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Rs895819 in *MIR27A* improves the predictive value of *DPYD* variants to identify patients at risk of severe fluoropyrimidine-associated toxicity

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The objective of this study was to determine whether genotyping of *MIR27A* polymorphisms rs895819A>G and rs11671784C>T can be used to improve the predictive value of *DPYD* variants to identify patients at risk of severe fluoropyrimidine-associated toxicity (FP-toxicity). Patients treated previously in a prospective study with fluoropyrimidine-based chemotherapy were genotyped for rs895819 and rs11671784, and *DPYD* c.2846A>T, c.1679T>G, c.1129-5923C>G and c.1601G>A. The predictive value of *MIR27A* variants for early-onset grade ≥3 FP-toxicity, alone or in combination with *DPYD* variants, was tested in multivariable logistic regression models. Random-effects meta-analysis was performed, including previously published data. A total of 1,592 patients were included. Allele frequencies of rs895819 and rs11671784 were 0.331 and 0.020, respectively. In *DPYD* wild-type patients, *MIR27A* variants did not affect risk of FP-toxicity (OR 1.3 for ≥1 variant *MIR27A* allele *vs.* none, 95% CI: 0.87–1.82, $p = 0.228$). In contrast, in patients carrying *DPYD* variants, the presence of ≥1 rs895819 variant allele was associated with increased risk of FP-toxicity (OR 4.9, 95% CI: 1.24–19.7, $p = 0.023$). Rs11671784 was not associated with FP-toxicity (OR 2.9, 95% CI: 0.47−18.0, $p = 0.253$). Patients carrying a *DPYD* variant and rs895819 were at increased risk of FP-toxicity compared to patients wild type for rs895819 and *DPYD* (OR 2.4, 95% CI: 1.27–4.37, $p = 0.007$), while patients with a *DPYD* variant but without a *MIR27A* variant were not (OR 0.3 95% CI: 0.06−1.17, $p = 0.081$). In meta-analysis, rs895819 remained significantly associated with FP-toxicity in *DPYD* variant allele carriers, OR 5.4 (95% CI: 1.83–15.7, $p = 0.002$). This study demonstrates the clinical validity of combined *MIR27A*/*DPYD* screening to identify patients at risk of severe FP-toxicity.

Tumor Markers and Signatures

Fluoropyrimidines are among the most frequently prescribed anticancer drugs for gastrointestinal, breast and head and neck cancers. Of the patients treated, 10–30% experiences severe, potentially lethal, fluoropyrimidine-associated toxicity.[1–4] The most well-established cause of intolerance to fluoropyrimidines is deficiency of the main 5-fluorouracil (5-FU) metabolic enzyme, dihydropyrimidine dehydrogenase (DPD).[5–8] DPD deficiency can be the result of polymorphisms in *DPYD*—the gene encoding DPD—and *DPYD* variants have therefore received wide-spread attention as predictors of fluoropyrimidine-associated toxicity.[3,9–12] Importantly, upfront screening for risk-associated *DPYD* variants and dose adaptation in patients carrying variant alleles has shown to be a feasible strategy to improve the safety of patients who carry *DPYD* variants.[13] At present, clinical validity has been demonstrated for four *DPYD* variants: c.1905 + 1G>A (*DPYD**2A, IVS14 + 1G>A, rs3918290), c.2846A>T (rs67376798), c.1679T>G (*DPYD**13, rs55886062) and c.1129–5923C>G (rs75017182; in complete linkage with the haplotype HapB3).[14,15] A fifth variant, c.1601G>A (*DPYD**4, rs1801158), has also been linked to altered DPD activity and fluoropyrimidine-associated toxicity, but the available evidence on clinical validity is less consistent.[9,15–17] In a recent meta-analysis, the c.1601G>A variant

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What's new?

Fluoropyrimidines are frequently used chemotherapeutic agents. Some patients however experience severe fluoropyrimidine-associated toxicity, with deficiency of the metabolic enzyme dihydropyrimidine dehydrogenase (DPD) as the most well-established cause of intolerance. DPD activity is regulated by microRNA-27-a, and polymorphisms in the encoding *MIR27A* gene have been shown to affect DPD activity. This study of 1592 patients suggests that the risk of early-onset severe toxicity in patients carrying *DPYD* variants is strongly affected by *MIR27A* rs895819 genotype. *MIR27A* variants can also be used to improve the predictive value of *DPYD* variants c.2846A›T, c.1679T›G, c.1129-5923C›G, and c.1601G›A for the identification of patients at risk.

was not found to be significantly associated with fluoropyrimidine-associated toxicity (relative risk: 1.52, 95% CI: 0.86–2.70, $p = 0.15$), but all analyzed studies had a relative risk above 1.0, suggesting some effect on toxicity risk.[15]

The positive predictive value (PPV) of *DPYD* variants to identify patients who will experience severe toxicity varies widely, and is typically 40–80%, depending on the *DPYD* variant, the population and the window in which toxicity is studied.[9,10,17] One factor that contributes to a PPV lower than 100% is the fact that in a proportion of *DPYD* variant allele carriers DPD activity is not found to be reduced to a clinically relevant extent.[7,18] This variability in the relationship between *DPYD* genotype and DPD phenotype can in part be explained by regulation of DPD at the posttranscriptional level. Recently, Offer *et al.* showed that DPD expression is regulated to a relevant extent by two microRNAs (miRs), miR-27a and miR-27b. These short, single-stranded RNAs associate with RNA-induced silencing complex (RISC) proteins and bind to *DPYD* mRNA, thereby inhibiting its translation and increasing degradation. Expression of miR-27a in murine liver was found to negatively correlate with *DPYD* mRNA level ($R^2 = 0.45$, $p = 0.0023$) and DPD activity ($R^2 = 0.49$, $p = 0.0012$).[19]

Polymorphisms in *MIR27A,* the gene encoding miR-27a, have been shown to influence miR-27a expression.[19,20] A common A>G polymorphism in *MIR27A*, rs895819, was found to increase miR-27a expression in lymphoblastoid cell lines, and was associated with reduced DPD activity in peripheral blood mononuclear cells (PBMCs) of human volunteers.[19] The latter suggests that rs895819 may have a relevant effect on 5-FU metabolism.[19]

We previously showed that among patients with *DPYD* risk-associated variants *DPYD**2A, c.2846A>T, c.1679T>G or c.1129–5923C>G, patients who also had the rs895819 variant (G) allele were at strongly increased risk of fluoropyrimidine-associated toxicity compared with patients without the rs895819 variant allele (OR 7.4 for each additional rs895819 variant allele present in combination with a *DPYD* variant, 95% CI: 1.7–31.9, $p = 0.0073$).[21] Importantly, there was a large difference in PPV for severe toxicity between patients with and without rs895819. For patients who carried both *DPYD* and rs895819 variant alleles the PPV was 71% compared with 25% for patients who carried only a *DPYD* variant. A second polymorphism in *MIR27A*, rs11671784, has also been associated with miR-27a expression, but its clinical relevance in patients treated with fluoropyrimidines remains unclear.[21–23]

If the predictive value of *MIR27A* polymorphisms in combination with *DPYD* variants can be confirmed, this has important implications for genetic screening strategies to identify patients at risk of fluoropyrimidine-associated toxicity. *MIR27A* genotyping might be of specific value in combination with *DPYD* variants that have a more modest effect on DPD activity, such as c.1129–5923C>G and possibly the c.1601G>A variant. The latter variant was not investigated in combination with *MIR27A* polymorphisms in the previous study by Amstutz *et al.*

Here, we undertook a study, based on a cohort of 1,592 patients treated previously in a prospective study, to determine the predictive value of *MIR27A* variants in combination with *DPYD* variants c.2846A>T, c.1679T>G and c.1129–5923C>G, as well as c.1601G>A, to identify patients at risk of fluoropyrimidine-associated toxicity.

Patients and Methods

Patients and study design

The basis for this study was a cohort of patients treated in a prospective, multicenter study of *DPYD**2A genotype-guided dosing of fluoropyrimidines, in which 1,631 patients were enrolled (clinicaltrials.gov identifier: NCT00838370).[13] In this study, 18 patients with the *DPYD**2A variant were treated with a reduced dose of the fluoropyrimidine; these patients were excluded from this analysis. No intervention was applied in the remaining 1,613 patients who proved to be wild type for *DPYD**2A; they were treated according to standard of care fluoropyrimidine-based anticancer regimens, *i.e.*, either fluoropyrimidines as single agent or combined with other chemotherapy, or radiotherapy. Toxicity was monitored and recorded during the entire treatment according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTC-AE) v3.0. All *DPYD**2A wild-type patients were considered eligible for inclusion in the analysis. Genomic DNA was collected before treatment. The study was approved by the Medical Ethics Committees of the Netherlands Cancer Institute and the two local study sites.

The 1,613 patients not carrying *DPYD**2A were previously included in a pharmacogenetic study in which we determined the clinical relevance of *DPYD* variants c.2846A>T (rs67376798), c.1679T>G (rs55886062), c.1129–5923C>G (rs75017182) and c.1601G>A (rs1801158) as predictors of severe fluoropyrimidine-associated toxicity[15] (details in Supporting Information). In the respective study, *DPYD* genotype data were acquired for all four *DPYD* variants in a total of 1,600 patients. The data from these 1,600 patients were the basis for this study.

The primary objective of this study was to determine whether rs895819 and rs11671784 in *MIR27A* can be used to improve the predictive value of *DPYD* variants c.2846A>T, c.1679T>G, c.1129–5923C>G and c.1601G>A to identify patients at risk of severe fluoropyrimidine-associated toxicity. Based on the report by Amstutz *et al.*,[21] we hypothesized that patients carrying both a *MIR27A* polymorphism and a *DPYD* variant would be at significantly higher risk of severe (CTC-AE grade $\geq$3) fluoropyrimidine-associated toxicity than patients carrying a *DPYD* variant alone, and that a combined screening strategy based on *DPYD* and *MIR27A* variants would be superior in terms of PPV compared with screening for *DPYD* alone.

Secondary objectives were to investigate the effect of rs895819 and rs11671784 on the risk of severe fluoropyrimidine-associated toxicity in patients without *DPYD* variants.

Determination of *DPYD* and *MIR27A* variants

DPYD variants were determined using standard PCR methods (details in Supporting Information). The genomic region containing *MIR27A* was amplified using PCR, followed by sequencing (Supporting Information).

Endpoints and data analysis

Endpoints. The primary endpoint of the study was severe, CTC-AE grade $\geq$3, fluoropyrimidine-associated toxicity during the first cycle of treatment. An analysis of the entire treatment duration was considered inadequate in view of the wide variation among patients in treatment duration in this heterogeneous daily-care patient population and the potential risk of attrition bias. The primary endpoint included the following toxicities: neutropenia, leukocytopenia, thrombocytopenia, diarrhea, mucositis, hand-foot syndrome and cardiac toxicity (only treatment-related events were scored). Cycle 1 toxicity score was dichotomized as absent to moderate (CTC-AE grade 0–2) *versus* severe (grade 3–5). Details on frequencies of toxicities have been reported previously.[13]

Data analysis. *MIR27A* variants were tested for deviation from Hardy–Weinberg equilibrium using the exact test, and allele frequencies were compared with previously reported frequencies. Linkage disequilibrium between rs895819 and rs11671784 was assessed by calculating D' (normalized linkage disequilibrium coefficient).

Associations with risk of severe toxicity were tested using logistic regression models with adjustment, in all analyses, for other factors known to be associated with toxicity, *i.e.*, age (continuous), gender and treatment regimen (capecitabine monotherapy, capecitabine plus platinum, capecitabine plus taxane, capecitabine-based triplet combination, capecitabine plus radiotherapy, capecitabine plus other drug or 5-FU-based chemotherapy). The planned starting dose of capecitabine was highly colinear with type of regimen and was not predictive of toxicity after adjustment for treatment regimen; it was therefore not included in the models. Also tumor type, disease stage or previous treatment were not predictive of toxicity and not included as covariates.

Based on available data regarding the effects of *MIR27A* polymorphisms on miR-27a expression, a dominant genetic model was used to analyze associations with risk of severe fluoropyrimidine-associated toxicity for both rs895819 and rs11671784.[19,22] Associations between *MIR27A* variants and risk of severe toxicity were first investigated in the overall population, by including both *MIR27A* variants in the logistic model, as well as the *DPYD* variants (as a dichotomized variable reflecting the presence of zero *vs.* $\geq$1 *DPYD* variant alleles). A statistical interaction term between *MIR27A* variants (zero *vs.* $\geq$1 variant alleles) and *DPYD* variants (zero *vs.* $\geq$1 variant alleles) was then analyzed, in view of the anticipated interaction and stronger effect of *MIR27A* variants in patients carrying *DPYD* variants.[21] Subsequently, the effect of *MIR27A* variants on risk of severe fluoropyrimidine-associated toxicity was investigated in the subgroups of patients with and without *DPYD* variants.

We subsequently evaluated the performance of potential screening strategies to identify patients at increased risk of early-onset toxicity, based on *DPYD* variants alone, *MIR27A* variants alone or the combination of *DPYD* and *MIR27A* variants. This was done by categorizing patients as follows: *DPYD* and *MIR27A* wild type ($DPYD^-/MIR27A^-$, the reference group), *DPYD* wild type and *MIR27A* variant ($DPYD^-/MIR27A^+$, *i.e.*, wild type for all *DPYD* variants tested and heterozygous or homozygous for either of the *MIR27A* variants), *DPYD* variant and *MIR27A* wild type ($DPYD^+/MIR27A^-$, *i.e.*, heterozygous or homozygous for c.2846A>T, c.1679T>G, c.1129–5923C>G or c.1601G>A and wild type for both *MIR27A* variants) or *DPYD* variant and *MIR27A* variant ($DPYD^+/MIR27A^+$, *i.e.*, heterozygous or homozygous for any of the *DPYD* variants and heterozygous or homozygous for either of the *MIR27A* variants). The PPVs of *DPYD* variants alone or in combination with *MIR27A* variants were compared.

Meta-analysis. To determine more accurately the effect size of rs895819 and rs11671784 on risk of toxicity in patients with *DPYD* c.2846A>T, c.1679T>G, c.1129–5923C>G or c.1601G>A, the results obtained in this study were meta-analyzed with the data by Amstutz *et al.*[21] In the published

study by Amstutz *et al.*, a different set of *DPYD* risk-associated variants had been considered, *i.e.*, *DPYD**2A, c.2846A>T, c.1679T>G and c.1129–5923C>G (not including c.1601G>A). For the purpose of the current analysis, to include the same *DPYD* variants in both datasets, patients carrying *DPYD**2A were excluded from the Amstutz *et al.* dataset, and the c.1601G>A variant was genotyped and included. Subsequently, the dataset from Amstutz *et al.* was analyzed using logistic regression analogous to the analysis of the primary dataset, with adjustment for the covariates that were adjusted for in the original study (*i.e.*, cisplatin/carboplatin co-administration and gender).[21] Only the prospectively enrolled patients from Amstutz *et al.* were included in the analysis ($n = 500$), because the remaining 14 patients were enrolled retrospectively after having experienced severe fluoropyrimidine-associated toxicity.

Subsequently, effect estimates from the two studies were pooled using random effects meta-analysis, according to DerSimonian and Laird.[24] A random-effects model was chosen because true differences in effect size between patient populations, as a result of differences in patient and treatment characteristics, were assumed.

The combined datasets were also used to investigate whether *MIR27A* variants differentially affected risk of toxicity depending on the type of *DPYD* variant present, as these differences could be relevant if patients were to be screened upfront for combinations of *DPYD* and *MIR27A* variants to individualize dosing of fluoropyrimidines. This was done by pooling both datasets and subsequently analyzing risk of toxicity for each *DPYD* variant, using logistic regression, with correction for age, gender and treatment regimen. Treatment regimen was recategorized in order to allow merging of the datasets, while maintaining adjustment for relevant concomitant chemotherapy (treatments were categorized as: 5-FU monotherapy, 5-FU doublet, 5-FU triplet, capecitabine monotherapy, capecitabine doublet and capecitabine triplet).

Associations with toxicity were reported as an odds ratio (OR) and 95% confidence interval (CI), with corresponding p values. A Bonferroni correction for testing for two polymorphisms was applied, and the threshold for significance was therefore set at $p < 0.025$. p Values are reported unadjusted. All statistical analyses were performed in R v3.1.0.[25]

Results

Patients and genotypes

A total of 1,592/1,600 patients (99.5%) were successfully genotyped for rs895819 and rs11671784, and these patients were included in the analysis (insufficient DNA was available for four patients, and genotype data could not be acquired for an additional four patients). The allele frequency was 0.331 for rs895819 and 0.020 for rs11671784, which is in line with the frequencies reported previously.[21,26] Neither for rs895819 or rs11671784 there was departure from Hardy–Weinberg equilibrium ($p = 0.336$ and $p = 1.000$, respectively), nor for any of the *DPYD* variants ($p > 0.1$). There was strong linkage disequilibrium between rs895819 and rs11671784 ($D' = 0.991$, $X^2 = 31.2$, $p < 0.00001$; $r = -0.099$; Supporting Information Table S2). The characteristics of the overall population are summarized in Table 1.

MIR27A polymorphisms are moderately associated with risk of fluoropyrimidine-associated toxicity in the overall population

We first investigated the effect of *MIR27A* polymorphisms on risk of fluoropyrimidine-associated toxicity in the overall population of 1,592 patients. In total, 165 of 1,592 patients (10%) developed CTC-AE grade $\geq$3 toxicity during the first cycle of treatment. There was a moderate association between rs895819 and fluoropyrimidine-associated toxicity in the overall population (OR 1.6, 95% CI: 1.10–2.22, $p = 0.012$). No significant effect of rs11671784 on risk of toxicity was found (OR 1.1, 95% CI: 0.49–2.62, $p = 0.777$). Also, the total number of *MIR27A* risk alleles ($\geq$1 risk-associated *MIR27A* alleles *vs.* wild type) was significantly associated with fluoropyrimidine-associated toxicity (OR 1.5, 95% CI: 1.06–2.15, $p = 0.022$).

Patients carrying *MIR27A* variants in combination with *DPYD* variants are at strongly increased risk of fluoropyrimidine-associated toxicity

An interaction term between *DPYD* and *MIR27A* status (any *MIR27A* allele *vs.* none) was statistically significant (OR 7.1, 95% CI: 1.44–34.5, $p = 0.016$). The direction of the effect suggested that the effect of *MIR27A* status had more influence on risk of toxicity in the presence of *DPYD* variants.

In *DPYD* wild-type patients ($n = 1{,}429$), there was no association with fluoropyrimidine-associated toxicity for rs895819 (OR 1.4, 95% CI: 0.94–1.97, $p = 0.101$) (Fig. 1a). Similarly, rs11671784 was not associated with toxicity in *DPYD* wild-type patients.

In contrast, in patients carrying *DPYD* variants ($n = 163$) there was a significant association between rs895819 and early severe fluoropyrimidine-associated toxicity (OR 4.9, 95% CI: 1.24–19.7, $p = 0.023$), and the magnitude of effect appeared to be similar for patients heterozygous and homozygous for rs895819 (Fig. 1b).

For carriers of rs11671784, risk of toxicity was not significantly increased (OR 2.9, 95% CI: 0.47–18.0, $p = 0.253$). Of the ten patients who carried rs11671784 in conjunction with a *DPYD* variant, five patients also carried rs895819. As preclinical data in gastric tumor samples have suggested that the combined presence of both variants might cancel out an effect on miR-27a expression,[23] we investigated patients who carried rs11671784 alone and patients who carried both *MIR27A* variants, separately. Compared with *DPYD* variant patients who did not carry rs11671784, patients carrying a *DPYD* variant and rs11671784 were at significantly increased risk of toxicity (OR 30.1, 95% CI: 2.29–396, $p = 0.010$), while of the five patients who carried both

Table 1. Patient characteristics according to *DPYD* genotype

	Overall population ($n = 1,592$)	Patients without *DPYD* variants[1] ($n = 1,429$)	Patients with *DPYD* variants ($n = 163$)
Age			
Median (range)	61 (21–89)	61 (21–89)	64 (28–87)
Gender			
Male	713 (45%)	631 (44%)	82 (50%)
Female	879 (55%)	798 (56%)	81 (50%)
Tumor type			
Colorectal cancer	846 (53%)	761 (53%)	85 (52%)
Gastric cancer	223 (14%)	197 (14%)	26 (16%)
Breast cancer	367 (23%)	334 (24%)	33 (20%)
Other	156 (10%)	137 (10%)	19 (12%)
Treatment			
Capecitabine monotherapy	423 (27%)	380 (27%)	43 (26%)
Capecitabine plus radiotherapy	434 (27%)	392 (27%)	42 (26%)
Capecitabine plus taxane	64 (4%)	57 (4%)	7 (4%)
Capecitabine plus platinum	373 (23%)	336 (24%)	37 (23%)
Capecitabine triplet combination	111 (7%)	100 (7%)	11 (7%)
Capecitabine plus other	22 (1%)	15 (1%)	7 (4%)
5-FU-based chemotherapy	165 (10%)	149 (10%)	16 (10%)
Origin			
Caucasian	1,526 (96%)	1,367 (96%)	159 (98%)
Other	66 (4%)	62 (4%)	4 (2%)
rs895819			
AA	704 (44%)	637 (45%)	67 (41%)
AG	723 (45%)	643 (45%)	80 (49%)
GG	165 (10%)	149 (10%)	16 (10%)
rs11671784			
CC	1,529 (96%)	1,376 (96%)	153 (94%)
CT	63 (4%)	53 (4%)	10 (6%)
TT	–	–	–
***MIR27A* risk alleles**[1]			
0	665 (42%)	603 (42%)	62 (38%)
1	738 (46%)	658 (46%)	80 (49%)
2	189 (12%)	168 (12%)	21 (13%)
***DPYD* variants**[2]			
Wild type	1,429 (90%)	1,429 (100%)	0 (0%)
c.2846A>T	19 (1%)	–	19 (12%)
c.1679T>G	3 (0.2%)	–	3 (2%)
c.1129-5923C>G (c.1236G>A)[3]	57 (4%)	–	57 (35%)
c.1601G>A (*DPYD**4)	84 (5%)	–	84 (52%)
Early fluoropyrimidine-associated toxicity			
Grade 0–2	1,427 (90%)	1,285 (90%)	142 (87%)
Grade ≥ 3	165 (10%)	144 (10%)	21 (13%)

[1]Sum of the no. of risk alleles present for rs895819 and rs11671784.
[2]Wild type indicates that patients were found to be wild type for *DPYD* c.2846A>T, c.1679T>G, c.1129–5923C>G and c.1601G>A (and *DPYD**2A, as these patients were excluded from the analysis because they were treated with a reduced dose of the fluoropyrimidine). There were no patients who carried more than one *DPYD* variant.
[3]All identified carriers of c.1236G>A were carriers of haplotype B3, *i.e.*, there was 100% linkage between c.1236G>A, c.1129–5923C>G and c.959–51T>G.

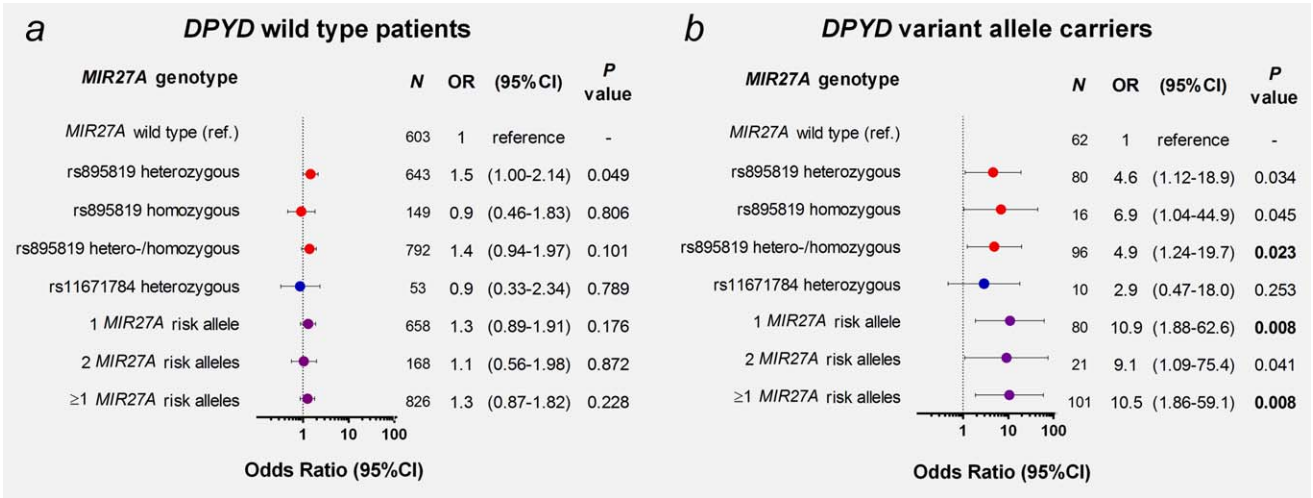

Figure 1. Associations between *MIR27A* variants and fluoropyrimidine-associated toxicity according to *DPYD* status. The figure shows the associations between *MIR27A* polymorphisms and risk of early severe fluoropyrimidine-associated toxicity in *DPYD* wild-type patients (*a*) and in patients who carry a *DPYD* variant (c.2846A>T, c.1679T>G, c.1129-5923C>G or c.1601G>A). (*b*) *MIR27A* = microRNA 27a (gene); *N* tox = number of patients experiencing severe toxicity/total number of patients; *DPYD* = dihydropyrimidine dehydrogenase (gene); OR = odds ratio; CI = confidence interval. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

MIR27A variants in combination with a *DPYD* variant, none experienced toxicity (and an OR could therefore not be estimated). We did not find a statistical interaction between rs11671784 and gender, as demonstrated by Amstutz *et al.*[21]

The effect of the individual *DPYD* variants on the observed association between rs895819 and severe fluoropyrimidine-associated toxicity in *DPYD* variant allele carriers was investigated by excluding patients carrying one specific *DPYD* variant at a time and repeating the analysis. This analysis revealed similar associations with severe toxicity for different combinations of *DPYD* variants, although the highest effect estimate was observed when patients carrying c.2846A>T, c.1679T>G or c.1129–5923C>G were included in the analysis (OR 9.8, 95% CI: 0.84–113.9, $p = 0.069$; Supporting Information Figure).

We also investigated the association between *MIR27A* variants and risk of severe toxicity in patients carrying *DPYD* variants when the entire treatment duration was taken into account, instead of only the first cycle. When the main analysis (as shown in Fig. 1) was repeated taking into account the full treatment duration, there was a trend toward an association between rs895819 and severe fluoropyrimidine-associated toxicity in patients carrying *DPYD* variants, OR 1.9 (95% CI: 0.81–4.57, $p = 0.140$). For rs11671784, the results were OR 0.7 (95% CI: 0.12–4.04, $p = 0.678$). When only c.2846A>T, c.1679T>G and c.1129–5923C>G were included, the results of the full-treatment analysis were OR 1.7 (95% CI: 0.48–6.15, $p = 0.405$) for rs895819; for rs11671784 an odds ratio could not be calculated because there were no toxicity events among the four patients carrying rs11671784.

For patients without *DPYD* variants, the results of the main analysis when taking into account the full treatment duration were: OR 1.3 (95% CI: 0.98–1.66, $p = 0.072$) for rs895819 and OR 1.1 (95% CI: 0.53–2.05, $p = 0.894$) for rs11671784.

A combined screening strategy for rs895819 and *DPYD* improves the PPV of upfront *DPYD* screening to identify patients at risk of fluoropyrimidine-associated toxicity

We then investigated the performance of potential screening strategies based on *DPYD* and *MIR27A* to identify patients at increased risk of fluoropyrimidine-associated toxicity. As shown in Figure 2a, patients who were $DPYD^-$/rs895819$^+$ were not at increased risk of severe toxicity compared with $DPYD^-$/$MIR27A^-$ patients, nor were $DPYD^+$/rs895819$^-$ patients. In contrast, $DPYD^+$/rs895819$^+$ patients were at significantly increased risk of toxicity (OR 2.4, 95% CI: 1.27–4.37, $p = 0.007$). For rs11671784 a similar pattern was observed (Fig. 2b), but the association with toxicity for $DPYD^+$/rs11671784$^+$ patients was not statistically significant (OR 2.3, 95% CI: 0.45–11.4, $p = 0.323$).

We further assessed the effect of different combinations of *MIR27A* and *DPYD* variants, by including all combinations as levels of one factor in a logistic regression model (Fig. 2c). This analysis confirmed the increased risk of toxicity for $DPYD^+$/rs895819$^+$ patients.

As shown in Supporting information, Table S3, the combined presence of *DPYD* and *MIR27A* variants had a much higher PPV compared with the presence of *DPYD* variants alone. Of the patients who carried a *DPYD* variant and either rs895819 or rs11671784, 20% (19/96) experienced early severe fluoropyrimidine-associated toxicity, while of the patients carrying only a *DPYD* variant allele and no *MIR27A* variant allele, 3% (2/62) experienced early severe fluoropyrimidine-associated toxicity ($p = 0.003$, Fisher's exact test).

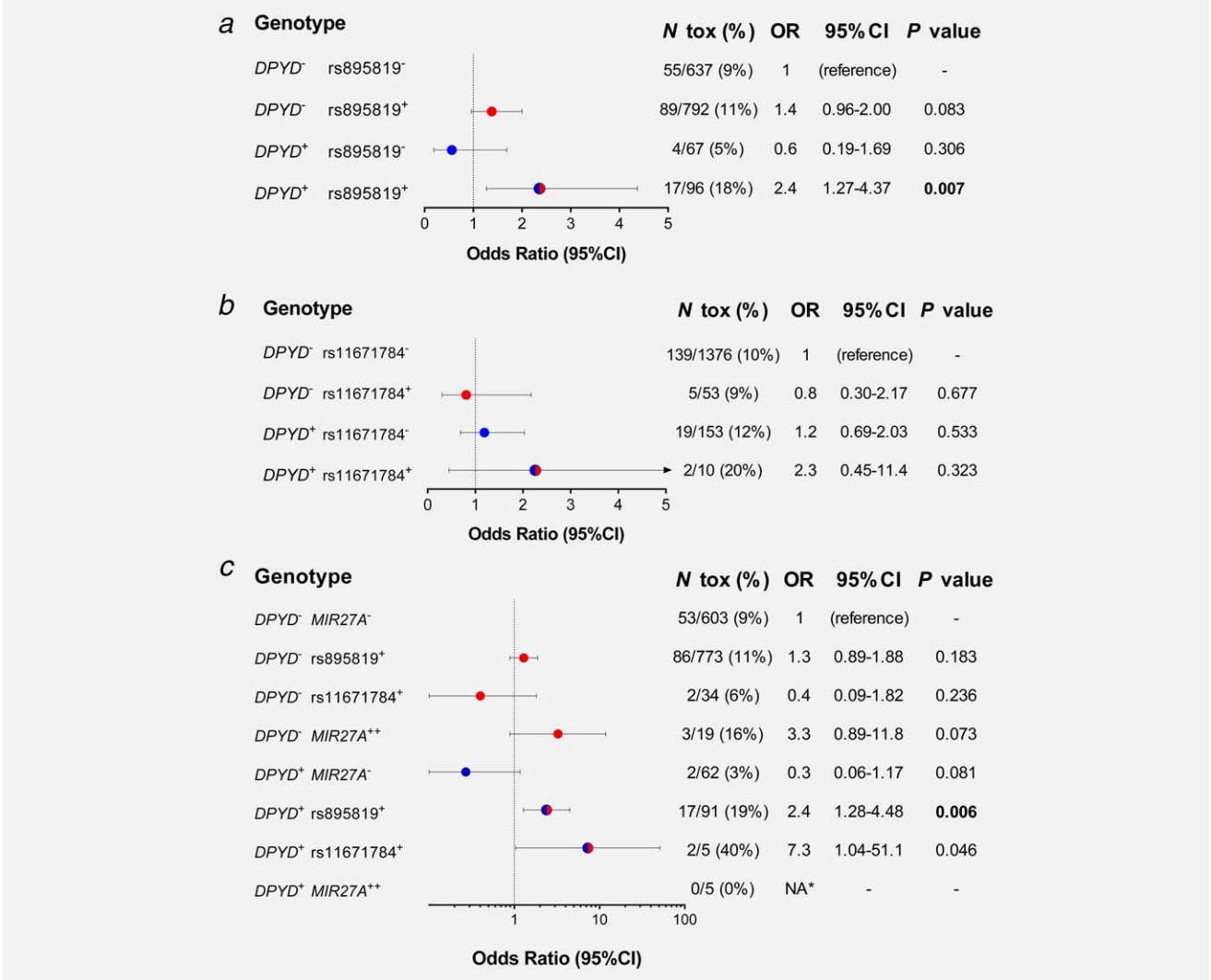

Figure 2. *DPYD* and *MIR27A* screening strategies to identify patients at risk of severe fluoropyrimidine-associated toxicity. The predictive value of *MIR27A* variants, alone or in combination with *DPYD* variants, to identify patients at risk of early severe fluoropyrimidine-associated toxicity. Results for rs895819 are displayed in (*a*) and results for rs11671784 in (*b*). In panel (*c*), the effect of combinations of *DPYD* and *MIR27A* genotypes on risk of severe fluoropyrimidine-associated toxicity is shown. The "+" sign indicates the presence of at least one *MIR27A* variant allele. The presence of *DPYD* c.2846A>T, c.1679T>G, c.1129-5923C>G or c.1601G>A is indicated as $DPYD^{+}$, while $MIR27A^{+}$ indicates the presence of at least one variant allele of rs895819 or rs11671784. *MIR27A* = microRNA 27a (gene); *DPYD* = dihydropyrimidine dehydrogenase (gene); OR = odds ratio; CI = confidence interval; *N* tox = number of patients experiencing severe toxicity/total number of patients; NA = not available (could not be calculated because 0/5 patients experienced severe toxicity). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Rs895819 is consistently associated with risk of severe toxicity in patients who carry *DPYD* variants in two independent cohorts, while data for rs11671784 remain inconclusive

Meta-analysis on the two studies was performed to determine more accurately the effect size of *MIR27A* variants on risk of severe toxicity in patients with *DPYD* variants c.2846A>T, c.1679T>G, c.1129–5923C>G or c.1601G>A (Fig. 3a), and to investigate the robustness of associations between *MIR27A* variants and risk of toxicity depending on the type of *DPYD* variant present (Fig. 3b). As shown in Figure 3a, the effect of rs895819 was consistent in both cohorts, and the summary effect size was OR 5.4 (95% CI: 1.83–15.7, $p = 0.002$), with no indications for heterogeneity ($Q = 0.035$, $p = 0.853$, $I^2 = 0\%$). The effect of rs11671784 was not found to be statistically significant in either of the studies or in meta-analysis.

Subsequently, we investigated the effect of MIR27A variants in combination with individual *DPYD* variants. This was done in the pooled datasets. As shown in Figure 3b, the presence of *MIR27A* variants predicted a significantly higher risk of severe toxicity in patients carrying c.1129–5923C>G ($n = 80$). In addition, there was a nominally, but not formally, significant association between *MIR27A* and severe

toxicity in patients carrying c.1601G>A ($n = 102$) with a similar effect size as for c.1129–5923C>G. For patients with c.2846A>T ($n = 22$) or c.1679T>G ($n = 5$), the presence of *MIR27A* variants also appeared to predict an increased risk of severe toxicity, but associations were not significant, possibly owing to the small numbers of patients who carried these variants.

Discussion

In this study, we show that *MIR27A* variants can be used to improve the predictive value of *DPYD* variants c.2846A>T, c.1679T>G, c.1129–5923C>G and c.1601G>A to identify patients at risk of severe fluoropyrimidine-associated toxicity. Compared with patients with a $DPYD^{+}/MIR27A^{-}$ genotype, patients carrying a $DPYD^{+}/MIR27A^{+}$ genotype were at much higher risk of early severe fluoropyrimidine-associated toxicity. The rs895819 variant was consistently found to be associated with fluoropyrimidine-associated toxicity in patients carrying *DPYD* variants in two independent studies (summary OR: 5.4, 95% CI: 1.83–15.7, $p = 0.002$), suggesting that combining rs895819 genotyping with *DPYD* genotyping can improve the predictive value of upfront *DPYD* screening.

The observed effect of rs895819 on risk of toxicity is in line with *in vitro* studies showing that the G allele is associated with increased miR-27a expression and with reduced DPD activity in PBMCs of human volunteers.[19] Our findings confirm the previous observation that this effect is primarily relevant in the presence of *DPYD* variants.[21] As we did not measure all deleterious *DPYD* variants, it is possible that the trend toward an association with toxicity observed in *DPYD* "wild-type" patients is the result of a stronger effect in a subgroup of these patients who carried other (rare) deleterious *DPYD* variants that were not measured, but this remains to be established.

The most well-established *DPYD* variants associated with severe fluoropyrimidine-associated toxicity are *DPYD**2A, c.2846A>T and c.1679T>G.[14,15] The relative risks for severe toxicity for these variants were recently found to be 2.9, 3.0 and 4.4, respectively, in a meta-analysis.[15] In the same analysis, it was shown that also c.1129–5923C>G was associated with fluoropyrimidine-associated toxicity, with a relative risk of 1.59 (95% CI: 1.29–1.97, $p < 0.0001$). The relative risk for c.1129–5923C>G was substantially lower than for *DPYD**2A, c.2846A>T or c.1679T>G, in line with a more subtle effect on DPD activity.[15,27] The current analysis shows that rs895819 status significantly affects the risk of severe toxicity in patients carrying c.1129–5923C>G (Fig. 3b). This indicates that combining rs895819 genotyping with genotyping of c.1129–5923C>G may lead to better determination of risk of fluoropyrimidine-associated toxicity in patients carrying c.1129–5923C>G.

The c.1601G>A variant, by itself, was not found to be significantly associated with fluoropyrimidine-associated toxicity in a recent meta-analysis of the published literature, although all included studies had a relative risk above 1.0, suggesting some effect on toxicity risk.[15] The current analysis indicates that in contrast to patients carrying c.1601G>A alone, carriers of c.1601G>A in combination with rs895819 are at increased risk of severe fluoropyrimidine-associated toxicity. This finding may thus explain previous conflicting results regarding the association of c.1601G>A with fluoropyrimidine-associated toxicity, and suggests that this may comprise a novel risk-associated genotype which requires dose adjustment. Combined with the results for c.1129–5923C>G,[21] these findings indicate that *MIR27A* genotype may be particularly useful to improve toxicity risk prediction in patients carrying *DPYD* variants with a moderate impact on DPD enzyme activity.

The relevance of rs11671784 remains inconclusive, as only a small number of patients carried this variant. Therefore, our findings regarding this variant should be interpreted with caution. As opposed to Amstutz *et al.*, we did not find a statistical interaction between rs11671784 and gender, and also no protective effect of *MIR27A* variants on risk of severe toxicity in *DPYD* wild-type patients.[21] Although we did not find a clear association between rs11671784 and fluoropyrimidine-associated toxicity, the observed effect size in the meta-analysis suggests that there might be an increased risk of toxicity in patients who carry this variant in combination with *DPYD* variants. Three preclinical investigations have shown that the rs11671784 T allele is associated with *reduced* miR-27a expression, as opposed to the increase in expression caused by rs895819.[20,22,23] This appears to contradict an increased risk of toxicity in carriers of this variant, as a reduction in miR-27a expression is expected to lead to higher DPD activity. However, great caution should be used in interpreting the findings from the three preclinical studies investigating the effect of rs11671784 on miR-27a expression.[20,22,23] The allele frequency of rs11671784 in this study, in the previous study by Amstutz *et al.* and according to the 1000 genomes project, is around 0.010–0.020 in European and American populations, and was reported to be 0.000 in Chinese populations.[21,26] Remarkably, the allele frequencies reported in the three preclinical investigations, all performed in China, were much higher, between 0.30 and 0.50. Importantly, the methods used for genotyping of rs11671784 in the respective studies have been shown to result in severe genotyping bias, and the results of these studies should therefore be interpreted with great caution.[28] The effect of rs11671784 on DPD activity in humans has not been investigated as far as we are aware. Additional studies are therefore required to establish the effect of rs11671784 on miR-27a expression, including an investigation of individuals carrying both *MIR27A* variants.

In our analysis of individual *DPYD* variants, we could establish that *MIR27A* variants were associated with risk of severe fluoropyrimidine-associated toxicity in patients with c.1129–5923C>G or c.1601G>A. Although patient numbers

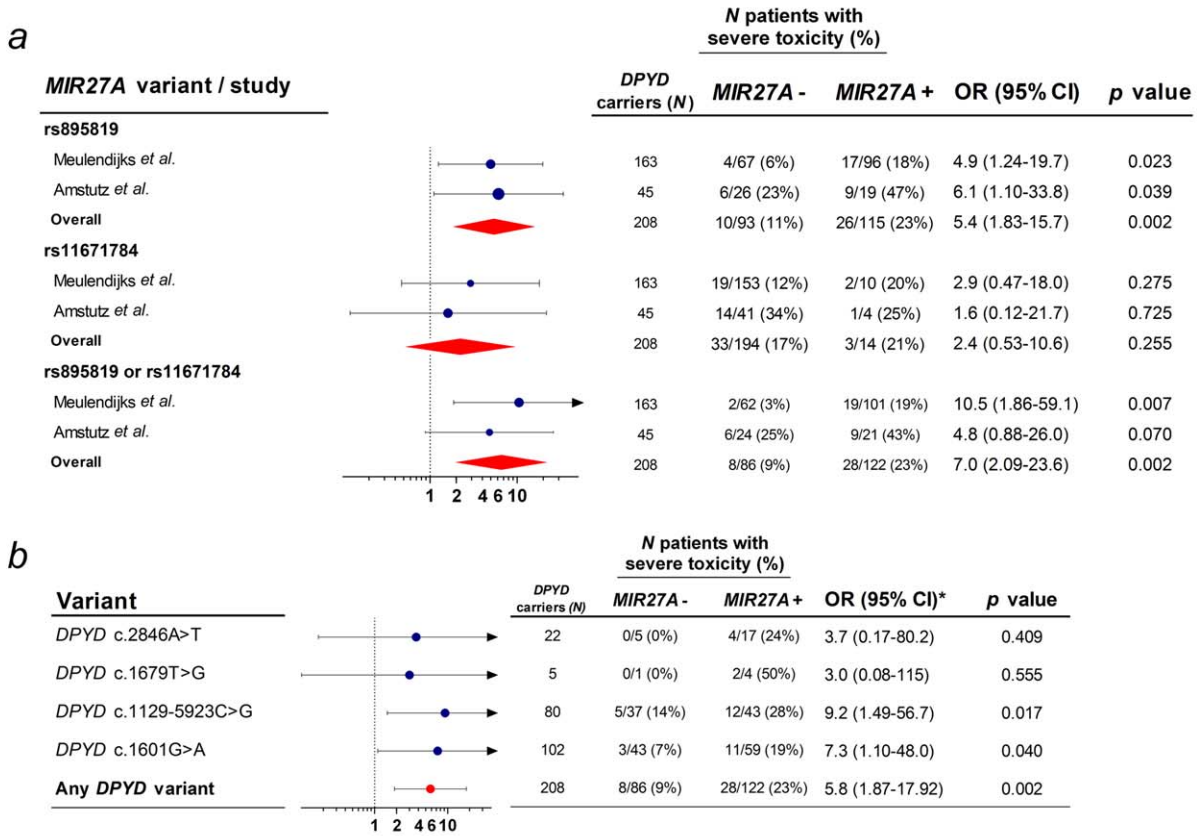

Figure 3. Results of meta-analysis to determine the effect of *MIR27A* variants in patients with *DPYD* variants, and pooled dataset analysis to determine the effect of individual *DPYD* variants. Panel (*a*) shows the results of the meta-analysis to determine the overall effect of *MIR27A* variants in patients who carry *DPYD* variants, based on this study and the study by Amstutz *et al.*[21] The *DPYD* variants tested in both Meulendijks *et al.* and Amstutz *et al.* in this meta-analysis were *DPYD* c.2846A>T, c.1679T>G, c.1129-5923C>G and c.1601G>A. The individual studies were analyzed using multivariable logistic regression. Random effects meta-analysis was subsequently performed according to DerSimonian and Laird.[24] Panel (*b*) shows the results of the pooled analysis to determine whether the effect of *MIR27A* variants on risk of toxicity in patients with *DPYD* variants was affected by the type of *DPYD* variant present. This was done by pooling of the current dataset with that of Amstutz *et al.* and performing logistic regression. *MIR27A*$^{+}$ indicates the presence of $\geq$1 variant *MIR27A* allele, *i.e.*, rs895819 and/or rs11671784, while *MIR27A*$^{-}$ indicates the presence of 0 variant alleles. *For c.2846A>T and c.1679T>G, univariable analysis was performed in view of small numbers of patients. The analyses for *DPYD* c.1129-5923C>G and c.1601G>A, and for all *DPYD* variants combined were adjusted for age, gender and treatment regimen. A 0.5 continuity correction was used when 0 toxicity events occurred in one *MIR27A* group to generate a finite OR (this was done for *DPYD* c.2846A>T and c.1679T>G; note that this leads to an underestimation of the true OR). *MIR27A* = microRNA 27a (gene); *DPYD* = dihydropyrimidine dehydrogenase (gene); OR = odds ratio; CI = confidence interval. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

were too low to enable definitive conclusions on c.2846A>T and c.1679T>G, similar effect sizes were observed, suggesting no major differences in the effect of *MIR27A* variants between the four investigated *DPYD* variants. Patients carrying the *DPYD**2A variant were not included in our analysis because these patients received an *a priori* reduced starting dose. It therefore remains to be confirmed whether genotyping of *MIR27A* variants is of added value in combination with *DPYD**2A. Observations in a limited number of *DPYD**2A carriers in the previous report by Amstutz *et al.* indicate, however, a similar effect of rs895819 also for this variant.[21] It should be noted that it could be that the differences in PPV that we demonstrated between genotyping for *DPYD* alone and the combination of *DPYD* and *MIR27A* are slightly different in a population which includes *DPYD**2A carriers.

The presented findings may have implications for studies investigating the relationship between *DPYD* variants and fluoropyrimidine-associated toxicity and, importantly, for studies aiming to improve patient safety by upfront screening for *DPYD* variants followed by dose adaptation in variant allele carriers. In conclusion, our findings suggest that the risk of severe early-onset toxicity in patients carrying *DPYD* variants is strongly affected by *MIR27A* rs895819 genotype. Specifically, we demonstrated this effect in patients carrying *DPYD* variants c.2846A>T, c.1679T>G, c.1129–5923C>G and c.1601G>A (*DPYD**2A was not included in our analysis). Patients with a *DPYD*$^{+}$/rs895819^{-} genotype are at a relatively low risk of toxicity, comparable to the average risk, while patients who have a *DPYD*$^{+}$/rs895819^{+} genotype are at strongly increased risk.

This suggests that the dose of fluoropyrimidines that these two groups of patients are able to tolerate is not equal. A two-stage screening strategy in which first *DPYD* variants are screened, followed by determination of rs895819 in patients carrying *DPYD* risk variants, could lead to better selection of patients who require a dose reduction and/or can be used to determine the extent of dose reduction required. This screening strategy requires further prospective validation.

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