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

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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, *67376798 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 X 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 < 0.0001), *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.

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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 adjuvant oxaliplatin/fluoropyrimidine 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, Hilden, Germany). The quality and quantity of the DNA were assessed spectrophotometrically using a Nanodrop 1000 spectrophotometer (Thermo Scientific). All DPYD gene variability identified in our analysis were only genotyped and not phenotyped. Three biallelic variants (DPD*1, DPD*2A, DPD*1X) and one variant (DPD*19X) were included in the MYRIAPOD ADMET 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
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 (Labcyber, 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 ≥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 m² per week.

According to the results of DPYD analysis, patients were categorised in three genotype groups: carriers of the homozygous wild type (p²); heterozygous (pq); and homozygous variant (q²). The possible association of DPYD variant with FAEs was analysed in the codominant model (p², pq, and q² genotypes considered separately) and in a dominant model with merged heterozygous (pq) and homozygous (q²) 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 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 of 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 $\chi^2$ 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 (Solé 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
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 introduced in TOSCA trial only during the late phase of accrual of randomised to FOLFOX-4 because option for XELOX regimen was excluded 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 genotype carriers of rs2297595 and *6 rs1801160. At multivariate analyses the associations with DPYD variants identified in the study population were consistent with the Hardy–Weinberg equilibrium (P > 0.05). Results of LD analyses are shown in Supplementary Table S2. From Table 1, analysis of dose intensity did not show differences across treatment arms.

Table 2. Demographic and clinical characteristics of the enrolled patients

| Arm, n (%) | All sample (N = 508) |
|------------|----------------------|
| 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) | Female sex—n (%) |
|------------|----------------|------------------|
| 0          | 488 (96.1)     | 217 (42.7)       |

| ECOG performance status, n (%) | 0 | 1 |
|-------------------------------|---|---|
| 23 (4.5)                     | 20 (3.9) |

| Tumour site, n (%) | Multiple site | Single site |
|--------------------|--------------|------------|
|                    | 23 (4.5)     | 485 (95.5) |

| Histology, n (%) | Adenocarcinoma | Mucoïd adenocarcinoma | Other |
|-----------------|----------------|-----------------------|-------|
| 437 (86.0)      | 65 (12.8)      | 6 (1.2)               |

| Stage, n (%) | II | III |
|--------------|----|-----|
| 184 (36.2)   | 324 (63.8) |

| Grade, n (%) | Gx | G1-2 | G3-4 | Missing |
|--------------|----|------|------|---------|
| 4 (0.8)      | 340 (67.6) | 159 (31.6) | 5       |

| T stage, n (%) | pT1 | pT2 | pT3 | pT4 |
|----------------|-----|-----|-----|-----|
| 1 (0.2)        | 12 (2.4) | 41 (6.1) | 380 (74.8) | 84 (16.5) |

| N stage, n (%) | pN0 | pN1 | pN2 |
|---------------|-----|-----|-----|
| 184 (36.2)    | 233 (45.9) | 91 (17.9) |

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

| All grade ≥ 3 adverse events | All sample N = 508 |
|-------------------------------|-------------------|
| 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)    |

Table 4. At univariate analysis, *6 rs1801160 (codominant model: FDR = 0.0083 in both the dominant and codominant models). No additional significant associations were detected (data not shown).

| Grade | First | Second |
|-------|-------|--------|
| ≥ 3   | 0.0136| 0.0006 |
|       | 0.0001| 0.0001 |

| Allele | Median TTT (95% CI) |
|--------|---------------------|
| *1A    | 0.78 (0.54–1.15)    |
| *2A    | 0.84 (0.59–1.23)    |
| *3A    | 1.24 (0.84–1.87)    |
| *6     | 2.13 (1.53–3.03)    |

| Allele | Median TTT (95% CI) |
|--------|---------------------|
| *1A    | 0.78 (0.54–1.15)    |
| *2A    | 0.84 (0.59–1.23)    |
| *3A    | 1.24 (0.84–1.87)    |
| *6     | 2.13 (1.53–3.03)    |

Abbreviations: FAEs: fluoropyrimidine-related adverse events.
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; Rosamarin 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 analysisa | Multivariate analysisb,c |
|----------------------|-------------------------|
|                      | HR (95% CI) | FDR | HR (95% CI) | FDR |
| **4 rs1801158**      |             |     |             |     |
| Overall codominant:  |             |     |             |     |
| G/A or A/G vs G/G    | 0.74 (0.30–1.80) | 0.9137 | 0.74 (0.30–1.80) | 0.5937 |
| Dominant:            |             |     |             |     |
| **5 rs1801159**      |             |     |             |     |
| Overall codominant:  |             |     |             |     |
| G/A or G/G vs A/A    | 0.76 (0.53–1.08) | 0.3509 | 0.76 (0.53–1.08) | 0.2837 |
| Dominant:            |             |     |             |     |
| **6 rs1801160**      |             |     |             |     |
| Overall codominant:  |             |     |             |     |
| G/G                  | 1.00        | 0.0054 | 1.00        | 0.0003 |
| 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:            |             |     |             |     |
| **9A rs1801265**     |             |     |             |     |
| Overall codominant:  |             |     |             |     |
| C/T or C/C vs T/T    | 1.00 (0.71–1.41) | 0.5133 | 1.00 (0.71–1.41) | 0.9847 |
| **rs2297595**        |             |     |             |     |
| Overall codominant:  |             |     |             |     |
| G/A or G/G vs A/A    | 1.55 (1.06–2.26) | 0.1661 | 1.55 (1.06–2.26) | 0.0958 |
| Dominant:            |             |     |             |     |
| **2A rs3918290**     |             |     |             |     |
| Overall codominant:  |             |     |             |     |
| G/A or G/G           | 10.74 (2.59–44.61) | 0.0054 | 14.72 (3.35–64.72) | 0.0004 |
| Dominant:            |             |     |             |     |
| **rs17376848**       |             |     |             |     |
| Overall codominant:  |             |     |             |     |
| C/T or C/C vs T/T    | 1.34 (0.73–2.49) | 0.6299 | 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           | 1.00        |     |             |     |

Abbreviations: 95% CI = confidence interval at 95; DPYD = dihydropyrimidine dehydrogenase; FDR = False Discovery Rate; HR = hazard ratio; TTT = time-to-toxicity.
aAdjusted for age, gender, stage, and treatment.
bAdjusted for age, gender, stage, and treatment.
cAdjusted 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 fluoropyrimidine is combined with other drugs.

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

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 diarrhea (24.4%) Any grade = 3 toxicity (85.3%) Hand-foot grade = 2 (43.1%) | rs3918290 (DPYD*2A), rs1801160 (DPYD*6), rs56038477 NO NO |
| NCCTG (Lee et al., 2014, 2016) | Adjuvant     | FOLFOX (2384) FOLFIRI (210) CT plus Cetux (1191) CT without Cetux (1403) | 25 + 1x  | Grade = 3 FAEs (33%) | rs3918290 (DPYD*2A), rs67376798 |
| PETACC-8 (Boige et al., 2016) | Adjuvant     | FOLFOX (780) FOLFIRI + Cetux (765)          | 25                               | Grade = 3 FAEs (49.5%) | rs1801160 (DPYD*6), rs67376798 |
| TRIBE (Del Re et al., 2015) | Metastatic  | FOLFOXIRI + Bev (220) FOLFOXIRI + Bev (220) | 2                               | Grade = 3 FAEs | rs3918290 (DPYD*2A) plus rs67376798 |
| 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; FOLFIRI = bolus/infusional 5-fluorouracil plus oxaplatin; FOLFOX = bolus/infusional 5-fluorouracil plus irinotecan; FOLFOXIRI = bolus/infusional 5-fluorouracil plus oxaplatin and irinotecan; N= number of patients; Oxa = oxaplatin.

A: the CAIRO-2 analysis, *2A rs3918290 G>G 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.

*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.

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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 mononuclear cells, Uracil breath test, endogenous plasma/urine Uracil/Dihydrouracil, sampling PK model after 5-fluorouracil test dose; van Staveren et al, 2013, 2016; Del Re et al, 2017). These phenotyping tests seem to possess better predictivity than genotyping for fluoropyrimidine toxicity (van Staveren 2017). These phenotyping tests seem to possess better predictivity than genotyping for fluoropyrimidine toxicity (van Staveren 2017). These phenotyping tests seem to possess better predictivity than genotyping for fluoropyrimidine toxicity (van Staveren 2017). These phenotyping tests seem to possess better predictivity than genotyping for fluoropyrimidine toxicity (van Staveren 2017). These phenotyping tests seem to possess better predictivity than genotyping for fluoropyrimidine toxicity (van Staveren 2017). These phenotyping tests seem to possess better predictivity than genotyping for fluoropyrimidine toxicity (van Staveren 2017).

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.

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

These authors contributed equally to this work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.
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