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MTHFR polymorphisms and capecitabine-induced toxicity in patients with metastatic colorectal cancer

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Abstract

Objective: The availability of current chemotherapeutic options for metastatic colorectal cancer (mCRC) has increased survival, but it is also accompanied by considerable morbidity. Fluoropyrimidines are the mainstay of systemic therapy. Germline pharmacogenetic markers involved in 5-fluorouracil pharmacodynamics could provide individualized pretreatment tools for predicting toxicity. Research on methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms and fluoropyrimidine treatment outcome has focused on intravenous 5-fluorouracil and has yielded inconclusive results. The literature on pharmacogenetics in capecitabine-based chemotherapy is scarce. Therefore, we analyzed the association of *MTHFR* gene polymorphisms and the occurrence of serious toxicity of first-line capecitabine monotherapy and combination therapy.

Methods: One hundred and twenty-seven patients treated with first-line monotherapy capecitabine and 141 patients on capecitabine–irinotecan combination therapy were recruited from the CAIRO trial, an open-label phase III randomized trial, comparing sequential versus combination chemotherapy with capecitabine, irinotecan and oxaliplatin in mCRC. All patients were genotyped for *MTHFR* 1298A > C and 677C > T polymorphisms and analyzed in both cohorts separately for the association between the *MTHFR* genotype and incidence of grade 3–4 overall toxicity and specific adverse events, as well as efficacy parameters.

Results: *MTHFR* 1298A > C and 677C > T genotypes were not associated with grade 3–4 overall toxicity, febrile neutropenia or hand–foot syndrome. *MTHFR* 1298CC homozygotes showed a borderline significantly higher incidence of grade 3–4 diarrhea compared with *MTHFR* 1298AC or AA individuals (25 vs. 5%, P = 0.041) in the monotherapy cohort. No significant association was found between the *MTHFR* genotypes and efficacy parameters in either treatment cohort.

Conclusion: *MTHFR* polymorphisms are not associated with toxicity or efficacy in mCRC patients treated with capecitabine-based chemotherapy.

Introduction

In recent years, chemotherapeutic options for the treatment of metastatic colorectal cancer (mCRC) have expanded and have improved overall survival (OS) considerably.[1;2] Fluoropyrimidines, such as 5-fluorouracil (5-FU) and the oral pro-drug capecitabine, are still the mainstay of systemic treatment. However, despite the significant progress made with systemic therapy, the prognosis for mCRC remains relatively poor, with a median OS time of 19–22 months after diagnosis.[3;4] At the same time, chemotherapeutic regimens used in mCRC may result in toxicity, causing morbidity and occasionally even mortality, and frequently necessitating dose reductions. Unfortunately, predictors for adverse drug events in mCRC are scarce. In addition to clinical parameters, such as age and sex[5], germline pharmacogenetic markers could provide pretreatment information on the risk of toxicity.[6;7]

Several pharmacogenetic studies examining genetic variants related to 5-FU therapy, such as methylenetetrahydrofolate reductase (MTHFR) and thymidylate synthase (TS), have been published, but without conclusive results. Although polymorphisms in the gene coding for dihydropyrimidine dehydrogenase, the main catabolic enzyme of 5-FU, are linked to increased toxicity, the low allele frequency of the most common polymorphism in this gene limits its clinical usefulness. In addition, the role of pharmacogenetic biomarkers in predicting the toxicity and efficacy of capecitabine is as yet largely unexplored.

Fluoropyrimidines act in two different ways.[8] First, 5-FU is incorporated into RNA, precluding protein synthesis, the preferential mode of action for 5-FU bolus infusion. In addition, when administered as a continuous infusion, 5-FU binds to TS. This prevents the conversion of 2'-deoxyuridine-5'-monophosphate into 2'-deoxythymidine-5'-monophosphate, the latter of which is an essential precursor for DNA synthesis. In forming this ternary complex, 5,10-methylenetetrahydrofolate (5,10-MTHF) is required as an essential cofactor. MTHFR catalyzes the irreversible conversion of 5,10-MTHF into 5-methyltetrahydrofolate, thereby reducing the amount of 5,10-MTHF available for binding to TS.

Although over 60 germline polymorphisms in the *MTHFR* gene have been described, only two have shown functional effects on enzyme activity.[9] A non-synonymous single nucleotide polymorphism (SNP) at base pair 677 (C>T, Ala222Val) and a second SNP at base pair 1298 (A>C, Glu428Ala) both reduce *MTHFR* enzyme activity.[10;11]

Functional polymorphisms have also been described for TS, including a variable number of tandem repeats (VNTR) polymorphism in the enhancer region (TSER) in the 5'-untranslated region and an SNP G > C at bp12 of the second repeat of this VNTR.[12;13] In addition, a polymorphic locus is found in the TS 3'-untranslated region, consisting of a 6 bp deletion at position 1494.[12;14]

It is hypothesized that by reducing enzyme activity, *MTHFR* polymorphisms enhance the stable formation of the TS/fluorodeoxyuridine monophosphate complex, thereby resulting in both greater effect and toxicity of fluoropyrimidines. Higher intratumoral TS-levels are considered to hinder cytotoxicity. These assumptions have been studied extensively for

intravenous 5-FU therapy, but with contradictory results.(Table 1) In both the adjuvant and the metastatic setting of CRC, capecitabine is often replacing 5-FU, both in monotherapy and in combination therapy. A schematic overview of the capecitabine pharmacodynamics is presented in Figure 1. To our knowledge, only one previous pharmacogenetic study of capecitabine monotherapy in mCRC patients has been published.[16]

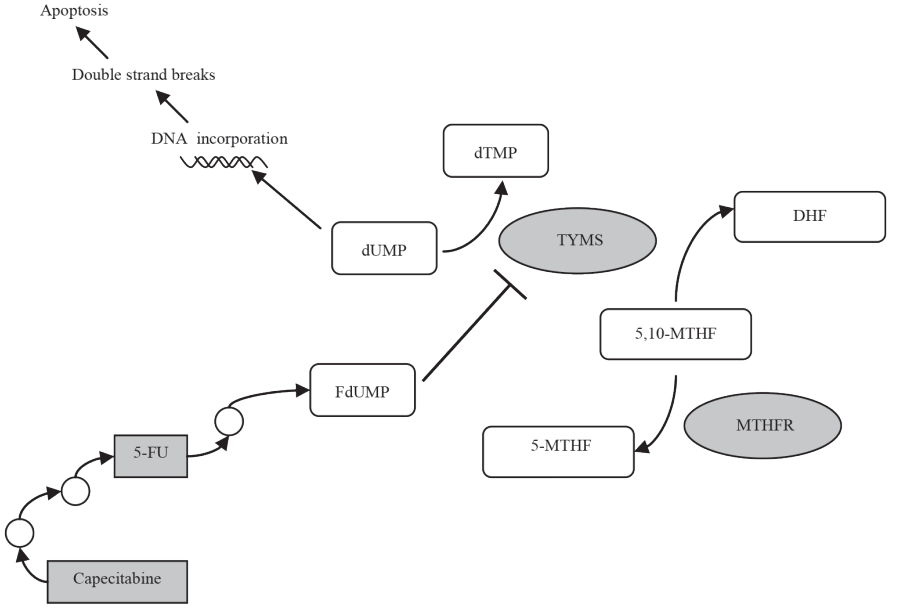

Figure 1. Schematic overview of enzymes involved in the cellular response of capecitabine

5-FU: 5-fluoro-uracil; DHF: dihydrofolate; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate; FdUMP: fluoro- deoxyuridine monophosphate; MTHF: methylene hydrofolate; MTHFR: methylene tetrahydrofolate reductase; TYMS: thymidylate synthase. Figure based on: Thorn C.F., et al.[15]

Therefore, in this multicenter study, we aimed to determine the effect of the germline polymorphisms *MTHFR* 677C > T and *MTHFR* 1298A > C on the toxicity and efficacy profile of capecitabine in patients with mCRC who started first-line palliative chemotherapy. Patients treated with two frequently used treatment schedules were studied: a cohort of patients treated with capecitabine monotherapy and a second cohort of patients treated with a combination therapy of capecitabine and irinotecan. Genetic variants in the gene encoding for TS were included as covariates.

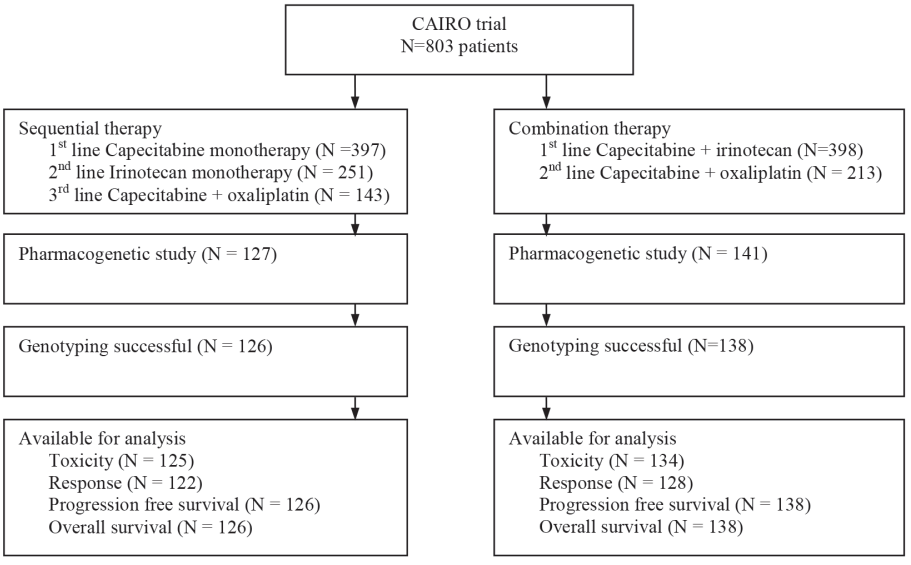

Figure 2. Study flowchart of the CAIRO study

Author, year (Ref.)	No of patients	Patient population	Treatment	Endpoint		Confirming hypothesis	Disproving hypothesis	
			5-FU bolus + LV		1298A>C	677C>T	1298A>C	677C>T
Afzal et al, 2009[1]	331	Stage II-III colon carcinoma (adjuvant)	5-FU bolus + LV	Toxicity			No effect of polymorphism	677CC more overall toxicity; and diarrhea
Afzal et al, 2011[2]	161 + 340	Stage II-III colon carcinoma (adjuvant) + mCRC	5-FU bolus + LV	Toxicity Efficacy			No effect of polymorphism*	No effect of polymorphism*
Castillo et al, 2010[3]	34	mCRC	5-FU bolus + LV	Efficacy	NA	TT longer TTP TT longer OS		
Derwinger et al, 2009[4]	142	Stage III colon carcinoma	5-FU bolus + LV (5% + oxali)	Toxicity Efficacy	NA	CT/TT more toxicity		CC better OS
Gusella et al, 2009[5]	130	Stage II-III colon carcinoma (adjuvant)	5-FU bolus + LV	Toxicity Efficacy		TT increased grade 3-4 toxicity	No effect of polymorphism	
Huang et al, 2008[6]	201	Stage II-III colon carcinoma (adjuvant)	5-FU bolus + LV	Efficacy			NA	No effect of polymorphism
Jakobsen et al, 2005[7]	88	mCRC	5-FU bolus + LV	Efficacy		TT higher RR	No effect of polymorphism	

Table 1. Overview of literature: MTHFR polymorphisms and cytotoxic effects of fluoropyrimidine-based chemotherapy in colorectal cancer, according to mode of administration

mCRC metastasized colorectal carcinoma; RR response rate; LV leucovorin; NA not assessed in this trial; pCR pathologic complete response; PFS progression free survival; DSS disease specific survival; OS overall survival; TTP time to progression; ACUP Adenocarcinoma of unknown primary.

*Compound heterozygotes or *MTHFR* 1298CC or *MTHFR* 677TT: less gastro-intestinal toxicity.

Only if associated with *TYMS* 3R3R

$ Other drugs included: platinum derivatives, gefitinib and irinotecan

Author, year (Ref.)	No of patients	Patient population	Treatment	Endpoint		Hypothesis confirming	Hypothesis disproving	
			Monotherapy fluoropyrimidine					
Balboa et al, 2010[8]	65	Pre-operative chemoradiation rectal carcinoma	5-FU bolus or cont. or capecitabine	Efficacy			No effect of polymorphism	No effect of polymorphism
Braun et al. 2009[9]	688	mCRC	5FU bolus, followed by cont.	Toxicity			No effect of polymorphism	No effect of polymorphism
Capitain et al, 2008[10]	76	mCRC	LV5FU2 or 5FU bolus + LV	Toxicity Efficacy	AA shorter OS#, CC more grade 3-4 toxicity	CC shorter OS#		
Cohen et al, 2003[11]	43	mCRC	Fluoro-pyrimidine	Toxicity Efficacy	NA	T allele higher RR		
Etienne et al, 2004[12]	98	mCRC	5-FU bolus or cont. + LV	Efficacy		TT higher RR	CC trend towards worse DSS	
Fernandez-Peralta et al, 2010[13]	143	Sporadic colorectal carcinoma	5-FU based chemotherapy	Efficacy		CT/TT better OS	AC/CC reduced OS	
Lamas et al. 2011[14]	93	Pre-operative chemoradiation rectal carcinoma	5-FU cont.	Efficacy			No effect of polymorphism	No effect of polymorphism
Pardini et al, 2011[15]	273	Colon carcinoma (adjuvant)	5-FU bolus + LV ± followed by cont.	Efficacy	AC/CC increased PFS			No effect of polymorphism
Schwab et al, 2008[16]	683	Colon cancer, breast cancer, other GI cancer, ACUP	5-FU bolus or cont. ± LV	Toxicity			No effect of polymorphism	No effect of polymorphism
Sharma et al, 2008[17]	54	mCRC	Capecitabine fixed-dose	Toxicity Efficacy	AA less toxicity		TT less toxicity	

Table 1. Continued

Author, year (Ref.)	No of patients	Patient population	Treatment	Endpoint		Hypothesis confirming	Hypothesis disproving	
			Combination therapy		1298A>C	677C>T	1298A>C	677C>T
Agostini et al, 2008[18]	42	Pre-operative chemoradiation rectal carcinoma (aged > 65y)	5-FU cont. or capecitabine ± oxaliplatin	Toxicity			No effect of polymorphism	No effect of polymorphism
Boigé et al, 2010[19]	349	mCRC	LV5FU2, FOLFIRI, FOLFOX	Toxicity Efficacy	CC higher RR only in FOLFOX			No effect of polymorphism
Budai et al, 2011[20]	85	mCRC	FOLFIRI + bevacizumab	Toxicity Efficacy			NA	No effect of polymorphism
Cecchin et al, 2011[21]	238	Pre-operative chemoradiation rectal carcinoma	Fluoro-pyrimidine ± other drug[$]	Efficacy			No effect of polymorphism	CT/TT lower RR
Chua et al, 2009[22]	118	mCRC	FOLFOX	Toxicity Efficacy		TT more grade 3-4 diarrhea	NA	
Etienne-Grimaldi et al, 2010[23]	117	mCRC	FOLFOX	Toxicity Efficacy	AC/CC higher RR	CT/TT higher RR		
Garcia-Aguilar et al, 2011[24]	132	Pre-operative chemoradiation rectal carcinoma	5-FU cont. ± followed by FOLFOX	Efficacy			NA	TT lower pCR
Glimelius et al, 2010[25]	140	mCRC	FLIRI or LV5FU2-IRI	Toxicity Efficacy			No effect of polymorphism	CT less early toxicity
Huang et al, 2010[26]	157	mCRC	FOLFOX	Efficacy			NA	No effect of polymorphism
Kristensen et al, 2010[27]	68	Stage II-II coloncarcinoma and mCRC	5-FU bolus / cont. or capecitabine ± oxaliplatin	Toxicity	CC increased toxicity			CT increased toxicity

Table 1. Continued

Author, year (Ref.)	No of patients	Patient population	Treatment	Endpoint		Hypothesis confirming	Hypothesis disproving	
			Combination therapy		1298A>C	677C>T	1298A>C	677C>T
Lamas et al, 2011[28]	72	mCRC	FOLFOX	Efficacy Toxicity			No effect of polymorphism	No effect of polymorphism
Lurje et al, 2008[29]	197	Stage II-III colon carcinoma (adjuvant)	5-FU bolus or cont.	Efficacy			No effect of polymorphism	No effect of polymorphism
Marcuello et al, 2006 [30]	94	mCRC	5-FU cont. + irinotecan or oxaliplatin/LV	Efficacy			No effect of polymorphism	No effect of polymorphism
Ruzzo et al, 2007[31]	166	mCRC	FOLFOX	Toxicity Efficacy			No effect of polymorphism	No effect of polymorphism
Ruzzo et al, 2008[32]	146	mCRC	FOLFIRI	Toxicity Efficacy			No effect of polymorphism	No effect of polymorphism
Suh et al, 2006[33]	54	mCRC	FOLFOX	Efficacy			NA	No effect of polymorphism
Terrazino et al, 2006[34]	125	Pre-operative chemoradiation rectal carcinoma	5-FU ± other drugs	Efficacy		CC higher RR	No effect of polymorphism	
Thomas et al, 2011[35]	96 + 35	Pre-operative chemoradiation rectal carcinoma	5-FU cont./ 5-FU cont. + irinotecan	Toxicity Efficacy			AA more mucositis/ diarrhea	No effect of polymorphism
Zhang et al, 2007[36]	318	mCRC	5-FU ± irinotecan ± oxaliplatin	Efficacy			♂: no effect ♂: AA longer OS	No effect of polymorphism

Table 1. Continued

Methods

Patients and treatment

In total, 127 patients who were treated with capecitabine monotherapy as the first-line treatment for mCRC were prospectively included in the study. In addition, a second cohort including 141 patients treated with first-line capecitabine–irinotecan combination therapy was studied. Patients were recruited from the CAIRO study, a multicenter open-label randomized phase III trial, comparing sequential versus combination chemotherapy with capecitabine, irinotecan and oxaliplatin in a total of 803 patients with mCRC.[17]

Patients with mCRC were enrolled in the CAIRO study between January 2003 and December 2004 by the Dutch Colorectal Cancer Group in 74 hospitals in the Netherlands. The study flowchart and number of patients available for analysis are shown in Figure 2. As this pharmacogenetic substudy was initiated later than the CAIRO clinical trial and not all study centers participated, the number of patients included in the pharmacogenetic analyses is limited to a total of 268 patients. The inclusion criteria were a WHO performance score of 0–2 and adequate renal, hepatic and bone marrow function. A history of previous adjuvant chemotherapy was allowed only if the last administration was 6 months before randomization. The main exclusion criteria were as follows: serious concomitant disease preventing the safe administration of chemotherapy and other malignancies in the past 5 years. Capecitabine (1250 mg/m^2, twice daily) was administered on days 1–14 in the monotherapy group every 3 weeks. In the combination therapy group, capecitabine (1000 mg/m^2, twice daily) was administered on days 1–14, and irinotecan (250 mg/m^2) on day 1, in a 3-weekly cycle. Tumor response was assessed by computed tomography scan, every 9 weeks, using the response evaluation criteria for solid tumors (version 1.0). Toxicity during first-line therapy was assessed at each visit by determining the patient's history, physical examination and hematology and biochemical laboratory tests. Toxic effects were classified following the US National Cancer Institute Common Toxicity Criteria, version 2.0. The CAIRO study protocol provided guidelines for dose modification in case of serious toxicity.

The study was approved both by the Central Committee on Research involving Human Subjects (CCMO) and by the local ethics committees of all participating centers. All patients included provided written informed consent before inclusion in the main study and the pharmacogenetic side study.

Genotyping data

Peripheral EDTA-blood samples were collected and stored at – 20°C before DNA isolation. Germline DNA was extracted with the Magnapure LC (Roche Diagnostics, Almere, the Netherlands) according to the manufacturer's instructions. *MTHFR* rs1801133 (677C > T) and *MTHFR* rs1801131 (1298A > C) genotypes were determined with TaqMan 7500 (Applied Biosystems, Nieuwerkerk aan de Ijssel, the Netherlands) according to the manufacturer's protocol using a predesigned assay. The call rate for the *MTHFR* genotypes was greater than

98%. Five per cent of samples were analyzed in duplicate, with 100% concordance. In addition, negative controls using water were included. To exclude confounding by other known pharmacogenetic determinants in 5-FU-based chemotherapy, we also assessed the *TYMS* genotype. The VNTR polymorphism in the *TYMS* 5'-untranslated region (TSER, rs34743033), including the additional G/C SNP in the second base pair for 3-repeat individuals (rs11540151), was determined by direct sequencing. The TYMS 1494del6bp polymorphism in the 3'-untranslated region (rs11280056) was determined with TaqMan 7500 (Applied Biosystems) according to the prescribed protocol. *DPYD**2A (IVS14 + 1G > A) was not included because of low expected population allele frequency.

Data and statistical analysis

The primary endpoints of this study were overall toxicity (i.e. grade 3 or higher) on first-line therapy, and specific adverse events, including hand–foot syndrome (HFS), diarrhea and febrile neutropenia. Toxicity was assessed for all patients who started treatment. The secondary endpoints were progression-free survival (PFS), OS and response rate. PFS was calculated from the date of randomization until the first observation of disease progression or death from any cause. OS was calculated as the interval from randomization until death from any cause or until the date of last follow-up. Survival data have been updated since the publication of the original CAIRO trial. Best response was assessed in all patients who completed at least three cycles of treatment. Clinical benefit was defined as either a complete or a partial response, or stable disease.

Genotype distributions were tested for agreement with those expected under Hardy–Weinberg equilibrium using the χ^2-test. The association between genotype or diplotype and the presence of overall toxicity of at least grade 3, specific toxicity parameters of at least grade 3 and clinical benefit were determined using a χ^2-test. Kaplan– Meier survival analysis and the log-rank test were used to test the relationship between genotype and OS/PFS. For *TYMS* polymorphisms, patients were further subdivided into three groups according to the expected level of TS expression: low (2R/2R or 2R/3RC or 3RC/3RC), intermediate (2R/3RG or 3RC/3RG) or high (3RG/3RG). *MTHFR* genotypes were grouped as follows: wild-type homozygote versus all other genotypes or variant-type homozygote versus all other genotypes. The association of genotype and OS or PFS was determined using the Mann–Whitney test. Fisher's exact test was used to determine the effect of grouped genotype on toxicity and clinical benefit. Multivariate analysis was carried out with *TYMS* genotype and sex. Because of the linkage disequilibrium between both *MTHFR* genotypes, we did not carry out multivariate analyses including both polymorphisms as independent covariates, but rather carried out separate analyses for *MTHFR* 1298A > C and 677C > T genotypes. On the basis of reports finding a difference in the effect of *MTHFR* genotype on 5-FU efficacy according to sex, subgroup analysis was carried out. Subgroup analysis was also carried out by subdividing patients according to previous adjuvant therapy. Patients with missing genotyping data were excluded from analysis. A two-sided significance level of P less than 0.01 was accepted for all analyses to compensate for multiple

testing. Analyses were carried out using the SPSS version 17.0 software (SPSS Inc., Chicago, Illinois, USA).

Results

Clinical data

The baseline characteristics of the studied patients are listed in Table 2. The median age at randomization for all patients was 61 years, ranging from 27 to 81 years. Patients were predominantly men (61%) and most patients had not received previous adjuvant chemotherapy (88%).

	Total N= 264	**Capecitabine monotherapy N= 126**	**Combination therapy N= 138**
Age			
Age at randomization, median (range)	61 years (27-81)	61 years (27-78)	62 years (37-81)
Sex			
Male	163 (62%)	77 (61%)	86 (62%)
Female	101 (38%)	49 (39%)	52 (38%)
Performance status			
PS 0	152 (58%)	77 (61%)	75 (54%)
PS 1	96 (36%)	42 (33%)	54 (40%)
PS 2	16 (6%)	7 (6%)	9 (6%)
LDH at randomization			
Normal	173 (66%)	82 (65%)	91 (66%)
>Upper limit of normal	91 (34%)	44 (35%)	47 (34%)
Site of primary tumor			
Colon	146 (55%)	73 (58%)	73 (53%)
Rectosigmoid	96 (37%)	40 (32%)	56 (41%)
Rectum	20 (8%)	11 (9%)	9 (6%)
Multiple tumors	1 (<1%)	1 (<1%)	0
Unknown	1 (<1%)	1 (<1%)	0
Predominant localization of metastases			
Liver	179 (68%)	83 (66%)	96 (70%)
Extra-hepatic	81 (31%)	39 (31%)	42 (30%)
Unknown	4 (1%)	4 (3%)	0
Previous adjuvant therapy			
Yes	31 (12%)	13 (10%)	18 (13%)
No	233 (88%)	113 (90%)	120 (87%)

Table 2. Baseline characteristics.

LDH, lactate dehydrogenase

Genotype frequencies

Genotype frequencies are listed in Table 3. Genotyping for *MTHFR* polymorphisms was successful for 126 of the 127 (99%) patients in the capecitabine monotherapy group. For the *MTHFR* 677C > T locus, we found that 51 (41%) patients were homozygote wild type, 14 (11%) were homozygote variant type and 61 (48%) were heterozygote. For *MTHFR* 1298A > C, 58 (46%) patients were homozygote wild type, 12 (10%) were homozygote variant type and 56 (44%) patients were heterozygote.

Genotyping was successful for 138 of the 141 (98%) patients in the combination therapy group. For *MTHFR* 677C > T, in this group 55 (40%) patients were homozygote wild type, 13 (9%) were homozygote variant type and 70 (51%) heterozygote. For *MTHFR* 1298A > C, 57 (42%) patients were homozygote wild type, 13 (9%) were homozygote variant type and 68 (49%) were heterozygote.

Allele frequencies for *MTHFR* 677C > T and for *MTHFR* 1298A > C in both groups were consistent with Hardy–Weinberg equilibrium (χ^2-test: $P > 0.05$). Genotype frequencies are similar to those reported by other authors.[18–20] No patients were found to be homozygous for both loci, consistent with the linkage disequilibrium between both polymorphisms described elsewhere.[11]

Genotyping for *TYMS* was successful for 112 (88%) patients in the monotherapy group and for 120 (85%) patients in the combination therapy group. In the capecitabine monotherapy group, *TSER*-genotype frequencies were 26 (23%) 2R2R, 31 (28%) 3R3R and 55 (49%) 2R/3R. A predicted low TS-expression genotype was present in 70 (62%) patients, an intermediate-expression genotype in 33 (30%) and a high-expression genotype in nine (8%) patients (see the Methods section for definition of expression level in individual genotypes). For the *TYMS* 1494del6bp genotype, 13 (12%) patients had the del/del genotype, 40 (36%) had the del/ins genotype and 59 (52%) had the ins/ins genotype.

In the combination therapy group, *TSER* genotypes were as follows: 28 (23%) 2R2R, 37 (31%) 3R3R and 55 (46%) heterozygote. TS expression genotypes were predicted to be low in 65 (54%) patients, intermediate in 46 (38%) and high in nine (8%) patients. The *TYMS* 1494 del/del genotype was found in 11 (9%) patients; 57 (48%) patients were heterozygote and 52 (43%) were ins/ins homozygote. The *TSER* genotype for patients in the monotherapy group was not consistent with the Hardy–Weinberg equilibrium ($\chi^2 = 12.2$, $P < 0.001$). However, because no deviation from the equilibrium was found (χ^2-test: $P > 0.05$) in the total population and in the combination therapy group, it is likely that this inconsistency was derived by chance. All other genotype frequencies were as expected under the Hardy– Weinberg equilibrium.

Genotype	Total	Capecitabine monotherapy	CapIri combination therapy
N=	264	126	138
***MTHFR* 677C>T**			
677CC	106 (40%)	51 (41%)	55 (40%)
677CT	131 (50%)	61 (48%)	70 (51%)
677TT	27 (10%)	14 (11%)	13 (9%)
***MTHFR* 1298A>C**			
1298AA	115 (44%)	58 (46%)	57 (42%)
1298AC	124 (47%)	56 (44%)	68 (49%)
1298CC	25 (9%)	12 (10%)	13 (9%)
Diplotype			
1298AA/677CC	26 (10%)	15 (12%)	11 (8%)
1298AA/677CT	64 (24%)	31 (25%)	33 (24%)
1298AA/677TT	25 (10%)	12 (10%)	13 (9%)
1298AC/677CC	57 (22%)	26 (21%)	31 (23%)
1298AC/677CT	65 (25%)	28 (22%)	37 (27%)
1298AC/677TT	2 (<1%)	2 (2%)	0
1298CC/677CC	23 (9%)	10 (8%)	13 (9%)
1298CC/677CT	2 (<1%)	2 (2%)	0
1298CC/677TT	0	0	0

Table 3. MTHFR genotype and diplotype frequencies

Correlation between MTHFR and TYMS genotypes/ diplotypes and toxicity

The results for toxicity analyses are listed in Table 3. No correlation was found between genotype and overall toxicity of at least grade 3 for the *MTHFR* 677C > T genotype and 1298A>C in patients treated with capecitabine monotherapy. Grouping genotypes according to the presence or absence of variant alleles did not show any statistically significant association with the incidence of severe overall toxicity. Diplotype analysis was carried out grouping patients according to the number of variant alleles (gene score). This did not result in a significant association between any gene score and overall toxicity of at least grade 3 (P = 0.838).

In addition, we carried out association analyses for the *MTHFR* genotype and specific adverse events. No significant association was found for the *MTHFR* 1298A > C genotype, the *MTHFR* 677C > T genotype or the *MTHFR* diplotype and the incidence of HFS of at least grade 3 or febrile neutropenia of at least grade 3. However, a trend towards a higher incidence of diarrhea of at least grade 3 was observed for *MTHFR* 1298CC homozygotes (AA and AC vs. CC: 5 vs. 25%, P = 0.041). No episodes of febrile neutropenia of at least grade 3 were observed in the capecitabine monotherapy group.

Next, all analyses were repeated for patients in the combination treatment arm. No associations were found between *MTHFR* 677C > T and 1298A > C genotypes and severe overall toxicity. These results remained similar after grouping genotypes according to the presence or absence of variant alleles. In terms of the effect of *MTHFR* polymorphisms on specific adverse events, we found no association for diarrhea or febrile neutropenia. *MTHFR*

1298CC individuals experienced a significantly higher incidence of HFS in the combination therapy group (*MTHFR* 1298AA 7% vs. AC 5% vs. CC 31%, P = 0.006; 1298AA and AC vs. CC: 6 vs. 31%, P = 0.011). No toxic deaths were observed in either treatment cohort.

The *TSER* genotype was found not to be associated with the incidence of overall toxicity of at least grade 3, diarrhea of at least grade 3 or febrile neutropenia of at least grade 3 in either treatment cohort (data not shown). However, a trend towards a protective effect of the *TSER* 2R allele on the incidence of HFS of at least grade 3 was found in the capecitabine monotherapy group (2R/2R 8% vs. 2R/3R 11% vs. 3R/3R 29%, P = 0.041; 2R2R and 2R/ 3R vs. 3R/3R: 10 vs. 29%, P = 0.019). This association was not found in the combination therapy group (2R/2R and 2R/3R vs. 3R/3R: 11 vs. 5%, P = 0.499). In both groups, no effect was found of the G > C SNP or the TYMS 1494 del6bp genotype on the incidence of overall or specific toxicity (data not shown).

TSER and *TYMS* 1494 del6bp genotypes were not associated with a difference in PFS or OS, and no significant interaction was found after combining *TSER* genotypes according to the expected level of TS expression on the basis of the G > C SNP. In addition, no association was found between clinical benefit and the *TYMS* 1494 del6bp or *TSER* (including G > C SNP) polymorphisms (data not shown). These results were found in both treatment groups. Only in the monotherapy group was there a non-significant trend towards a longer PFS for high TS-expression (3RG/3RG) individuals (3RG/3RG vs. all other genotypes, 10.2 vs. 6.2 months, P = 0.022), as well as for *TYMS* 1494del/del individuals (1494del/del vs. del/ins and ins/ins: 10.2 vs. 6.1 months, P = 0.017).

Subgroup and multivariate analysis

Multivariate analyses including the *MTHFR* genotype, *TYMS* genotype and sex as covariates did not yield any significant results. No patients with the *MTHFR* 1298CC genotype in first-line capecitabine monotherapy had received previous adjuvant chemotherapy. This is probably because of chance (χ^2-test: P = 0.453). In either treatment group, very few *MTHFR* 677TT individuals had received previous chemotherapy. Therefore, multivariate analysis for the association of *MTHFR* genotype with efficacy and toxicity parameters according to previous adjuvant treatment could not be carried out.

Subdividing our population according to sex showed no significant association between the incidence of adverse events and any *MTHFR* genotype or diplotype (data not shown). Male patients with the *MTHFR* 1298AA genotype had slightly, but non-significant, shorter PFS than patients with at least one variant allele (median PFS 5.7 vs. 6.9 months, P = 0.043).

	Capecitabine	*p*-value		*p*-value		*p*-value		*p*-value
	Overall toxicity ≥ grade 3		**Diarrhea ≥ grade 3**		**HFS ≥ grade 3**		**Febrile neutropenia**	
Overall	37%		7%		16%		0%	
MTHFR 677C>T								
MTHFR 677CC	40%		12%		29%		-	
MTHFR 677CT	33%		5%		7%		-	
MTHFR 677TT	43%	*0.649*	0%	*0.193*	24%	*0.018*	-	*n.a.*
MTHFR 677CC	40%		12%		24%		-	
MTHFR 677CT + 677TT	35%	*0.574*	4%	*0.154*	11%	*0.079*	-	*n.a.*
MTHFR 677CT + 677CC	36%		8%		14%		-	
MTHFR 677TT	43%	*0.770*	0%	*0.596*	29%	*0.237*	-	*n.a.*
MTHFR 1298A>C								
MTHFR 1298AA	37%		7%		19%		-	
MTHFR 1298AC	34%		4%		11%		-	
MTHFR 1298CC	50%	*0.578*	25%	*0.033*	25%	*0.309*	-	*n.a.*
MTHFR1298AA	37%		7%		19%		-	
MTHFR 1298AC + 1298CC	37%	*1.000*	7%	*1.000*	13%	*0.464*	-	*n.a.*
MTHFR 1298AC + 1298AA	35%		5%		15%		-	
MTHFR1298CC	50%	*0.355*	25%	*0.041*	25%	*0.406*	-	*n.a.*
MTHFR diplotype*								
No variant alleles	43%		14%		36%		-	
One variant allele	33%		5%		12%		-	
Two variant alleles	38%		8%		14%		-	
Three variant alleles	50%	*0.838*	0%	*0.630*	25%	*0.170*	-	*n.a.*

Table 4. MTHFR genotype and adverse events of first-line chemotherapy

HFS, hand-foot syndrome

*No variant alleles, 1298AA/677CC; one variant allele, 1298AC/677CC or 1298AA/677CT; two variant alleles, 1298CC/677CC or 1298AA/677TT or 1298AC/677CT; three variant alleles, 1298CC/677CT or 1298AC/67TT.

	Combination therapy	***p*-value**		***p*-value**		***p*-value**		***p*-value**
	Overall toxicity ≥ grade 3		**Diarrhea ≥ grade 3**		**HFS ≥ grade 3**		**Febrile neutropenia**	
Overall	50%		23%		8%		6%	
MTHFR 677C>T								
MTHFR 677CC	47%		22%		11%		4%	
MTHFR 677CT	51%		27%		6%		7%	
MTHFR 677TT	54%	*0.862*	8%	*0.297*	8%	*0.581*	8%	*0.665*
MTHFR 677CC	47%		22%		6%		4%	
MTHFR 677CT + 677TT	52%	*0.728*	24%	*0.838*	11%	*0.348*	7%	*0.475*
MTHFR 677CT + 677CC	50%		25%		8%		6%	
MTHFR 677TT	54%	*1.000*	8%	*0.298*	8%	*1.000*	8%	*0.560*
MTHFR 1298A>C								
MTHFR 1298AA	54%		16%		7%		7%	
MTHFR 1298AC	44%		29%		5%		6%	
MTHFR 1298CC	62%	*0.355*	23%	*0.199*	31%	*0.006*	0%	*0.621*
MTHFR1298AA	54%		16%		7%		7%	
MTHFR 1298AC + 1298CC	47%	*0.489*	28%	*0.103*	9%	*0.762*	5%	*0.719*
MTHFR 1298AC + 1298AA	49%		23%		6%		7%	
MTHFR1298CC	62%	*0.562*	23%	*1.000*	31%	*0.011*	0%	*1.000*
MTHFR diplotype*								
No variant alleles	36%		0%		9%		0%	
One variant allele	53%		27%		5%		8%	
Two variant alleles	49%		24%		11%		5%	
Three variant alleles	-	*0.582*	-	*0.154*	-	*0.391*	-	*0.536*

Table 4. Continued

	Capecitabine monotherapy	*p*-value		*p*-value		*p*-value
	PFS in months (95%CI)		OS in months (95%CI)		Clinical benefit	
	median		median			
Overall	6.3 (5.8-6.9)		19.5 (16.7-22.2)		85%	
MTHFR 677C>T						
MTHFR 677CC	6.6 (5.1-8.2)		19.5 (15.6-23.3)		90%	
MTHFR 677CT	6.1 (5.1-7.1)		18.1 (12.8-23.4)		78%	
MTHFR 677TT	6.6 (5.5-7.7)	*0.148*	24.0 (19.9-28.1)	*0.857*	100%	*0.067*
MTHFR 677CC	6.6 (5.1-8.2)		19.5 (15.6-23.3)		90%	
MTHFR 677CT + 677TT	6.1 (5.3-6.9)	*0.190*	19.5 (13.1-25.8)	*0.609*	82%	*0.310*
MTHFR 677CT + 677CC	6.3 (5.7-6.8)		19.2 (16.3-22.0)		83%	
MTHFR 677TT	6.6 (5.5-7.7)	*0.807*	24.0 (19.9-28.1)	*0.270*	100%	*0.127*
MTHFR 1298A>C						
MTHFR 1298AA	6.1 (5.7-6.5)		19.4 (11.4-27.4)		93%	
MTHFR 1298AC	6.9 (5.0-8.8)		19.8 (12.1-27.4)		78%	
MTHFR 1298CC	6.5 (4.2-8.8)	*0.610*	18.7 (17.6-19.8)	*0.534*	83%	*0.097*
MTHFR1298AA	6.1 (5.7-6.5)		19.4 (11.4-27.4)		93%	
MTHFR 1298AC + 1298CC	6.7 (5.1-8.4)	*0.582*	19.5 (15.4-23.5)	*0.957*	79%	*0.042*
MTHFR 1298AC + 1298AA	6.3 (5.8-6.8)		18.7 (17.6-19.8)		86%	
MTHFR1298CC	6.5 (4.2-8.8)	*0.904*	19.5 (13.5-25.4)	*0.758*	83%	*0.691*
MTHFR diplotype*						
No variant alleles	6.3 (5.8-6.8)		21.0 (13.5-28.6)		100%	
One variant allele	6.7 (4.7-8.6)		19.4 (10.9-27.9)		87%	
Two variant alleles	6.0 (4.5-7.5)		19.1 (17.2-21.1)		78%	
Three variant alleles	8.3 (6.2-10.5)	*0.470*	21.4 (12.6-30.2)	*0.942*	100%	*0.157*

Table 5. MTHFR genotype and efficacy of first-line chemotherapy

CI, confidence interval; OS, overall survival; PFS, progression-free survival.
*No variant alleles, 1298AA/677CC; one variant allele, 1298AC/677CC or 1298AA/677CT; two variant alleles, 1298CC/677CC or 1298AA/677TT or 1298AC/677CT; three variant alleles, 1298CC/677CT or 1298AC/67TT.

	Combination therapy	*p*-value		*p*-value		*p*-value
	PFS in months (95%CI)		OS in months (95%CI)		Clinical benefit	
	median		median			
Overall	8.3 (7.6-9.1)		20.0 (16.9-23.1)		93%	
MTHFR 677C>T						
MTHFR 677CC	9.2 (7.9-10.5)		19.8 (14.1-25.4)		94%	
MTHFR 677CT	7.8 (6.6-8.9)		20.0 (16.0-24.0)		93%	
MTHFR 677TT	6.3 (3.5-9.1)	*0.047*	23.1 (12.4-33.8)	*0.923*	92%	*0.964*
MTHFR 677CC	9.2 (7.9-10.5)		19.8 (14.1-25.4)		94%	
MTHFR 677CT + 677TT	7.8 (6.5-9.0)	*0.052*	20.5 (16.7-24.3)	*0.888*	93%	*1.000*
MTHFR 677CT + 677CC	8.3 (7.5-9.1)		20.0 (16.6-23.4)		93%	
MTHFR 677TT	6.3 (3.5-9.1)	*0.625*	23.1(12.4-33.8)	*0.994*	92%	*1.000*
MTHFR 1298A>C						
MTHFR 1298AA	7.1 (5.8-8.4)		17.9 (15.0-20.9)		94%	
MTHFR 1298AC	8.8 (7.9-9.7)		22.3 (18.8-25.9)		92%	
MTHFR 1298CC	9.4 (5.8-13.0)	*0.107*	22.4 (14.9-29.9)	*0.529*	90%	*0.848*
MTHFR1298AA	7.1 (5.8-8.4)		17.9 (15.0-20.9)		94%	
MTHFR 1298AC + 1298CC	9.0 (8.1-10.0)	*0.033*	22.4 (18.9-25.8)	*0.148*	92%	*0.735*
MTHFR 1298AC + 1298AA	8.3 (7.7-8.9)		20.0 (16.6-23.4)		93%	
MTHFR1298CC	9.4 (5.8-13.0)	*0.425*	22.4 (14.9-29.9)	*0.916*	90%	*0.531*
MTHFR diplotype*						
No variant alleles	6.5 (5.7-7.3)		14.4 (6.9-21.9)		90%	
One variant allele	8.4 (7.7-9.2)		19.8 (15.3-24.2)		97%	
Two variant alleles	8.3 (7.1-9.6)		22.3 (18.9-25.8)		90%	
Three variant alleles	-	*0.216*	-	*0.165*	-	*0.353*

Table 5. Continued

Discussion

The present study is the second and the largest to address *MTHFR* pharmacogenetics of capecitabine-based therapy in mCRC. No significant association was found between the *MTHFR* 677C > T or the 1298A > C genotype or diplotype and the incidence of severe chemotherapy-induced adverse events for either monotherapy or combination therapy with capecitabine. *MTHFR* 1298CC homozygotes showed a non-significant increase in grade 3–4 diarrhea when treated with capecitabine monotherapy, in accordance with our hypothesis. No effect was found of these genotypes on clinical response or survival statistics in our populations.

Because of the prospective accrual of patients in the CAIRO study, there is homogeneity in the treatment protocol for all patients, thereby obviating the risk of confounding by dosage or mode of 5-FU administration. Publications to date, including many clinical trials[3;18;19;21–39] and two recent meta-analyses[40;41], could not show a convincing effect of *MTHFR* polymorphisms on fluoropyrimidine-induced toxicity or treatment benefit in mCRC. However, whereas all these studies have focused on intravenous 5-FU therapy, capecitabine is increasingly being incorporated into the first-line treatment for mCRC, making the existing literature on 5-FU pharmacogenetics less relevant. Although capecitabine has comparable efficacy to 5-FU as monotherapy or in combination therapy[1], toxicity profiles differ. Capecitabine leads to a higher incidence of HFS than a 5-FU bolus injection.[42] This side effect is also found more frequently in 5-FU continuous infusion and suggests a difference in 5-FU pharmacodynamics depending on the mode of administration.[5] As a result, polymorphisms involved in the folate pathway may have a different effect on efficacy and toxicity according to the treatment schedule and mode of administration, and pharmacogenetic studies with 5-FU cannot be extrapolated to capecitabine. Only one previous small clinical trial studying pharmacogenetics of capecitabine monotherapy in mCRC patients has been published.[16] In this study, *MTHFR* 677TTand *MTHFR* 1298AA genotypes were associated with a lower incidence of grade 2–3 toxicity. Although the results for *MTHFR* 1298AA individuals confirm the hypothesis that *MTHFR* polymorphisms enhance capecitabine cytotoxicity, the results for *MTHFR* 677TT are contrary to what was expected. As there is no obvious pharmacological explanation for this incongruence, the results may have been affected by the small sample size. By choosing a stricter significance level, we reduced the risk of false-positive results because of multiple testing and found no association of the *MTHFR* genotype and capecitabine-induced adverse events. Furthermore, we focused only on the occurrence of severe toxicity (i.e. grade 3 or 4) because the goal of pretreatment testing is the prevention of serious adverse events. Indeed, an increase in grade 2 toxicity will not lead to pre-emptive dose reduction and therefore may not be considered clinically relevant.

As capecitabine cytotoxicity is the result of many interdependent enzymatic reactions, not only including *MTHFR* but also *TS*, the effects of one aberrant enzyme may be obscured by those of another. Therefore, addressing only polymorphisms in one gene may be an oversimplification of reality and this was the motivation to also include genetic variants in *TYMS* and *TSER*. The importance of the variants is supported by two studies, showing that patients with *TYMS*

3R/3R and either the *MTHFR* 1298CC or the 677TT genotype had a higher response rate to 5-FU-based chemotherapy, and longer OS or time to progression.[18;34] We univariately and multivariately evaluated the contributing effect of *TYMS* polymorphisms, without any effect on study outcome. We then identified the patients in our cohort carrying the *TYMS* 3R/3R genotype and a homozygote variant genotype for at least one of the *MTHFR* polymorphisms, in an attempt to replicate the two above-mentioned studies. In our population, however, only a few patients carried the *TYMS* 3R/3R-*MTHFR* 677TT or *TYMS* 3R/3R-*MTHFR* 1298CC genotype (five and four patients in the monotherapy group, respectively). Although we found no significant associations of these genotypes and the clinical outcome parameters, analyses are limited by the small number of affected patients (data not shown).

In early phase I trials, capecitabine was combined with oral leucovorin. This addition showed no effect on capecitabine pharmacokinetics, but appeared to reduce the maximum tolerable dose.[43;44] Currently, capecitabine therapy is not combined with leucovorin, in contrast to intravenous 5-FU therapy and tegafur. It can be hypothesized that in case of high serum levels of active folate, either by diet or by administration of leucovorin, the effects of *MTHFR* polymorphisms are masked. Folate intake and serum folate levels differ according to the geographical location of the population.[45;46] The folate levels in a Dutch population are on average lower than those for other European populations.[45] Therefore, in our population, folate level does not seem to explain the lack of effect of *MTHFR* polymorphisms.

In many modern chemotherapy regimens, capecitabine is combined with other chemotherapeutic agents, such as irinotecan or oxaliplatin. In combination therapy, toxicity caused by one of the agents may lead to dose reduction, and this may affect the possible pharmacogenetic associations of the other drugs. Our study is unique in the fact that it studies *MTHFR* pharmacogenetics in both capecitabine monotherapy and combined therapy. In the combination therapy group, however, the results may have been biased by the known pharmacogenetic effects of *UGT1A1*. Indeed, in Caucasians, the incidence of diarrhea and specifically febrile neutropenia because of irinotecan have been shown to be influenced by *UGT1A1**28 genotype.[6;31] We found no significant association between *MTHFR* genotypes and the incidence of febrile neutropenia or diarrhea in the combination treatment cohort. Therefore, inclusion of the *UGT1A1* genotype in multivariate analysis was not deemed contributory. To fully exclude an interference of the *UGT1A1* genotype, we carried out toxicity analyses for all patients in the combination therapy cohort responding to the *UGT1A1* homozygote wild-type genotype. In this subgroup of 57 patients, we found a preventive effect of the *MTHFR* 677TT genotype on the occurrence of severe diarrhea. None of eight patients with the *MTHFR* 677TT genotype developed grade 3 or 4 diarrhea, versus 11 out of 49 patients with the *MTHFR* 677CT or TT genotype (0 vs 22%, P = 0.009, data not shown). As no statistically significant effect was found in the monotherapy group, these results would suggest an effect of *MTHFR* 677C > T on irinotecan toxicity, for which there is no obvious pharmacologic explanation. Furthermore, introducing additional subgroup analysis should lead to the acceptance of an even stricter significance level, thereby making the outcome statistically non-significant.

Therefore, we conclude that this remarkable result was because of multiple testing, rather than a pharmacogenetic effect.

In a recent study involving neoadjuvant chemoradiation treatment for rectal carcinoma, *MTHFR* polymorphisms were predictive of grade 3–4 diarrhea and mucositis in patients receiving 5-FU monotherapy, but not in patients receiving 5-FU in combination with irinotecan.[20] However, because patients were prospectively assigned to either 5-FU monotherapy or 5-FU/irinotecan on the basis of the *TYMS* genotype, it cannot be excluded that the difference between the two groups was caused by the *TYMS* genotype, rather than the addition of irinotecan.

It has been proposed that the conflicting results of pharmacogenetic studies with 5-FU in mCRC are related to sex differences. Zhang et al.[39] reported a better OS for the *MTHFR* 1298AA genotype only for women in a heavily pre-treated cohort of mCRC patients. Another study found that *MTHFR* 1298AC heterozygote women had a shorter OS.[47] However, a sex-specific effect could not be confirmed by others.[21;25–27;48] Our data show a slightly, albeit non-significant, shorter PFS for male patients with the *MTHFR* 1298AA genotype. In our opinion, these conflicting data suggest that the difference between sexes may be the result of multiple testing in increasingly small groups.

Interestingly, epigenetic changes may act in concert with genetic variations. Cancer cell lines expressing the *MTHFR* 1298CC homozygous genotype show a higher number of methylated genes compared with heterozygotes or wild-type homozygotes.[49] The *MTHFR* 1298C allele was associated with a longer doubling time in cancer xenografts, with the shortest doubling time for 1298AA homozygotes, independent of 5-FU.[50]

Conversely, colorectal cancer cells and xenografts transfected with variant *MTHFR* 677T showed an accelerated growth rate compared with non-transfected cells, but were also inhibited more effectively by 5-FU plus leucovorin.[51] *MTHFR* polymorphisms may therefore be a prognostic, rather than a predictive marker. Indeed, Fernandez-Peralta et al.[29] found that the *MTHFR* 1298C variant allele was associated with shorter OS in sporadic colorectal cancer patients, even in the absence of 5-FU-containing chemotherapy.

Although we studied a larger group of patients on capecitabine monotherapy than any previous study, our sample size remains relatively small. The small number of homozygote variant individuals in our population limits the statistical power of this study to detect small effects of pharmacogenetics on clinical outcome. However, if *MTHFR* genotypes were strongly associated with toxicity or efficacy parameters, these effects would have been found even in a relatively small cohort. Other polymorphisms have been described for *MTHFR*. It cannot be excluded that a full haplotype analysis, including all known *MTHFR* polymorphisms, would show an effect on fluoropyrimidine toxicity. However, as *MTHFR* 677C > T and *MTHFR* 1298A > C are the polymorphisms showing functional importance, we believe that the chances of finding an effect on 5-FU or capecitabine toxicity are small. Therefore, we conclude that *MTHFR* 677C > T and 1298A > C polymorphisms are not related to the occurrence of severe toxicity (and efficacy) of capecitabine-based chemotherapy in mCRC.

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