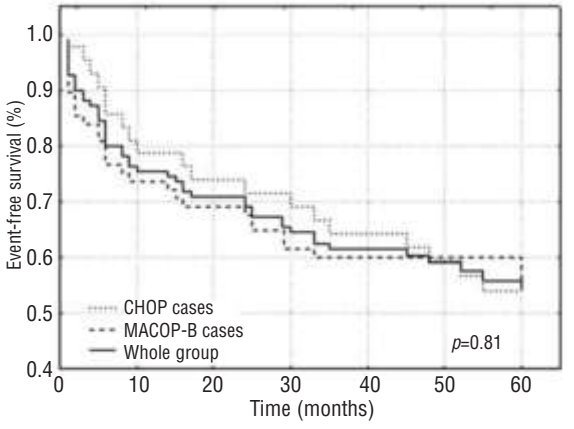

Figure 1. Kaplan-Meier analysis of EFS in the whole NHL group and in the two treatment subgroups.

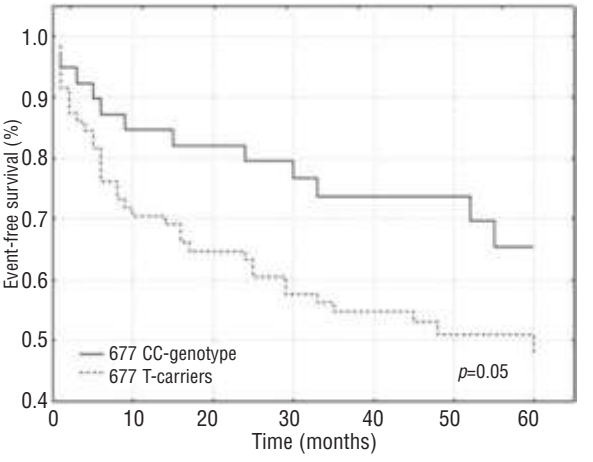

Figure 2. Kaplan-Meier analyses of EFS in the whole group of NHL cases stratified by *MTHFR* C677T genotype.

ering any grade of toxicity or severe toxicity. An unexpected underrepresentation of 677CT heterozygotes was found among patients with mucositis, associated with discordant but non-significant risk results. These data could be explained, in part, by the known partial linkage disequilibrium between 677 and 1298 alleles responsible for an unequal mutual distribution of the two allelic counterparts.[44] As regards the 1298 variant, effects were seen exclusively for mucositis both in the whole group and in the MACOP-B-treated subgroup, with associated risks increased by about 5- and 9-fold, respectively: the risks increased further when only severe mucositis was considered. Conversely, among patients with NHL treated with CHOP, no type or grade of toxicity was associated with *MTHFR* genotypes. Therefore, the role of *MTHFR* variants found in the whole group is mainly ascribable to their effects in MACOP-B-treated patients. Some recent studies showed increased toxicity in 677T-carriers treated with methotrexate[19-21] although other studies did not confirm such an association.[24,26,27] In particular, this association was not observed in pediatric patients with either NHL or acute lymphoblastic leukemia.[23,26] However, in adult NHL, we found strong chemotherapy toxicity associated with the 677TT-genotype. Different methotrexate doses and schemes and also diverse nutritional/folate status between adult and pediatric NHL patients might account in part for these discrepant results. The particularly evident association found in the MACOP-B subgroup could be ascribed to the inclusion of methotrexate in this combination of chemotherapeutic agents. *MTHFR* gene variants may increase sensitivity to methotrexate, perhaps through an imbalance of folate isoforms. Pharmacologically induced low levels of 5-methyltetrahydrofolate and constitutively low availability of this substrate in 677T-carriers, together with predictable effects on homocysteine concentrations, may account for the observed exacerbated toxicity.[21,45] The association between survival and folate pathway gene variants in cancer patients treated with antifolates is less investigated and still controversial. It seems that a diminished survival is present in cases carrying those alleles responsible for an imbalance of folate isoforms.[22,23,26] We found that 677T-carriers had a lower probability of EFS at 5 years of follow-up when compared to patients with the other genotypes, both in the whole group and in the MACOP-B-treated subgroup. Specifically, 677T carriers had an about 2-fold increased risk of adverse events. This was particularly evident in the MACOP-B-treated subgroup and when 1298CC homozygotes were excluded from the reference group. This would imply that the 1298C allele also has negative effects on survival, although previous studies in patients with acute lymphoblastic leukemia did not find such an association.[22] To a lesser extent than 677TT-carriers, individuals with the 1298CC genotype have decreased MTHFR activity and slightly raised homocysteine levels.[2] In addition, because of partial linkage disequilibrium, the coexistence of 677T and 1298C alleles in *cis* is possible, but very rare, supporting the hypothesis that triple mutations (i.e. 677TT/1298AC or 677CT/1298CC) or double homozygous conditions (i.e. 677TT/1298CC) are probably *de novo* recombinant events.[44] This is consistent with the fact that virtually all 677TT subjects have wild-type 1298 alleles. For these reasons, it is hard to observe a clear allele-dosage effect for the 1298 variant being better accounted for in homozygous conditions. This could, in part, justify the difficulty in ascribing effects on survival to the 1298-variant itself and also account for the improved probability of EFS observed in 677-wild-type carriers when the 1298CC homozygotes were excluded from the analysis. Among NHL cases treated with CHOP, no significantly different survival rates or risks were associated with particular *MTHFR* genotypes. This could be explained in part by the very low number of cases investigated, or alternatively, as

for the toxicity data, might be mainly due to the absence of methotrexate in this chemotherapy regimen. Thus, *MTHFR*-dependent survival might partially depend on treatment type and composition. We cannot, however, exclude that different mean ages or gender compositions of the two treatment subgroups might have accounted for the different results. It should be noted that when genotype was not considered very much closer survival profiles were found in the two subgroups (Figure 1). That said, the main purpose of our study was not to compare toxicity or survival patterns between two groups of NHL patients treated with different protocols, but rather to determine whether different *MTHFR*-genotypes have a role in the clinical outcome of such patients or particular subgroups of patients.

How folate unbalancing influences cancer remains to be established. It is currently believed that it may act by altering DNA methylation and/or synthesis.[46-48] Therefore, by affecting folate balance, folate pathway gene variants might modulate cancer risk and influence the effects of chemotherapy. In particular, 677T- and/or 1298C-carriers, who have more 5,10-methylene-tetrahydrofolate may have enhanced thymidylate synthase activity, interfering in turn with the therapeutic target of methotrexate. This might favor residual neoplastic clone expansion. At the same time, 677T- and/or 1298C-carriers, who have less 5-methyl-tetrahydrofolate, may have raised levels of homocysteine, increasing the toxicity of methotrexate. On the other hand, these polymorphisms, as well as other folate pathway gene variants, have been described to protect against the development of cancer.[12,14,16,18,49,50] This means that subjects carrying such variants may have dual but opposite effects from the polymorphism. They may have reduced susceptibility to cancer but increased drug-related toxicity and even reduced survival rates. The same mechanisms (e.g. more efficient thymidylate synthesis) may act beneficially in the healthy subjects but detrimentally in patients with cancer. Such gene variants might be considered *Judas-alleles* acting as friend in the healthy subjects but as a foe in the cancer patients.

In conclusion, our study ascribes *MTHFR* gene variants an important role in the outcome of patients with NHL, possibly by interfering with methotrexate as a part of a chemotherapy combination. We are aware of the limits of our study due to the small sample size and the fact that two gene variants partially account for these complex mechanisms. It is strongly recommended that folate levels are assessed in future studies, because this substrate could affect the efficacy of chemotherapy. Definitive conclusions should nevertheless be drawn with extreme caution, and further larger studies and/or multicenter analyses are needed to address these issues properly and to confirm the present findings.

Authors' Contributions

DG working hypothesis, designed the study, wrote the article and obtained funding support; AO interpreted and analyzed data, ST, LC and FF experimental and molecular biology work, made some important conceptual suggestions; EM and MDP collection, management and analyses of all hematologic and clinical data, DC and AB analyses and assessing of hematologic and non-hematologic toxicity, GG and AP statistical analyses, AC and GLS senior authors, clinical care of patients monitoring therapies, MDM interesting suggestion for the design of the manuscript and the discussion section, revised the manuscript critically. All authors took part in the revision of the manuscript and approved the final version.

Conflict of Interest

The authors reported no potential conflicts of interest.

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