FULL PAPER

BJC

British Journal of Cancer (2017) 117, 1269–1277 | doi: 10.1038/bjc.2017.289 BJC OPEN

Keywords: colorectal cancer; pharmacogenetics; toxicity; chemotherapy; dihydropyrimidine dehydrogenase; polymorphisms; 5-fluorouracil; capecitabine

Dihydropyrimidine dehydrogenase pharmacogenetics for predicting fluoropyrimidine-related toxicity in the randomised, phase III adjuvant TOSCA trial in high-risk colon cancer patients

A Ruzzo*[,1,23], F Graziano*[,2,23], Fabio Galli[3], Francesca Galli[3], E Rulli[3], S Lonardi[4], M Ronzoni[5], B Massidda[6], V Zagonel[4], N Pella[7], C Mucciarini[8], R Labianca[9], M T Ionta[6], I Bagaloni[1], E Veltri[10], P Sozzi[11], S Barni[12], V Ricci[5], L Foltran[13], M Nicolini[14], E Biondi[15], A Bramati[16], D Turci[17], S Lazzarelli[18], C Verusio[19], F Bergamo[4], A Sobrero[20], L Frontini[21], M Menghi[22] and M Magnani[1]

[1]*Department of Biomolecular Sciences, Università degli Studi di Urbino 'Carlo Bo', Urbino 61032, Italy;* [2]*Azienda Ospedaliera 'Ospedali Riuniti Marche Nord', Department of Oncology, Pesaro 61122, Italy;* [3]*Laboratory of Methodology for Clinical Research, Department of Oncology, IRCCS-Istituto di Ricerche Farmacologiche 'Mario Negri', Milano 20156, Italy;* [4]*IOV- IRCCS, Padova 35128, Italy;* [5]*Ospedale San Raffaele, Department of Oncology, Milano 20132, Italy;* [6]*Azienda Ospedaliera Universitaria di Cagliari, P.O. Monserrato, Monserrato 09042, Italy;* [7]*Azienda Ospedaliera S. Maria della Misericordia, Department of Oncology, Udine 33010, Italy;* [8]*Ospedale 'B. Ramazzini', Department of Oncology, Carpi 41012, Italy;* [9]*Ospedale Papa Giovanni XXIII, Department of Oncology, Bergamo 24127, Italy;* [10]*Ospedale di Gaeta (ASL Latina), Department of Oncology, Gaeta 04024, Italy;* [11]*Ospedale degli Infermi di Biella, Department of Oncology, Biella 13875, Italy;* [12]*Ospedale 'Treviglio-Caravaggio', Department of Oncology, Treviglio 24047, Italy;* [13]*Azienda Ospedaliera Santa Maria degli Angeli, Department of Oncology, Pordenone 33170, Italy;* [14]*Azienda Ospedaliera Ospedale 'Cervesi', Department of Oncology, Cattolica 47841, Italy;* [15]*Ospedale 'F, Renzetti', Department of Oncology, Lanciano 66034, Italy;* [16]*Azienda Ospedaliera Fatebenefratelli, Department of Oncology, Milano 20121, Italy;* [17]*AUSL Ospedale di Ravenna, Department of Oncology, Ravenna 48121, Italy;* [18]*Azienda Ospedaliera di Cremona, Department of Oncology, Cremona 26100, Italy;* [19]*Ospedale di Saronno, Department of Oncology, Saronno 21047, Italy;* [20]*Azienda Ospedaliera 'Ospedale San Martino', Department of Oncology, Genova 16132, Italy;* [21]*Fondazione GISCAD, Parabiago 20015, Italy and* [22]*Diatech Pharmacogenetics, Jesi 60035, Italy*

Background: Dihydropyrimidine dehydrogenase (DPD) catabolises ~85% of the administered dose of fluoropyrimidines. Functional *DPYD* gene variants cause reduced/abrogated DPD activity. *DPYD* variants analysis may help for defining individual patients' risk of fluoropyrimidine-related severe toxicity.

Methods: The TOSCA Italian randomised trial enrolled colon cancer patients for 3 or 6 months of either FOLFOX-4 or XELOX adjuvant chemotherapy. In an ancillary pharmacogenetic study, 10 *DPYD* variants (*2A rs3918290 G>A, *13 rs55886062 T>G, rs67376798 A>T, *4 rs1801158 G>A, *5 rs1801159 A>G, *6 rs1801160 G>A, *9A rs1801265 T>C, rs2297595 A>G, rs17376848 T>C, and rs75017182 C>G), were retrospectively tested for associations with ⩾grade 3 fluoropyrimidine-related adverse events (FAEs). An association analysis and a time-to-toxicity (TTT) analysis were planned. To adjust for multiple testing, the Benjamini and Hochberg's False Discovery Rate (FDR) procedure was used.

Results: FAEs occurred in 194 out of 508 assessable patients (38.2%). In the association analysis, FAEs occurred more frequently in *6 rs1801160 *A* allele carriers (FDR = 0.0083). At multivariate TTT analysis, significant associations were found for *6 rs1801160 *A* allele carriers (FDR < 0.0001), *2A rs3918290 *A* allele carriers (FDR < 0.0001), and rs2297595 *GG* genotype carriers (FDR = 0.0014). Neutropenia was the most common FAEs (28.5%). *6 rs1801160 (FDR < 0.0001), and *2A rs3918290 (FDR = 0.0004) variant alleles were significantly associated with time to neutropenia.

Conclusions: This study adds evidence on the role of *DPYD* pharmacogenetics for safety of patients undergoing fluoropyrimidine-based chemotherapy.

*Correspondence: Dr A Ruzzo; E-mail: annamaria.ruzzo@uniurb.it or Dr F Graziano; E-mail: frada@tin.it

Received 20 March 2017; revised 28 June 2017; accepted 28 July 2017; published online 24 August 2017

The pyrimidine analog 5-fluorouracil (5-FU) and its oral pro-drug capecitabine are among the most prescribed anti-cancer chemotherapeutic agents. Up to one-third of patients exposed to these drugs experience early-onset severe or life-threatening toxicity (Meulendijks *et al*, 2016). The narrow therapeutic index may be even more unfavorable when 5-FU and capecitabine are used in the adjuvant setting, where potentially cured patients undergo a prophylactic treatment strategy.

Dihydropyrimidine dehydrogenase (DPD) catabolises ~85% of the administered dose of fluoropyrimidines and its activity is highly variable (~8–21-fold) in the population (van Kuilenburg *et al*, 1999). Functional *dihydropyrimidine dehydrogenase* (*DPYD*) gene variants have been found to be associated with reduced/abrogated DPD activity (Meulendijks *et al*, 2016). Retrospective and prospective pharmacogenetic studies have emphasised the possible predictive role of *DPYD* variants for 5-FU and capecitabine toxicity. This information and the prediction of an individual patients' risk of severe toxicity could allow for an adequate monitoring and improve overall management and quality of care (Meulendijks *et al*, 2016).

To date, three *DPYD* genetic variants have been consistently associated with fluoropyrimidine risk of toxicity (Caudle *et al*, 2013): *2A rs3918290 G>A, which causes the skipping of the entire exon 14; *13 rs55886062 T>G, which causes an Ile56Ser aminoacid change in a flavine binding domain of DPD; and the rs67376798 A>T, which results in a Asp949Val aminoacid change near an iron-sulfur motif. In a recent review with clinical practice guidelines, fluoropyrimidine dose omission or reductions were recommended in carriers of homozygous and heterozygous carriers of these three 'core' variants (Caudle *et al*, 2013). Because of the very low frequency of these risk alleles there is still debate on their relevance and cost-effectiveness in a 'real world' pre-treatment screening strategy (Deenen *et al*, 2016). Also, the frequencies of the risk 'core' variants in the general population are ~0.1–1%, but these figures cannot explain the estimated 10–15% of DPD-linked fluoropyrimidine-related adverse events (FAEs; Caudle *et al*, 2013; Meulendijks *et al*, 2016). Therefore, additional *DPYD* risk variants need to be investigated for broadening the spectrum of *DPYD* genotyping in the clinical practice. The analyses from randomised clinical trial represent a unique opportunity for evaluating association between genetic variants and clinical outcomes and they are necessary for confirming the predictive role for toxicity of candidate polymorphisms. Three or six colon adjuvant (TOSCA) is a large randomised trial addressing the role of a shorter duration of an adjuvant oxaliplatin/fluoropyrimidines regimen in surgically resected stage III and high-risk stage II colorectal cancer (Lonardi *et al*, 2016).

In 2006, we planned an ancillary pharmacogenetic study to the TOSCA clinical trial for investigating genetic variants with possible predictive role for chemotherapy-related toxicity. The early study plan did not include the analysis of *DPYD* genetic variants. Patients from the main clinical trial were accrued in the ancillary pharmacogenetic study, which evaluated 17 polymorphisms in 11 genes (Ruzzo *et al*, 2014). In 2014, we planned an additional retrospective analysis in the original study population and devoted to *DPYD* genetic variants for fluoropyrimidine-related toxicity.

MATERIALS AND METHODS

The TOSCA trial. Patients included in this study represent a subgroup of the 3.759 patients with surgically resected, stage III and high-risk stage II colorectal cancer recruited in TOSCA trial between 2007 and 2011 (Lonardi *et al*, 2016). This is an Italian intergroup, multicentre, randomised, non-inferiority phase III study in high-risk stage II and stage III colon cancer patients treated with 3 or 6 months of either FOLFOX-4 (intravenous oxaliplatin 85 mg/m^2 on day 1 and a 2-hour infusion of L-folinic acid 100 mg/m^2 followed by bolus 5-FU 400 mg/m^2 and a 22-hour continuous infusion of 5-FU 600 mg/m^2 for two consecutive days with treatment repeated every two weeks) or XELOX (intravenous oxaliplatin 130 mg/m^2 on day 1, followed by capecitabine 1000 mg/m^2 per os twice daily on days 1–14 with cycles were repeated every 21 days) adjuvant chemotherapy, sponsored by GISCAD (Italian Group For The Study Of Gastrointestinal Cancer) and supported by Italian Medicines Agency (AIFA; Lonardi *et al*, 2016). Selected haematologic and non-haematologic toxicities (anaemia, leukopenia, neutropenia, thrombocytopenia, asthenia, diarrhoea, mucositis stomatitis, vomiting, nausea, hepatic toxicity, skin toxicity, and neurotoxicity) were assessed at the start of each cycle using Common Toxicity Criteria for Adverse Events (CTCAE) version 3.0.

All adverse events at any time were monitored and reported. Toxicity was managed as follows; in case of grade ⩾3 haematologic toxicity or persistent grade 2 the dose of all drugs was reduced by 25%. In case of grade ⩾3 non-haematologic toxicity the dose of the related drugs was reduced by 50%. In case of grade ⩾3 or persistent grade 2 neurotoxicity, oxaliplatin dose was reduced by 20%. Oxaliplatin was definitely stopped if grade ⩾2 neurosensory symptoms persisted between cycles.

Patients eligible for the TOSCA study were asked to give further and specific written informed consent to be enrolled in the pharmacogenetic studies. All experiments were performed in accordance with relevant guidelines and regulations and the Local Ethics Committee of each Institution approved the Study.

DPYD assessments. The retrospective *DPYD* analysis in the ancillary pharmacogenetic study to the TOSCA clinical trial was planned in 2014 after the publication of the pharmacogenetic analysis in the QUASAR2 study (Rosmarin *et al*, 2014). Genetic markers of toxicity for capecitabine monotherapy were selected after systemic review and then investigated in the QUASAR2 patients population of Caucasian individuals. We aimed at re-evaluating the *DPYD* panel of the QUASAR2 study (*4 rs1801158, *5 rs1801159, *6 rs1801160, *9A rs1801265, rs2297595, *2A rs3918290, *13 rs55886062, and rs67376798) in the homogeneous population of patients of the TOSCA trial who underwent adjuvant fluoropyrimidine/oxaliplatin combination chemotherapy. At the time of the planning of our *DPYD* analysis, two additional variants, rs17376848 and rs75017182, showed promising predictive role for fluoropyrimidine-related toxicity (van Kuilenburg *et al*, 2010; Teh *et al*, 2013; Froehlich *et al*, 2015). These genetic variants were included in our panel (Table 1) considering that: (A) the polymorphisms had some degree of likelihood to alter the structure or the expression of the gene in a biologically relevant manner; (B) the 'q' allele frequency was expected to be >1%; and (C) the polymorphisms were established and well-documented.

Genomic DNA was extracted from 2 ml whole blood by using the QiaAmp kit (Qiagen, Valencia, CA, USA). rs75017182 was analysed by Real-Time PCR assay using the Easy *DPYD* kit (Diatech Pharmacogenetics, Jesi, Italy), while the other nine *DPYD* variants were all included in the MYRIAPOD ADMET kit (Diatech Pharmacogenetics), and analysed on the MassARRAY System (Agena Bioscience). The MassARRAY protocol is characterised by three main steps: polymerase chain reaction (PCR), single-base primer extension (SBE), and separation of the products on a matrix-loaded silicon chip by matrix-assisted laser desorption ionization time of life mass spectrometry (MALDI-TOF MS). After the amplification of the region of interest, a primer extension reaction with oligos that bind adjacent to the targeted polymorphic site and all four nucleotide terminators (iPLEX) was carried out. The extension reaction generated different products for different alleles: primers extended with the terminator dNTP

Table 1. Characteristics of the *DPYD* studied variants with observed genotypes

					Genotype (number of patients)			Allele frequency	
rs_numbers	Nucleotide change	Effect	CPIC code	*N*	p^2	pq	q^2	*p*	*q*
rs1801158	1601 G>A	Ser 534 Asn	DPYD*4	497	472	24	1	0.974	0.026
rs1801159	1627 A>G	Ile 543 Val	DPYD*5	496	318	156	22	0.798	0.202
rs1801160	2194 G>A	Val 732 Ile	DPYD*6	497	427	65	5	0.924	0.075
rs1801265	85 T>C	Cys 29 Arg	DPYD*9A	497	311	169	17	0.796	0.204
rs2297595	496 A>G	Met 166 Val	—	493	395	94	4	0.897	0.103
rs3918290	1905 + 1G>A	Exon skipping	DPYD*2A	494	491	3	0	0.997	0.003
rs17376848	1896 T>C	Phe 632 Phe	—	497	465	31	1	0.967	0.033
rs55886062	1679 T>G	Ile 56 Ser	DPYD*13	496	496	0	0	1	0
rs67376798	2846 A>T	Asp 949 Val	—	497	491	6	0	0.994	0.006
rs75017182	1129–5923 C>G	Aberrant splicing	—	504	494	10	0	0.99	0.01

Abbreviations: CPIC code = Clinical Pharmacogenetics Implementation Consortium (https://cpicpgx.org); *N* = number of patients; p^2 = major allele homozygous genotype; pq = heterozygous genotype; q^2 = minor allele homozygous genotypes; rs_number = reference SNP ID number.

complementary to the targeted polymorphic site. All iPLEX products, each with its unique mass, were then identified using mass spectrometry. PCR and SBE reactions were performed in a thermal cycler (Labcycler, SensoQuest), whereas the extension products were analysed using the MALDI-TOF MassARRAY Analyzer 4 (Agena Bioscience), according to the MYRIAPOD ADMET kit's instructions for use and using all reagents and consumables contained in the SQ TYPING 960 Kit (Diatech Pharmacogenetics). The genotype call was performed with the iGENETICS MYRIAPOD software (Diatech Pharmacogenetics).

All laboratory analyses were performed blind to the patients' treatment and clinical outcomes. Genetic data were then transferred to and independently analysed at IRCCS Istituto di Ricerche Farmacologiche 'Mario Negri'.

Statistics. Conforming to previously FAEs definition (Lee *et al*, 2014; Boige *et al*, 2016) and to the planned management of toxicity in the TOSCA trial, grade $\geqslant 3$ neutropenia, diarrhoea, asthenia, nausea, vomiting, leukopenia, thrombocytopenia, mucositis, stomatitis, and skin toxicity were deemed as severe FAEs. The treatment compliance was described in terms of treatment interruption and dose intensity, defined as the dose given in mg per m^2 per week.

According to the results of *DPYD* analysis, patients were categorised in three genotype groups: carriers of the homozygous wild type (p^2); heterozygous (pq); and homozygous variant (q^2). The possible association of *DPYD* variant with FAEs was analysed in the codominant model (p^2, pq, and q^2 genotypes considered separately) and in a dominant model with merged heterozygous (pq) and homozygous (q^2) risk variant genotype carriers.

To test the effect of *DPYD* genotypes on toxicity, two analyses were planned: an association analysis and a time-to-toxicity (TTT) analysis. This choice was made because a conventional analysis with a binary outcome describing only the occurrence of severe toxicity may be inaccurate in the case of few observations (due to the rarity of some genotypes), and it may not capture potential clinically meaningful differences also in terms of time of toxicity onset (Thanarajasingam *et al*, 2016). The association analysis compared the rate of FAEs across *DPYD* genotypes by means of a Fisher's test in contingency tables. The TTT was defined as the time from date of randomisation in TOSCA trial to the date of severe FAEs occurrence. Subjects without severe FAEs at the time of analysis were censored at the date they were last known to be event-free while on treatment. TTT curves were estimated using the Kaplan–Meier method. Cox proportional hazard models stratified for treatment duration (6 or 3 months) were used to assess the effects of *DPYD* genotypes on TTT. Multivariate analysis stratified for treatment duration was performed to adjust the identified effect for age, gender, stage and treatment (FOLFOX-4 or XELOX). Results were provided as the hazard ratio (HR) with 95% confidence interval (95% CI).

All reported *P*-values were two-sided with $P<0.05$ value considered statistically significant. However, to adjust the analyses for multiple testing, the Benjamini and Hochberg's False Discovery Rate (FDR) procedure was used, considering both the dominant and codominant model.

Assuming the prevalence of a high-risk allele of at least 10% and FAEs in about one-third of the study population, 188 events would allow the detection of a HR of at least 2 associated to the group with unfavorable genotypes (90% power and 5% type I error in a bilateral test). Detection of significant association for the three 'core' variants (*2A rs3918290, *13 rs55886062, and rs67376798) would require higher HR values given the expected frequencies of their risk alleles below 10%.

A χ^2 test was used for checking the Hardy–Weinberg equilibrium. Linkage disequilibrium (LD), defined as a non-random association of alleles adjacent loci, was assessed and both D' and r^2 measures were provided. D' can take any value from 0 (random co-inheritance of alleles) to 1 (complete LD); r^2 also ranges from 0 (random co-inheritance of alleles) to 1 (perfect LD). Values of $r^2<0.33$ suggest absence of strong LD (Ardlie *et al*, 2002). Analyses were performed with SAS 9.4 (SAS Institute, Cary, NC, USA) and the SNPStats package (Solè *et al*, 2006).

RESULTS

Patient characteristics and toxicity. From July 2007 to October 2011, 534 patients from 26 experimental centers entered the study. This figure represents 81% of patients randomised in the same period and by the same centers in the main study. Twenty-six patients were not assessable for the following reason: 5 patients were never treated, for 2 patients the treatment data were unavailable, and for 19 patients the blood sampling was not assessable due to technical problems. Therefore, the analysis was conducted in 508 patients.

Characteristics of the 508 patients are shown in Table 2. Patients' baseline characteristics were consistent with those of the

Table 2. Demographic and clinical characteristics of the enrolled patients

	All sample (*N* = 508)
Arm, *n* (%)	
Folfox-4 (6 months)	183 (36.0)
Folfox-4 (3 months)	187 (36.8)
Xelox (24 weeks)	70 (13.8)
Xelox (12 weeks)	68 (13.4)
Age, years	
Median (Q1–Q3)	64.2 (57.4–70.7)
Female sex—*n* (%)	217 (42.7)
ECOG performance status, *n* (%)	
0	488 (96.1)
1	20 (3.9)
Tumour site, *n* (%)	
Multiple site	23 (4.5)
Single site:	485 (95.5)
Ascending colon	137 (28.3)
Sigmoid-rectum colon	201 (41.4)
Descending colon	66 (13.6)
Trasverse colon	32 (6.6)
Splenic flexure	27 (5.6)
Hepatic flexure	22 (4.5)
Histology, *n* (%)	
Adenocarcinoma	437 (86.0)
Mucoid adenocarcinoma	65 (12.8)
Other	6 (1.2)
Stage, *n* (%)	
II	184 (36.2)
III	324 (63.8)
Grade, *n* (%)	
Gx	4 (0.8)
G1-2	340 (67.6)
G3-4	159 (31.6)
Missing	5
T stage, *n* (%)	
pTx	1 (0.2)
pT1	12 (2.4)
pT2	41 (6.1)
pT3	380 (74.8)
pT4	84 (16.5)
N stage, *n* (%)	
pN0	184 (36.2)
pN1	233 (45.9)
pN2	91 (17.9)

Abbreviations: *n* = number; Q1 = first quartile; Q3 = third quartile.

Table 3. Grade ⩾3 adverse events occurred in the study population

	All sample *N* = 508
All grade >3 adverse events	
Neutropenia	145 (28.5)
Grade ⩾2 neurological toxicity	131 (25.8)
Diarrhoea	33 (6.5)
Asthenia	16 (3.1)
Nausea	14 (2.8)
Vomiting	11 (2.2)
Leukopenia	11 (2.2)
Thrombocytopenia	6 (1.2)
Hepatic toxicity	6 (1.2)
Mucositis	4 (0.8)
Stomatitis	2 (0.4)
Anaemia	2 (0.4)
Skin toxicity	1 (0.2)
First grade ⩾3 FAEs occurred	194 (38.2)
Neutropenia	130 (67.0)
Diarrhoea	25 (12.9)
Leukopenia	10 (5.2)
Asthenia	8 (4.1)
Nausea	8 (4.1)
Thrombocytopenia	4 (2.1)
Mucositis	4 (2.1)
Vomiting	3 (1.6)
Stomatitis	1 (0.5)
Skin toxicity	1 (0.5)

Abbreviations: FAEs: fluoropyrimidine-related adverse events.

whole trial population (Lonardi *et al*, 2016). Most patients were randomised to FOLFOX-4 because option for XELOX regimen was introduced in TOSCA trial only during the late phase of accrual of this ancillary study. Toxicity related to adjuvant chemotherapy is reported in Table 3. Again, the spectrum and the frequency of toxicities did not differ from those observed in whole trial population (Lonardi *et al*, 2016). One hundred ninety-four (38.2%) patients experienced at least one FAE. Neutropenia was the commonest among FAEs occurring in 145 patients (28.5%). As shown in Supplementary Table S1, analysis of dose intensity did not show differences across treatment arms.

Genetic assessments. Table 1 lists the studied genetic variants and the distribution of genotypes of patients successfully assessed for each polymorphism. Consistent with previous observations, genotype frequency did not differ from those observed in Caucasian population. The *13 rs55886062 G allele was not found in the studied population and therefore, this variant was excluded from subsequent analyses. Allele frequencies of the remaining polymorphisms were consistent with the Hardy–Weinberg equilibrium ($P > 0.05$). Results of LD analyses are shown in Supplementary Table S2.

DPYD variants and FAEs. The prevalence of *DPYD* high-risk alleles was heterogeneous, ranging from 0% of the *13 rs55886062 G allele to 37.5% of the *9A rs1801265 C allele. Therefore, 194 events would allow detection of an HR of at least 8.3 and an HR of at least 1.5 for a prevalence of a high-risk allele equal to 1% and to 35%, respectively (power of 90% and a I type error of 5%, for a bilateral test). A statistically significant association was found between *6 rs1801160 genotypes and FAEs (FDR = 0.0083 in both the dominant and codominant models). No additional significant associations were detected (data not shown).

Results about the effect of *DPYD* variants on TTT are shown in Table 4. At univariate analysis, *6 rs1801160 (codominant model: FDR = 0.0022), rs2297595 (codominant model: FDR = 0.0413), *2A rs3918290 (codominant model: FDR = 0.0001) correlated with TTT. Specifically, *6 rs1801160 *GA* genotype carriers and *A* allele carriers were at risk for shorter TTT (HR 1.99, 95% CI 1.38–2.86, FDR = 0.0002 and HR 2.01, 95% CI 1.42–2.86, FDR = 0.0006, respectively). Median TTT for *6 rs1801160 *GG, GA* and *AA* genotype carriers were 7.0, 3.0 and 2.1 months, respectively. Also, the rs2297595 *GG* genotype (HR 4.28, 95% CI 1.35–13.55, FDR = 0.0136) and the *2A rs3918290 *GA* genotype (HR 15.34, 95% CI 4.72–49.89, FDR = 0.0001) showed a shorter TTT. Median TTT for rs2297595 *AA, AG* and *GG* genotype carriers were 7.0, 6.6 and 1.2 months, respectively. Median TTT for *2A rs3918290 *GG* and *GA* genotype carriers were 7.0 and 0.9 months, respectively. Figure 1 depicts Kaplan–Meier curves of 'q' allele carriers *vs* 'p^2' genotype carriers of rs2297595 and *6 rs1801160. At multivariate analyses the associations with *DPYD* variants identified in the univariate analyses were confirmed.

Neutropenia was the commonest FAEs, occurring in 145 patients (28.5%). The second one was diarrhoea, which occurred

Table 4. Effect of DPYD variants on TTT for FAEs

	Univariate analysis[a]		Multivariate analysis[a,b]	
	HR (95% CI)	FDR	HR (95% CI)	FDR
***4 rs1801158**				
Overall codominant:		0.9831		
Dominant:				
G/A or A/A *vs* G/G	0.87 (0.43–1.78)	0.8874		
***5 rs1801159**				
Overall codominant:		0.2910		
Dominant:				
G/A or G/G *vs* A/A	0.85 (0.63–1.15)	0.4400		
***6 rs1801160**				
Overall codominant:		0.0022		0.0002
G/G	1.00		1.00	
G/A	1.99 (1.38–2.86)	0.0002	2.06 (1.43–2.96)	0.0001
A/A	2.40 (0.76–7.60)	0.1375	2.53 (0.79–8.09)	0.1189
Dominant:				
G/A or A/A *vs* G/G	2.01 (1.42–2.86)	0.0006	2.09 (1.47–2.97)	<0.0001
***9A rs1801265**				
Overall codominant:		0.2181		
Dominant:				
C/T or C/C *vs* T/T	1.10 (0.82–1.47)	0.7640		
rs2297595				
Overall codominant:		0.0413		0.0032
A/A	1.00		1.00	
G/A	1.40 (0.99–1.97)	0.0569	1.26 (0.89–1.78)	0.1950
G/G	4.28 (1.35–13.55)	0.0136	6.77 (2.10–21.84)	0.0014
Dominant:				
G/A or G/G *vs* A/A	1.46 (1.05–2.05)	0.0722	1.33 (0.95–1.87)	0.0942
***2A rs3918290**				
Overall codominant:				
G/A *vs* G/G	15.34 (4.72–49.89)	0.0001	14.98 (4.39–51.09)	<0.0001
rs17376848				
Overall codominant:		0.9652		
Dominant:				
C/T or C/C *vs* T/T	1.15 (0.65–2.02)	0.8386		
rs67376798				
Overall codominant:				
T/A *vs* A/A	3.02 (1.12–8.16)	0.0722		
rs75017182				
Overall codominant:				
C/G *vs* Cs/C	0.99 (0.37–2.67)	0.9831		

Abbreviations: 95% CI = confidence interval at 95%; DPYD = dihydropyrimidine dehydrogenase; FAEs = fluoropyrimidine-related adverse events; FDR = False Discovery Rate; HR = hazard ratio; TTT = time-to-toxicity.
[a]Cox proportional hazard models.
[b]Adjusted for age, gender, stage, and treatment.

in 33 patients (6.5%). Therefore, univariate and multivariate Cox analyses to address the effect of *DPYD* variants on TTT for specific FAEs were performed only for neutropenia (Table 5). At univariate analysis, associations with time to neutropenia were found for *6 rs1801160 and *2A rs3918290. In detail, *6 rs1801160 *GA* genotype carriers in the codominant model and *A* allele carriers in the dominant model were at risk for shorter time to neutropenia (HR 2.19, 95% CI 1.46–3.28, FDR = 0.0002 and HR 2.18, 95% CI 1.47–3.24, FDR = 0.0024, respectively). The codominant model analysis for *2A rs3918290 showed significant association with short time to neutropenia for *GA* variant genotype carriers (HR 10.74, 95% CI 2.59–44.61, FDR = 0.0054). The impact of all this *DPYD* variants was confirmed at multivariate analysis.

DISCUSSION

As shown in Table 6, this study is added to previous pharmacogenetic analyses for *DPYD*, which were incorporated in randomised clinical trials of fluoropyrimidine-based chemotherapy in colorectal cancer (Deenen *et al*, 2011; Lee *et al*, 2014; Rosmarin *et al*, 2014; Del Re *et al*, 2015; Boige *et al*, 2016; Lee *et al*, 2016). These studies offer a unique opportunity for performing pharmacogenetics in an optimal setting, where the genotyped patient population is well characterised and uniformly assessed for clinical/pathologic characteristics and the monitoring of toxicity. Unfortunately, these studies cannot be uniformly evaluated because of the substantial differences in disease stage (adjuvant *vs* metastatic), chemotherapy protocols (often with biologics), panels of *DPYD* variants, and methodology for assessing putative pharmacogenetics associations. To this regard, we introduced the TTT analysis in addition to a standard genotypes/FAEs distribution analysis, which was commonly adopted in studies listed in Table 6. The TTT analysis for detecting pharmacogenetic associations with FAEs may help to disclose potential clinical impact of *DPYD* variants, which could be lost in a common binary analysis of genotype frequencies in contingency tables. The TTT analysis adds the dimension of time, and therefore, it allows for detection of 'more and early' toxicity events (Thanarajasingam *et al*, 2016). In fact, if severe toxicity occurs after multiple cycles of chemotherapy, it may also represent a cumulative effect and the stress of the system after several doses of the drugs. On the contrary, if severe toxicity events occur early, they are more likely related to innate defects, often linked with catabolic pathways (Sahota *et al*, 2016). Notably, some clinical analyses on *DPYD* variants and fluoropyrimidine-related toxicity were based on FAEs occurring within the first 3 cycles of therapy (Gross *et al*, 2008; Deenen *et al*, 2011, Froehlich *et al*, 2015). The TTT approach avoids the need of defining such a cut-point and it may better characterise a gene-linked toxicity profile. Also, it should be considered that some functional *DPYD* variants may not induce a dramatic loss of enzyme function like the *2A rs3918290, and therefore, in these cases, TTT analysis may be more sensitive for detecting the risk of toxicity determined by *DPYD* variants with moderate functional effects.

In the present study population, potential baseline confounders for early toxicity could be excluded since the administration of adjuvant combination chemotherapy was per-protocol proposed to high-risk colon cancer patients without evidence of metastatic disease, no major comorbidity, long life expectancy, and good performance status. Furthermore, only 2 patients interrupted treatment due to disease progression and in these patients no fluoropyrimidine-related toxicity was observed.

In our population of patients, the observed frequencies of the rare deleterious *DPYD* variant alleles *2A rs3918290, *13 rs55886062, and rs67376798 were 0.6%, 0%, and 1.2%, respectively. Only *2A rs3918290 showed significant association with FAEs in the TTT analysis achieving an HR equal to 14.98, and a significant impact on time to neutropenia (Tables 4 and 5, respectively). However, even if they all had shown significant HRs for FAEs, they

Table 5. Effect of DPYD variants on TTT for neutropenia

	Univariate analysis[a]		Multivariate analysis[a,b]	
	HR (95% CI)	FDR	HR (95% CI)	FDR
***4 rs1801158**				
Overall codominant:		0.9137		
Dominant:				
G/A or A/A *vs* G/G	0.74 (0.30–1.80)	0.5937		
***5 rs1801159**				
Overall codominant:		0.3509		
Dominant:				
G/A or G/G *vs* A/A	0.76 (0.53–1.08)	0.2837		
***6 rs1801160**				
Overall codominant:		0.0054		0.0003
G/G	1.00		1.00	
G/A	2.19 (1.46–3.28)	0.0002	2.30 (1.53–3.46)	<0.0001
A/A	2.07 (0.51–8.45)	0.3107	2.00 (0.49–8.26)	0.3364
Dominant:				
G/A or A/A *vs* G/G	2.18 (1.47–3.24)	0.0024	2.28 (1.53–3.40)	<0.0001
***9A rs1801265**				
Overall codominant:		0.5133		
Dominant:				
C/T or C/C *vs* T/T	1.00 (0.71–1.41)	0.9847		
rs2297595				
Overall codominant:		0.1661		
Dominant:				
G/A or G/G *vs* A/A	1.55 (1.06–2.26)	0.0958		
***2A rs3918290**				
Overall codominant:				
G/A *vs* G/G	10.74 (2.59–44.61)	0.0054	14.72 (3.35–64.72)	0.0004
rs17376848				
Overall codominant:		0.6299		
Dominant:				
C/T or C/C *vs* T/T	1.34 (0.73–2.49)	0.5133		
rs67376798				
Overall codominant:				
T/A *vs* A/A		0.5133		
rs75017182				
Overall codominant:				
C/G *vs* C/C		0.3509		

Abbreviations: 95% CI = confidence interval at 95; DPYD = dihydropyrimidine dehydrogenase; FDR = False Discovery Rate; HR = hazard ratio; TTT = time-to-toxicity.
[a]Cox proportional hazard models.
[b]Adjusted for age, gender, stage, and treatment.

cannot explain the overall estimated contribution of functional *DPYD* variants in causing severe fluoropyrimidine toxicity. DPD deficiency has been described in ~40–60% of patients with ⩾3 grade fluoropyrimidine-induced toxicity (Meulendijks *et al*, 2015). However, DPD deficiency cannot always be traced back to a currently known *DPYD* variant associated with reduced enzyme activity (Meulendijks *et al*, 2015). Therefore, other detrimental variants should be identified to improve sensitivity of *DPYD* genotyping (Gentile *et al*, 2016). Indeed, among the seven additional *DPYD* studied variants, two (*6 rs1801160 and rs2297595) showed associations with FAEs.

The *DPYD* *6 rs1801160 was analysed within the *DPYD* panel of three studies listed in Table 6 (Deenen *et al*, 2011; Rosmarin *et al*, 2014; Boige *et al*, 2016). Notably, in the large PETACC-8 study, *6 rs1801160 showed statistically significant association with grade 3 or greater FAEs and neutropenia in particular (Boige *et al*, 2016). In the QUASAR2 (Rosmarin *et al*, 2014) and the CAIRO-2 (Deenen *et al*, 2011) studies, *6 rs1801160 did not show predictive role for FAEs. However, it should be considered that the QUASAR2 analysis (Rosmarin *et al*, 2014) was performed in patients treated with capecitabine mono-chemotherapy only. As far as the CAIRO-2 is concerned, the high probability of developing FAEs (85%) was considered as a major reason for not detecting significant associations between FAEs and all tested *DPYD* variants in this study (Deenen *et al*, 2011). If we look at risk associations between *6 rs1801160 and FAEs in the present study and the PETACC-8 study (Boige *et al*, 2016), it should be noted a significant but moderate effect size attributed to the *6 rs1801160 A risk allele. Results from the pharmacogenetics analysis by Kleibl *et al* suggested an impact of the *6 rs1801160 A allele in determining fluoropyrimidine toxicity especially in the context of specific *DPYD* haplotypes (Kleibl *et al*, 2009). Notably, in the whole *DPYD* panel, the *6 rs1801160 locus did not show strong LD, thus excluding that the association of the variant with toxicity may be only the results of LD with a neighboring etiologic variant. These aspects would suggest direct but mild impact on phenotype of the *6 rs1801160, which cumulates with other variants and/or emerges in specific chemotherapy regimen because of toxicity synergy between fluoropyrimidine and other drugs (i.e., oxaliplatin; Offer and Diasio, 2016). In the sub-type analysis of FAEs, the *6 rs1801160 variant showed detrimental effect on time to neutropenia. We observed grade ⩾3 neutropenia in the 28.5% of patients and this figure is slightly lower than the toxicity rates previously reported in patients treated with XELOX and FOLFOX regimens (up to 40%; Eng (2009)). These figures would exceed the expected frequency of ⩾3 grade neutropenia if the sum of neutropenia rates in single-agent studies of oxaliplatin, capecitabine and bolus/infusional 5-FU (<10% of patients) would be applied for prediction. The array of interactions and synergisms between fluoropyrimidines and oxaliplatin in humans may explain this discrepancy. In this context, a *DPYD* variant, which depresses, but does not abrogate the enzyme function may significantly increase the risk of severe toxicity (neutropenia) when the fluorpyrimidine is combined with other drugs.

DPYD pharmacogenetics in the PETACC-8 study (Boige *et al*, 2016) included the rs2297595, but without detecting significant associations with FAEs. In the present study, the homozygous rs2297595 *GG* genotype was associated with a significant relatively large effect (HR 6.77) in the TTT analysis, whereas the heterozygous genotype did not. This behavior would suggest an 'allele-dosage' effect and a clinically meaningful DPD deficient phenotype in carriers of the 'q^2' genotype. This hypothesis parallels previous findings in a retrospective pharmacogenetic study by Gross *et al* (2008), and it is compatible with the putative functional effect of the rs2297595 variant. The methionine-valine exchange, as consequence of the non-synonymous sequence variation occurs in a highly conserved site during evolution, which may be critical to enzyme structure and function (Mattison *et al*, 2002). Moreover, LD analyses showed that the *6 rs1801160 and rs2297595 loci are not co-inherited, and therefore they may act independently.

The analysis of the median TTT values contributes to the understanding of the clinical impact of the *6 rs1801160, *2A

Table 6. Summary of randomised controlled clinical trials with dedicated *DPYD* pharmacogenetic analyses

Trial (reference)	Setting	Treatment arms (*N*)	Number of *DPYD* studied variants	Toxicity outcomes (%)	Significant associations
QUASAR2 (Rosmarin *et al*, 2014)	Adjuvant	Cap (436) Cap + Bev (491)	12	Grade ⩾3 FAEs (32.4%)	rs67376798
CAIRO-2 (Deenen *et al*, 2011)	Metastatic	Cap/Oxa/Bev (281) Cap/Oxa/Bev/Cetux (287)	29	Grade ⩾3 diarrhoea (24.4%) Any grade ⩾3 toxicity (85.3%) Hand-foot grade ⩾2 (43.1%)	rs3918290 (DPYD*2A), rs1801160 (DPYD*6), rs56038477 no[a] no
NCCTG (Lee *et al*, 2014, 2016)	Adjuvant	FOLFOX (2384) FOLFIRI (210) CT plus Cetux (1191) CT without Cetux (1403)	25 + 1[b]	Grade ⩾3 FAEs (33%)	rs3918290 (DPYD*2A), rs67376798
PETACC-8 (Boige *et al*, 2016)	Adjuvant	FOLFOX (780) FOLFOX + Cetux (765)	25	Grade ⩾3 FAEs (49.5%)	rs1801160 (DPYD*6), rs67376798
TRIBE (Del Re *et al*, 2015)	Metastatic	FOLFOXIRI + Bev (220) FOLFIRI + Bev (220)	2	Grade ⩾3 FAEs	rs3918290 (DPYD*2A) plus rs67376798[c]
TOSCA—ancillary	Adjuvant	FOLFOX (370) Cap/Oxa (138)	10	Grade ⩾3 FAEs (32.4%)	rs3918290 (DPYD*2A), rs1801160 (DPYD*6), rs2297595

Abbreviations: Bev = bevacizumab; Cap = capecitabine; Cetux = cetuximab; DPYD = dihydropyrimidine dehydrogenase; FAEs = fluoropyrimidine-related adverse events; FOLFOX = bolus/infusional 5-fluorouracil plus oxaliplatin; FOLFIRI = bolus/infusional 5-fluorouracil plus Irinotecan; FOLFOXIRI = bolus/infusional 5-fluorouracil plus oxaliplatin and irinotecan; *N* = number of patients; Oxa = oxaliplatin.

[a]In the CAIRO-2 analysis, *2A rs3918290 G > A did not meet criteria for statistical significant thresholds in the overall analysis of toxicity, but all carriers of the *2A rs3918290 A allele developed grade 3–4 toxicity with 1 death possibly related to the capecitabine treatment.

[b]A second pharmacogenetic assessment in the NCCTG trial added to the original 25 *DPYD* genotypes the novel rs75017182 C > G genetic variant.

[c]A combined analysis of the two genotypes for association with FAE was performed.

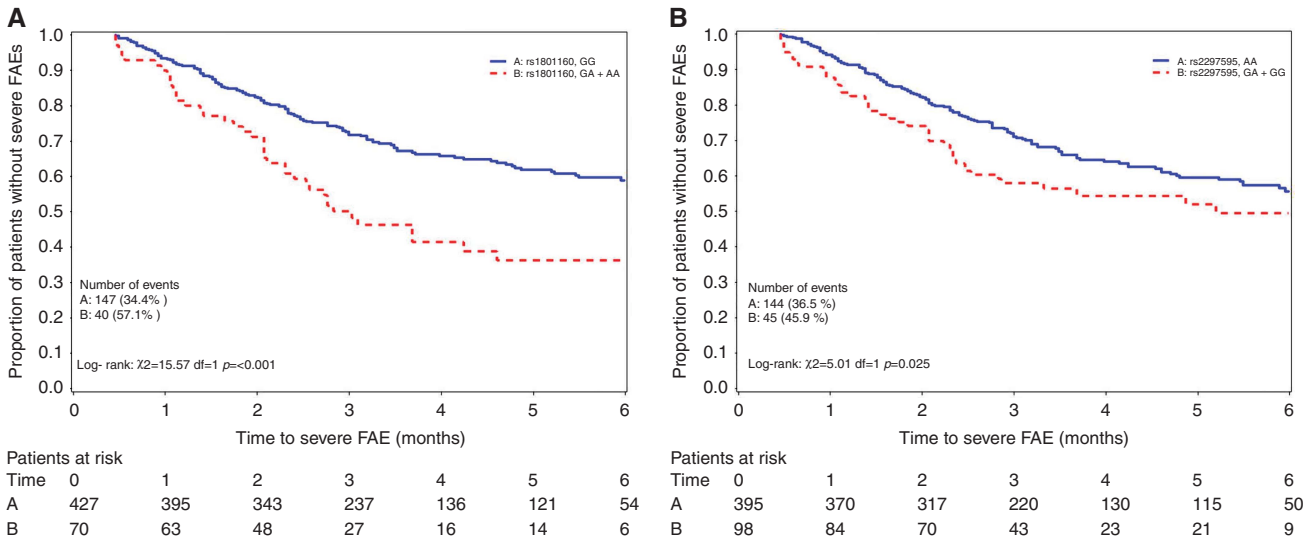

Figure 1. Kaplan-Meier curves. (**A**) TTT curves of the *6 rs1801160 minor A allele carriers (merged heterozygous plus homozygous minor allele carriers) and homozygous GG genotype carriers. (**B**) TTT curves of the rs2297595 minor G allele carriers (merged heterozygous plus homozygous minor allele carriers) and homozygous AA genotype carrier.

rs3918290, and rs2297595 variants. Median TTT was 7 months among common homozygous genotypes carriers, whereas it was significantly shortened (between 0.9 and 2.1 months) in carriers of the homozygous variant *6 rs1801160 and rs2297595 and the *2A rs3918290 heterozygous genotypes. Notably, shortened TTT was detectable in *6 rs1801160, but not rs2297595 heterozygous genotype carriers, thus corroborating the hypothesis of a different effect of the two variants in depressing/altering the DPD function. The early onset of toxicity corroborates the hypothesis of an underlying enzymatic defect and the opportunity of verifying *DPYD* variants/DPD status in patients with early severe FAEs after fluoropyrimidine exposure.

As far as ethnicity is concerned, the frequency of the *6 rs1801160 A risk allele seems comparable in Caucasian, Middle-Eastern, and African-American, whereas it seems less frequent in Asian populations (Caudle *et al*, 2013). The clinical impact of the rs2297595 variant may be more relevant to populations of African ancestry, where its frequency seems to double in comparison with Caucasian populations (Aminkeng *et al*, 2014).

It is still matter of debate whether *DPYD* genotyping should be incorporated in the routine pre-treatment screening of patients undergoing fluoropyrimidine-based chemotherapy. To this regard, the recent guidelines of the European Society for Medical Oncology (ESMO) consider the testing as an option, which is indicated in the case of patients who experience severe toxicity and before the fluoropyrimidine is re-introduced (van Cutsem *et al*, 2016). We disagree with statement, especially when possible cautions could be adopted in treatment settings with narrow therapeutic window. Since the *DPYD* assessment was not incorporated in our original study plan, we could not perform a reliable cost-effectiveness analysis. However, available analyses suggest that *DPYD*-genotype guided dosing according to *2A rs3918290 (Deenen *et al*, 2016), or *2A rs3918290, *13 rs55886062, and rs67376798 (Cortejoso *et al*, 2016) may significantly improve safety of fluoropyrimidine therapy and being cost saving.

It should be considered that additional tests have been developed for assessing the activity of the DPD enzyme (DPD activity in peripheral blood monuclear cells, Uracil breath test, endogenous plasma/urine Uracil/Dihydrouracil, sampling PK model after 5-fluoruracil test dose; van Staveren *et al*, 2013, 2016; Del Re *et al*, 2017). These phenotyping tests seem to possess better predictivity then genotyping for fluoropyrimidine toxicity (van Staveren *et al*, 2013, 2016). In a recent analysis in 550 patients, Meulendijks *et al* found that high pre-treatment uracil concentrations were strongly associated with severe fluoropyrimidine-related toxicity, whereas *DPYD* genotypes did not (Meulendijks *et al*, 2017). However, in this study, *DPYD* genotyping was limited to rs67376798, *13 rs55886062, rs75017182, and *4 rs1801158. In general, as with the genotyping strategy, the phenotyping tests suffer from suboptimal sensitivity and specificity. Notably, a test for detecting DPD deficiency and preventing fluoropyrimidine toxicity requires high sensitivity. On the other side, low specificity may cause unnecessary dose reduction and suboptimal exposure to effective chemotherapy. To this end, as pointed out by Boisdron-Celle *et al* (2007), *DPYD* genotyping and DPD phenotyping tests could be integrated in a two-step strategy for screening selected patients.

In conclusion, this study remarks the role of *DPYD* *2A rs3918290 for fluoropyrimidine-related toxicity. It also indicates that *6 rs1801160 and rs2297595 produce additional *DPYD* genotypes, which may be predictive of toxicity in the same setting. TTT analysis in pharmacogenetic studies may help to characterise the clinical impact of risk alleles causing reduced DPD function.

ACKNOWLEDGEMENTS

This work was partially supported by the TERPAGE Project 'POR MARCHE FESR 2007–2013'. AR, Francesco G, and MM conceived and performed the study design, performed the manuscript preparation and data interpretation. Fabio G performed coordination study. Francesca G, Fabio G, and ER performed statistical analysis, data interpretation, and manuscript preparation. SL, MR, BM, VZ, NP, CM, RL, MTI, EV, PS, SB, V R, IB, LF, MN, EB, AB, DT, SL, CV, FB,AS, and LF collected samples and patients' data, and commented the manuscript. RL, LF, and AS participated in the study design and data interpretation, and helped to draft the manuscript. All authors reviewed the manuscript.

This paper is dedicated to the memory of our friend and colleague, Irene Floriani.

[23]These authors contributed equally to this work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Aminkeng F, Ross CJ, Rassekh SR, Brunham LR, Sistonen J, Dube MP, Ibrahim M, Nyambo TB, Omar SA, Froment A, Bodo JM, Tishkoff S, Carleton BC, Hayden MR (2014) Canadian Pharmacogenomics Network for Drug Safety Consortium. Higher frequency of genetic variants conferring increased risk for ADRs for commonly used drugs treating cancer, AIDS and tuberculosis in persons of African descent. *Pharmacogenomics J* **14**(2): 160–170.

Ardlie KG, Kruglyak L, Seielstad M (2002) Patterns of linkage disequilibrium in the human genome. *Nat Rev Genet* **3**: 299–309.

Boige V, Vincent M, Alexandre P, Tejpar S, Landolfi S, Le Malicot K, Greil R, Cuyle PJ, Yilmaz M, Faroux R, Matzdorff A, Salazar R, Lepage C, Taieb J, Laurent-Puig P (2016) DPYD genotyping to predict adverse events following treatment with flourouracil-based adjuvant chemotherapy in patients with stage III colon cancer: a secondary analysis of the PETACC-8 randomized clinical trial. *JAMA Oncol* **2**: 655–662.

Boisdron-Celle M, Remaud G, Traore S, Poirier AL, Gamelin L, Morel A, Gamelin E (2007) 5-Fluorouracil-related severe toxicity: a comparison of different methods for the pretherapeutic detection of dihydropyrimidine dehydrogenase deficiency. *Cancer Lett* **249**(2): 271–282.

Caudle KE, Thorn CF, Klein TE, Swen JJ, McLeod HL, Diasio RB, Schwab M (2013) Clinical pharmacogenetics implementation consortium guidelines for dihydropyrimidine dehydrogenase genotype and fluoropyrimidine dosing. *Clin Pharmacol Ther* **94**: 640–645.

Cortejoso L, García-González X, García MI, García-Alfonso P, Sanjurjo M, López-Fernández LA (2016) Cost-effectiveness of screening for DPYD polymorphisms to prevent neutropenia in cancer patients treated with fluoropyrimidines. *Pharmacogenomics* **17**(9): 979–984.

Deenen MJ, Tol J, Burylo AM, Doodeman VD, de Boer A, Vincent A, Guchelaar HJ, Smits PH, Beijnen JH, Punt CJ, Schellens JH, Cats A (2011) Relationship between single nucleotide polymorphisms and haplotypes in DPYD and toxicity and efficacy of capecitabine in advanced colorectal cancer. *Clin Cancer Res* **17**: 3455–3468.

Deenen MJ, Meulendijks D, Cats A, Sechterberger MK, Severens JL, Boot H, Smits PH, Rosing H, Mandigers CM, Soesan M, Beijnen JH, Schellens JH (2016) Upfront genotyping of DPYD*2A to individualize fluoropyrimidine therapy: a safety and cost analysis. *J Clin Oncol* **34**: 227–234.

Del Re M, Cremolini C, Loupakis F, Marmorino F, Citi V, Palombi M, Bergamo F, Schirripa M, Rossini D, Cortesi E, Tomasello G, Spadi R, Buonadonna A, Amoroso D, Vitello S, Di Donato S, Granetto C, D'Amico M, Danesi R, Falcone A (2015) DPYD c.1905 + 1G > A and c.2846A > T and UGT1A1*28 allelic variants as predictors of toxicity: Pharmacogenetic translational analysis from the phase III TRIBE study in metastatic colorectal cancer. *J Clin Oncol* **33**, (suppl): abstr 3532. http://meetinglibrary.asco.org/record/112386/abstract.

Del Re M, Restante G, Di Paolo A, Crucitta S, Rofi E, Danesi R (2017) Pharmacogenetics and metabolism from science to implementation in clinical practice: the example of dihydropyrimidine dehydrogenase. *Curr Pharm Des* **23**(14): 2028–2034.

Eng C (2009) Toxic effects and their management: daily clinical challenges in the treatment of colorectal cancer. *Nat Rev Clin Oncol* **6**(4): 207–218.

Froehlich TK, Amstutz U, Aebi S, Joerger M, Largiadèr CR (2015) Clinical importance of risk variants in the dihydropyrimidine dehydrogenase gene for the prediction of early-onset fluoropyrimidine toxicity. *Int J Cancer* **136**(3): 730–739.

Gentile G, Botticelli A, Lionetto L, Mazzuca F, Simmaco M, Marchetti P, Borro M (2016) Genotype-phenotype correlations in 5-fluorouracil metabolism: a candidate DPYD haplotype to improve toxicity prediction. *Pharmacogenomics J* **16**: 320–325.

Gross E, Busse B, Riemenschneider M, Neubauer S, Seck K, Klein HG, Kiechle M, Lordick F, Meindl A (2008) Strong association of a common dihydropyrimidine dehydrogenase gene polymorphism with fluoropyrimidine-related toxicity in cancer patients. *PLoS One* **3**(12): e4003.

Kleibl Z, Fidlerova J, Kleiblova P, Kormunda S, Bilek M, Bouskova K, Sevcik J, Novotny J (2009) Influence of dihydropyrimidine dehydrogenase gene (DPYD) coding sequence variants on the development of fluoropyrimidine-related toxicity in patients with high-grade toxicity and

patients with excellent tolerance of fluoropyrimidine-based chemotherapy. *Neoplasma* **56**: 303–316.

Lee AM, Shi Q, Pavey E, Alberts SR, Sargent DJ, Sinicrope FA, Berenberg JL, Goldberg RM, Diasio RB (2014) DPYD variants as predictors of 5-fluorouracil toxicity in adjuvant colon cancer treatment (NCCTG N0147). *J Natl Cancer Inst* **106**: dju298.

Lee AM, Shi Q, Alberts SR, Sargent DJ, Sinicrope FA, Berenberg JL, Grothey A, Polite B, Chan E, Gill S, Kahlenberg MS, Nair SG, Shields AF, Goldberg RM, Diasio RB (2016) Association between DPYD c.1129-5923 C > G/hapB3 and severe toxicity to 5-fluorouracil-based chemotherapy in stage III colon cancer patients: NCCTG N0147(Alliance). *Pharmacogenet Genomics* **26**: 133–137.

Lonardi S, Sobrero A, Rosati G, Di Bartolomeo M, Ronzoni M, Aprile G, Scartozzi M, Banzi M, Zampino MG, Pasini F, Marchetti P, Cantore M, Zaniboni A, Rimassa L, Ciuffreda L, Ferrari D, Barni S, Zagonel V, Maiello E, Rulli E, Labianca R (2016) TOSCA (Three or Six Colon Adjuvant) Investigators. Phase III trial comparing 3-6 months of adjuvant FOLFOX4/XELOX in stage II-III colon cancer: safety and compliance in the TOSCA trial. *Ann Oncol* **27**: 2074–2081.

Mattison LK, Johnson MR, Diasio RB (2002) A comparative analysis of translated dihydropyrimidine dehydrogenase cDNA; conservation of functional domains and relevance to genetic polymorphisms. *Pharmacogenetics* **12**(2): 133–144.

Meulendijks D, Cats A, Beijnen JH, Schellens JH (2016) Improving safety of fluoropyrimidine chemotherapy by individualizing treatment based on dihydropyrimidine dehydrogenase activity—ready for clinical practice? *Cancer Treat Rev* **50**: 23–34.

Meulendijks D, Henricks LM, Sonke GS, Deenen MJ, Froehlich TK, Amstutz U, Largiadèr CR, Jennings BA, Marinaki AM, Sanderson JD, Kleibl Z, Kleiblova P, Schwab M, Zanger UM, Palles C, Tomlinson I, Gross E, van Kuilenburg AB, Punt CJ, Koopman M, Beijnen JH, Cats A, Schellens JH (2015) Clinical relevance of DPYD variants c.1679T > G, c.1236G > A/HapB3, and c.1601G > A as predictors of severe fluoropyrimidine-associated toxicity: a systematic review and meta-analysis of individual patient data. *Lancet Oncol* **16**: 1639–1650.

Meulendijks D, Henricks LM, Jacobs BAW, Aliev A, Deenen MJ, de Vries N, Rosing H, van Werkhoven E, de Boer A, Beijnen JH, Mandigers CMPW, Soesan M, Cats A, Schellens JHM (2017) Pretreatment serum uracil concentration as a predictor of severe and fatal fluoropyrimidine-associated toxicity. *Br J Cancer* **116**(11): 1415–1424.

Offer SM, Diasio RB (2016) Biomarkers of fluorouracil toxicity: insight from the PETACC-8 trial. *JAMA Oncol* **2**: 662–663.

Rosmarin D, Palles C, Church D, Domingo E, Jones A, Johnstone E, Wang H, Love S, Julier P, Scudder C, Nicholson G, Gonzalez-Neira A, Martin M, Sargent D, Green E, McLeod H, Zanger UM, Schwab M, Braun M, Seymour M, Thompson L, Lacas B, Boige V, Ribelles N, Afzal S, Enghusen H, Jensen SA, Etienne-Grimaldi MC, Milano G, Wadelius M, Glimelius B, Garmo H, Gusella M, Lecomte T, Laurent-Puig P, Martinez-Balibrea E, Sharma R, Garcia-Foncillas J, Kleibl Z, Morel A, Pignon JP, Midgley R, Kerr D, Tomlinson I (2014) Genetic markers of toxicity from capecitabine and other fluorouracil-based regimens: investigation in the QUASAR2 study, systematic review, and meta-analysis. *J Clin Oncol* **32**: 1031–1039.

Ruzzo A, Graziano F, Galli F, Giacomini E, Floriani I, Galli F, Rulli E, Lonardi S, Ronzoni M, Massidda B, Zagonel V, Pella N, Mucciarini C, Labianca R, Ionta MT, Veltri E, Sozzi P, Barni S, Ricci V, Foltran L, Nicolini M, Biondi E, Bramati A, Turci D, Lazzarelli S, Verusio C, Bergamo F, Sobrero A, Frontini L, Magnani M (2014) Genetic markers for toxicity of adjuvant oxaliplatin and fluoropyrimidines in the phase III TOSCA trial in high-risk colon cancer patients. *Sci Rep* **5**: 6828.

Sahota T, Danhof M, Della Pasqua O (2016) Pharmacology-based toxicity assessment: towards quantitative risk prediction in humans. *Mutagenesis* **31**: 359–374.

Solè X, Guinò E, Valls J, Iniesta R, Moreno V (2006) SNPStats: a web tool for the analysis of association studies. *Bioinformatics* **22**: 1928–1929.

Teh LK, Hamzah S, Hashim H, Bannur Z, Zakaria ZA, Hasbullani Z, Shia JK, Fijeraid H, Md Nor A, Zailani M, Ramasamy P, Ngow H, Sood S, Salleh MZ (2013) Potential of dihydropyrimidine dehydrogenase genotypes in personalizing 5-fluorouracil therapy among colorectal cancer patients. *Ther Drug Monit* **35**(5): 624–630.

Thanarajasingam G, Atherton PJ, Novotny PJ, Loprinzi CL, Sloan JA, Grothey A (2016) Longitudinal adverse event assessment in oncology clinical trials: the Toxicity over Time (ToxT) analysis of Alliance trials NCCTG N9741 and 979254. *Lancet Oncol* **17**: 663–670.

van Cutsem E, Cervantes A, Adam R, Sobrero A, Van Krieken JH, Aderka D, Aranda Aguilar E, Bardelli A, Benson A, Bodoky G, Ciardiello F, D'Hoore A, Diaz-Rubio E, Douillard JY, Ducreux M, Falcone A, Grothey A, Gruenberger T, Haustermans K, Heinemann V, Hoff P, Köhne CH, Labianca R, Laurent-Puig P, Ma B, Maughan T, Muro K, Normanno N, Österlund P, Oyen WJ, Papamichael D, Pentheroudakis G, Pfeiffer P, Price TJ, Punt C, Ricke J, Roth A, Salazar R, Scheithauer W, Schmoll HJ, Tabernero J, Taïeb J, Tejpar S, Wasan H, Yoshino T, Zaanan A, Arnold D (2016) ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. *Ann Oncol* **27**(8): 1386–1422.

van Kuilenburg AB, van Lenthe H, Blom MJ, Mul EP, Van Gennip AH (1999) Profound variation in dihydropyrimidine dehydrogenase activity in human blood cells: major implications for the detection of partly deficient patients. *Br J Cancer* **79**(3–4): 620–626.

van Kuilenburg AB, Meijer J, Gökcay G, Baykal T, Rubio-Gozalbo ME, Mul AN, de Die-Smulders CE, Weber P, Mori AC, Bierau J, Fowler B, Macke K, Sass JO, Meinsma R, Hennermann JB, Miny P, Zoetekouw L, Roelofsen J, Vijzelaar R, Nicolai J, Hennekam RC (2010) Dihydropyrimidine dehydrogenase deficiency caused by a novel genomic deletion c.505_513del of DPYD. *Nucleosides Nucleotides Nucleic Acids* **29**: 509–514.

van Staveren MC, Guchelaar HJ, van Kuilenburg AB, Gelderblom H, Maring JG (2013) Evaluation of predictive tests for screening for dihydropyrimidine dehydrogenase deficiency. *Pharmacogenomics J* **13**(5): 389–395 Review. Erratum in: (2014) Pharmacogenomics J **14**(4): 400.

van Staveren MC, van Kuilenburg AB, Guchelaar HJ, Meijer J, Punt CJ, de Jong RS, Gelderblom H, Maring JG (2016) Evaluation of an oral uracil loading test to identify DPD-deficient patients using a limited sampling strategy. *Br J Clin Pharmacol* **81**(3): 553–561.

Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)