Individualized dosing of fluoropyrimidines by genotyping and phenotyping of dihydropyrimidine dehydrogenase

Geïndividualiseerd doseren van fluoropyrimidines door middel van genotypering en fenotypering van dihydropyrimidine dehydrogenase
(met een samenvatting in het Nederlands)

Proefschrift

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Contents

PREFACE

Fluoropyrimidine anticancer drugs

The class of fluoropyrimidine anticancer drugs includes 5-fluorouracil (5-FU), which is given intravenously, and the oral prodrugs capecitabine and tegafur (also a component of S-1, Teysuno®). These drugs are used by an estimated two million patients yearly worldwide[1] and are the cornerstone of chemotherapeutic treatment of several solid tumor types, including colorectal, breast, gastric and head- and neck cancer.

5-FU was developed in the '50s,[2] thus it has been in use for over sixty years, and capecitabine has been approved by the European regulatory authorities in 2001. In many countries, 5-FU is more and more replaced by capecitabine, due to the at least equal efficacy, a favorable safety profile, and higher patient convenience as a result of the oral formulation.[3–6]

While fluoropyrimidine drugs are highly valuable treatment options, fluoropyrimidine-related toxicity is a major clinical limitation. Common toxicities associated with fluoropyrimidine therapy include diarrhea, mucositis, myelosuppression and hand-foot syndrome. Around 30% of the patients develops severe toxicity,[5,6] which is usually associated with interruption or discontinuation of potentially effective anticancer therapy, often requires hospitalization, and is even fatal in up to 0.5-1% of patients.[7,8]

Metabolism of fluoropyrimidines

After administration of capecitabine, this prodrug is rapidly converted in the liver to 5-FU by a three-step conversion. First 5'-deoxy-5-fluorocytidine (5'-dFCR) is formed, then 5'-deoxy-5-fluoro-uridine (5'-dFUR), which is then further converted to 5-FU.[9] The largest proportion of formed 5-FU is then rapidly catabolized in the liver by the enzyme dihydropyrimidine dehydrogenase (DPD) into the inactive metabolite dihydro-5-fluorouracil (FUH_2). This is further converted into other inactive metabolites and finally excreted via the urine.[10] As over 80% of 5-FU is inactivated by DPD, and also a proportion of 5-FU is directly excreted via the urine, only a small fraction of 5-FU (1-5%) is available for intracellular conversion into active metabolites that possess anti-tumor activity by inhibiting DNA and RNA synthesis.[11]

Deficiency of dihydropyrimidine dehydrogenase

Although fluoropyrimidines have been on the market for over sixty years, only in the last decades it has become clear that safety of patients treated with fluoropyrimidines is strongly affected by inter-individual variability in the DPD enzyme. DPD activity varies widely between patients, with an estimated 3 to 8% of the population having reduced DPD activity.[12,13] DPD deficiency results in reduced 5-FU clearance, and as a direct consequence, increased risk of severe treatment-related toxicity.[13,14] Multiple studies showed that around 39 to 61% of patients with severe fluoropyrimidine-associated toxicity were found to have decreased DPD activity.[15–17]

DPD deficiency is often the result of polymorphisms in *DPYD*, the gene encoding DPD. Currently there are four *DPYD* variants considered to be clinically relevant (*DPYD**2A, c.1679T>G, c.2846A>T and c.1236G>A), as it has been shown that patients carrying one of these variants are at significantly increased risk of developing severe treatment-related toxicity when treated with a standard dose of fluoropyrimidines. Prospective screening and dose reductions in heterozygous carriers of *DPYD* variants is considered to be a useful strategy to improve patient safety.[18]

However, not all variation in DPD enzyme activity can be explained by one of these four *DPYD* variants, and therefore not all toxicity can be prevented by *DPYD* genotyping. Identification of patients at risk of severe fluoropyrimidine-related toxicity remains challenging, and therefore other methods to identify patients at risk are being investigated, mostly focusing on DPD phenotyping. Several phenotyping methods have been developed, including *ex vivo* quantification of DPD activity in peripheral blood mononuclear cells (PBMCs), quantification of plasma levels of uracil, the endogenous substrate of DPD, or determining uracil clearance after an uracil loading dose.[19–21]

Outline of this thesis

This thesis describes several studies that aimed to identify the key factors that can improve safety of fluoropyrimidine-based chemotherapy.

In the first part of the thesis the implementation of *DPYD*-genotype guided dosing in clinical practice is discussed. For this implementation clear guidelines on how to apply dose adjustments for different *DPYD* variants are necessary. In **Chapter 1** the gene activity score is described which can be a useful tool to calculate the amount of dose reduction for a certain *DPYD* variant. **Chapter 2** focuses on the international guideline on fluoropyrimidine dosing based on *DPYD* genotype by the Clinical Pharmacogenetics Implementation Consortium (CPIC). As the evidence on the association between DPD deficiency and increased risk of severe fluoropyrimidine-associated toxicity is numerous, we believe that *DPYD*-genotype guided dosing should be implemented as standard of care, as is described in **Chapter 3**. Implementation of *DPYD*-guided dosing would benefit from including recommendations on *DPYD*-guided dosing in the drug label of capecitabine and 5-fluorouracil as well. This call for a drug label update is outlined in **Chapter 4**.

The second part of this thesis presents several studies on *DPYD* genotyping. **Chapter 5** describes a meta-analysis investigating the clinical relevance of the *DPYD* variants c.1679T>G, c.1236G>A and c.1601G>A. In **Chapter 6**, **Chapter 7** and **Chapter 8** patients with a rare *DPYD* genotype (a homozygous or compound heterozygous *DPYD* genotype) resulting in significantly reduced or even absent DPD enzyme activity are described. **Chapter 9** investigates whether genotyping of *MIR27A* polymorphisms (the gene encoding miR-27a, known to regulate DPD activity) can be used to improve the predictive value of *DPYD* genotyping. **Chapter 10** focuses on a retrospective study of patients heterozygous for *DPYD**2A in which it was determined if the applied dose reductions did not have a negative effect on effectiveness of the fluoropyrimidine treatment. **Chapter 11** describes the results of a large prospective trial where patients were prospectively screened for four *DPYD* variants and dose reductions based on *DPYD* genotype were applied.

In the third part several studies focusing on DPD phenotyping are discussed. In **Chapter 12** it was studied whether endogenous uracil concentrations are a useful predictor of severe fluoropyrimidine-related toxicity. **Chapter 13** focuses on uracil concentrations as well, and describes a study where the effect of food intake on uracil levels was determined. In **Chapter 14** the same prospective study as Chapter 11 is described, but now focusing on the results of a comparison between different DPD phenotyping methods in the study population.

Finally, in the **Conclusions and perspectives** section, the main conclusions of the research described in this thesis are summarized and results are put in a broader perspective.

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Implementation of *DPYD* genotype-guided dosing

1

Translating *DPYD* genotype into DPD phenotype: using the *DPYD* gene activity score

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SUMMARY

The dihydropyrimidine dehydrogenase enzyme (DPD, encoded by the gene *DPYD*) plays a key role in the metabolism of fluoropyrimidines. DPD deficiency occurs in 4–5% of the population and is associated with severe fluoropyrimidine-related toxicity. Several single nucleotide polymorphisms (SNPs) in *DPYD* have been described that lead to absent or reduced enzyme activity, including *DPYD**2A, *DPYD**13, c.2846A>T and c.1236G>A/haplotype B3. Since these SNPs differ in their effect on DPD enzyme activity, a differentiated dose adaption is recommended. We propose the gene activity score for translating *DPYD* genotype into phenotype, accounting for differences in functionality of SNPs. This method can be used to standardize individualized fluoropyrimidine dose adjustments, resulting in optimal safety and effectiveness.

INTRODUCTION

The fluoropyrimidine anticancer drug 5-fluorouracil (5-FU) and its oral prodrug capecitabine are frequently used in the treatment of a variety of cancers, including breast, colorectal, head and neck and gastric cancer. The dihydropyrimidine dehydrogenase enzyme (DPD), encoded by the gene *DPYD*, plays a key role in the metabolism of fluoropyrimidines. Over 80% of the administered dose of 5-FU is metabolized by DPD in the liver into the inactive metabolite 5,6-dihydro-5-fluorouracil, which makes DPD the rate-controlling enzyme for inactivation of 5-FU.[1] DPD deficiency occurs in 4–5% of the population and results in decreased inactivation of 5-FU. This can lead to an increase in active metabolites of 5-FU which is associated with an increased risk of severe and even fatal toxicity.[2–4] Toxicity could be limited by exposing DPD-deficient patients to a decreased dose of fluoropyrimidines, to keep plasma levels of 5-FU and its metabolites at a therapeutic level for these patients. Over 30 genetic polymorphisms in *DPYD* have been described among which several lead to reduced function or a non-functional DPD enzyme.[4–6] Polymorphisms can appear in heterozygous form (one SNP on one allele), homozygous form (two identical SNPs on two alleles) or double heterozygous form (two different SNPs on either one or two alleles, the latter is also called compound heterozygous). Two SNPs on two alleles lead to a larger decrease in DPD enzyme activity, compared with the heterozygous form. An example of a *DPYD* polymorphism is the splice-site variant *DPYD**2A (IVS14+1G>A; c.1905+1G>A; rs3918290), which leads to deletion of exon 14 and hence a non-functional DPD enzyme and is the most studied polymorphism in *DPYD*.

In recent years, genotyping costs have dropped significantly and pre-emptive testing for single or multiple SNPs to guide treatment with fluoropyrimidines has become accessible. Upfront genotype-directed dose-adaptation of fluoropyrimidines is feasible and has been shown to increase safety for patients and to be cost-effective for *DPYD**2A.[7,8] However, only a minority of institutions have implemented screening programs as standard of care.[9–11] Some physicians are reluctant to implement upfront genotype-guided dosing due to a lack of results from prospective randomized studies comparing genotype-guided and traditional dosing. The only prospective randomized study was terminated prematurely for ethical reasons as one patient in the control arm died due to 5-FU-related toxicity.[12]

In addition to *DPYD**2A, other SNPs in *DPYD* have been described to result in decreased DPD enzyme activity, including *DPYD**13 (c.1679T>G; I560S; rs55886062), c.2846A>T (D949V; rs67376798) and c.1236G>A (E412E; rs56038477, in haplotype B3).[13–15] However, not all of these SNPs result in a similar decrease in DPD enzyme activity as *DPYD**2A.[3,14,16] As a result of the growing number of alleles and their range of activity, deriving DPD phenotype from genotype is increasingly challenging. In the near future the number of alleles will increase even further, since genetic testing is developing fast and single SNP testing might be replaced by testing SNP panels, whole exome sequencing or even whole genome sequencing. Consequently, there is a need for an individualized recommendation of dose adjustment of fluoropyrimidines, taking into account the specific genetic variants and their resulting reductions in DPD enzyme activity. In this paper, we describe a method for translation of *DPYD* genotype into DPD phenotype making use of the gene activity score. This method accounts for the differences in functionality of the SNPs in *DPYD*, which results in a more differentiated dose adjustment and thus in optimal safety and effectiveness.

PREVIOUS GUIDELINES & RECOMMENDATIONS

According to the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) capecitabine and 5-FU are contraindicated in patients with a known DPD deficiency.[17,18] However, no recommendations are given for upfront screening for DPD deficiency and no distinction is made between heterozygous or homozygous DPD deficient patients. Also the American Society of Clinical Oncology (ASCO), European Society for Medical Oncology (ESMO) and National Comprehensive Cancer Network (NCCN) do not state any genotyping guidelines or recommendations prior to fluoropyrimidine treatment. In the guideline of the Clinical Pharmacogenetics Implementation Consortium (CPIC, a network that provides guidelines on the translation of genetic laboratory tests into actionable prescribing decisions) patients heterozygous for *DPYD**2A, *DPYD**13 or c.2846A>T are considered to have intermediate or partial DPD enzyme activity and recommended for these patients is an initial dose reduction of at least 50% (no dosing recommendations are given for other SNPs, including c.1236G>A, because evidence on these variants was considered weak or conflicting).[19] Also the Pharmacogenetics Working Group of the Royal Dutch Society for the Advancement of Pharmacy (KNMP) has provided guidelines. They recently updated their online guidelines for dose adjustments for fluoropyrimidines from a 50% dose reduction for heterozygous carriers to more specified dose reductions of 25 or 50% in heterozygous carriers of a SNP in *DPYD* (depending on the specific SNP), and 50, 75 or 100% in patients carrying more than one SNP in *DPYD.*[20,21] We consider the dosing guidance of the CPIC and KNMP very useful and would like to add the gene activity score to these guidelines. With the gene activity score we can facilitate in a more specific dose-adjustment in fluoropyrimidine treatment using current knowledge on differences in DPD enzyme activity due to *DPYD* variants.

KNOWN *DPYD* ALLELES & THEIR EFFECT ON DPD ENZYME ACTIVITY

DPYD*2A (rs3918290)

*DPYD**2A is the most widely studied polymorphism in *DPYD*. The SNP was first described by Vreken *et al*. in a case series of two unrelated patients[22] and McLeod *et al*. named it *DPYD**2A in an article in which the nomenclature for a series of *DPYD* SNPs was defined.[23] Allele frequencies of *DPYD**2A have been reported to vary between ~0.1 and 1.0% in African-American and Caucasian populations, respectively.[13,19,24,25] *DPYD**2A leads to skipping of the entire exon 14 and deletion of 165 base pairs which results in a truncated protein that is catalytically inactive.[22,26] This was recently confirmed in a study by Offer *et al.* where in an *in vitro* model of DPD activity several *DPYD* variants were homozygously expressed in mammalian cells and the enzymatic activity of expressed protein was completely absent.[27] This indicates that in heterozygous carriers of this variant, who have one dysfunctional allele and one functional allele, ~50% of the normal DPD enzyme activity will remain. Furthermore, a correlation between the *DPYD**2A variant and reduced enzyme activity in peripheral blood mononuclear cells (PBMCs) was found in several *ex vivo* studies that confirmed decreased function of *DPYD**2A.[26,28–30] and consequently an association was also found between *DPYD**2A and reduction in fluoropyrimidine clearance in patients.[31,32] In numerous studies an association between *DPYD**2A allele carriership and the increased risk of toxicity related to fluoropyrimidine treatment was confirmed.[4,24,31,33–45] For example, in a meta-analysis by Terrazzino *et al*. a strong correlation between the *DPYD**2A allele and overall grade ≥3 toxicity was found (odds ratio (OR) 5.42, *P*<0.001).[33] Deenen *et al.* described a mean capecitabine dose reduction of 50%, guided by toxicity, in patients carrying *DPYD**2A, compared with a mean dose reduction of

10% in wild-type patients.[42] Also, an initial dose reduction of capecitabine or 5-FU of 50% of standard dose has proven to decrease the risk of severe toxicity in *DPYD**2A carriers.[7,8] The above-mentioned *in vitro, ex vivo and in vivo* studies provide solid evidence for the non-functionality of *DPYD**2A and a 50% reduced function in patients heterozygous for *DPYD**2A.

c.2846A>T (rs67376798)

The c.2846A>T variant allele was first described by van Kuilenburg *et al*. in 2000.[28] The c.2846A>T polymorphism leads to a structural change in the DPD enzyme that interferes with cofactor binding or electron transport.[16] Reported allele frequencies of c.2846A>T vary from 0.1% to 1.1% in African-Americans and Caucasians respectively.[13,19,24,46] *In vitro* data show that homozygous expression of the c.2846A>T variant results in an activity of 59% compared with wild-type (*P*=0.0031).[13] Although the enzyme activity of c.2846A>T is significantly impaired, it is not comparable to the extent observed for *DPYD**2A, where homozygous expression resulted in a completely non-functional enzyme.[27] This finding that homozygous expression of c.2846A>T results in ~50% reduction, suggests that a heterozygous carrier would have around 25% reduction in DPD activity. Furthermore, also in clinical practice a difference between the effect of the *DPYD**2A variant and the c.2846A>T variant has been observed. Deenen *et al*. described an average 25% dose reduction for c.2846A>T heterozygous patients in response to fluoropyrimidine-related toxicity, compared with 50% for *DPYD**2A heterozygous patients.[42] Although there are less publications for c.2846A>T than for *DPYD**2A, several studies and two meta-analyses found an association between the c.2846A>T variant and increased risk of severe fluoropyrimidine-associated toxicity, which indicates that a dose reduction is warranted.[4,24,33,36,41,42,44,45,47] In the study by Rosmarin *et al.* an OR of 9.35 (*P*=0.0043) was found between c.2846A>T and capecitabine-related severe (≥grade 3) toxicity.[47] The evidence described above shows that c.2846A>T has rest-activity left, but that a dose reduction would still be required to prevent toxicities that would occur using a full dose of fluoropyrimidines. Therefore, based upon the available evidence we can assume that a dose reduction of 25% is most rational.

DPYD*13 (rs55886062)

*DPYD**13 was first described by Collie-Duguid *et al*. as "T1679G".[48] The allele frequency was found to vary from 0.07 to 0.1% in Caucasians.[19,24] The precise functional consequences of the *DPYD**13 variant have not yet been unraveled, but are thought to be related to destabilization of a sensitive region of the protein.[16] *DPYD**13 has been found in patients with decreased enzyme activity, not in patients showing normal DPD enzyme activity.[29] Homozygous expression of this variant resulted in a 75% reduction of DPD enzyme activity compared with wild-type, as reported in an *in vitro* study by Offer *et al.*[27] This suggests that this variant almost completely inactivates the protein. Decreased DPD enzyme activity in patients with the *DPYD**13 variant was determined only in a limited number of *ex vivo* studies using PBMCs.[16,29,30,48] A major variation of enzyme activity was found, ranging from 1.7 times to 500 times decreased compared with the normal enzyme activity and once the enzyme activity was undetectable,[30] although it must be mentioned that these results could be influenced by other copresent *DPYD* variants. Patients with *DPYD**13 showed severe toxic side effects in several studies.[4,24,29,44,48,49] Also dose adjustments were described by two groups.[4,24] Morel *et al.* described a heterozygous patient that experienced severe grade 4 toxicity. After a 6-week treatment interruption, 5-FU was safely reintroduced with individual pharmacokinetic

adjustment, based on 5-FU plasma levels.[4] The above mentioned studies show that *DPYD**13 results in an almost non-functional enzyme and consequently low enzyme activity levels. Without a dose reduction, toxicities are likely to develop, however safe use of 5-FU is still possible with a dose adjustment. We suggest a starting dose of 50% for patients carrying *DPYD**13 to ensure safe and effective use of fluoropyrimidines.

c.1236G>A/HapB3 (rs56038477)

The c.1236G>A variant was first described by Seck *et al*., as a silent mutation that displays normal DPD enzyme activity.[46] The c.1236G>A polymorphism occurs in exon 11 and is a synonymous variant that is in complete linkage with c.483+18G>A, c.680+139G>A, c.959-51T>G and c.1129-5923C>G;[14] these variants in linkage have been termed haplotype B3.[14,15] The c.1129-5923C>G intronic polymorphism (rs75017182) results in aberrant splicing and is likely to be the responsible variant for the effect on DPD enzyme activity.[3,14] The frequency of heterozygous patients in Caucasian populations was reported to vary between 2.6% and 6.3%.[14,15,42,49,50] DPD enzyme activity for c.1236G>A carriers was measured in PBMCs in two studies.[14,46] Enzyme activities were reported to be 2.9, 4.2, 6.2 and 1.6 nmol/(mg*h) (normal value = 9.6 ± 2.6 nmol/(mg*h)) for one homozygous and three heterozygous carriers of c.1236G>A, respectively.[14] In addition, a heterozygous patient in another study was found to have an enzyme activity of 10.2 nmol/(mg*h), which was reported as 'normal activity', since the enzyme activity of the population ranged from 4.8 to 15 nmol/(mg*h).[46] Unfortunately, data on c.1236G>A and enzyme activity are limited and not consistent. The homozygous patient still had 30% DPD activity remaining.[14] Furthermore we observed two homozygous patients with this variant in our own institute with a relevant DPD enzyme activity left of around 50%, showing that this variant does not result in a completely nonfunctional enzyme.[62] In the study of Sistonen *et al.* the ratio between endogenous dihydrouracil (DHU) and uracil (U) was measured in patients carrying the c.1129-5923C>G variant.[50] This ratio can be used as a phenotyping marker for DPD enzyme activity, as described in several studies.[51–55] Sistonen *et al.* found a statistically significant decrease in DHU/U ratio compared with wild type patients (*P*=0.044). However, no significant effect for the other *DPYD* risk variants (*DPYD**2A, *DPYD**13 and c.2846A>T) was observed, which might be caused by the small sample size of patients with those variants. The c.1236G>A/HapB3 variant has been associated with severe and lethal toxicity.[14,15,42,49,56] For example, Froehlich *et al.* found a relative risk of 3.74 (*P*=0.00002) in c.1236G>A/HapB3 carriers for severe toxicity (grade 3–5).[49] In contrast, no significant effect of the c.1236G>A/HapB3 variant was found in two other studies.[44,47] A dose reduction to prevent toxicity may be advantageous since multiple studies found a correlation with severe toxicity; however the degree of dose reduction cannot easily be determined with the enzyme activity from only two published studies and conflicting results in clinical studies. In heterozygous patients, a dose reduction of 50% would be too large since c.1236G>A/HapB3 does not result in a completely non-functional enzyme. No dose reduction at all would be in contradiction to the correlation found between this variant and toxicity. Therefore a more cautious dose reduction of 25% seems appropriate, to avoid both increased risk of toxicity and prevent underdosing.

Also our own experimental data support the differentiation between various SNPs in *DPYD*. We determined the endogenous pretreatment ratio between DHU and U in a large cohort of patients (*N*=539) treated with capecitabine or 5-FU.[63] This cohort is a subset of patients participating in a prospective multicenter trial of *DPYD**2A-guided dosing of fluoropyrimidines (clinicaltrials.gov identifier: NCT00838370).[7,8] The DHU and U levels were measured in pretreatment serum samples using a validated LC-MS/MS method;[64] chromatographic separation was performed on an Acquity UPLC® HSS T3 column (150 x 2.1 mm ID, particle size 1.8 µm), and a triple quadruple mass spectrometer (API5500, AB Sciex, USA) was used for quantification of U and DHU. The method was validated over a concentration range of 1–100 ng/mL for U and 10–1000 ng/mL for DHU. Genotyping for *DPYD* variants was performed using standard PCR methods. A distinction was made between patients heterozygous for *DPYD**2A, c.2846A>T, *DPYD**13 or c.1236G>A and wild type patients (Figure 1). For patients heterozygous for *DPYD**2A, c.2846A>T, *DPYD**13 and c.1236G>A the median relative DHU/U ratio compared with wild-type is 52, 68, 50 and 101% respectively. These results confirm that DPD enzyme activity differs between carriers of certain *DPYD* polymorphisms and points toward a differentiated dose reduction for each individual SNP.

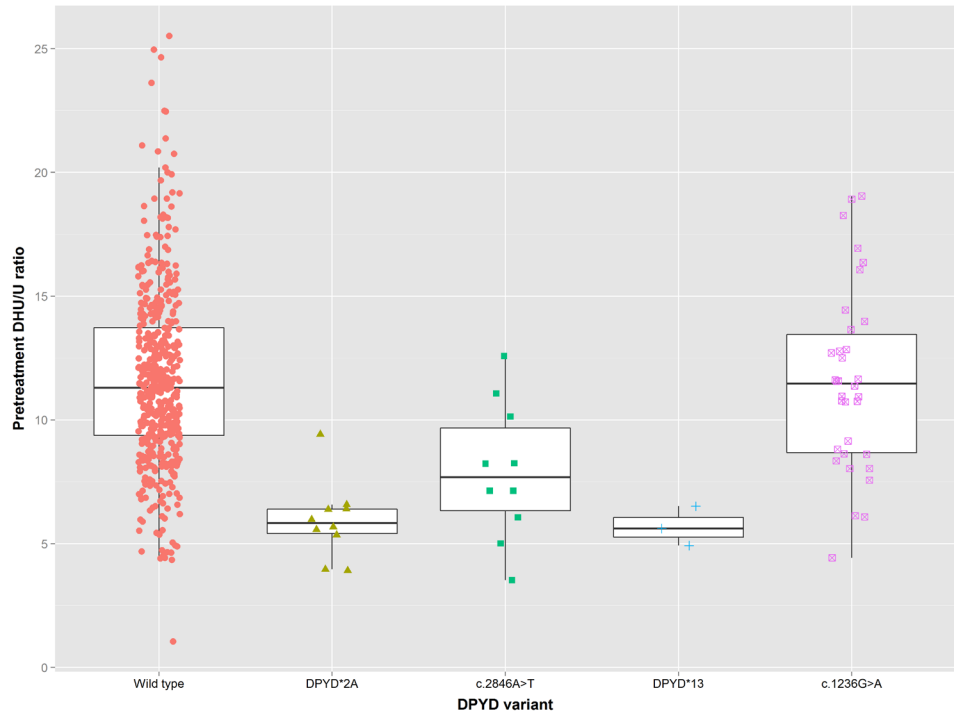

Figure 1. DHU/U ratio according to *DPYD* genotype.
Shown are individual values and a box plot with the median of the DHU/U ratio for patients with a *DPYD* polymorphism or *DPYD* wild type patients. *Abbreviations:* DHU: dihydrouracil; U: uracil.

GENE ACTIVITY SCORE

The gene activity score method is based on the principle that variant alleles can differ in the extent to which they influence enzyme activity. Such a method was first described by Steimer *et al.* where a 'quantitative functional gene dose' is assigned to alleles of the gene *CYP2D6*, a highly polymorphic gene that is involved in the metabolism of various clinically used drugs, including antidepressants, antipsychotics and opioids.[57] Thereafter Gaedigk *et al.* introduced the 'activity score' and divided *CYP2D6* alleles in three categories, consisting of fully functional alleles (value of 1), reduced activity alleles (value of 0.5) and nonfunctional alleles (value of 0).[58] The values for both alleles of a patient are summed, leading to an individual gene activity score that represents the enzymatic phenotype of the patient. This method results in a uniform way of describing phenotypes and can be used for adjusting the dose of a drug. For *CYP2D6* it has been demonstrated that the gene activity score is valid and easy-to-use for translating genotype and predicted phenotype.[58] The gene activity score may also be useful to properly interpret different DPD enzyme activities, translate these into a phenotype and thus personalize fluoropyrimidine treatment according to *DPYD* genotype. With this tool a more precise distinction between non-active and reduced activity alleles can be made and it also provides the possibility to include novel SNPs which may be identified in the near future using whole exome and whole genome sequencing. The activity score as proposed by Gaedigk *et al.* has proven beneficial for *CYP2D6*, for which a large number of polymorphisms are known.

We have fully investigated and described four SNPs in *DPYD* (*DPYD**2A, c.2846A>T, *DPYD**13, c.1236G>A/HapB3). This literature review describes what DPD enzyme activities are to be expected in patients with a certain SNP in *DPYD*. In addition to that, we have shown additional data of pretreatment DHU/U ratio in correlation to *DPYD**2A, c.2846A>T, *DPYD**13 and c.1236G>A. We focus on these four SNPs because, based on the available literature data, we believe they are the most relevant. Additional SNPs can be easily added to the gene activity score in the future when sufficient data are available. An outline for the suggested assigned values to various alleles of *DPYD* is given in Table 1. So far only the four SNPs described above are included, because sufficient evidence is available that they result in low DPD enzyme activity and severe fluoropyrimidine-related toxicity. Consequently, following the calculated gene activity scores for *DPYD* an individualized dose recommendation for fluoropyrimidines can be given, as is shown in Table 2. This is a recommendation for a starting dose; after the first or second cycle the dose can be titrated according to tolerance. Wild-type patients have two fully functional alleles, are allocated the maximal gene activity score of 2 and will receive the standard starting dose.

Table 1. Values for activity assigned to alleles of *DPYD*.

Activity value	Alleles	References
0	*DPYD**2A (rs3918290)	4,7,8,16,24,26–44
	*DPYD**13 (rs55886062)	4,16,27,29,30,44,48,49
0.5	c.2846A>T (rs67376798)	4,13,24,33,36,41,42,44,47
	c.1236G>A/HapB3 (rs56038477)	14,15,42,44,46,47,49,50,56
1	*DPYD**1 (wild-type)	

These values for both alleles of a patient are summed, leading to an individual gene activity score.

Table 2. Initial dose recommendation for *DPYD* gene activity score.

Gene activity score	% of standard dose
0	Alternative drug
0.5	25%
1	50%
1.5	75%
2	100%

Patients heterozygous for *DPYD**2A or *DPYD**13 have one non-functional allele and one fully functional allele, will therefore have an expected DPD enzyme activity of 50% and receive a gene activity score of 1. The recommended dose reduction of capecitabine or 5-FU for those patients is 50%. Patients carrying one allele with the c.2846A>T or c.1236G>A/HapB3 variant will have one decreased activity allele and one fully functional allele, which results in DPD enzyme activity of ~75% of normal. They are allocated a gene activity score of 1.5, for which a recommended starting dose of 75% of the standard dose applies.

DISCUSSION & CONCLUSION

There is ample evidence that shows that DPD-deficient patients develop severe toxicities when treated with a normal dose of fluoropyrimidines. Even though this relation is widely known, there is no global systematic approach to prevent severe toxic side effects using *DPYD* polymorphisms as predictive markers. Upfront *DPYD**2A screening has been implemented in a limited number of institutions and other SNPs are increasingly added to the standard genetic screening. Testing for an increasing number of SNPs that result in different DPD enzyme activities makes it harder to derive a dosing advice. The gene activity score is a new method for translating *DPYD* genotype into DPD phenotype. It can be used to standardize the process of describing DPD enzyme activity, which stimulates uniformity. In the CPIC guideline a dose recommendation of 50% is advised for *DPYD**2A, *DPYD**13 and c.2846A>T.[19] In the gene activity score as proposed in this manuscript we adopt these recommendations for *DPYD**2A and *DPYD**13, but deviate in the dose advice for c.2846A>T and include a dose advice for c.1236G>A/HapB3. We have summarized *in vitro, ex vivo* and *in vivo* studies to determine the appropriate dose recommendation for these SNPs. In addition, we have shown our own experimental data. Our data are in agreement with previous data and show a 50% reduced DPD enzyme activity in patients heterozygous for *DPYD**2A and *DPYD**13 and a ~25% decreased activity for heterozygous patients with c.2846A>T. Unfortunately, our data on c.1236G>A do not correspond and additional data containing DPD enzyme activity measurements in patients with c.1236G>A/HapB3 are scarce and not in agreement. Including our study, three out of four studies suggest that c.1236G>A results in an enzyme activity close to normal levels. However, Sistonen *et al.* showed a significant reduction in DHU/U ratio in patients carrying this variant[50] and associations with the development of severe toxic side effects have also been described. The toxicity data point out that a dose reduction for c.1236G>A/HapB3 is required, but a dose reduction of 50% would be too large considering the measured enzyme activities. Therefore a dose reduction to 75% of the normal dose for heterozygous patients seems appropriate in order to prevent toxicity

as well as to prevent underdosing. After the initial dose reduction the patient should be closely monitored and the dose can be adjusted according to occurring toxicity.

Currently only four SNPs in *DPYD* are allocated a gene activity score, since we consider these variants are the most relevant polymorphisms. It has been described before that 13[59] to 19[60] variants are expected to result in DPD deficiency. However, more research is necessary on the effect of these other SNPs on DPD enzyme activity before they can be included in the gene activity score. With the gene activity score approach it is possible to continuously keep adding variant alleles or updating the values of the gene activity score that are assigned to variant alleles. When new information on effects on enzyme activity is published, this can be included, while the currently proposed gene activity score can already be used in clinical practice. In addition, more research is needed with regard to compound heterozygous patients (patients who carry two different SNPs) and homozygous patients. These patients would benefit from an additional phenotyping test to measure the DPD enzyme activity as to determine the optimal dose adjustment or decide to treat with an alternative drug.

Both genotyping and phenotypic biomarkers have been proposed in order to predict and reduce toxicity in patients. However, the gold standard of phenotyping (measuring DPD enzyme activity in PBMCs) is not easy to implement as a routine test and other phenotyping methods, such as uracil test dose, endogenous DHU/U ratio and 2-^{13}C-uracil breath test, have not yet been fully validated or standardized.[61] Compared with phenotyping methods, genotyping methods are faster, easier and less expensive, so it is expected that it will be implemented more often as standard of care for patients undergoing fluoropyrimidine treatment.

The dose recommendations described in this article will be implemented in an upcoming large prospective clinical trial (NCT02324452) in the Netherlands where upfront genotypic assessment of *DPYD* will be performed for around 1250 patients treated with capecitabine or 5-FU. Simultaneously, our work was recently implemented by the Dutch Pharmacogenetics Working Group by using the gene activity score for translating *DPYD* genotype into DPD phenotype.[21]

To conclude, we propose using the gene activity score for the translation of *DPYD* genotype into a numeric value that can be easily used to describe DPD phenotype and to advise an individualized dose adjustment for the use of fluoropyrimidines.

FUTURE PERSPECTIVE

We expect that in the future more knowledge will be gained regarding relevant SNPs in *DPYD* other than the ones described in this article. Currently there are 13–19 SNPs expected to result in DPD deficiency. In addition, SNPs in other genes involved in fluoropyrimidine metabolism or mRNA could influence the DPD enzyme activity and could thus in the future be added to the activity score. The design of the gene activity score makes it possible to add other *DYPD* SNPs while maintaining a uniform method for describing DPD activity using a score table and for deriving individualized dose adjustments.

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2

Clinical Pharmacogenetics Implementation Consortium (CPIC) guideline for dihydropyrimidine dehydrogenase genotype and fluoropyrimidine dosing: 2017 update

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SUMMARY

The purpose of this guideline is to provide information for the interpretation of clinical dihydropyrimidine dehydrogenase (*DPYD*) genotype tests so that the results can be used to guide dosing of fluoropyrimidines (5-fluorouracil and capecitabine). Detailed guidelines for the use of fluoropyrimidines, their clinical pharmacology,[1] as well as analyses of cost-effectiveness are beyond the scope of this document. The Clinical Pharmacogenetics Implementation Consortium (CPIC®) guidelines consider the situation of patients for which genotype data are already available[2] (updates available at https://cpicpgx.org/guidelines/guideline-for-fluoropyrimidines-and-*DPYD*/).

FOCUSED LITERATURE REVIEW

A systematic literature review focused on *DPYD* genotype and 5-fluorouracil, capecitabine, and tegafur was conducted (see Supplement), with reviews used as summaries of earlier literature.

GENE: *DPYD*

Background

DPYD, the gene encoding dihydropyrimidine dehydrogenase (DPD), the rate-limiting enzyme for fluoropyrimidine catabolism, spans 950 kb on chromosome 1p22 with 4,399 nucleotides in 23 coding exons.[3] Numerous genetic variants in *DPYD* are known that alter the protein sequence or mRNA splicing (see *DPYD* Allele Frequency Table available at the Clinical Pharmacogenetics Implementation Consortium (CPIC) website).[4] Some of these variants, based on current knowledge, do not affect DPD activity in a clinically relevant manner (e.g., c.85T>C, *9A, rs1801265, p.C29R; c.1627A>G, *5, rs1801159, p.I543V; c.2194G>A, *6, rs1801160, p.V732I), whereas others result in reduced enzyme function. In the context of 5-fluorouracil, four decreased function *DPYD* variants are of primary relevance due to their population frequency and established impact on enzyme function and toxicity risk: c.1905+1G>A (rs3918290, also known as *DPYD**2A, *DPYD*:IVS14+1G>A), c.1679T>G (rs55886062, *DPYD**13, p.I560S), c.2846A>T (rs67376798, p.D949V), and c.1129–5923C>G (rs75017182, HapB3). Of these variants, c.1905+1G>A and c.1679T>G have the most deleterious impact on DPD activity, whereas c.2846A>T and c.1129–5923C>G result in moderately reduced DPD activity (see further details below in *Linking genetic variability to variability in drug-related phenotypes*). The most well-studied *DPYD* variant, c.1905+1G>A (*2A), is located at the intron boundary of exon 14 and results in skipping of the entire exon and a nonfunctional protein.[5] The variant c.1129–5923C>G, located deep in intron 10, introduces a cryptic splice site and the partial production of a nonfunctional transcript.[6] This single nucleotide polymorphism (SNP) is the likely underlying causal variant of a *DPYD* haplotype (HapB3) spanning intron 5 to exon 11.[7] The synonymous variant c.1236G>A (rs56038477) is in perfect linkage disequilibrium with c.1129–5923C>G (r^2=1.0, D'=1.0 in 1000 Genomes Project), and thus a proxy for this variant in Europeans. The variants c.1679T>G and c.2846A>T are missense mutations that affect protein function.[8]

In Europeans, HapB3 with c.1129–5923C>G is the most common decreased function *DPYD* variant (see *DPYD* Allele Frequency Table available at the CPIC website)[4] with carrier frequencies of 4.7%, followed by c.1905+1G>A (carrier frequency: 1.6%) and c.2846A>T (carrier frequency: 0.7%). Considering all four variants combined, ~7% of Europeans carry at least one decreased function *DPYD* variant. In individuals with African ancestry, the decreased function variant c.557A>G (rs115232898, p.Y186C) is relatively common (3–5% carrier frequency). Most other *DPYD* variants of phenotypic consequence are very rare (summarized in the *DPYD* Allele Frequency Table available at the CPIC website)[4] and were not observed even in large cohort studies.[9–11]

Nomenclature

While some *DPYD* variants have been assigned a star (*) allele, only a minority of known variants has such a designation. Furthermore, the (*) allele nomenclature is used for other drug-metabolizing enzymes to designate haplotypes consisting of more than one variant. Due to the size of *DPYD* and the low frequency of most variants, reliable haplotype inference across the entire gene is not possible. Therefore, the preferred nomenclature for *DPYD* variants is the use of Human Genome Variation Society (HGVS) nomenclature or rsID (see Supplement for further details).

Genetic test interpretation

Evidence supporting DPD function associated with known *DPYD* variants is summarized in the *DPYD* Allele Functionality Table available at the CPIC website.[4] The relationship between *DPYD* genotype and phenotype has only been clearly established for a few variants, whereas the functional impact of many rare variants has been only assessed *in vitro*. Thus, the *DPYD* Allele Functionality Table available at the CPIC website[4] was divided into sections according to the strength of evidence supporting the assigned allele function: Strong evidence supporting function (from both *in vitro* and clinical studies); moderate evidence supporting function (from *in vitro* and clinical/*ex vivo* studies); *in vitro* data only and/or limited clinical/*ex vivo* data supporting function; uncertain function (conflicting or insufficient evidence supporting function, currently not considered actionable). For each variant, an activity score similar to that described in Henricks *et al.*[12] was applied: 1 for normal function, 0.5 for decreased function, and 0 for no function variants (including variants with minimal DPD activity).

Table 1 summarizes the likely DPD phenotype based on genotype. The DPD phenotype is assigned using a gene activity score (*DPYD*-AS), calculated as the sum of the activity scores of the two *DPYD* variants with the lowest variant activity score (based on the *DPYD* Allele Functionality Table available at the CPIC website).[4] Briefly, carriers of two no function variants are classified as *DPYD* poor metabolizers (*DPYD*-AS: 0); carriers of one no function or decreased function variant are considered *DPYD* intermediate metabolizers (*DPYD*-AS: 1 or 1.5), and those with only normal function variants are classified as *DPYD* normal metabolizers (*DPYD*-AS: 2). If two different decreased/no function variants are present, they are presumed to be on different gene copies. Irrespective of the presence of decreased/no function variants, patients may carry multiple normal function variants. Common normal function variants may be located on the same gene copy as other normal function variants or decreased/no function variants (see Supplement for further details). For example genotype to phenotype interpretations see the Genotype-Phenotype Table available at the CPIC website.[4]

To ensure correct test interpretation for the transversion variants c.1129–5923C>G and c.2846A>T, the strand to which alleles are assigned needs to be considered. In this guideline, allele designations are relative to the coding DNA reference sequence (NM_000110.3) and thus the decreased function (i.e., minor) alleles are c.1129–5923G and c.2846T, respectively.

Table 1. Assignment of likely DPD phenotypes based on DPYD genotypes.

Likely phenotype	Activity score[a]	Genotypes[b]	Examples of genotypes[c]
DPYD normal metabolizer	2	An individual carrying two normal function alleles.	c.[=];[=], c.[85T>C];[=], c.[1627A>G];[=]
DPYD intermediate metabolizer	1 or 1.5	An individual carrying one normal function allele plus one no function allele or one decreased function allele, or an individual carrying two decreased function alleles.	c.[1905+1G>A];[=], c.[1679T>G]; [=], c.[2846A>T];[=], c.[1129–5923C>G];[=][d], c.[1129–5923C>G]; [1129–5923C>G][d], c. [2846A>T];[2846A>T]
DPYD poor metabolizer	0 or 0.5	An individual carrying two no function alleles or an individual carrying one no function plus one decreased function allele.	c.[1905+1G>A];[1905+1G>A], c.[1679T>G];[1679T>G], c.[1905+1G>A];[2846A>T], c.[1905+1G>A]; [1129-5923C>G][d]

[a]Calculated as the sum of the two lowest individual variant activity scores. See text for further information.
[b]Allele definitions, assignment of allele function and references can be found on the CPIC website (*DPYD* Allele Functionality Table available at the CPIC website).[4]
[c]HGVS nomenclature using the reference sequence NM_000110.3.
[d]Likely HapB3 causal variant. See *DPYD* Allele Functionality Table available at CPIC website[4] for other HapB3 proxy SNPs.

Available genetic test options

Testing options for *DPYD* genotype range from targeted analysis of selected variants to resequencing of the complete coding regions. In the context of 5-fluorouracil toxicity, at present most tests focus on the four most common and well-established risk variants (c.1905+1G>A, c.1679T>G, c.2846A>T, c.1129–5923C>G) or a subset thereof. Additional information about commercially available genetic testing options can be found at the Genetic Testing Registry website (http://www.ncbi.nlm.nih.gov/gtr/).

Incidental findings

Individuals who harbor one copy of a no function *DPYD* variant can be considered to have carrier status for an inborn error of metabolism and consideration should be given to its potential effects on offspring. Patients homozygous for inactivating variants of *DPYD* have complete dihydropyrimidine dehydrogenase deficiency, a clinically heterogeneous autosomal recessive disorder of pyrimidine metabolism that shows wide variability of clinical presentations, ranging from no symptoms to severe convulsive disorders with motor and mental retardation.[13,14]

Other considerations

Some of the testing options for 5-fluorouracil toxicity also include testing for other gene variants in *TYMS* and *MTHFR*. To date, however, the clinical utility of these genotypes is unclear (see further details in Supplement), and predictive dosing strategies have yet to be successfully applied. For a summary of pharmacogenomics studies of 5-fluorouracil, see the PGx Research tab at http://www.pharmgkb.org/ drug/PA128406956.

There are alternative or complementary tests to *DPYD* genotyping that assess DPD activity directly in peripheral mononuclear cells or indirectly through the endogenous dihydrouracil/uracil ratio (UH_2/U) in plasma, or using a uracil loading test.[15] See Meulendijks *et al*.[16] for a review of these methods. The application of a combined genotype/phenotype approach including selected *DPYD* risk variants has been shown to reduce toxicity in a prospective study.[17] However, such tests are not widely available. Furthermore, the mean and range of the pretherapeutic endogenous UH_2/U ratio varied widely between studies, limiting its practical use, and several studies did not observe a strong correlation between the UH_2/U ratio and 5-fluorouracil plasma concentrations.[18]

DRUGS: FLUOROPYRIMIDINES

Background

The fluoropyrimidines 5-fluorouracil and capecitabine are widely used in the treatment of solid tumors including colorectal and breast cancer, and cancers of the aerodigestive tract. Each year, over 2 million patients are newly diagnosed with tumors that are commonly treated with

fluoropyrimidines, mostly in combination with other antineoplastic drugs.[19] Approximately 10–40% of fluoropyrimidine-treated patients develop severe and sometimes life-threatening toxicity (neutropenia, nausea, vomiting, severe diarrhea, stomatitis, mucositis, hand-foot syndrome).[7,11,20] 5-fluorouracil has a narrow therapeutic window, resulting in a small difference between minimum efficacious and maximum tolerable dose. Only 1–3% of the administered 5-fluorouracil is metabolized to cytotoxic metabolites, with ~80% of the administered dose being degraded and the rest excreted in the urine. DPD is the first and rate-limiting step in the catabolic pathway converting 5-fluorouracil to dihydrofluorouracil (DHFU) (for further details, see the 5-fluorouracil pathway at http://www.pharmgkb.org/pathway/PA150653776). DPD levels show high inter- and intra-individual variation, which influences 5-fluorouracil exposure.[21] Reduced activity of DPD results in reduced clearance and increased half-life of 5-fluorouracil, and can cause profound dose-related toxicities.[22,23] Capecitabine is a prodrug of 5-fluorouracil, being converted to 5-fluorouracil and also metabolized by DPD. Therefore, toxic effects are similar in patients with decreased/no function *DPYD* variants.[9,24]

Linking genetic variability to variability in drug-related phenotypes

There is substantial evidence linking *DPYD* genotype with variability in DPD enzyme activity, 5-fluorouracil clearance, and 5-fluorouracil toxicity (summarized in Supplementary Table 1), which provides the basis for the dosing recommendations (Table 2). In a meta-analysis combining data from eight cohort studies (*N*=7,365 patients), the association of four *DPYD* variants with severe fluoropyrimidine-related toxicity was demonstrated: c.1905+1G>A (*2A), c.2846A>T, c.1679T>G (*13), and c.1129–5923C>G (HapB3) with relative risks for toxicity of 2.9 (95% confidence interval (CI): 1.8–4.6), 3.0 (2.2–4.1), 4.4 (2.1–9.3), and 1.6 (1.3–2.0), respectively.[20] For all of these variants, an impact on DPD activity (assessed in PBMCs or using the UH_2/U ratio) has been shown (Supplementary Table 1).[6] The strongest impact on DPD activity was observed for c.1905+1G>A and c.1679T>G, with a 50% and 68% reduction in heterozygous carriers, respectively.[6] A moderate reduction in DPD activity was observed in heterozygous carriers of c.2846A>T and c.1129–5923C>G (30% and 35% reduced activity, respectively).[6] Two homozygous carriers of c.1129–5923C>G had 41% and 55% DPD activity compared to controls, consistent with a partial DPD deficiency.[25] Homozygous expression *in vitro* resulted in dramatically reduced DPD activity (<25% of wildtype activity) for c.1905+1G>A and c.1679T>G, and in reduced DPD activity (39–59% of wildtype activity) for c.2846A>T.[26,27] In heterozygous carriers of c.1905+1G>A, c.2846A>T, and c.1679T>G, 5-fluorouracil clearance was reduced by 40–80% compared to non-carriers.[23,28] For heterozygous carriers of c.557A>G (p.Y186C), commonly observed in individuals of African ancestry, a 46% reduction in PBMC DPD activity compared to non-carriers was observed.[29]

Prescribing recommendations

Table 2 summarizes the genetics-based dosing recommendations for fluoropyrimidines using the calculated *DPYD* activity score (*DPYD*-AS). The strength of the prescribing recommendations is based on the known impact of some variants (c.19051+G>A, c.1679T>G, c.2846A>T, c.1129–5923C>G) on DPD activity, the demonstrated relationship between DPD activity and 5-fluorouracil clearance, and between 5-fluorouracil exposure and its toxic effects. Patients who are heterozygous for *DPYD* decreased/no function variants demonstrate partial DPD deficiency and should receive reduced starting doses. Prospective genotyping of c.1905+1G>A followed by a 50% dose reduction

Table 2. Recommended dosing of fluoropyrimidines[a] by DPD phenotype.

Phenotype	Implications for phenotypic measures	Dosing recommendations	Classification of recommendations[b]
DPYD normal metabolizer	Normal DPD activity and "normal" risk for fluoropyrimidine toxicity.	Based on genotype, there is no indication to change dose or therapy. Use label-recommended dosage and administration.	Strong
DPYD intermediate metabolizer	Decreased DPD activity (leukocyte DPD activity at 30% to 70% that of the normal population) and increased risk for severe or even fatal drug toxicity when treated with fluoropyrimidine drugs.	Reduce starting dose based on activity score followed by titration of dose based on toxicity[c] or therapeutic drug monitoring (if available). **Activity score 1:** Reduce dose by 50%. **Activity score 1.5:** Reduce dose by 25% to 50%.	**Activity score 1:** Strong **Activity score 1.5:** Moderate
DPYD poor metabolizer	Complete DPD deficiency and increased risk for severe or even fatal drug toxicity when treated with fluoropyrimidine drugs.	**Activity score 0.5:** Avoid use of 5-fluorouracil or 5-fluorouracil prodrug-based regimens. In the event, based on clinical advice, alternative agents are not considered a suitable therapeutic option, 5-fluorouracil should be administered at a strongly reduced dose[d] with early therapeutic drug monitoring.[e] **Activity score 0:** Avoid use of 5-fluorouracil or 5-fluorouracil prodrug-based regimens.	Strong

[a]5-fluorouracil or capecitabine.
[b]Rating scheme described in Supplement.
[c]Increase the dose in patients experiencing no or clinically tolerable toxicity in the first two cycles to maintain efficacy; decrease the dose in patients who do not tolerate the starting dose to minimize toxicities.
[d]If available, a phenotyping test (see main text for further details) should be considered to estimate the starting dose. In the absence of phenotyping data, a dose of <25% of the normal starting dose is estimated assuming additive effects of alleles on 5-FU clearance.
[e]Therapeutic drug monitoring should be done at the earliest time point possible (e.g., minimum time point in steady state) in order to immediately discontinue therapy if the drug level is too high.

in heterozygous carriers resulted in a rate of severe toxicity comparable to non-carriers.[30] This study thus demonstrated that *DPYD* genetic testing can reduce the occurrence of severe fluoropyrimidine-related toxicity, and that a dose reduction of 50% is suitable for heterozygous carriers of no function variants (*DPYD*-AS: 1). For decreased function variants, evidence is limited regarding the optimal degree of dose reduction. For c.2846A>T, a small retrospective study observed that the average capecitabine dose in heterozygous carriers was reduced by 25% compared to non-carriers.[24] In a small prospective study, five patients carrying c.1236G>A (proxy for c.1129–5923C>G) were safely treated with a 25% reduced capecitabine starting dose.[31] This suggests that heterozygous carriers of decreased function variants (*DPYD*-AS: 1.5) may tolerate higher doses compared to carriers of no function variants (*DPYD*-AS: 1). In patients with *DPYD*-AS

of 1.5, the individual circumstances of a given patient should therefore be considered to determine if a more cautious approach (50% starting dose followed by dose titration), or an approach maximizing potential effectiveness with a potentially higher toxicity risk (25% dose reduction) is preferable. Of note, both studies indicating the suitability of a 25% dose reduction in decreased function variant carriers included only patients receiving capecitabine and no data are currently available for infusional 5-fluorouracil.

Given that some patients carrying decreased or no function variants tolerate normal doses of 5-fluorouracil, to maintain effectiveness, doses should be increased in subsequent cycles in patients experiencing no or clinically tolerable toxicity in the first two chemotherapy cycles or with subtherapeutic plasma concentrations. Similarly, doses should be decreased in patients who do not tolerate the starting dose.

In *DPYD* poor metabolizers (*DPYD*-AS: 0.5 or 0), it is strongly recommended to avoid use of 5-fluorouracil-containing regimens. However, if no fluoropyrimidine-free regimens are considered a suitable therapeutic option, 5-fluorouracil administration at a strongly reduced dose combined with early therapeutic drug monitoring may be considered for patients with *DPYD*-AS of 0.5. It should be noted, however, that no reports of the successful administration of low-dose 5-fluorouracil in *DPYD* poor metabolizers are available to date. Assuming additive effects of decreased and no function alleles (*DPYD*-AS: 0.5), it is estimated that a dose reduction of at least 75% would be required (i.e., starting dose <25% of normal dose). Furthermore, in such cases a phenotyping test (see the section *Gene: DPYD: Other Considerations*) is advisable to estimate DPD activity and a starting dose. The US Food and Drug Administration (FDA) and the Health Canada Santé Canada (HCSC) have added statements to the drug labels for 5-fluorouracil and capecitabine that warn against use in patients with DPD deficiency, and prescribing recommendations for 5-fluorouracil, capecitabine, and tegafur are also available from the Dutch Pharmacogenetics Working Group.[32]

Tegafur

Tegafur (not available in the United States), is a prodrug of 5-fluorouracil administered in combination with uracil (UFT) or with gimeracil and oteracil (S-1, Teysuno). For these therapies, evidence regarding the impact of *DPYD* variants on toxicity risk is very limited. Given the inhibition of DPD by the co-administered uracil or gimeracil, dose requirements of patients carrying decreased/no function *DPYD* variants are currently unknown. The dosing recommendations provided here currently apply only to 5-fluorouracil and capecitabine. As such, tegafur is rated as a CPIC "no recommendation" (see Supplement for definition).

Pediatrics

At the time of this writing, data on the possible role of *DPYD* genetic variation in 5-fluorouracil toxicity in pediatric patient populations are extremely scarce; however, there is no evidence to suggest that 5-fluorouracil pharmacokinetics differ from adult patients,[33] and thus no evidence that *DPYD* variants would affect 5-fluorouracil metabolism differently in children.

Recommendations for incidental findings

Symptoms of DPD deficiency generally present in childhood and, in the majority of patients, within the first year of life. Currently, a correlation between symptom severity and DPD function and/or genetics has not been established. However, early phenotypic (e.g., urine screening of uracil

and its degradation products) and/or genetic testing (pre- or postnatal) of offspring of *DPYD* no function variant carriers could aid in early diagnosis[14] to avoid a lengthy diagnostic odyssey.

Other considerations

Recently, a common polymorphism (rs895819A>G) in the *DPYD*-regulatory microRNA miR-27a was associated with lower DPD activity[34] and with fluoropyrimidine-related toxicity in patients carrying decreased function *DPYD* variants.[35,36] This suggests that this *MIR27A* variant may allow further stratification of *DPYD* risk variant carriers. However, pharmacokinetic studies combining *DPYD* and *MIR27A* genotype are needed before dosing recommendations that incorporate *MIR27A* genotype can be made.

Other genetic variation and patient characteristics such as sex and age have also been associated with 5-fluorouracil toxicity; however, the clinical utility of these associations are not fully understood (see Supplement for more information). Disease and treatment regimens may influence the overall risk of toxicity, and thus also the absolute risk of toxicity in carriers of *DPYD* decreased/no function variants. However, the association of *DPYD* variants with 5-fluorouracil-related toxicity has been found to be fairly consistent across treatment regimens.[9,20]

Pharmacokinetically guided dosing of 5-fluorouracil has been shown to result in an increase in the proportion of patients with 5-fluorouracil exposure (AUC) within the targeted therapeutic range and a reduced number of 5-fluorouracil-related adverse effect.[37–39] In particular, to avoid underdosing of patients with genotype-based dose reductions who tolerate higher 5-fluorouracil doses, follow-up therapeutic drug monitoring is recommended.

Implementation of this guideline

The guideline supplement contains resources that can be used within electronic health records (EHRs) to assist clinicians in applying genetic information to patient care for the purpose of drug therapy optimization (see *Resources to incorporate pharmacogenetics into an electronic health record with clinical decision support* sections of the Supplement).

POTENTIAL BENEFITS AND RISKS FOR THE PATIENT

The benefit of *DPYD* genotyping has been demonstrated in a prospective study, which showed a reduced occurrence of severe 5-fluorouracil-related toxicity and no toxicity-related deaths in carriers of c.1905+1G>A after genotype-guided dose reduction.[30] Conversely, not all carriers of *DPYD* decreased/no function variants develop severe toxicity at standard doses.[20,28] As a consequence, some carriers of such variants may not receive the full benefit of fluoropyrimidine therapy with the recommended dose reductions. To maintain efficacy, it is important to increase the dose in patients experiencing no or clinically tolerable toxicity or with subtherapeutic 5-fluorouracil plasma concentrations. Patients who proceed with 5-fluorouracil therapy may still experience acceptable lower-grade toxicity that may even be necessary in order to achieve efficacy. A possible risk is the misreporting or misinterpretation of genetic test results.

CAVEATS: APPROPRIATE USE AND/OR POTENTIAL MISUSE OF GENETIC TESTS

The presence of decreased or no function variants does not always result in toxicity. Overall, ~50% of decreased function *DPYD* variant carriers develop severe 5-fluorouracil-related toxicity with standard doses,[20,28,40] with estimates varying depending on the overall frequency of toxicity for a given treatment regimen and the number of treatment cycles evaluated.[7,11,28,40,41] At the same

time, patients without a *DPYD* decreased/no function variant may still experience severe toxicity due to other genetic, environmental, or other factors.

The sensitivity of *DPYD* genetic testing depends on the number of variants investigated. By combining the *DPYD* variants c.1905+1G>A, c.2846A>T, c.1679T>G, c.1129–5923C>G, 20–30% of early-onset 5-fluorouracil toxicities can be explained.[7] However, a test that includes only a subset of those *DPYD* variants (e.g., only c.1905+1G>A) has a reduced sensitivity. Finally, given the existence of many additional rare deleterious *DPYD* variants, a genetic test investigating only selected decreased/no function variants does not fully rule out DPD defects.

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SUPPLEMENT

Guideline updates

The Clinical Pharmacogenetics Implementation Consortium (CPIC) guideline for *DPYD* genotypes and the dosing of fluoropyrimidines is published in full on https://cpicpgx.org/guidelines/guideline-for-fluoropyrimidines-and-dpyd/[1] and at pharmgkb.org. Relevant information will be reviewed periodically and updated guidelines published online.

Literature review

2013 guideline

A literature search of the PubMed® database (1966 to March 2013) using the keywords ((DPD OR DPYD OR Dihydropyrimidine Dehydrogenase) AND (fluorouracil OR 5-FU OR fluoropyrimidines OR capecitabine OR tegafur) AND genotype) was performed and results were limited to those available in English. Further articles were found via the reference sections of reviews. Using these search terms, 104 publications were identified. Study inclusion criteria included publications that included analyses for the association between *DPYD* genotypes (c.1905+1G>A , c.1679T>G, and c.2846A>T) and metabolism of dihydropyrimidines and adverse drug events or clinical outcomes. Non-English manuscripts were excluded. Following application of these inclusion criteria, 30 publications were reviewed and included in the evidence tables.

2017 guideline

We searched PubMed® database as described above between 1966 and March 2017. The 2013 literature review was repeated to include all known *DPYD* genotypes. Using these search terms, 150 publications were identified. Following application of the inclusion criteria, 49 publications were reviewed and included in the evidence tables. An additional 42 studies were identified from the reference sections of reviews and other published paper, and included in the evidence tables, bringing the total included studies to 91 (Supplementary Table 1).

Genetic test interpretation

While some *DPYD* variants have been assigned a (*) allele, this nomenclature has not been updated to include more recently identified decreased function variants. As a consequence, only a minority of *DPYD* variants has a (*) allele designation. Furthermore, the (*) allele nomenclature is used for other drug metabolizing enzymes to designate haplotypes. Due to the size of *DPYD*, the gene encompasses several haplotype blocks[2,3] with low linkage disequilibrium between variants located in different haplotype blocks. As a consequence, it is not possible to reliably determine full haplotypes that incorporate genotypes for common polymorphisms (e.g. c.85T>C, c.2194G>A) across the entire gene. Therefore, any (*) alleles used for *DPYD* generally do not refer to haplotypes but only to a genotype at one specific SNP locus. To avoid confusion with (*) allele nomenclature used for haplotypes of other drug metabolizing enzymes, the preferred nomenclature for *DPYD* variants is therefore the use of rs# or HGVS nomenclature.

Test results for *DPYD* do not report a diplotype for the entire gene, but genotypes for individual SNP loci. Importantly, however, all currently established toxicity-associated decreased/no function *DPYD* variants have a low population frequency (<5%) and are observed most frequently in individuals without a second decreased/no function variant. Therefore, in patients who carry two

different decreased/no function variants, for the test interpretation, it is assumed that the two variants with an impact on DPD activity are located on different gene copies. For patients, in whom novel *DPYD* variants with suspected deleterious impact are detected in combination with known decreased/no function variants, this assumption may not be correct. In such a case, a phenotyping test may be helpful to determine enzyme activity, or genotyping of relatives (parents, siblings, and offspring) to determine segregation patterns. In addition, a genetic test may also include genotyping of other, common *DPYD* variants (e.g. c.85T>C, c.1627A>G, c.2194G>A). If this is case, a patient may be heterozygous for multiple of these variants and it cannot be determined which alleles are located on the same gene copy. However, based on current data, none of these common variants have a clinically relevant impact in the context of 5-fluorouracil related toxicity. The exact haplotype configuration of these normal function variants is thus not required for the test interpretation. Therefore, to calculate the *DPYD* gene activity score, only the variant activity scores for the two variants with the lowest activity score is considered. For example, if a patient is a heterozygous carrier of a decreased function variant (e.g. c.1129–5923C>G) and two normal function variants (e.g. c.85T>C and c.1627A>G), the variant activity score of 0.5 for c.1129–5923C>G would be considered for one gene copy, and an activity score of 1 for the second gene copy, resulting in a total gene activity score of 1.5.

The dosing recommendations in this guideline are specific for variant alleles in which there are clear data linking the *DPYD* genotype to fluoropyrimidine toxicity (c.1905+1G>A, c.1679T>G, c.2846A>T, c.1129–5923C>G) (Supplementary Table 1). Several other variants have been reported to be associated with reduced enzyme activity and/or linked to fluoropyrimidine toxicity, albeit with somewhat weaker evidence (see *DPYD* Allele Functionality Table[4], "moderate evidence supporting function"). While most of these variants are rare (see *DPYD* Allele Frequency Table[4]), the decreased function variant rs115232898 (c.557T>C, p.Y186C) is relatively common in individuals of African ancestry and has been observed in case reports of patients with severe 5-fluorouracil related toxicity.[5,6]

On the other hand, several *DPYD* variants that are relatively common in the population have strong or moderate evidence that they do not impact DPD function in a clinically relevant manner in the context of 5-fluorouracil related toxicity. For rs1801159 (*5, c.1627A>G, p.I543V) and rs1801265 (*9A, c.85T>C, p.C29R) none of the large cohort and case-control studies observed a significant association with toxicity or reduced DPD activity (see Supplementary Table 1). For other variants, associations with toxicity have been observed in single studies, but could not be reproduced in a majority of studies (rs1801160, *6, c.2194G>A, p.V732I; rs2297595, c.496T>C, p.M166V) or by meta-analysis (rs1801158, *4, c.1601G>A, p.S534N) (see Supplementary Table 1). Based on current knowledge, a fluorouracil dose adaptation in carriers of these variants is thus not warranted.

Many of the variants listed in the "*in vitro* data only and/or limited clinical/*ex vivo* data" category (see *DPYD* Allele Functionality Table[4]) as decreased or no function variants have a very low (<0.5%) allele frequency in the populations studied (see *DPYD* Allele Frequency Table[4]) and to date, there are no studies linking these variant alleles directly to toxicity related to fluoropyrimidines. Their functional effect was evaluated by comparison of their in vitro activity to the *in vitro* activity of known toxicity-associated *DPYD* variants: All variants with *in vitro* activity similar to c.1905+1G>A and c.1679T>G were categorized as "no function" variants; variants with *in vitro* activity greater than that of known "no function" variants but equal to or lower than the *in vitro* activity of c.2846A>T were classified as "decreased function" variants.

Several variants listed in the "unclear or conflicting data supporting function" category had *in vitro* DPD activity (i.e. homozygous expression of the variant) that was significantly lower than wildtype activity, but the magnitude of the decrease was smaller than for any established toxicity-associated variant. For these variants, it is currently not known if the decrease in DPD activity observed *in vitro* has a clinically relevant impact on 5-fluorouracil toxicity. At the time of writing, these variants would thus not be actionable for a reduction of the starting dose in fluoropyrimidine-based therapies.

Other considerations

Several other genes may influence responses to 5-fluorouracil,[7,8] in particular genes of the folate pathway. The most well-studied of these are *MTHFR* and *TYMS*, although to date for *TYMS* the underlying causal variants of associations[9] and their clinical utility[10] are unclear, and associations have been inconsistent for *MTHFR*.[10] Therefore, predictive dosing strategies for these genes have yet to be successfully applied. Similarly, a recently identified association of a variant (rs17822471) in *ABCC11*, a transporter of 5-fluorouracil metabolites, with fluoropyrimidine-related leukopenia requires further investigation.[11,12] In the context of capecitabine-based therapies, genes in the capecitabine activation pathway have also been studied, most notably *CDA*, *CES1* and *CES2*.[13,14]

While some associations have been reported, these results have not been sufficiently replicated to determine potential genotype-based therapeutic strategies. Furthermore, Fernandez-Rozadilla *et al.* performed a genome-wide association study on 221 colorectal cancer patients (including a validation set of 791 patients) that had been treated with a 5-fluorouracil-based regimen.[15] Seven SNPs (rs16857540 (*NLGN1*), rs2465403 (*COLEC10*), rs10876844 (*OR10AE3P*, *PSMB2*P), rs10784749, rs17626122 (*PARD3B*), rs7325568 and rs4243761) showed evidence of association with adverse drug reactions. They also evaluated the association signals for seven SNPs that had been linked to 5-fluorouracil-related toxicity in the literature (rs1801159 and rs1801265 (*DPYD)*, rs18010919 (*UMPS*), rs1801133 (*MTHFR*), rs34743033, rs34489327 (*TYMS*), rs1695 (*GSTP1*)). Four of these variants had good proxy SNPs in the study, but none of them showed a statistically significant association. Some of the identified associations underscore the potential importance of other genes that may contribute increased risk of toxicity of 5-fluorouracil, although further studies are needed to determine their clinical utility.

Level of evidence

The evidence summarized in Supplementary Table 1 is graded using a scaled modified slightly from Valdes *et al.*[16]

High: Evidence includes consistent results from well-designed, well-conducted studies.

Moderate: Evidence is sufficient to determine effects, but the strength of the evidence is limited by the number, quality, or consistency of the individual studies; generalizability to routine practice; or indirect nature of the evidence.

Weak: Evidence is insufficient to assess the effects on health outcomes because of limited number or power of studies, important flaws in their design or conduct, gaps in the chain of evidence, or lack of information

Strength of recommendation

CPIC's dosing recommendations (Table 2, main manuscript) are based on weighting the evidence from a combination of preclinical functional and clinical data, as well as on some existing disease-

specific consensus guidelines. Some of the factors that are taken into account include *in vivo* clinical outcome for reference drug, *in vivo* PK/PD for reference drug, and *in vitro* enzyme activity with probe substrate only.

Overall, the dosing recommendations are simplified to allow rapid interpretation by clinicians. We chose to use a slight modification of a transparent and simple system for just three categories for recommendations adopted from the rating scale for evidence-based recommendations on the use of retroviral agents (http://aidsinfo.nih.gov/contentfiles/AdultandAdolescentGL.pdf):

Strong recommendation for the statement: "The evidence is high quality and the desirable effects clearly outweigh the undesirable effects."

Moderate recommendation for the statement: "There is a close or uncertain balance" as to whether the evidence is high quality and the desirable clearly outweigh the undesirable effects.

Optional recommendation for the statement: The desirable effects are closely balanced with undesirable effects, or the evidence is weak or based on extrapolations. There is room for differences in opinion as to the need for the recommended course of action.

No recommendation: There is insufficient evidence, confidence, or agreement to provide a recommendation to guide clinical practice at this time.

The strength of the 5-fluorouracil dosing recommendations (Table 2, main manuscript) is based on the fact that some variants (c.1905+1G>A, c.1679T>G, c.2846A>T, c.1129–5923C>G) clearly affect DPD activity, and DPD activity is clearly related to 5-fluorouracil clearance, and 5-fluorouracil exposure is associated with its toxic effects. Therefore, reduction of 5-fluorouracil dosage in patients with these variants can prevent severe and possibly life-threatening toxicities, as has been demonstrated for c.1905+1G>A.[17] The strength of the capecitabine dosing recommendations is based on the fact that this prodrug of 5- fluorouracil is metabolized by DPD in the same manner.

Resources to incorporate pharmacogenetics into an electronic health record with clinical decision support

Clinical decision support (CDS) tools integrated within electronic health records (EHRs) can help guide clinical pharmacogenetics at the point of care.[18-22] See https://cpicpgx.org/guidelines/guideline-for-fluoropyrimidines-and-dpyd/ for resources to support the adoption of CPIC guidelines within an EHR. Based on the capabilities of various EHRs and local preferences, we recognize that approaches may vary across organizations. Our intent is to synthesize foundational knowledge that provides a common starting point for incorporating the use of *DPYD* genotype results to guide fluoropyrimidine dosing in an EHR.

Effectively incorporating pharmacogenetic information into an EHR to optimize drug therapy should have some key attributes. Pharmacogenetic results, an interpreted phenotype, and a concise interpretation or summary of the result must be documented in the HER.[23,24] To incorporate a phenotype in the EHR in a standardized manner, genotype test results provided by the laboratory must be consistently translated into an interpreted phenotype (Table 1, main manuscript). Because clinicians must be able to easily find the information, the interpreted phenotype may be documented as a problem list entry or in a patient summary section; these phenotypes are best stored in the EHR at the "person level" rather than at the date-centric "encounter level". Additionally, results should be entered as standardized and discrete terms to facilitate using them to provide point-of-care CDS.[18,25]

Because pharmacogenetic results have lifetime implications and clinical significance, results should be placed into a section of the EHR that is accessible independent of the test result date to allow clinicians to quickly find the result at any time after it is initially placed in the EHR. To facilitate this process, CPIC is providing gene-specific information figures and tables that include full diplotype to phenotype tables, diagram(s) that illustrate how *DPYD* pharmacogenetic test results could be entered into an EHR, example EHR consultation/genetic test interpretation language and widely used nomenclature systems for genes relevant to the CPIC guideline (see https://cpicpgx.org/guidelines/guideline-for-fluoropyrimidines-and-dpyd/).[26]

Point-of-care CDS should be designed to effectively notify clinicians of prescribing implications at any time after the test result is entered into the EHR. CPIC is also providing gene-drug specific tables that provide guidance to achieve these objectives with diagrams that illustrate how point-of-care CDS should be entered into the EHR, example pre- and post-test alert language, and widely used nomenclature systems for drugs relevant to the CPIC guideline (see https://cpicpgx.org/guidelines/guideline-for-fluoropyrimidines-and-dpyd/).

Supplementary Table 1. Evidence linking *DPYD* genotype with DPD phenotype and dihydropyrimidine toxicity.

***DPYD* *allele rsID nucleotide change[a] protein change[b]**	**Parameter**	**Major findings**	**References**	**Type of experimental model**	**Level of Evidence[c,d]**
*2A rs3918290 c.1905+1G>A	Activity	AG is associated with decreased DPD activity as compared to GG	**Supports Statement: *Statistically Significant:*** Wei, *et al.* (1996)[27], Kuilenburg, *et al.* (2016)[28], Nie, *et al.* (2017)[29] ***Same Direction of Association:*** Sistonen, *et al.* (2014)[30]	Clinical, ex vivo	Moderate
		Within cell lines, AA is associated with decreased DPD activity as compared to GG	**Supports Statement: *Statistically Significant:*** Offer, *et al.* (2013)[31]	In vitro	High
		AA + AG were observed in individuals with decreased DPD activity	**Supports Statement:** Holopainen, *et al.* (1997)[32], Vreken, *et al.* (1997)[33], Van Kuilenburg, *et al.* (1997)[34], Ridge, *et al.* (1998)[35], Van Kuilenburg, *et al.* (1999)[36], van Kuilenburg, *et al.* (2001)[37], Johnson, *et al.* (2002)[38], Maring, *et al.* (2002)[39], Van Kuilenburg, *et al.* (2002)[40], Van Kuilenburg, *et al.* (2002)[41], Al-Sanna'a, *et al.* (2005)[42], Ezzeldin, *et al.* (2005)[43], Largillier, *et al.* (2006)[44], Magne, *et al.* (2007)[45], Loganayagam, *et al.* (2010)[46], Van Kuilenburg, *et al.* (2010)[47], Thomas, *et al.* (2016)[48] **Does Not Support Statement:** Collie-Duguid, *et al.* (2000)[49], Loganayagam, *et al.* (2010)[6]	Clinical, ex vivo	High
	Dose	AG is associated with decreased capecitabine dose as compared to GG	**Supports Statement: *Statistically Significant:*** Deenen, *et al.* (2011)[50]	Clinical	Moderate
		Individuals with AG received a decreased dose of capecitabine	**Supports Statement:** Joerger, *et al.* (2015)[51]	Clinical	Weak
	Efficacy	AG is associated with a decreased acute lymphoblastic leukemia complete remission rate as compared to GG	**Supports Statement: *Statistically Significant:*** Zhao, *et al.* (2016)[52]	Clinical	Weak
		AA + AG are not associated with progression-free, disease-free, event-free or overall survival times or confirmed response rate as compared to GG	McLeod, *et al.* (2010)[53], Deenen, *et al.* (2011)[50], Cai, *et al.* (2014)[54], Zhao, *et al.* (2016)[52]	Clinical	Moderate
	Metabolism	AG is associated with decreased metabolism of fluorouracil as compared to GG	**Supports Statement: *Statistically Significant:*** Boisdron-Celle, *et al.* (2007)[55], Van Kuilenburg, *et al.* (2008)[56], Gentile, *et al.* (2016)[57]	Clinical, ex vivo	High
		Individuals with AG were observed to have decreased metabolism of fluorouracil	**Supports Statement:** Maring, *et al.* (2002)[58], Joerger, *et al.* (2015)[51]	Clinical	Moderate

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
	Toxicity	AA + AG are associated with increased risk or severity of fluoropyrimidine toxicity as compared to GG	**Supports Statment:** ***Statistically Significant (overall toxicity):*** Van Kuilenburg, *et al.* (2002)[59], Salgueiro, *et al.* (2004)[60], Boisdron-Celle, *et al.* (2007)[55], Schwab, *et al.* (2008)[61], Deenen, *et al.* (2011)[50], Lee, *et al.* (2014)[62], Toffoli, *et al.* (2015)[63] ***Statistically Significant (myelosuppression):*** Schwab, *et al.* (2008)[61], Kleibl, *et al.* (2009)[64], Kristensen, *et al.* (2010)[65], Rosmarin, *et al.* (2014)[66], Cai, *et al.* (2014)[54] ***Statistically Significant (hand-foot syndrome):*** Cai, *et al.* (2014)[54] ***Statistically Significant (diarrhea):*** Deenen, *et al.* (2011)[50], Cai, *et al.* (2014)[54] ***Statistically Significant (mucositis):*** Schwab, *et al.* (2008)[61], Kleibl, *et al.* (2009)[64] ***Statistically Significant (hepatotoxicity):*** Zhao, *et al.* (2016)[52] ***Statistically Significant (infection):*** Zhao, *et al.* (2016)[52] ***Same Direction of Association (overall toxicity)*:** Braun, *et al.* (2009)[67], Dhawan, *et al.* (2013)[68], Rosmarin, *et al.* (2014)[66], Rosmarin, *et al.* (2015)[9], Froehlich, *et al.* (2015)[3], Boige, *et al.* (2016)[69] ***Same Direction of Association (neutropenia):*** McLeod, *et al.* (2010)[53] **Does Not Support Statement:** Amstutz, *et al.* (2009)[2], McLeod, *et al.* (2010)[53]	Clinical	High
	Toxicity	AA + AG were observed in individuals with fluoropyrimidine toxicity	**Supports Statement:** Wei, *et al.* (1996)[27], Van Kuilenburg, *et al.*(1997)[34], Van Kuilenburg, *et al.* (2000)[70], Van Kuilenburg, *et al.* (2001)[37], Raida, *et al.* (2001)[71], Johnson, *et al.* (2002)[38], Maring, *et al.* (2002)[58], Van Kuilenburg, *et al.* (2002)[41], Steiner, *et al.* (2005)[72], Ezzeldin, *et al.* (2005)[43], Largillier, *et al.* (2006)[44], Morel, *et al.* (2006)[73], Saif, *et al.* (2007)[74], Salgado, *et al.* (2007)[75], Magne, *et al.* (2007)[45], Sulzyc-Bielicka, *et al.*(2008)[76], Gross, *et al.* (2008)[77], Loganayagam, *et al.* (2010)[46], Boige, *et al.* (2010)[78], Ceric, *et al.* (2010)[79], Van Kuilenburg, *et al.* (2010)[47], Savva-Bordalo, *et al.* (2010)[80], Cellier, *et al.* (2011)[147], Loganayagam, *et al.* (2013)[13], Suarez Martinez-Falero, *et al.* (2014)[81], Joerger, *et al.* (2015)[51], Thomas, *et al.* (2016)[48], Roberto, *et al.* (2017)[82]	Clinical	High
*13 rs55886062 c.1679T>G p.I560S	Activity	GT is associated with decreased DPD activity as compared to TT	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.*(2013)[5], Nie, *et al.* (2017)[29] ***Same Direction of Association:*** Sistonen, *et al.* (2014)[30]	Clinical, ex vivo	Moderate
		Within cell lines, GG is associated with decreased DPD activity was compared to TT	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2013)[31]	In vitro	High
		GT was observed in individuals with decreased DPD activity	**Supports Statement:** Collie-Duguid, *et al.* (2000)[49], Johnson, *et al.* (2002)[38], Van Kuilenburg, *et al.* (2002)[40], Ezzeldin, *et al.* (2005)[43], Thomas, *et al.* (2016)[48]	Clinical, ex vivo	High
	Toxicity	GT is associated with increased risk or severity of fluoropyrimidine toxicity as compared to TT	**Supports Statement:** ***Same Direction of Association (overall toxicity):*** Rosmarin, *et al.* (2015)[9], Froehlich, *et al.* (2015)[3], Lee, *et al.* (2014)[62], Toffoli, *et al.* (2015)[63] **Does Not Support Statement:** Amstutz, *et al.* (2009)[2], Deenen, *et al.* (2011)[50], Boige, *et al.* (2016)[69]	Clinical	High
		GT was observed in individuals with fluoropyrimidine toxicity	**Supports Statement:** Collie-Duguid, *et al.* (2000)[49], Johnson, *et al.* (2002)[38], Ezzeldin, *et al.* (2005)[43], Morel, *et al.* (2006)[73], Loganayagam, *et al.* (2010)[46], Cellier, *et al.* (2011)[83], Loganayagam, *et al.* (2013)[13], Dhelens, *et al.* (2016)[84], Thomas, *et al.* (2016)[48]	Clinical	High

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
rs67376798 c.2846A>T p.D949V	Activity	The AT genotype/the T allele is associated with decreased DPD activity as compared to the AA genotype	**Supports Statement:** ***Statistically Significant:*** Seck, *et al.* (2005)[85] ***Same Direction of Association:*** Sistonen, *et al.* (2014)[30], Van Kuilenburg, *et al.* (2016)[28], Nie, *et al.* (2017)[29] **Does Not Support Statement:** Offer, *et al.* (2013)[5]	Clinical, ex vivo	Moderate
		Within cell lines, the T allele is associated with decreased DPD activity as compared to AA	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86], Van Kuilenburg, *et al.* (2016)[28]	In vitro	High
		The AT genotype/the T allele was observed in individuals with decreased DPD activity	**Supports Statement:** Van Kuilenburg, *et al.* (2002)[40], Loganayagam, *et al.* (2010)[46], Thomas, *et al.* (2016)[48] **Does Not Support Statement:** Loganayagam, *et al.* (2010)[46]	Clinical, ex vivo	Moderate
	Dose	The AT genotype is associated with decreased capecitabine dose as compared to the AA genotype	**Supports Statement:** ***Statistically Significant:*** Deenen, *et al.* (2011)[50]	Clinical	Moderate
	Efficacy	The AT genotype is not associated with disease-free survival as compared to the AA genotype	Lee, *et al.* (2014)[62]	Clinical	Moderate
	Metabolism	The AT genotype is associated with decreased metabolism of fluorouracil as compared to the AA genotype	**Supports Statement:** ***Statistically Significant:*** Boisdron-Celle, *et al.* (2007)[55] ***Same Direction of Association:*** Gentile, *et al.* (2016)[57]	Clinical, ex vivo	Moderate
	Toxicity	The AT genotype/the T allele is associated with increased risk or severity of fluoropyrimidine toxicity as compared to the AA genotype	**Supports Statement:** ***Statistically Significant (overall toxicity):*** Boisdron-Celle, *et al.* (2007)[55], Schwab, *et al.* (2008)[61], Deenen, *et al.* (2011)[50], Rosmarin, *et al.* (2014)[66], Lee, *et al.* (2014)[62], Toffoli, *et al.* (2015)[63], Boige, *et al.* (2016)[69] ***Statistically Significant (diarrhea):*** Deenen, *et al.* (2011)[50], Joerger, *et al.* (2015)[51] ***Same Direction of Association (overall toxicity)*:** Rosmarin, *et al.* (2015)[9], Froehlich, *et al.* (2015)[3]	Clinical	High
		The AT genotype/the T allele was observed in individuals with fluoropyrimidine toxicity	**Supports Statement:** Van Kuilenburg, *et al.* (2000)[70], Morel, *et al.* (2006)[73], Gross, *et al.* (2008)[77], Loganayagam, *et al.* (2010)[46], Obi, *et al.* (2011)[87], Cellier, *et al.* (2011)[83], Loganayagam, *et al.* (2013)[13], Thomas, *et al.* (2016)[48], Van Kuilenburg, *et al.* (2016)[28] **Does Not Support Statement:** Kristensen, *et al.* (2010)[65]	Clinical	High
HapB3 rs75017182 + rs56038477 + rs56276561 c.1129-5923C>G + c.1236G>A (p.E412E) + c.483+18G>A	Activity	HapB3 is associated with decreased DPD activity	**Supports Statement:** ***Statistically Significant:*** Sistonen, *et al.* (2014)[30], Nie, *et al.* (2017)[29] ***Same Direction of Association:*** Offer, *et al.* (2013)[5]	Clinical, ex vivo	Moderate
		HapB3 was observed in individuals with decreased DPD activity	**Supports Statement:** Van Kuilenburg, *et al.* (2010)[47], Meulendijks, *et al.* (2016)[88] **Does Not Support Statement:** Seck, *et al.* (2005)[85]	Ex vivo	Moderate
	Dose	HapB3 was observed in individuals who required a fluoropyrimidine dose reduction	**Supports Statement:** Meulendijks, *et al.* (2016)[88] **Does Not Support Statement:** Meulendijks, *et al.* (2016)[88]	Clinical	Weak

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
	Toxicity	HapB3 is associated with increased risk or severity of fluoropyrimidine toxicity	**Supports Statement:** ***Statistically Significant (overall toxicity):*** Amstutz, *et al.* (2009)[2], Van Kuilenburg, *et al.* (2010)[47], Froehlich, *et al.* (2015)[3] ***Statistically Significant (diarrhea):*** Deenen, *et al.* (2011)[50] ***Statistically Significant (neutropenia):*** Lee, *et al.* (2016)[89] ***Same Direction of Association (overall toxicity):*** Schwab, *et al.* (2008)[61], Deenen, *et al.* (2011)[50], Rosmarin, *et al.* (2014)[66], Rosmarin, *et al.* (2015)[9], Lee, *et al.* (2016)[89], Boige, *et al.* (2016)[69] **Does Not Support Statement:** Kleibl, *et al.* (2009)[64], Loganayagam, *et al.* (2013)[13], Falvella, *et al.* (2015)[90]	Clinical	High
		HapB3 was observed in individuals with fluoropyrimidine toxicity	**Supports Statement:** Van Kuilenburg, *et al.* (2010)[47], Meulendijks, *et al.* (2016)[88] **Does Not Support Statement:** Meulendijks, *et al.* (2016)[88]	Clinical	Weak
*2A (rs3918290) + rs67376798 (c.2846A>T) + *13 (rs55886062)	Toxicity	When the 1905+1 A allele and 2846 T allele are assessed together, with or without the 1679 G allele, they are associated with increased risk or severity of fluoropyrimidine toxicity	**Supports Statement:** ***Statistically Significant (overall toxicity):*** Morel, *et al.* (2006)[73], Saif, *et al.* (2013)[91] ***Statistically Significant (gastrointestinal events):*** Capitain, *et al.* (2008)[92]	Clinical	High
*2A (rs3918290) + rs67376798 (c.2846A>T) + *13 (rs55886062) + HapB3	Toxicity	When the 1905+1 A allele, the rs67376798 T allele and the HapB3 haplotype are assessed together, with or without the 1679 G allele, they are associated with increased risk or severity of fluoropyrimidine toxicity	**Supports Statement:** ***Statistically Significant (overall toxicity):*** Jennings, *et al.* (2013)[93] Froehlich, *et al.* (2015)[3]	Clinical	High
*2A (rs3918290) + rs67376798 (c.2846A>T) + *13 (rs55886062) + *4 (rs1801158)	Toxicity	When the 1905+1 A allele, 2846 T allele, 1679 G allele and 1601 A allele are assessed together they are associated with increased risk or severity of fluoropyrimidine toxicity	**Supports Statement:** ***Statistically Significant (overall toxicity):*** Loganayagam, *et al.* (2013)[13]	Clinical	Moderate
*2A (rs3918290) + rs67376798 (c.2846A>T) + HapB3	Activity	When the 1905+1 A allele, 2846 T allele and the HapB3 haplotype are assessed together they are associated with decreased DPD activity	**Supports Statement:** ***Statistically Significant:*** Sistonen, *et al.* (2014)[30], Kuilenburg, *et al.* (2016)[28]	Clinical, ex vivo	Moderate
*4 rs1801158 c.1601G>A p.S534N	Activity	AG/the A allele is associated with decreased DPD activity as compared to GG	**Supports Statement:** ***Statistically Significant:*** Seck, *et al.* (2005)[85] ***Same Direction of Association:*** Sistonen, *et al.* (2014)[30], Van Kuilenburg, *et al.* (2016)[28] **Does Not Support Statement:** Offer, *et al.* (2013)[5]	Clinical, ex vivo	Weak
		AG was observed in individuals with decreased DPD activity	**Supports Statement:** Collie-Duguid, *et al.* (2000)[49], Gross, *et al.* (2003)[94], Thomas, *et al.* (2016)[48] **Does Not Support Statement:** Ridge, *et al.* (1998)[35]	Clinical, ex vivo	Weak
		Within cell lines, the A allele is associated with decreased DPD activity as compared to GG	**Supports Statement:** ***Statistically Significant:*** Van Kuilenburg, *et al.* (2016)[28]	In vitro	Weak
		Within cell lines AA is associated with increased DPD activity as compared to the G allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2013)[31]	In vitro	Weak
	Metabolism	AG is not associated with altered fluorouracil metabolism as compared to GG	Gentile, *et al.* (2016)[57]	Ex vivo	Weak
	Toxicity	The A allele is associated with increased risk or severity of fluoropyrimidine toxicity as compared to GG	**Supports Statement**: ***Same Direction of Association:*** Froehlich, *et al.* (2015)[3], Loganayagam, *et al.* (2013)[13], Rosmarin, *et al.* (2014)[66], Rosmarin, *et al.* (2015)[9] **Does Not Support Statement:** Schwab, *et al.* (2008)[61], Kleibl, *et al.* (2009)[64], Amstutz, *et al.* (2009)[2], Deenen, *et al.* (2011)[50], Froehlich, *et al.* (2015)[3], Toffoli, *et al.* (2015)[63], Boige, *et al.* (2016)[69]	Clinical	Weak
		AG was observed in individuals with fluoropyrimidine toxicity	**Supports Statement:** Collie-Duguid, *et al.* (2000)[49], Gross, *et al.* (2003)[94], Lazar, *et al.* (2004)[9], Van Kuilenburg, *et al.* (2010)[47]	Clinical	Weak
*5 rs1801159 c.1627A>G p.I543V	Activity	AG + GG are not associated with altered DPD activity as compared to AA	He, *et al.* (2008)[96], Offer, *et. al.* (2013)5, Sistonen, *et al.* (2014)[30], Van Kuilenburg, *et al.* (2016)[28]	Clinical, ex vivo	High
		Within cell lines, the GG or the G allele are not associated with altered DPD activity as compared to AA	Offer, *et al.* (2013)[31], Van Kuilenburg, *et al.* (2016)[28]	In vitro	High
		AG + GG were observed in individuals with decreased DPD activity	**Supports Statement:** Collie-Duguid, *et al.* (2000)[49], Gross, *et al.* (2003)[94], Ezzeldin, *et al.* (2005)[43], Thomas, *et al.* (2016)[48] **Does Not Support Statement:** Ridge, *et al.* (1998)[35], Collie-Duguid, *et al.* (2000)[49], Seck, *et al.* (2005)[85], Ezzeldin, *et al.* (2005)[43]	Clinical, ex vivo	Weak
	Metabolism	AG + GG are associated with decreased metabolism of fluorouracil as compared to AA	**Supports Statement:** ***Statistically Significant:*** Zhang, *et al.* (2007)[97], Teh, *et al.* (2013)[98] ***Same Direction of Association:*** Gentile, *et al.* (2016)[57] **Does Not Support Statement:** Rudek, *et al.* (2013)[99]	Clinical, ex vivo	Weak
	Efficacy	AG + GG are associated with increased overall survival time, or increased response to fluoropyrimidine treatment as compared to AA	**Supports Statement:** ***Statistically Significant:*** Grau, *et al.* (2008)[100], Joerger, *et al.* (2015)[51] **Does Not Support Statement:** McLeod, *et al.* (2010)[53]	Clinical	Weak
		AG + GG are associated with decreased response to fluoropyrimidine treatment as compared to AA	**Supports Statement:** ***Statistically Significant:*** Zhang, *et al.* (2012)[101]	Clinical	Weak
		AG + GG are not associated with progression-free survival time as compared to AA	McLeod, *et al.* (2010)[53], Farina-Sarasqueta, *et al.* (2010)[102]	Clinical	Weak

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
	Toxicity	AG + GG are associated with risk or severity of fluoropyrimidine toxicity as compared to AA	**Supports Statement:** ***Statistically Significant (nausea/vomiting):*** Zhang, *et al.* (2007)[97] ***Statistically Significant (leukopenia):*** Zhang, *et al.* (2007)[97] ***Same Direction of Association:*** Rosmarin, *et al.* (2014)[66] **Does Not Support Statement:** Gross, *et al.* (2003)[94], Cho, *et al.* (2007)[103], Schwab, *et al.* (2008)[61], Kleibl, *et al.* (2009)[64], Amstutz, *et al.* (2009)[2], McLeod, *et al.* (2010)[53] Deenen, *et al.* (2011)[50], Zhang, *et al.* (2012)[101], Teh, *et al.* (2013)[98], Rosmarin, *et al.* (2015)[9], Froehlich, *et al.* (2015)[3], Joerger, *et al.* (2015)[51], Toffoli, *et al.* (2015)[63], Boige, *et al.* (2016)[69]	Clinical	Weak
		AG + GG were observed in individuals with fluoropyrimidine toxicity	**Supports Statement:** Collie-Duguid, *et al.* (2000)[49], Van Kuilenburg, *et al.* (2000)[70], Lazar, *et al.* (2004)[95], Ezzeldin, *et al.* (2005)[43], Kim, *et al.* (2010)[104], Van Kuilenburg, *et al.* (2010)[47], Zaanan, *et al.* (2014)[105], Thomas, *et al.* (2016)[48] **Does Not Support Statement:** Collie-Duguid, *et al.* (2000)[49]	Clinical	Weak
*6 rs1801160 c.2194G>A p.V732I	Activity	AG/the A allele is associated with decreased DPD activity as compared to GG	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2013)[5] ***Same Direction of Association:*** Kuilenburg, *et al.* (2016)[28]	Ex vivo	weak
		Within cell lines, the AA genotype or the A allele are associated with decreased DPD activity as compared to GG	**Supports Statement:** ***Statistically Significant:*** Van Kuilenburg, *et al.* (2016)[28] **Does Not Support Statement:** Offer, *et al.* (2013)[31]	In vitro	weak
		AA + AG were observed in individuals with decreased DPD activity	**Supports Statement:** Collie-Duguid, *et al.* (2000)[49], Thomas, *et al.* (2016)[48] **Does Not Support Statement:** Collie-Duguid, *et al.* (2000)[49], Seck, *et al.* (2005)[85]	Clinical, ex vivo	weak
	Metabolism	AA + AG are associated with decreased metabolism of fluorouracil as compared to GG	**Supports Statement:** ***Statistically Significant:*** Gentile, *et al.* (2016)[57]	Ex vivo	weak
	Efficacy	AA + AG are not associated with complete remission rate, event-free survival or response to fluoropyrimidine treatment as compared to GG	Zhang, *et al.* (2012)[101], Zhao, *et al.* (2016)[52]	Clinical	weak
	Toxicity	AA + AG are associated with increased risk or severity of fluoropyrimidine toxicity as compared to GG	**Supports Statement:** ***Statistically Significant (overall toxicity):*** Boige, *et al.* (2016)[69] ***Statistically Significant (myelosuppression):*** Kleibl, *et al.* (2009)[64], Boige, *et al.* (2016)[69] ***Statistically Significant (diarrhea):*** Deenen, *et al.* (2011)[50] **Does Not Support Statement:** Schwab, *et al.* (2008)[61], Amstutz, *et al.* (2009)[2], Deenen, *et al.* (2011)[50], Zhang, *et al.* (2012)[101], Rosmarin, *et al.* (2015)[9], Froehlich, *et al.* (2015)[3], Toffoli, *et al.* (2015)[63], Zhao, *et al.* (2016)[52]	Clinical	weak
		AA + AG were observed in individuals with fluoropyrimidine toxicity	**Supports Statement:** Collie-Duguid, *et al.* (2000)[49], Van Kuilenburg, *et al.* (2000)[70], Thomas, *et al.* (2016)[48], Del Re, *et al.* (2015)[106] **Does Not Support Statement:** Thomas, *et al.* (2016)[48]	Clinical	weak

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
*9A rs1801265 c.85T>C p.C29R	Activity	The CC + CT genotypes are associated with increased DPD activity as compared to TT	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2013)[5], Sistonen, *et al.* (2014)[30] **Does Not Support Statement:** He, *et al.* (2008)[96], Van Kuilenburg, *et al.* (2016)[28]	Clinical, ex vivo	weak
		Within cell lines, CC is associated with increased DPD activity as compared to TT	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2013)[31]	In vitro	weak
		Within cell lines, the C allele is associated with decreased DPD activity as compared to TT	**Supports Statement:** ***Statistically Significant:*** Van Kuilenburg, *et al.* (2016)[28]	In vitro	weak
		CC + CT were observed in individuals with decreased DPD activity	**Supports Statement:** Vreken, *et al.* (1997)[33], Van Kuilenburg, *et al.*(1999)[36], Van Kuilenburg, *et al.* (1999)[107], Collie-Duguid, *et al.* (2000)[49], Van Kuilenburg, *et al.* (2002)[40], Gross, *et al.* (2003)[94], Ezzeldin, *et al.* (2005)[43], Thomas, *et al.* (2016)[48] **Does Not Support Statement:** Collie-Duguid, *et al.* (2000)[49], Johnson, *et al.* (2002)[38], Gross, *et al.* (2003)[94], Seck, *et al.* (2005)[85], Ezzeldin, *et al.* (2005)[43]	Clinical, ex vivo	weak
	Metabolism	CC is associated with decreased metabolism of fluorouracil as compared to CT + TT	**Supports Statement:** Gentile, *et al.* (2016)[57] **Does Not Support Statement:** Boisdron-Celle, *et al.* (2007)55, Zhang, *et al.* (2007)[97]	Clinical, ex vivo	weak
	Efficacy	CC + CT are associated with decreased event-free survival time and decreased response to fluorouracil treatment as compared to TT	**Supports Statement:** ***Statistically Significant:*** Zhao, *et al.* (2016)[52]	Clinical	weak
		CC + CT is not associated with overall survival, progression-free survival or response to fluoropyrimidine treatment as compared to TT	Grau, *et al.* (2008)[100], McLeod, *et al.* (2010)[53], Joerger, *et al.* (2015)[51]	Clinical	moderate
	Toxicity	CC are associated with risk or severity of fluoropyrimidine toxicity as compared to TT	**Supports Statement:** ***Statistically Significant (nausea/vomiting):*** Zhang, *et al.* (2007)[97] ***Statistically Significant (hand-foot syndrome):*** Joerger, *et al.* (2015)[51] ***Statistically Significant (diarrhea):*** Joerger, *et al.* (2015)[51] ***Statistically Significant (infection):*** Zhao, *et al.* (2016)[52] ***Statistically Significant (nephrotoxicity):*** Zhao, *et al.* (2016)[52] ***Statistically Significant (hepatotoxicity):*** Zhao, *et al.* (2016)[52] ***Same Direction of Association (overall toxicity):*** Froehlich, *et al.* (2015)[3] **Does Not Support Statement:** Gross, *et al.* (2003)[94], Boisdron-Celle, *et al.* (2007)[55], Morel, *et al.*(2006)[73], Schwab, *et al.* (2008)[61], Amstutz, *et al.* (2009)[2], McLeod, *et al.* (2010)[53], Deenen, *et al.* (2011)[50], Dhawan, *et al.* (2013)[68], Rosmarin, *et al.* (2014)[66], Rosmarin, *et al.* (2015)[9], Boige, *et al.* (2016)[69]	Clinical	weak
		CC + CT are associated with decreased risk of gastrointestinal toxicity as compared to TT	**Supports Statement:** ***Statistically Significant:*** Kleibl, *et al.* (2009)[64]	Clinical	weak

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
		CC + CT were observed in individuals with fluoropyrimidine toxicity	**Supports Statement:** Collie-Duguid, *et al.* (2000)[49], Van Kuilenburg, *et al.* (2000)[70], Lazar, *et al.* (2004)[95], Kim, *et al.* (2010)[104], Van Kuilenburg, *et al.* (2010)[47], Kristensen, *et al.* (2010)[65], Zaanan, *et al.* (2014)[105], Saif, *et al.* (2014)[6], Baskin, *et al.* (2015)[108], Thomas, *et al.* (2016)[48], Del Re, *et al.* (2015)[106] **Does Not Support Statement:** Kristensen, *et al.* (2010)[65], Thomas, *et al.* (2016)[48]	Clinical	weak
rs2297595 c.496A>G p.M166V	Activity	The AG + GG genotypes are not associated with altered DPD activity as compared to the AA genotype	Seck, *et al.* (2005)[85], Offer, *et al.* (2013)[5], Van Kuilenburg, *et al.* (2016)[28]	Ex vivo	weak
		Within cell lines, the G allele is associated with decreased DPD activity as compared to the AA genotype	**Supports Statement:** ***Statistically Significant:*** Van Kuilenburg, *et al.* (2016)[28]	In vitro	weak
		Within cell lines, the G allele is associated with increased DPD activity as compared to the A allele	**Supports Statement:** ***Statistically Significant :*** Offer, *et al.* (2014)[86]	In vitro	weak
		The AG genotype was observed in individuals with decreased DPD activity	**Supports Statement:** Gross, *et al.* (2003)[94], Thomas, *et al.* (2016)[48] **Does Not Support Statement:** Johnson, *et al.* (2002)[38]	Clinical, ex vivo	weak
	Metabolism	The AG + GG genotypes are associated with decreased metabolism of fluorouracil as compared to the AA genotype	**Supports Statement:** ***Statistically Significant:*** Gentile, *et al.* (2016)[57]	Ex vivo	weak
	Efficacy	The AG genotype is not associated with response to fluoropyrimidine treatment as compared to the AA genotype	Zhang, *et al.* (2012)[101]	Clinical	weak
	Toxicity	The AG + GG genotypes are associated with risk or severity of fluoropyrimidine toxicity as compared to the AA genotype	**Supports Statement:** ***Statistically Significant (overall toxicity):*** Gross, *et al.* (2008)[77], Falvella, *et al.* (2015)[90] ***Statistically Significant (diarrhea):*** Deenen, *et al.* (2011)[50] ***Statistically Significant (hand-foot syndrome):*** Deenen, *et al.* (2011)[50] ***Same Direction of Association:*** Deenen, *et al.* (2011)[50] **Does Not Support Statement for overall toxicity:** Schwab, *et al.* (2008)[61], Amstutz, *et al.* (2009)[2], Zhang, *et al.* (2012)[101], Loganayagam, *et al.* (2013)[13], Rosmarin, *et al.* (2014)[66], Rosmarin, *et al.* (2015)[9], Froehlich, *et al.* (2015)[3], Toffoli, *et al.* (2015)[63], Boige, *et al.* (2016)[69]	Clinical	weak
		The AG + GG genotypes are associated with a decreased risk of neutropenia as compared to the AA genotype	**Supports Statement:** ***Statistically Significant:*** Kleibl, *et al.* (2009)[64]	Clinical	weak
		The AG genotype/the G allele was observed in individuals with fluoropyrimidine toxicity	**Supports Statement:** Van Kuilenburg, *et al.* (2000)[70], Gross, *et al.* (2003)[94], Van Kuilenburg, *et al.* (2010)[47], Kristensen, *et al.* (2010)[65], Thomas, *et al.* (2016)[48] **Does Not Support Statement:** Kristensen, *et al.* (2010)[65], Thomas, *et al.* (2016)[48]	Clinical	weak
rs115232898 c.557A>G p.Y186C	Activity	The AG genotype is associated with decreased DPD activity as compared to the AA genotype	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2013)[5]	Ex vivo	Moderate

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
		Within cell lines, the GG genotype/the G allele is associated with decreased DPD activity as compared to the AA genotype	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[109]**,** Offer, *et al.* (2014)[86]	In vitro	Moderate
		The AG genotype/the G allele was observed in individuals with decreased DPD activity	**Supports Statement:** Ezzeldin, *et al.* (2005)[43] Zaanan, *et al.* (2014)[105]	Clinical, ex vivo	Moderate
	Toxicity	The AG genotype was observed in individuals with fluoropyrimidine toxicity	**Supports Statement:** Zaanan, *et al.* (2014)[105], Saif, *et al.* (2014)[6]	Clinical	Weak
rs61622928 c.1218G>A p.M406I	Activity	The AG genotype/the A allele is not associated with altered DPD activity as compared to the GG genotype	Offer, *et al.* (2013)[5], Van Kuilenburg, *et al.* (2016)[28]	Ex vivo	weak
		Within cell lines, the A allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86], Van Kuilenburg, *et al.* (2016)[28]	In vitro	weak
		The AG genotype/the A allele was observed in an individual with decreased DPD activity	**Supports Statement:** Ezzeldin, *et al.* (2005)[43], Thomas, *et al.* (2016)[48]	Clinical, ex vivo	weak
rs17376848 c.1896T>C p.F632F	Activity	The CC + CT genotypes are not associated with altered DPD activity as compared to the TT genotype	He, *et al.* (2008)[96], Offer, *et al.* (2013)[5]	Clinical, ex vivo	weak
		The CT genotype/the C allele were observed in individuals with decreased DPD activity	**Supports Statement:** Collie-Duguid, *et al.* (2000)[49], Ezzeldin, *et al.* (2005)[43] **Does Not Support Statement:** Collie-Duguid, *et al.* (2000)[49]	Ex vivo	weak
	Metabolism	The CC + CT genotypes are associated with decreased metabolism of fluorouracil as compared to the TT genotype	**Supports Statement:** ***Statistically Significant:*** Teh, *et al.* (2013)[98]	Clinical	weak
	Toxicity	The CT genotype/the C allele is associated with risk or severity of fluoropyrimidine toxicity as compared to the TT genotype	**Supports Statement:** ***Statistically Significant (overall toxicity):*** Kristensen, *et al.* (2010)[65], Falvella, *et al.* (2015)[90] ***Statistically Significant (neutropenia):*** Teh, *et al.* (2013)[98] ***Statistically Significant (stomatitis):*** Joerger, *et al.* (2015)[51] **Does Not Support Statement:** Schwab, *et al.* (2008)[61], Kleibl, *et al.* (2009)[64], Amstutz, *et al.* (2009)[2], Deenen, *et al.* (2011)[50], Froehlich, *et al.* (2015)[3], Toffoli, *et al.* (2015)[63], Boige, *et al.* (2016)[69]	Clinical	weak
		The CT genotype was observed in individuals with fluoropyrimidine toxicity	**Supports Statement:** Gross, *et al.* (2003)[94]	Clinical	weak

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
*2B rs1801159 + rs3918290 1627A>G (I543V) + 1905+1G>A	Activity	The *2B/*4 genotype was observed in an individual with decreased DPD activity	**Supports Statement:** Ridge, *et al.* (1998)[35]	Ex vivo	weak
	Toxicity	The *2B/*4 genotype was observed in an individual with fluoropyrimidine toxicity	**Supports Statement:** Ridge, *et al.* (1998)[35]	Clinical	weak
*3 rs72549303 c.1898delC p.P633Qfs	Activity	Within cell lines, the del allele is associated with decreased DPD activity as compared the C allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
		The C/del + del/del genotypes were observed in individuals with decreased DPD activity	**Supports Statement:** Vreken, *et al.* (1997)[110], Vreken, *et al.* (1997)[33], Van Kuilenburg, *et al.*(1999)[36]	Clinical, ex vivo	weak
*7 rs72549309 c.295_298delTCAT p.F100Sfs	Activity	Within cell lines, the del allele is associated with decreased DPD activity as compared to the TCAT allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
		The del/del genotype was observed in individuals with DPYD deficiency	**Supports Statement:** Van Kuilenburg, *et al.* (1999)[36]	Clinical	weak
*8 rs1801266 c.703C>T p.R235W	Activity	Within cell lines, the T allele is associated with decreased DPD activity as compared to the C allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
		The T allele was observed in individuals with decreased DPD activity	**Supports Statement:** Vreken, *et al.* (1997)[110], Vreken, *et al.* (1997)[33], Van Kuilenburg, *et al.* (1999)[36]	Clinical, ex vivo	weak
	Toxicity	The T allele was observed in an individual without fluoropyrimidine toxicity	**Supports Statement:** Kristensen, *et al.* (2010)[65]	Clinical	weak
*9B rs1801267 + rs1801265 c.2657G>A + c.85T>C p.R886H + p.C29R	Activity	*9B/*9B/the *9B allele was observed in individuals with decreased DPD activity	**Supports Statement:** Vreken, *et al.* (1997)[110], Vreken, *et al.* (1997)[33], Van Kuilenburg, *et al.*(1999)[36]	Clinical, ex vivo	weak
		Within cell lines, the A allele of the rs1801267 variant (part of *9B) is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	weak

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
	Toxicity	The A allele of the rs1801267 variant (part of *9B) is not associated with risk of fluoropyrimidine toxicity as compared to the G allele	Boige, *et al.* (2016)[69]	Clinical	weak
*10 rs1801268 c.2983G>T p.V995F	Activity	Within cell lines, the T allele is associated with decreased DPD activity as compared to the G allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
		The TT genotype was observed in an individual with decreased DPD activity	**Supports Statement:** Van Kuilenburg, *et al.* (1999)[36]	Clinical	weak
*11 rs72549306 c.1003G>T p.V335L	Activity	The T allele was observed in individuals with decreased DPD activity	**Supports Statement:** Kouwaki, *et al.* (1998)[111]	Ex vivo	weak
		The T allele was observed to result in decreased DPD activity in *E.coli* lysates	**Supports Statement:** Kouwaki, *et al.* (1998)[111]	In vitro	weak
		Within cell lines, the T allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	weak
	Toxicity	The T allele was observed in an individual with fluoropyrimidine toxicity	**Supports Statement:** Kouwaki, *et al.* (1998)[111]	Clinical	weak
*12 rs80081766 + rs78060119 c.62G>A + c.1156G>T p.R21Q + p.E386X	Activity	The *12 allele was observed in individuals with decreased DPD activity		Ex vivo	weak
		The T allele of the rs78060119 variant (part of *12) was observed to result in undetectable DPD activity in *E.coli* lysates	**Supports Statement:** Kouwaki, *et al.* (1998)[111]	In vitro	weak
		The A allele of the rs80081766 variant (part of *12) was not observed to result in altered DPD activity in *E.coli* lysates	Kouwaki, *et al.* (1998)[111]	In vitro	weak
		Within cell lines, the T allele of the rs78060119 variant (part of *12) is associated with decreased DPD activity as compared to the G allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
		Within cell lines, the A allele of the rs80081766 variant (part of *12) is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	weak

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
	Toxicity	The *12 allele was observed in an individual with fluoropyrimidine toxicity	**Supports Statement:** Kouwaki, *et al.* (1998)[111]	Clinical	weak
		The GG genotype of the rs78060119 variant (part of *12) is not associated with risk or severity of fluoropyrimidine toxicity as compared to the TT or GT genotypes	Zhao, *et al.* (2016)[52]	Clinical	weak
rs111858276 c.1484A>G p.D495G	Activity	Within cell lines, the G allele is associated with decreased DPD activity as compared to the A allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs112766203 c.2279C>T p.T760I	Activity	Within cell lines, the T allele is associated with decreased DPD activity as compared to the C allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs114096998 c.3067C>A p.P1023T	Activity	Within cell lines, the A allele is associated with increased DPD activity as compared to the C allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs115632870 c.151-69G>A	Activity	The AG genotype is associated with decreased DPD activity as compared to the GG genotype	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2013)[5]	Ex vivo	weak
rs12022243 1906-14763G>A	Toxicity	The A allele is associated with increased severity of fluoropyrimidine toxicity as compared to the G allele	**Supports Statement:** ***Statistically Significant:*** Rosmarin, *et al.* (2015)[9]	Clinical	weak
s12132152 g.97523004G>A[e]	Toxicity	The A allele is associated with increased severity of fluoropyrimidine toxicity as compared to the G allele	**Supports Statement:** ***Statistically Significant:*** Rosmarin, *et al.* (2015)[9]	Clinical	weak
rs76387818 g.97539400G>A[e]	Toxicity	The A allele is associated with increased severity of fluoropyrimidine toxicity as compared to the G allele	**Supports Statement:** ***Statistically Significant:*** Rosmarin, *et al.* (2015)[9]	Clinical	weak
rs7548189 c.1906-19696G>T	Toxicity	The T allele is associated with increased severity of fluoropyrimidine toxicity as compared to the G allele	**Supports Statement:** ***Same Direction of Association:*** Rosmarin, *et al.* (2015)[9]	Clinical	weak
rs137999090 c.2021G>A p.G674D	Activity	Within cell lines, the A allele is associated with decreased DPD activity as compared to the G allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs138616379 c.1775G>A p.R592Q	Activity	Within cell lines, the A allele is associated with decreased DPD activity as compared to the G allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs141044036 c.2872A>G p.K958E	Activity	Within cell lines, the G allele is associated with decreased DPD activity as compared to the A allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
rs143154602 c.1057C>T p.R353C	Activity	Within cell lines, the T allele is associated with decreased DPD activity as compared to the C allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs143986398 c.274C>G p.P92A	Activity	Within cell lines, the G allele is associated with decreased DPD activity as compared to the C allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs145773863 c.1777G>A p.G593R	Activity	Within cell lines, the A allele is associated with decreased DPD activity as compared to the G allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs146356975 c.868A>G p.K290E	Activity	Within cell lines, the G allele is associated with decreased DPD activity as compared to the A allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs147601618 c.1796T>C p.M599T	Toxicity	The CT genotype was observed in an individual with fluoropyrimidine toxicity	**Supports Statement:** Ofverholm, *et al.* (2010)[112]	Clinical	weak
	Activity	Within cell lines, the C allele is not associated with DPD activity as compared to the T allele	Offer, *et al.* (2014)[86]	In vitro	weak
rs183105782 c.910T>C p.Y304H	Activity	Within cell lines, the C allele is associated with decreased DPD activity as compared to the T allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs183385770 c.1024G>A p.D342N	Activity	Within cell lines, the T allele is associated with decreased DPD activity as compared to the C allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs186169810 c.1314T>G p.F438L	Activity	Within cell lines, the G allele is associated with decreased DPD activity as compared to the T allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs188052243 c.2678A>G p.N893S	Activity	Within cell lines, the G allele is associated with decreased DPD activity as compared to the A allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs190577302 c.1054C>G p.L352V	Activity	Within cell lines, the G allele is associated with decreased DPD activity as compared to the C allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs200687447 c.2482G>A p.E828K	Activity	Within cell lines, the A allele is associated with increased DPD activity as compared to the G allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs367619008 187A>G p.K63E	Toxicity	The AG genotype was observed in an individual with fluoropyrimidine toxicity	**Supports Statement:** Kleibl, *et al.* (2009)[64]	Clinical	weak

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
rs376073289 c.623G>A p.R208Q	Toxicity	The AG genotype was observed in an individual with fluoropyrimidine toxicity	**Supports Statement:** Thomas, *et al.* (2016)[48]	Clinical	weak
		The A allele is not associated with risk of fluoropyrimidine toxicity	Schwab, *et al.* (2008)[61]	Clinical	weak
rs45589337 c.775A>G p.K259E	Activity	Within cell lines, the G allele is not associated with altered DPD activity as compared to the A allele	Offer, *et al.* (2014)[86]	In vitro	weak
		The G allele is not associated with altered DPD activity as compared to the AA genotype	Sistonen, *et al.* (2014)[30]	Clinical	weak
		The AG genotype was observed in an individual without altered DPD activity	**Supports Statement:** Gross, *et al.* (2003)[94]	Ex vivo	weak
	Toxicity	The AG genotype was observed in an individual with fluoropyrimidine toxicity	**Supports Statement:** Gross, *et al.* (2003)[94]	Clinical	weak
		The AG genotype/the G allele is not associated with risk or severity of fluoropyrimidine toxicity as compared to the AA genotype	Schwab, *et al.* (2008)[61], Rosmarin, *et al.* (2015)[9], Froehlich, *et al.* (2015)[3]	Clinical	weak
rs55674432 c.2639G>T p.G880V	Activity	Within cell lines, the T allele is associated with decreased DPD activity as compared to the G allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs56293913 c.1129-15T>C	Activity	The CC + CT genotypes are not associated with altered DPD activity as compared to the TT genotype	Offer, *et al.* (2013)[5], Sistonen, *et al.* (2014)[30]	Clinical, ex vivo	weak
	Toxicity	The CC + CT genotypes are associated with increased severity of fluoropyrimidine toxicity as compared to the TT genotype	**Supports Statement:** ***Statistically Significant:*** Gross, *et al.* (2008)[77] ***Same Direction of Association:*** Amstutz, *et al.* (2009)[2], Deenen, *et al.* (2011)[50]	Clinical	weak
rs568132506 c.257C>T p.P86L	Activity	The CT + TT genotypes were observed in individuals with decreased DPD activity	**Supports Statement:** Van Kuilenburg, *et al.* (2002)[40], Thomas, *et al.* (2016)[48]	Clinical, ex vivo	weak
	Toxicity	The CT genotype was observed in an individual with fluoropyrimidine toxicity	**Supports Statement:** Thomas, *et al.* (2016)[48]	Clinical	weak
rs59086055 c.1774C>T p.R592W	Activity	Within cell lines, the T allele is associated with decreased DPD activity as compared to the C allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs60139309 c.2582A>G p.K861R	Activity	Within cell lines, the G allele is associated with increased DPD activity as compared to the A allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
rs61757362 c.2948C>T p.T983I	Activity	Within cell lines, the T allele is associated with decreased DPD activity as compared to the C allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs72547601 c.2933A>G p.H978R	Activity	Within cell lines, the G allele is associated with decreased DPD activity as compared to the A allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
		The GG genotype was observed in an individual with decreased DPD activity	**Supports Statement:** Van Kuilenburg, *et al.* (2002)[40]	Ex vivo	weak
rs72549304 c.1475C>T p.S492L	Activity	Within cell lines, the T allele is associated with decreased DPD activity as compared to the C allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
		The TT genotype was observed in an individual with decreased DPD activity	**Supports Statement:** Van Kuilenburg, *et al.* (2002)[40]	Ex vivo	weak
rs72549305 c.1108A>G p.I370V	Activity	Within cell lines, the G allele is not associated with DPD activity as compared to the A allele	Offer, *et al.* (2014)[86]	In vitro	weak
		The GG genotype was observed in an individual with decreased DPD activity	**Supports Statement:** Van Kuilenburg, *et al.* (2002)[40]	Ex vivo	weak
rs72549307 c.632A>G p.Y211C	Activity	Within cell lines, the G allele is associated with decreased DPD activity as compared to the A allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
		The GG genotype was observed in an individual with decreased DPD activity	**Supports Statement:** Van Kuilenburg, *et al.* (2002)[40]	Ex vivo	weak
	Toxicity	The G allele is not associated with risk or severity of fluoropyrimidine toxicity	Froehlich, *et al.* (2015)[3]	Clinical	weak
rs72549308 c.601A>C p.S201R	Activity	Within cell lines, the C allele is associated with decreased DPD activity as compared to the A allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs72549310 c.61C>T p.R21X	Activity	Within cell lines, the T allele is associated with decreased DPD activity as compared to the C allele	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2014)[86]	In vitro	weak
rs72728438 c.1974+75A>G	Activity	The AG genotype is associated with decreased DPD activity as compared to the AA genotype	**Supports Statement:** ***Statistically Significant:*** Offer, *et al.* (2013)[5]	Ex vivo	weak
rs777425216 c.1651G>A p.A551T	Toxicity	The AG genotype was observed in an individual with fluoropyrimidine toxicity	**Supports Statement:** Rosmarin, *et al.* (2015)[9]	Clinical	weak
rs150036960 c.46C>G p.L16V	Activity	Within cell lines, the G allele is not associated with altered DPD activity as compared to the C allele	Offer, *et al.* (2014)[86]	In vitro	

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
rs150385342 c.313G>A p.A105T	Activity	Within cell lines, the A allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	
rs141462178 c.343A>G p.M115V	Activity	Within cell lines, the G allele is not associated with altered DPD activity as compared to the A allele	Offer, *et al.* (2014)[86]	In vitro	
rs200562975 c.451A>G p.N151D	Activity	Within cell lines, the G allele is not associated with altered DPD activity as compared to the A allele	Offer, *et al.* (2014)[86]	In vitro	
rs139834141 c.498G>A p.M166I	Activity	Within cell lines, the A allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	
rs150437414 c.929T>C p.L310S	Activity	Within cell lines, the C allele is not associated with altered DPD activity as compared to the T allele	Offer, *et al.* (2014)[86]	In vitro	
rs145112791 c.934C>T p.L312F	Activity	Within cell lines, the T allele is not associated with altered DPD activity as compared to the C allele	Offer, *et al.* (2014)[86]	In vitro	
rs201018345 c.967G>A p.A323T	Activity	Within cell lines, the A allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	
rs143815742 c.1181G>T p.R394L	Activity	Within cell lines, the T allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	
rs140602333 c.1180C>T p.R394W	Activity	Within cell lines, the T allele is not associated with altered DPD activity as compared to the C allele	Offer, *et al.* (2014)[86]	In vitro	
rs200064537 c.1260T>A p.N420K	Activity	Within cell lines, the A allele is not associated with altered DPD activity as compared to the T allele	Offer, *et al.* (2014)[86]	In vitro	
rs764666241 c.1278G>T p.M426I	Activity	Within cell lines, the T allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	
rs142512579 c.1294G>A p.D432N	Activity	Within cell lines, the A allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	
rs72975710 c.1349C>T p.A450V	Activity	Within cell lines, the T allele is not associated with altered DPD activity as compared to the C allele	Offer, *et al.* (2014)[86]	In vitro	

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
rs144395748 c.1358C>G p.P453R	Activity	Within cell lines, the G allele is not associated with altered DPD activity as compared to the C allele	Offer, *et al.* (2014)[86]	In vitro	
		The GG genotype is not associated with altered DPD activity as compared to the CG genotype	Offer, *et al.* (2013)[5]	Ex vivo	
rs199549923 c.1403C>A p.T468N	Activity	Within cell lines, the A allele is not associated with altered DPD activity as compared to the C allele	Offer, *et al.* (2014)[86]	In vitro	
rs138391898 c.1519G>A p.V507I	Activity	Within cell lines, the A allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	
rs148994843 c.1543G>A p.V515I	Activity	Within cell lines, the A allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	
rs190951787 c.1577C>G p.T526S	Activity	Within cell lines, the G allele is not associated with altered DPD activity as compared to the C allele	Offer, *et al.* (2014)[86]	In vitro	
rs142619737 c.1615G>A p.G539R	Activity	Within cell lines, the A allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	
rs201615754 c.1682G>T p.R561L	Activity	Within cell lines, the T allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	
rs3918289 c.1905C>G p.N635K	Activity	Within cell lines, the G allele is not associated with altered DPD activity as compared to the C allele	Offer, *et al.* (2014)[86]	In vitro	
rs55971861 c.1906A>C p.I636L	Activity	Within cell lines, the C allele is not associated with altered DPD activity as compared to the A allele	Offer, *et al.* (2014)[86]	In vitro	
rs138545885 c.1990G>T p.A664S	Activity	Within cell lines, the T allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	
rs145548112 c.2161G>A p.A721T	Activity	Within cell lines, the A allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	
rs146529561 c.2186C>T p.A729V	Activity	Within cell lines, the T allele is not associated with altered DPD activity as compared to the C allele	Offer, *et al.* (2014)[86]	In vitro	

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
rs60511679 c.2195T>G p.V732G	Activity	Within cell lines, the G allele is not associated with altered DPD activity as compared to the T allele	Offer, *et al.* (2014)[86]	In vitro	
rs56005131 c.2303C>A p.T768K	Activity	Within cell lines, the A allele is not associated with altered DPD activity as compared to the C allele	Offer, *et al.* (2014)[86]	In vitro	
rs199634007 c.2336C>A p.T779N	Activity	Within cell lines, the A allele is not associated with altered DPD activity as compared to the C allele	Offer, *et al.* (2014)[86]	In vitro	
rs201035051 c.2623A>C p.K875Q	Activity	Within cell lines, the C allele is not associated with altered DPD activity as compared to the A allele	Offer, *et al.* (2014)[86]	In vitro	
rs147545709 c.2656C>T p.R886C	Activity	Within cell lines, the T allele is not associated with altered DPD activity as compared to the C allele	Offer, *et al.* (2014)[86]	In vitro	
rs145529148 c.2915A>G p.Q972R	Activity	Within cell lines, the G allele is not associated with altered DPD activity as compared to the A allele	Offer, *et al.* (2014)[86]	In vitro	
rs72547602 c.2921A>T p.D974V	Activity	Within cell lines, the T allele is not associated with altered DPD activity as compared to the A allele	Offer, *et al.* (2014)[86]	In vitro	
rs139459586 c.2978T>G p.L993R	Activity	Within cell lines, the G allele is not associated with altered DPD activity as compared to the T allele	Offer, *et al.* (2014)[86]	In vitro	
rs202144771 c.2977C>T p.L993F	Activity	Within cell lines, the T allele is not associated with altered DPD activity as compared to the C allele	Offer, *et al.* (2014)[86]	In vitro	
rs140114515 c.3049G>A p.V1017I	Activity	Within cell lines, the A allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	
rs148799944 c.3061G>C p.V1021L	Activity	Within cell lines, the C allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2014)[86]	In vitro	
rs6670886 c.525G>A p.S175S	Activity	The A allele is not associated with altered DPD activity as compared to the G allele	Offer, *et al.* (2013)[5]	Ex vivo	
rs3790387 c.763-118A>G	Activity	The AG + GG genotypes are not associated with altered DPD activity as compared to the AA genotype	Offer, *et al.* (2013)[5], Van Kuilenburg, *et al.* (2016)[28]	Ex vivo	

DPYD *allele rsID nucleotide change[a] protein change[b]	Parameter	Major findings	References	Type of experimental model	Level of Evidence[c,d]
		The G allele is not associated with altered DPD activity as compared to the A allele	Sistonen, *et al.* (2014)[30]	Clinical	
rs112550271 c.850+41T>C	Activity	The CT genotype is not associated with altered DPD activity as compared to the TT genotype	Offer, *et al.* (2013)[5], Van Kuilenburg, *et al.* (2016)[28]	Ex vivo	
rs2811202 c.958+134T>G	Activity	The GG + GT genotypes are not associated with altered DPD activity as compared to the TT genotype	Offer, *et. al.* (2013)[5]	Ex vivo	
rs61789183 c.1340-106T>A	Activity	The AA + AT genotypes are not associated with altered DPD activity as compared to the TT genotype	Offer, *et al.* (2013)[5], Van Kuilenburg, *et al.* (2016)[28]	Ex vivo	
rs57918000 c.1371C>T p.N457N	Activity	The CT genotype is not associated with altered DPD activity as compared to the CC genotype	Offer, *et al.* (2013)[5]	Ex vivo	
rs2786783 c.1740+39C>T	Activity	The CT + TT genotypes are not associated with altered DPD activity as compared to the CC genotype.	Offer, *et al.* (2013)[5], Sistonen, *et al.* (2014)[30]	Ex vivo, clinical	
rs2811178 1740+40A>G	Activity	The AG + GG genotypes are not associated with altered DPD activity as compared to the AA genotype	Offer, *et al.* (2013)[5], Sistonen, *et al.* (2014)[30]	Ex vivo, clinical	
rs12137711 c.2300-39G>A	Activity	The AG genotype is not associated with altered DPD activity as compared to the GG genotype	Offer, *et al.* (2013)[5]	Ex vivo	
rs41309171 c.234-123G>C	Activity	The CG genotype is not associated with altered DPD activity as compared to the GG genotype	Sistonen, *et al.* (2014)[30]	Clinical	
rs138924556 c.850+91C>T	Activity	The CT genotype is not associated with altered DPD activity as compared to the CC genotype	Sistonen, *et al.* (2014)[30]	Clinical	
rs368600943 c.1129-28G>T	Activity	The GT genotype is not associated with altered DPD activity as compared to the GG genotype	Sistonen, *et al.* (2014)[30]	Clinical	

[a]Nucleotide changes according to reference sequence NM_000110.3 unless otherwise specified.
[b]Protein changes according to reference sequence NP_000101.2.
[c]Rating Scheme for Quality of Evidence as per ref.[16]
[d]Some of the small case series, although not strong individually, collectively do support a strong recommendation.
[e]Nucleotide changes according to NC_000001.10
[f]Likely HapB3 causal variant. Proxy SNPs are c.1236G>A (rs56038477, E412E), c.483+18G>A (rs56276561) and c.959-51T>G (rs115349832). c.680+139G>A (rs6668296) is not exclusive to HapB3 and therefore not a suitable proxy.

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3

Prospective *DPYD* genotyping to reduce the risk of fluoropyrimidine-induced severe toxicity: ready for prime time

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SUMMARY

5-Fluorouracil (5-FU) and capecitabine are among the most frequently prescribed anticancer drugs. They are inactivated in the liver by the enzyme dihydropyrimidine dehydrogenase (DPD). Up to 5% of the population is DPD-deficient and these patients have a significantly increased risk of severe and potentially lethal toxicity when treated with regular doses of 5-FU or capecitabine. DPD is encoded by the gene *DPYD* and variants in *DPYD* can lead to a decreased DPD activity. Although prospective *DPYD* genotyping is a valuable tool to identify patients with DPD deficiency, and thus those at risk for severe and potential life-threatening toxicity, prospective genotyping has not yet been implemented in daily clinical care. Our goal was to present the available evidence in favor of prospective genotyping, including discussion of unjustified worries on cost-effectiveness, and potential underdosing.

CASE: FATAL TOXICITY FOLLOWING TREATMENT WITH CAPECITABINE

A 52-year-old woman with human epidermal growth factor receptor 2 (HER2)-positive metastasized breast cancer was treated with capecitabine 1250 mg/m^2 twice daily, for 14 days every 3 weeks, plus intravenous trastuzumab on day 1. The first cycle was fully completed; at day 18 of treatment mild diarrhea and a herpes zoster infection located at her mouth were noticed during routine outpatient visit. Due to low hematological laboratory values (leucocytes, neutrophils CTC grade 2, and thrombocytes CTC grade 3), the second cycle was planned to be deferred by 1 week. However, 3 days later she returned to the hospital with now severe diarrhea (CTC grade 4), sepsis, neutropenic fever, severe leucopenia and life-threatening thrombocytopenia and mucositis, for which she was admitted to the intensive care unit. A long and intensive hospitalization period followed, but despite optimal treatment and supportive care, the patient did not recover from severe toxicity and deteriorated even further. At day 34 of admission the patient deceased as a result of this severe toxicity. Genetic testing revealed that the patient was heterozygous for *DPYD**2A, a variant allele known to result in dihydropyrimidine dehydrogenase deficiency.[1] In case screening would have been performed prior to start of therapy, capecitabine dosage could have been reduced by 50%, thereby possibly preventing fatal capecitabine-induced toxicity.[2]

INTRODUCTION

5-Fluorouracil (5-FU) and its oral pro-drug capecitabine belong to the group of the fluoropyrimidine drugs, and are among the most frequently used anticancer drugs in the treatment of common cancer types such as colorectal, stomach, breast, head and neck and skin cancer.[3–7] 5-FU has a relatively narrow therapeutic index and, depending on type of treatment regimen, around 15-30% of patients suffer from severe toxicity such as diarrhea, nausea, mucositis, stomatitis, myelosuppression, neurotoxicity and hand-foot syndrome.[4,8–12] These side-effects lead to mortality in approximately 0.5–1% of patients using 5-FU and capecitabine.[4,13]

The enzyme dihydropyrimidine dehydrogenase (DPD) plays a key role in the catabolism of 5-FU. It is the rate-limiting enzyme degrading over 80% of the drug to its inactive metabolite 5-fluoro-5,6-dihydrouracil.[9,14,15] Because of this, DPD is an important factor for efficacy,[16,17] as well as the development of toxicity.[10] DPD is encoded by the gene *DPYD*, which consists of 23 exons on chromosome 1p22.[18] More than 160 single nucleotide polymorphisms (SNPs) are known within this gene, some resulting in altered enzyme activity.[19] Eighty *DPYD* variants were experimentally tested for their enzyme activity[20] and *DPYD* variants may result in an absolute or a partial DPD-deficiency (0.5% versus 3–5% of the population, respectively).[21,22] About 30–50% of the patients treated with a fluoropyrimidine drug who suffer from severe or life-threatening toxicity (grade 3–5) have no or decreased DPD enzyme activity, and 50–88% of patients carrying a variant in *DPYD* suffer from grade ≥3 fluoropyrimidine-related toxicity.[6,10,11,21,23–25]

Although pharmacogenomic tests in general have the potential to improve clinical outcome by increasing efficacy and decreasing toxicity, and the potential to decrease the cost of health care, their use in routine clinical practice is still limited.[26] This also holds true for the use of *DPYD* genotyping prior to start of treatment with fluoropyrimidines.[27,28] Other DPD deficiency screening methods (e.g. phenotyping) have been described,[29] and are currently being investigated (NCT02324452), but we feel are not ready yet for clinical application. In the current paper, we present an overview on the evidence for prospective *DPYD* genotyping and discuss critical questions related to its implementation. Associations of *DPYD* variants with fluoropyrimidine-induced toxicity, prevention of severe toxicity upon *DPYD* testing, cost consequences and existing guidelines will be discussed.

AVAILABLE EVIDENCE FOR THE ASSOCIATION OF DPYD VARIANTS AND 5-FU-INDUCED SEVERE TOXICITY

The relationship between *DPYD* variants and 5-FU induced severe toxicity is widely acknowledged. Recently, data have been summarized in three separate meta-analyses.[8,9,30] Terrazzino *et al*. evaluated 4094 patients (15 studies) for *DPYD**2A (IVS14+1G>A; rs3918290) and 2308 patients for c.2846A>T (D949V, rs67376798). They confirmed the clinical validity of these SNPs as risk factors for the development of fluoropyrimidine-associated severe toxicities (details in Table 1).[9] The second meta-analysis, performed by Rosmarin *et al.,* included data of 4855 patients (17 studies). They describe eight *DPYD* variants of which *DPYD**2A and c.2846A>T also showed convincing evidence of an association with toxicity (Table 1).[8] The third meta-analysis of Meulendijks *et al.,* included data of 7365 patients (eight studies) and confirmed the association between severe toxicity and the variants *DPYD**2A and c.2846A>T, but also for *DPYD**13 (I560S; c.1679T>G; rs55886062) and c.1236G>A/HapB3 (E412E; rs56038477) (Table 1).[30]

Very recently, three additional papers, not part of the three meta-analyses, have confirmed significant associations between *DPYD* variants and toxicity (Table 1).[4,31,32] Although multiple variants of *DPYD* have been described, *DPYD**2A, *DPYD**13, c.2846A>T and c.1236G>A/HapB3 are the variants that are most extensively studied and convincingly associated with fluoropyrimidine-related severe toxicity.[8,9,30]

The HuGE risk translator[33] is an online tool to calculate test characteristics for the evaluation of the predictive ability of genetic markers. Data (e.g. odds ratio) from two of three meta-analyses described above could be entered as a 'two-risk genotype' for *DPYD**2A and c.2846A>T, resulted in low (~10 to ~25%) sensitivity and positive predictive values and high (>96%) specificity and negative predictive values (NPV). The number needed to screen (i.e. genotype) appears to be 210–250 patients and the number needed to treat (i.e. apply dose adjustments) is five or six patients (Table 2).

Important to note is that values for diagnostic test criteria of a pharmacogenomic test based on SNPs in *DPYD* can never reach 100%, because not all DPD deficiencies and toxicity can be explained by variants in *DPYD*.[34] It must also be said that the high specificity (±98%) and high NPV (±96.5%) in this setting are most important, when the goal is to treat all patients with a variant (including false-positives). The consequence of a (false) positive result is a relatively low-risk dose reduction for the first of many cycles, which can be adjusted in safe conditions in the second cycle and onwards if no toxicity occurs. The consequence of a false negative result may be much larger since it could result in a too high systemic drug exposure that subsequently leads to severe, potentially lethal toxicity, which is associated with long-lasting hospital and/or intensive care unit (ICU) admissions.

In a previous study approximately 10% of the *DPYD**2A variant allele carriers treated with the standard fluoropyrimidine dose deceased as a result of drug-induced severe toxicity.[35] The approach of pretreatment genotyping followed by a reduced starting dose plus tolerance-guided dose titration could prevent the occurrence of severe toxicities in *DPYD* variant allele carriers, resulting in a direct safer use with minimum risk of underdosing. The above mentioned test characteristics are reached using the two most investigated SNPs and these values will probably improve when a larger panel of *DPYD* SNPs is probed. Costs are not likely to increase substantially when adding SNPs because genotyping costs continue to decrease.[36,37] Although more *DPYD* variants that alter DPD enzyme activity are continuously discovered and studied, the perfect set of SNPs has not been defined yet. Currently we feel there is substantial evidence to support dose recommendations for at least four variants (*DPYD**2A, c.2846A>T, *DPYD**13 and c.1236G>A/HapB3).[38] Another possibility for prospective screening could be the more informative, but hugely more expensive genotyping of the entire coding region of *DPYD*. However we have focused on genotyping SNPs. To date, SNP genotyping has been most extensively studied, is technically feasible in a general hospital setting and multiple guidelines providing SNP-based dose recommendations are available.

Table 1. Toxicity associations of *DPYD* variants.
Brief summary of a few selected studies showing the results of *DPYD* variants and their associations with 5-FU and/or capecitabine induced severe toxicity. Included are three meta-analyses and three more recent papers. Results originating with only 5-FU or only capecitabine (CAP) are explicitly marked. Rosmarin et al. have also tested 5-FU infusion (in) and 5-FU bolus (bo) separately. Meulendijks *et al*. have described RR values, not OR values, as shown by *.

Group	*DPYD* variant	Association with 5-FU and/or capecitabine grade ≥3 toxicity (OR/*RR [95% CI], P-value)
Terrazzino *et al.* 2013 [9]	*DPYD**2A (rs3918290)	Overall toxicity (5.42 [2.79–10.52], *P*<0.001) Diarrhoea (5.54 [2.31–13.29], *P*<0.001) Haematological toxicity (15.77 [6.36–39.06], *P*<0.001) Mucositis (7.48 [3.03–18.47], *P*<0.001)
	c.2846A>T (rs67376798)	Overall toxicity (8.18 [2.65–25.25], *P*<0.001) Diarrhoea (6.04 [1.77–20.66], *P*=0.004)
Rosmarin *et al.* 2014 [8]	*DPYD**2A (rs3918290)	Overall toxicity (6.71 [1.66-27.1], *P*=0.0075) (5-FU in.) Diarrhoea (7.71 [1.61-36.9], *P*=0.011) (5-FU in.) Mucositis/stomatitis (7.15 [1.75-29.1], *P*=0.0061) (5-FU bo.) Neutropenia (12.90 [3.13-53.3], *P*=0.00040) (5-FU bo.)
	c.2846A>T (rs67376798)	Overall toxicity (9.35 [2.01-43.4], *P*=0.0043) (CAP) Diarrhoea (3.14 [0.82- 11.9], *P*=0.093) (CAP) Hand-foot syndrome (1.31 [0.35-4.96], *P*=0.69) (CAP)
	*DPYD**2A (rs3918290) c.2846A>T (rs67376798)	Overall toxicity (5.51 [1.95-15.51], *P*=0.0013) (CAP)
Meulendijks *et al.* 2015 [30]	*DPYD**2A (rs3918290)	Overall toxicity (*2.85 [1.75-4.62], *P*<0.0001)
	c.2846A>T (rs67376798)	Overall toxicity (*3.02 [2.22-4.10], *P*<0.0001)
	*DPYD**13 (rs55886062)	Overall toxicity (*4.40 [2.08-9.30], *P*<0.0001) Gastrointestinal toxicity (*5.72 [1.40-23.33], *P*=0.015) Haematological toxicity (*9.76 [3.03-31.48], *P*=0.00014)
	c.1236G>A/HapB3 (rs56038477)	Overall toxicity (*1.59 [1.29-1.97], *P*<0.0001) Gastrointestinal toxicity (*2.04 [1.49-2.78], *P*<0.0001) Haematological toxicity (*2.07 [1.17-3.68], *P*=0.013)
Rosmarin *et al.* 2015 [4]	rs12132152 (AF:0.03)	Overall toxicity (3.83 [3.26–4.40], $P=4.31*10^{-6}$) (CAP) Hand-foot syndrome (6.12 [5.48–6.76], $P=3.29*10^{-8}$) (CAP) Diarrhoea (0.44 [0–1.32], *P*=0.065) (CAP)
	rs12022243 (AF: 0.22)	Overall toxicity (1.69 [1.45–1.94], $P=2.55*10^{-5}$) (CAP) Hand-foot syndrome (1.43 [1.16–1.7], *P*=0.0096) (CAP) Diarrhoea (1.79 [1.54–2.05], $P=9.86*10^{-6}$) (CAP)
	rs76387818	Overall toxicity (4.05 [3.47–4.62], $P=2.11*10^{-6}$) (CAP) Hand-foot syndrome (6.44 [5.79–7.09], $P=1.75*10^{-8}$) (CAP) Diarrhoea (0.44 [0–1.33], *P*=0.071) (CAP)
	rs7548189	Overall toxicity (1.67 [1.43–1.91], $P=3.79*10^{-5}$) (CAP) Hand-foot syndrome (1.42 [1.15–1.69], *P*=0.011) (CAP) Diarrhoea (1.21 [0.84–1.58], *P*=0.0015) (CAP)
Falvella *et al.* 2015 [32]	c.496A>G (rs2297595)	Overall toxicity (5.94 [1.29–27.22], *P*=0.022) (CAP)
	c.1896T>C (rs17376848)	Overall toxicity (14.53 [1.36–155.20], *P*=0.027) (CAP)
Joerger *et al.* 2015 [31]	c.1896T>C (rs17376848) c.85T>C (rs1801265) c.2846A>T (rs67376798)	Diarrhoea (*P*<0.05) (CAP) Hand-foot syndrome (*P*<0.02) (CAP)

Table 2. Test characteristics of genotyping for *DPYD2A and c.2846A>T.**
Clinical utility test characteristics of genotyping for *DPYD**2A and c.2846A>T, calculated using "The HuGE Risk translator"[33] for Terrazzino *et al.*[9] and Rosmarin *et al.*[8]

Test characteristics	Terrazzino *et al.*[9]	Rosmarin *et al.*[8]
sensitivity	14.5%	11.8%
specificity	97.6%	98.4%
positive predictive value	19.8%	23.6%
negative predictive value	96.5%	96.4%
number needed to screen (i.e. genotype)	210 patients	251 patients
number needed to treat (i.e. apply dose adjustments)	6 patients	5 patients

WHAT IS NEEDED FOR IMPLEMENTATION OF *DPYD* GENOTYPING IN DAILY ROUTINE CLINICAL CARE?

Clinical implementation of a biomarker test such as *DPYD* pharmacogenomics is hampered due to the ongoing discussion on whether a randomized clinical trial (RCT) is considered necessary to provide the required evidence before clinical implementation.[26,29,37,39–45] Despite the fact that RCTs are considered the gold standard study design to prove effectiveness, adequate evidence can also be provided by small-scale, innovative, prospective interventional studies.[40] However, with the available evidence favoring upfront genotyping, it may not be ethically feasible to randomize patients, and patients may not be willing to be included in the control arm with an increased risk for severe toxicity. Indeed, the only attempt at a prospective randomized study was performed in France. Boisdron-Celle *et al*. presented a multicenter prospective cohort study of upfront DPD deficiency screening executed from 2008 until 2012.[46] The purpose of the study was to confirm the medical and economic aspect of upfront DPD deficiency screening in a prospective way as was done retrospectively by Traoré *et al*.[47] Patients using 5-FU based chemotherapy were included in one of two parallel patient cohorts (arm A and arm B). Patients in arm A were prospectively screened for DPD deficiency (a combined genotyping and phenotyping approach), and patients in arm B were retrospectively tested. A total of 1130 patients were included (arm A: 720 patients, arm B: 410 patients). One patient died due to 5-FU early-onset toxicity and it was retrospectively confirmed that this patient was DPD deficient (arm B). The enrolment of patients was prematurely closed for ethical reasons, because of the proven 5-FU-induced toxic death of this patient.[46,48] Against this background, we conclude that evidence from a randomized prospective clinical trial on *DPYD* genotyping will never be acquired for ethical reasons. In addition, some predictive biomarkers were previously implemented without evidence from an RCT. Clinical use of (K)RAS selection for epidermal growth factor receptor (EGFR) therapy was influenced by updated registration texts for EGFR inhibitors from the Food and Drug Administration (FDA)[49] and European Medicines Agency (EMA) after retrospective analyses of three studies (CRYSTAL trial, OPUS trial and CA225025).[50–52] Also hormone receptor status for hormone therapy in breast cancer has never been proven in a prospective randomized study.

CLINICAL IMPLEMENTATION OF *DPD* DEFICIENCY TESTING

Advantages and disadvantages of phenotyping and genotyping as possible DPD deficiency

screening methods were described previously[29] and several institutes[53–59] have executed (prospective) screening of *DPYD* variants or DPD deficiency in a study context. Unfortunately, available literature of clinical implementation remains limited to only a few centers in France, Germany, the Netherlands, Ireland and the United States of America (USA).[44,53,60,61] An established and well-recognized *DPYD* clinical implementation program is that of the 'Institut de Cancerologie de l'Ouest' in Angers (France) where screening for DPD deficiency has been a regular procedure for over 10 years. Besides this institute, over 100 centers in France use the 'Onco Drug Personalized Medicine' or ODPM Tox™ and 2000 patients are being screened with this approach every year.[62,63] Boisdron-Celle *et al*. describe a large trial in which 11,104 patients were prospectively screened (combining genotyping and phenotyping) and patients with a *DPYD* variant or decreased DPD activity received an individual dose adjustment. Genotyping in the trial consisted of 24 mutations in *DPYD* and phenotyping included the DHU/U ratio. Two hundred forty seven patients with grade 3–5 toxicity were retrospectively tested. In total, 3% of all patients carried one or more mutations. Twenty seven out of 247 retrospectively tested patients died of whom 16 (59%) and 24 (89%) were identified with genotyping or phenotyping, respectively. The combined approach would have identified 98% of grade 3–4 toxicity patients and 100% of mortalities.[63]

(COST-)EFFECTIVENESS OF DPD DEFICIENCY TESTING

A prospective, multicenter study was conducted by Deenen *et al*., in which 2038 patients were screened for *DPYD**2A prior to start with 5-FU or capecitabine.[64] Twenty two patients (1.1%) were heterozygous carriers of *DPYD**2A and patients received an initial dose reduction of 50% when starting therapy, followed by dose titration based on clinical tolerance. Toxicity results showed that the risk of grade ≥3 toxicity was significantly reduced to 28% compared to 73% in historical controls (P<0.001). Drug-induced death reduced from 10% to 0%. This study convincingly shows that pre-treatment genotyping of *DPYD**2A followed by dose adjustment in carrier patients improves patient safety. A cost-analysis was executed using a decision analytic model from a health care payer perspective, including only direct medical costs. Genotyping costs were €75 per test. The average total treatment cost per patient was slightly lower for screening (€2,772) than for non-screening (€2,817). The approach was shown to be feasible in routine clinical practice.[64] Ahmed *et al*. presented a cost-analysis of a retrospective screening for four *DPYD* variants in 31 patients who experienced grade 3–4 toxicity. Five patients carried a variant and were admitted to the ICU due to toxicity. The costs of hospital admission (€155,083) were much higher than the screening costs of all patients starting with fluoropyrimidine therapy for CRC during the study period (€26,800).[53] Another retrospective study of 48 patients shows cost-effectiveness with *DPYD* screening costs for four variants being almost nine times lower than hospital admissions of four patients (£1,776 versus £15,525; approximately €2,500 versus €21,500).[58] We must bear in mind that genotyping technology is developing fast and prices continue to decline.[37] Phenotyping tests have been recently reviewed by Van Staveren *et al.,* and to our knowledge, to date no additional cost-effectiveness analysis for a phenotyping test has been published.[29]

RECOMMENDATIONS AND GUIDELINES OF *DPYD* PHARMACOGENOMICS

Warnings or contraindications for using 5-FU/capecitabine in DPD deficient patients are stated by the FDA and EMA.[65,66] This is meaningless without knowing, and thus testing a patient for DPD deficiency. No formal recommendations on pre-therapeutic (upfront) screening for DPD deficiency

are given by health authorities, regulatory agencies or guideline committees from the National Comprehensive Cancer Network or American Society of Clinical Oncology. The European Society for Medical Oncology explicitly states that they do not recommend upfront routine testing for DPD deficiency despite the risk of severe and potential lethal toxicity.[67] It is unknown to us what arguments underlie this recommendation. Only in cases of severe toxicity due to 5-FU treatment DPD deficiency screening is strongly recommended, and exposure to standard dose of 5-FU is contraindicated in proven DPD deficiency patients, according to guidelines published in 2012.[67]
The lack of official recommendations on pre-therapeutic genotyping is limiting the process of implementation. One of the reasons may be that such a recommendation is drug-specific and not tumor-type specific while oncology guidelines are traditionally tumor-type specific (e.g. KRAS mutation, human epidermal growth factor receptor 2 (HER2) expression).
The Clinical Pharmacogenetics Implementation Consortium and the Dutch Pharmacogenetics Working Group of the Royal Dutch Pharmacists Association provide evidence-based guidelines and recommendations what dose adjustments to apply in *DPYD* variant allele carriers.[37,68,69] Recommendations depend on the *DPYD* allele and carrier status (heterozygous, homozygous), and are guided by the gene activity score. After initial reduction dosages can be further titrated based on clinical tolerance. Dose reductions are 75, 50 or 25% for gene activity scores of 0.5, 1 and 1.5, respectively. The gene activity score varies from 0 (no DPD activity) to 2 (normal DPD activity).[38,69]

BARRIERS FOR CLINICAL IMPLEMENTATION

Potential barriers hampering the clinical implementation of prospective *DPYD* testing are:

(1) 'Perceived lack of scientific evidence';
The evidence for the association of DPYD variants and severe fluoropyrimidine-induced toxicity has been discussed and is considered convincing. Furthermore, an RCT is considered unethical and unnecessary.

(2) 'There is a lack of laboratory facilities and there is no reimbursement';
The number of laboratories that offer genetic testing for DPYD is continuously increasing, techniques are easier to operate and prices for genetic testing will continue to decrease.[37] The cost of a DPYD genetic test is currently in the range of €50 to €100. These amounts are negligible compared to the costs of treatment that could easily reach €10,000 or more.[70] This genetic test (which is a once-in-a-lifetime test when no additional SNPs are added) should be as normal as testing for other contraindications for drugs such as liver enzymes, renal function or physical condition. Laboratories usually offer the test with a turnaround time of 2–3 days which is acceptable and does not result in treatment delay, which is a serious concern of clinicians and patients.

(3) 'There is not enough guidance on how to use the test';
Peer reviewed guidance on how to use the outcomes of the genetic test is well covered.[37,38,68,69]

(4) 'There is a risk of underdosing patients';
Guidelines advise to reduce the dose of fluoropyrimidines in the first cycle in patients carrying DPYD variants associated with decreased DPD activity to create similar systemic drug levels compared to wild-type patients. In the following cycles tolerance-guided dose titration is used to create the most optimal treatment. This strategy minimizes the risk for underdosing. In addition, 5-FU and capecitabine are often used in combination with other anti-cancer drugs, so only a fraction of the total therapy is reduced.

(5) 'Phenotyping tests are more specific';
Phenotyping tests measuring DPD enzyme activity directly are more closely predicting DPD deficiency as compared to *DPYD* genotyping. However, DPD enzyme measurements are also more expensive, more time consuming, have dreadful logistics (can be time-dependent), high turnaround-times (>1 week) and only a very limited number of laboratories provide the tests. For these reasons DPD enzyme activity measurements are less likely to be implemented as a routine clinical test compared to the genotyping test.

(6) 'Genetic screening does not predict DPD deficiency perfectly';
Patients who do not carry a *DPYD* variant can still develop severe side-effects and patients carrying a *DPYD* variant do not necessarily develop toxicity. Clearly, as with other drugs, other patient and treatment characteristics also influence the risk of severe toxicity. The sensitivity and specificity shall for this reason never reach 100% as discussed above. In the USA, with a population of 300 million, there are 1300 deaths each year due to 5-FU induced toxicity.[71] More than half of the deceased patients could have been identified using genotyping according to Boisdron-Celle *et al*.[63]

CONCLUSION

Although pharmacogenomics in general has the potential to result in safer use of drugs by supporting individualized therapy, this unfortunately has not resulted in clinical implementation of *DPYD* screening in the oncology field. Based on the available evidence, we argue that upfront *DPYD* screening using a pharmacogenomics test in patients planned to be treated with a fluoropyrimidine should become the standard of care. Treatment with fluoropyrimidines has been the cornerstone chemotherapy for several oncological indications for more than 50 years, and will probably continue to stay so. With the increasing incidence of cancer the number of patients who are likely to be treated with a fluoropyrimidine drug will increase, as well as the number of patients that would be saved from 5-FU or capecitabine induced severe toxicity when using pre-treatment genetic screening. In 2010 Ciccolini *et al*. already pointed out that it was time to mandate the integration of systematic prospective testing for *DPYD* as part of routine clinical practice in oncology.[10] Based on the arguments given above we truly believe it is time to add upfront *DPYD* genotyping to the current guidelines and to start implementation of *DPYD* screening without further delay. When upfront testing followed by dose adjustments is fully functional as part of routine clinical practice we can expect that grade ≥3 fluoropyrimidine-related toxicity substantially decreases without the risk of underdosing.

CONFLICT OF INTEREST

The authors declare no conflict of interest. CATC Lunenburg and this study were supported by an unrestricted grant from Roche Pharmaceuticals. There was no involvement in the study design, data collection, analysis or interpretation of the data.

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DPYD genotype-guided dose individualization to improve patient safety of fluoropyrimidine therapy: call for a drug label update

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SUMMARY

The fluoropyrimidine anticancer drugs, especially 5-fluorouracil (5-FU) and capecitabine, are frequently prescribed for several types of cancer, including breast, colorectal, head and neck and gastric cancer. In the current drug labels of 5-FU and capecitabine in the European Union and the United States of America no adaptive dosing strategies are incorporated for polymorphic metabolism of 5-FU.

Although treatment with fluoropyrimidines is generally well tolerated, a major clinical limitation is that a proportion of the treated population experiences severe, sometimes life-threatening, fluoropyrimidine-related toxicity. This toxicity is strongly affected by interindividual variability in activity of dihydropyrimidine dehydrogenase (DPD), the main metabolic enzyme for inactivation of fluoropyrimidines, with an estimated 3-8% of the population being partially DPD deficient. A reduced functional or abrogated DPD enzyme is often caused by genetic polymorphisms in *DPYD*, the gene encoding for DPD, and heterozygous carriers of such *DPYD* polymorphisms have a partial DPD deficiency. When these partially DPD deficient patients are treated with a full dose of fluoropyrimidines, they are generally exposed to toxic levels of 5-FU and its metabolites, and the risk of developing severe treatment-related toxicity is therefore significantly increased.

Currently, functional and clinical validity is well established for four *DPYD* variants (*DPYD**2A, c.2846A>T, c.1679T>G and c.1236G>A), as those variants have retrospectively and in a large population study prospectively been shown to be associated with increased risk of fluoropyrimidine-associated toxicity. Patient safety of fluoropyrimidine treatment can be significantly improved by pre-emptive screening for *DPYD* genotype variants and dose reductions in heterozygous *DPYD* variant allele carriers, thereby normalizing 5-FU exposure. Based on the critical appraisal of currently available data, adjusting the labels of capecitabine and 5-FU by including recommendations on pre-emptive screening for *DPYD* variants and *DPYD* genotype-guided dose adjustments should be the new standard of care.

INTRODUCTION

Fluoropyrimidines, a group of anticancer drugs including 5-fluorouracil (5-FU) and its oral prodrugs capecitabine (Xeloda®) and tegafur (active compound of S-1, Teysuno®), have been the backbone of anticancer treatment in a variety of cancers, including breast, colorectal, head and neck, anal, pancreas and gastric cancer, for over 50 years. These drugs are among the most frequently prescribed anticancer drugs, as an estimated two million patients are treated with fluoropyrimidines each year.[1,2] Despite convincing evidence, no dose individualization strategies are recommended for polymorphic metabolism of 5-FU in the current drug labels of the fluoropyrimidines drugs 5-FU and capecitabine.

Although the majority of patients can be treated safely with capecitabine and 5-FU, a substantial proportion experiences severe fluoropyrimidine-related toxicity.[3,4] For example, in phase III studies in metastatic colorectal cancer, 30%–40% of the patients treated with 5-FU or capecitabine monotherapy experienced severe (grade ≥3) treatment-related toxicity, mainly consisting of diarrhea, mucositis, bone marrow suppression and hand-foot syndrome.[5,6] Treatment of severe toxicity is usually associated with interruption or even discontinuation of potentially effective anticancer therapy, and often requires hospitalization, which also increases healthcare costs. Furthermore, severe fluoropyrimidine-related toxicity leads to mortality in ~0.5%–1% of patients.[5–8] This indicates that fluoropyrimidine-associated toxicity is a substantial clinical problem.

In this review, the current knowledge on metabolism of fluoropyrimidines, available methods to test for dihydropyrimidine dehydrogenase (DPD) activity and associations between DPD deficiency and fluoropyrimidine-related toxicity are described. The benefits of fluoropyrimidine dose individualization based on genotyping for *DPYD* polymorphisms that reduce DPD activity are outlined, supporting our call for an update of the drug label of the fluoropyrimidine drugs capecitabine and 5-FU and to include dose individualization strategies based on *DPYD* genotype.

METABOLISM OF FLUOROPYRIMIDINES

After administration, the prodrug capecitabine is stepwise converted into 5-FU. Only a small fraction of 5-FU (1%–5%) is converted intracellularly into the cytotoxic metabolites fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP; Figure 1).[9,10] Besides this, the DPD enzyme converts ~80% of the administered dose of 5-FU into the inactive metabolite 5,6-dihydro-5-fluorouracil (FUH_2), which makes DPD the rate-controlling enzyme for inactivation of 5-FU (Figure 1).[9–11] The amount of 5-FU available for conversion into cytotoxic metabolites is therefore primarily determined by systemic DPD activity. The DPD enzyme is mainly expressed in the liver, the main site of 5-FU metabolism. DPD activity varies widely between patients, with an estimated 3%–8% of the population being partially DPD deficient, having an approximately up to 50% lower enzymatic activity.[12,13] Complete DPD deficiency (~0% enzyme activity) is much rarer than partial deficiency, with an estimated incidence of 0.1%.[12,14–16] When partially DPD deficient patients are treated with standard doses of fluoropyrimidines, the reduced DPD activity will result in decreased inactivation of 5-FU, thereby increasing levels of active metabolites of 5-FU, which is associated with a strongly increased risk of severe and even fatal toxicity.[17–19] Several studies showed that around 39%–61% of patients with severe fluoropyrimidine-associated toxicity were found to have decreased DPD activity.[20–22] DPD deficiency is most often the result of genetic polymorphisms in *DPYD*, the gene encoding the DPD enzyme. *DPYD* is a highly polymorphic gene with over 35 genetic polymorphisms in *DPYD*

described, among which several lead to reduced function or a non-functional DPD enzyme, such as *DPYD**2A, c.2846A>T, c.1679T>G, and c.1236G>A.[19,23,24] It is estimated that around half of the DPD deficient cases can be identified by these four *DPYD* variants.[25]
Carriers of functionally relevant *DPYD* variants are likely to be subject to increased 5-FU exposure when treated with standard dose of fluoropyrimidines. By upfront screening for *DPYD* variants followed by adjusting the fluoropyrimidine starting dose in partially DPD deficient patients, high 5-FU exposure in these patients can be avoided and therapeutic exposure achieved, thereby reducing risk of severe treatment-related toxicity.[26–28]

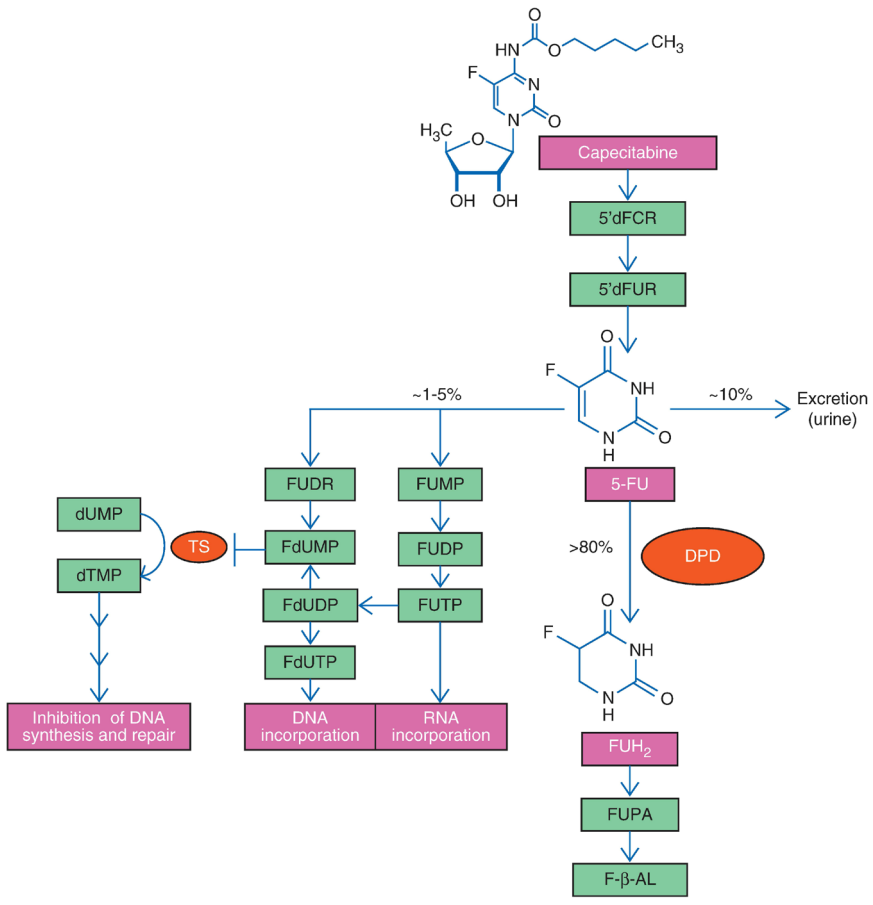

Figure 1. Metabolic pathway of fluoropyrimidines.
Abbreviations: 5'dFCR: 5'-deoxy-5-fluorocytidine; 5'dFUR: 5'-deoxy-5-fluorouridine; 5-FU: 5-fluorouracil; DPD: dihydropyrimidine dehydrogenase; dTMP: deoxythymidine monophosphate; dUMP: deoxyuridine monophosphate; FdUDP: fluorodeoxyuridine diphosphate; FdUMP: fluorodeoxyuridine monophosphate; FdUTP: fluorodeoxyuridine triphosphate; FUDP: fluorouridine diphosphate; FUDR: fluorodeoxyuridine; FUH_2: 5,6-dihydro-5-fluorouracil; FUMP: fluorouridine monophosphate; FUPA: fluoro-ß-ureidopropionate; FUTP: fluorouridine triphosphate; F-ß-AL: fluoro-ß-alanine; TS: thymidylate synthase.

METHODS FOR TESTING FOR DPD DEFICIENCY

Several strategies for testing for DPD deficiency have been developed, which are based on either genotyping of *DPYD* or measurement of the DPD phenotype. A genotype-based approach consists of testing for single nucleotide polymorphisms in *DPYD* and advantages include high diagnostic accuracy, since results are unambiguous and the test is not influenced by environmental factors. Furthermore, this test is relatively easy to implement in diagnostic laboratories of hospitals.[25] Evidence-based dose recommendations are available on how to dose fluoropyrimidines in the case a patient carries a *DPYD* polymorphism[29,30] and this will be described in more detail in the paragraph on dose individualization.
Other strategies for determination of DPD deficiency are methods measuring the DPD phenotype. These methods include measuring DPD enzyme activity in peripheral blood mononuclear cells (PBMCs), or measurement of *in vivo* concentrations of the endogenous substrate of DPD, uracil, and its metabolic product dihydrouracil, either after a loading dose of uracil or the endogenous plasma levels of dihydrouracil and uracil.[25,31] Compared with a genotype-based approach, these methods are considered more difficult to implement as a routine diagnostic test, as these methods are often more time-consuming and materials and equipment might not be available in every hospital. In addition, limited information is available on how to adjust the dose in patients with DPD deficiency identified by DPD phenotyping tests, and additional studies on clinical validity and utility are required prior to clinical implementation.[25,31] A promising aspect of DPD phenotyping tests is that sensitivity to identify DPD deficiency might be increased when used in addition to *DPYD* genotyping tests, as currently not all DPD deficiency can be attributed to a genetic alteration in *DPYD*.

EXAMPLES OF CURRENT INFORMATION ON DPD DEFICIENCY IN FLUOROPYRIMIDINE DRUG LABELS

In the European Union, DPD deficiency is mentioned in the current version of the summary of product characteristics (SPC) of capecitabine in the sections *Contraindications* and *Special warnings and precautions for use*.[32] It is stated that capecitabine is contraindicated in patients with known complete absence of DPD activity. As a special warning it is mentioned that severe, life-threatening, or fatal adverse reactions have been attributed to DPD deficiency. However, no obligations or recommendations on pre-emptive testing for DPD deficiency are mentioned in the SPC and it is also stated that there is insufficient data to recommend specific dose reductions in patients with partial DPD activity. Similar information on DPD deficiency is provided in the United States by the Food and Drug Administration (FDA) in the package insert of capecitabine.[33] The same accounts for the SPC and FDA label of 5-FU.[34,35]
Despite strong evidence linking *DPYD* variants to severe toxicity, pharmacogenetic testing for *DPYD* polymorphisms is currently not described in the drug label. We recently informed health authorities (European Medicines Agency (EMA), Dutch Medicines Evaluation Board (MEB), and FDA) and encouraged companies holding marketing authorization applications to request adjusting the drug labels of capecitabine and 5-FU. Recently published studies[27,36] add to the numerous available studies published over many years, supporting pre-emptive *DPYD* genotyping and dose individualization. Patient safety of fluoropyrimidine therapy can be substantially improved, if dose adjustments based on *DPYD* genotype are finally implemented worldwide.

DOSE INDIVIDUALIZATION BASED ON DPYD GENOTYPE

DPYD*2A genotype

*DPYD*2A (IVS14+1G>A; c.1905+1G>A; rs3918290) is the most widely studied polymorphism in *DPYD* and was the first variant described as being functionally relevant.[37] Allele frequencies of *DPYD*2A have been reported to vary between ~0.1% and 1.0% in African-American and Caucasian populations, respectively.[24,29,38,39] *DPYD*2A leads to skipping of the entire exon 14 and deletion of 165 base pairs which results in a truncated protein that is catalytically inactive.[37,40] This is supported by an *in vitro* study by Offer *et al.* in which several *DPYD* variants were homozygously expressed in mammalian cells and the enzymatic activity of expressed protein was completely absent when expressing *DPYD*2A[41] and patients with complete DPD deficiency that were identified as homozygous *DPYD*2A variant allele carriers.[37,39,42] This suggests that in heterozygous carriers of this variant, who have one dysfunctional allele and one functional allele, ~50% of the normal DPD enzyme activity will be retained. The *DPYD*2A variant has been associated with ~50% decreased DPD enzyme activity in PBMCs in several *ex vivo* studies.[20,40,43,44] This DPD activity in PBMCs is well correlated with DPD activity in the liver, the main site of 5-FU metabolism.[45] In addition, in heterozygous *DPYD*2A* carriers 5-FU clearance was found to be significantly reduced, resulting in increased exposure to 5-FU and active metabolites.[46–48]

Clinical validity and utility of DPYD*2A genotype-guided dosing

Data on *DPYD*2A and toxicity from retrospective studies are numerous. In many studies and two recent meta-analyses increased risk of toxicity related to fluoropyrimidine treatment in *DPYD*2A variant allele carriers was confirmed.[19,36,38,46,49–61]

The clinical utility of *DPYD*2A-guided dosing to prevent severe toxicity was recently investigated in the prospective clinical trial by Deenen *et al.*[27,28] Patients intended to be treated with fluoropyrimidine-based chemotherapy were genotyped for *DPYD*2A before start of therapy. In view of the predictable effect on DPD activity and 5-FU pharmacokinetics, *DPYD*2A variant allele carriers received an initial dose reduction of 50%. This initial dose reduction could be followed by dose-titration based on tolerance. Of the 1631 patients screened and treated with fluoropyrimidines, 18 patients (1.1%) were found to be a heterozygous *DPYD*2A carrier.[27] Toxicity data for variant allele carriers treated with a reduced dose were compared with historical controls from literature, i.e. *DPYD*2A variant allele carriers receiving standard dose. Given the strong association between *DPYD*2A and increased risk of severe and lethal toxicity, a randomized clinical trial, in which *DPYD*2A carriers would receive a full fluoropyrimidine dose, was considered unethical. The study showed that risk of severe (grade ≥3) treatment-related toxicity was significantly lower in *DPYD*2A variant allele carriers undergoing genotype-guided dosing than in the historical controls, respectively, 28% and 73% (*P*<0.001). Drug-related death was reduced from 10% in historical controls to 0% in this study. The *DPYD*2A genotype-guided dosing strategy resulted in comparable severe toxicity risk when compared with patients wild-type for *DPYD*2A given standard-dose therapy (23%, *P*=0.64).[27]

c.2846A>T, c.1679T>G, and c.1236G>A genotype

Because the frequency of *DPYD*2A in Caucasian patients is around 1%, it provides only limited sensitivity to identify patients at risk of severe toxicity. Additional *DPYD* variants have been identified that are associated with DPD deficiency. Clinical validity has currently been established

for three other *DPYD* variants (c.2846A>T, c.1679T>G, and c.1236G>A).

The c.2846A>T variant allele (D949V; rs67376798) was first described by van Kuilenburg *et al*. in 2000.[20] The c.2846A>T polymorphism leads to a structural change in the DPD enzyme that interferes with cofactor binding or electron transport.[62] Reported allele frequencies of c.2846A>T vary from 0.1% to 1.1% in African-Americans and Caucasians respectively.[24,29,38,63] *In vitro* data from Offer *et al.* show that homozygous expression of the c.2846A>T variant results in an activity of 59% compared with wild-type (*P*=0.0031).[24] Although the enzyme activity of c.2846A>T is significantly impaired, it is not comparable to the extent observed for *DPYD*2A, where homozygous expression resulted in a completely non-functional enzyme.[41] This finding that homozygous expression of c.2846A>T results in ~50% reduction, implies that a heterozygous carrier would have around 25% reduction in DPD activity, and would benefit from a 25% dose reduction.[30]

The *DPYD* variant c.1679T>G (*DPYD*13*; I560S; rs55886062) is a very rare *DPYD* variant, with an allele frequency found to vary from 0.07% to 0.1% in Caucasians.[29,38] Homozygous expression of this variant resulted in a 75% reduction of DPD enzyme activity compared with wild-type, as reported in an *in vitro* study by Offer *et al.*[41] This suggests that this variant almost completely inactivates the protein, and that heterozygous carriers would have around 50% reduction in DPD enzyme activity.[30] Decreased DPD enzyme activity in patients carrying the c.1679T>G variant was determined in a limited number of *ex vivo* studies using PBMCs.[43,44,62,64]

The c.1236G>A polymorphism (E412E; rs556038477) occurs in exon 11 and is a synonymous variant that is in complete linkage with c.483+18G>A, c.680+139G>A, c.959-51T>G, and c.1129-5923C>G; these variants in linkage have been termed haplotype B3 (HapB3).[65,66] The c.1129-5923C>G intronic polymorphism (rs75017182) results in aberrant splicing and is likely to be responsible for the effect on DPD enzyme activity.[18,66] The frequency of heterozygous patients in Caucasian populations was reported to vary between 2.6% and 6.3%.[58,65–68] DPD enzyme activity in PBMCs in c.1236G>A/HapB3 carriers was found to be reduced.[66] As DPD activity is not completely absent in homozygous carriers of this *DPYD* polymorphism, it is expected that a 25% dose reduction for heterozygous carriers is suitable.[30,69]

Clinical validity of c.2846A>T, c.1679T>G, and c.1236G>A genotype-guided dosing

Meulendijks *et al*. have investigated associations between *DPYD* variants and severe fluoropyrimidine-associated toxicity to determine clinical validity in a recent systematic review and meta-analysis using individual patient data.[36,70] A total of 7356 patients from 8 studies were included in the analysis. *DPYD* c.1679T>G was found to be significantly associated with fluoropyrimidine-induced severe toxicity (relative risk 4.40, 95%CI 2.08-9.30, *P*<0.0001). Also c.1236G>A was significantly associated with fluoropyrimidine-induced severe toxicity (relative risk 1.59, 95%CI 1.29-1.97, *P*<0.0001). For c.2846A>T a significant association with severe toxicity was found as well (relative risk 3.02, 95%CI 2.22-4.10, *P*<0.0001).[36] These results show that c.1679T>G, c.1236G>A, and c.2846A>T are clinically relevant predictors of fluoropyrimidine-associated toxicity, in addition to the *DPYD*2A variant.

Judging from the data on functional and clinical relevance of these variants, it can be expected, in line with recommended dose adjustments for heterozygous *DPYD*2A carriers, that initial dose reductions in heterozygous carriers of these three other *DPYD* polymorphisms (as described in Table 1) will result in normalization of 5-FU exposure and reduction in the risk of severe toxicity as well.

For the *DPYD* variants c.2846A>T and c.1679T>G, in addition to *DPYD**2A, initial dose reductions are recommended in the guidelines of the Clinical Pharmacogenetics Implementation Consortium (CPIC).[29] c.1236G>A/HapB3 is not yet included in this guideline (published in 2013).
The dose recommendations for these four *DPYD* variants are considered initial recommendations. If treatment is considered safe after two cycles of chemotherapy, based on clinical and laboratory assessments, individual dose up titration can be applied, to achieve maximum safe exposure in all patients.

Cost-effectiveness of DPYD genotype-guided dosing
The occurrence of severe fluoropyrimidine-related toxicity does not only have an impact on the patient itself, but can also result in significant health care costs, with high costs for medication to treat side-effects and hospitalization. If *DPYD* genotyping (costs around €75 to €100 per patient) and individualized dosing can decrease the incidence of severe toxicity, it can be expected that costs of health care will decrease as well. This was investigated in two studies.[27,71]
The prospective study by Deenen *et al.* included a model-based cost-analysis, from a health care payer perspective (including only direct medical costs).[27] The average total treatment costs per patient were shown to be slightly lower (€2772) for upfront *DPYD**2A-screening than for non-screening (€2817).[27]
In a study by Mercier *et al.* a comparison was made between 74 patients with head and neck cancer receiving standard dosage of 5-FU and 74 patients that received a dose reduction if considered DPD-deficient (tested phenotypically).[71] Direct and indirect costs for managing treatment-related toxicities were taken into account. It was shown that there was a large reduction in treatment costs for toxicity per patient if applying individualized dosing guided by DPD-status, from $6279 in the group with standard dosing versus $294 in the group with adaptive dosing based on DPD-status.[71] Based on these studies, dose individualization based on *DPYD* genotype or DPD phenotype is shown to be cost-saving.

BENEFIT–RISK ASSESSMENT AND UNCERTAINTIES IN *DPYD* GENOTYPE-GUIDED DOSING

Beneficial effects of DPYD genotype-guided dosing of fluoropyrimidines
An important aspect of the strategy of *DPYD* genotype-guided dose individualization is that dose reductions in *DPYD* variant allele carriers should not result in underdosing of fluoropyrimidines, as this might result in less effective treatment. No clinical trials have been conducted that formally investigated if dose reductions based on *DPYD* genotype result in similar efficacy as the standard dose in patients with a wildtype *DPYD* genotype. However, in the study of Deenen *et al*. pharmacokinetic analyses showed that the dose-normalized (normalized to a dose of 1250 mg/m^2) area under the plasma concentration–time curve (AUC) of 5-FU proved to be twice as high in patients with the *DPYD**2A genotype compared with the exposure in the wild-type control population.[27] These results show that adequate systemic exposure to 5-FU is achieved following a 50% dose reduction, and therefore it is unlikely that efficacy is negatively influenced by dose reductions in patients with partial DPD deficiency. Furthermore, several studies have been carried out that investigated the effect on efficacy of dose individualization based on DPD phenotyping tests. Although these studies all had a small sample size and results should therefore be interpreted with caution, in all studies, reducing the dose in patients with partial DPD deficiency

Table 1. Initial dose recommendations for heterozygous *DPYD* variant allele carriers.[30]

***DPYD* variant**	**% of standard fluoropyrimidine dose[a]**
*DPYD**2A (rs3918290)	50%
c.1679T>G (rs55886062)	50%
c.2846A>T (rs67376798)	75%
c.1236G>A/HapB3 (rs56038477)	75%

[a] For patients with complete DPD deficiency (for example homozygous *DPYD* variant allele carriers) selection of alternative treatment is recommended.

did not negatively affect treatment efficacy.[71–73] These studies used different methods than the *DPYD* genotyping proposed here, but as there is a clear correlation between *DPYD* genotype and DPD phenotype, these clinical reports support the expectation that efficacy is unlikely to be negatively influenced by dose reductions in patients with partial DPD deficiency.

Risks of DPYD genotype-guided dosing of fluoropyrimidines
Risk of severe fluoropyrimidine-related toxicity can be significantly reduced when applying an initial dose reduction in *DPYD* variant allele carriers. However, not all toxicity can be prevented with this strategy, as there are other factors influencing DPD enzyme activity as well, and not all treatment-related toxicity can be explained by DPD deficiency. Nevertheless, if with upfront *DPYD* genotype-guided dose individualization risk of severe fluoropyrimidine-related toxicity in *DPYD* variant allele carriers can be reduced until background risk of toxicity (risk for *DPYD* wildtype patients), patient safety will be significantly improved.

Uncertainties of DPYD genotype-guided dosing and benefit–risk balance
Dose recommendations for *DPYD* variant allele carriers (Table 1) are based on *in vitro* and *in vivo* data, and are expected to be suitable for the majority of patients. However, as DPD activity is known to have a high interindividual variability, these dose recommendations may not be the ideal recommendation for all patients. This limitation can partly be prevented if individual dose titration (upward or downward) based on tolerance is applied after the first two cycles of treatment.
Most studies focus on heterozygous carriers of *DPYD* variants. Much rarer are patients who are homozygous carriers of a specific *DPYD* variant or carriers of multiple *DPYD* variants simultaneously on different alleles (the so-called compound heterozygous), and therefore recommendations for a suitable dose for such patients are more difficult. Patients with a homozygous *DPYD* variant genotype for variants such as *DPYD**2A are expected to have no residual DPD activity, and therefore treatment with a fluoropyrimidine is discouraged. For a homozygous *DPYD* genotype of a variant with a more modest effect on DPD activity (such as c.1236G>A) or a compound heterozygous *DPYD* genotype, the effect on the DPD phenotype is more difficult to predict. When a patient with such a rare genotype is identified, we advise to perform additional tests, such as determining enzyme activity in PBMCs, before deciding on an individual treatment plan.
The *DPYD* variants described in this review are especially relevant for Caucasians, as most studies focused on patients of this ethnic origin. For ethnicities other than Caucasians, more research on

the frequency and clinical relevance of these and other *DPYD* variants is recommended.
Currently, most evidence on clinical validity and utility is available for individualized dosing based on *DPYD* genotype. However, several DPD phenotyping tests are being developed that have the potential to increase sensitivity to identify DPD deficient patients. Before these tests could be implemented as standard of care, further studies are required.
As described, the number of prospective studies investigating clinical utility of *DPYD* genotype-guided dosing is still limited. However, based on the wealth of evidence from retrospective studies, it can be concluded that there is a clear correlation between *DPYD* genotype, 5-FU metabolism and fluoropyrimidine-associated toxicity, which makes *DPYD* genotype-guided dose individualization the logical next step.
Weighing the available data on efficacy of *DPYD* genotype-guided dosing against the substantial decrease in risk of severe fluoropyrimidine-related toxicity that can be achieved, it is concluded to recommend *DPYD* genotype-guided screening and dose individualization of fluoropyrimidines.

CONCLUSIONS AND RECOMMENDATIONS ON A FLUOROPYRIMIDINE LABEL UPDATE

As fluoropyrimidine drugs such as capecitabine and 5-FU are the cornerstone in anticancer treatment of a variety of cancers, fluoropyrimidine-related toxicity remains a major clinical problem. It is known that carriers of the *DPYD* variants are at significantly increased risk of developing severe fluoropyrimidine-related toxicity. Strong evidence is currently available for an association with increased risk of severe toxicity for four *DPYD* variants (*DPYD**2A, c.1679T>G, c.2846A>T, and c.1236G>A/HapB3). Dose recommendations for these variants are available. When applying an initial dose reduction of fluoropyrimidine therapy, this risk of severe toxicity can be strongly reduced.
Despite a wealth of evidence, upfront testing for DPD deficiency in patients treated with fluoropyrimidines and dose individualization based on *DPYD* genotype is still not officially recommended and not included in the drug label. Also guidelines, including the ESMO consensus guideline for the management of patients with metastatic colorectal cancer, are reluctant in recommending upfront screening for DPD deficiency.[74] However, risk on severe fluoropyrimidine-related toxicity substantially decreases, without risk of underdosing, when upfront *DPYD* genotype screening followed by dose adjustments in variant allele carriers will become part of routine clinical practice. In this way, important anti-cancer drugs such as 5-FU and capecitabine do not have to be withheld from partially DPD deficient patients.
Based on the review of available data, adjusting the drug labels of capecitabine and 5-FU products is recommended. It is time to change standard of care and screening for DPD deficiency should be carried out before start of treatment and included in the label of fluoropyrimidine drugs. After reviewing our proposal, the EMA has now asked the involved pharmaceutical companies to update the SPC of fluoropyrimidines by including information on *DPYD*-genotyping and genotype-guided dosing.
Based on the currently available literature there is convincing evidence for genotype-guided screening for four *DPYD* variants (*DPYD**2A, c.2846A>T, c.1679T>G, and c.1236G>A). *DPYD*-genotyping is a pre-emptive test that can be easily carried out in clinical laboratories. In case of heterozygous carriers of these *DPYD* polymorphisms (partial DPD deficiency) an initial dose reduction should be recommended, consisting of dose recommendations for these four

polymorphisms, as shown in Table 1. These dose recommendations apply both to fluoropyrimidine monotherapy as well as combination therapy with other chemotherapeutic agents or radiotherapy. After initially reducing the fluoropyrimidine starting dose, further individual dose titration (upward or downward) based on tolerance is recommended, to guarantee maximum safe drug exposure in all patients. For patients with a complete DPD deficiency (no residual DPD activity, for example homozygous *DPYD**2A variant allele carriers) fluoropyrimidine treatment should remain contra-indicated and selection of alternative treatment is recommended.

DISCLOSURE

The authors have declared no conflicts of interest.

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Genotyping of dihydropyrimidine dehydrogenase

5

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

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SUMMARY

Background

The best-known cause of intolerance to fluoropyrimidines is dihydropyrimidine dehydrogenase (DPD) deficiency, which can result from deleterious polymorphisms in the gene encoding DPD (*DPYD*), including *DPYD**2A and c.2846A>T. Three other variants—*DPYD* c.1679T>G, c.1236G>A/HapB3, and c.1601G>A—have been associated with DPD deficiency, but no definitive evidence for the clinical validity of these variants is available. The primary objective of this systematic review and meta-analysis was to assess the clinical validity of c.1679T>G, c.1236G>A/HapB3, and c.1601G>A as predictors of severe fluoropyrimidine-associated toxicity.

Methods

We did a systematic review of the literature published before Dec 17, 2014, to identify cohort studies investigating associations between *DPYD* c.1679T>G, c.1236G>A/HapB3, and c.1601G>A and severe (grade ≥3) fluoropyrimidine-associated toxicity in patients treated with fluoropyrimidines (fluorouracil, capecitabine, or tegafur-uracil as single agents, in combination with other anticancer drugs, or with radiotherapy). Individual patient data were retrieved and analyzed in a multivariable analysis to obtain an adjusted relative risk (RR). Effect estimates were pooled by use of a random-effects meta-analysis. The threshold for significance was set at a *P* value of less than 0.0167 (Bonferroni correction).

Findings

7365 patients from eight studies were included in the meta-analysis. *DPYD* c.1679T>G was significantly associated with fluoropyrimidine-associated toxicity (adjusted RR 4.40, 95% CI 2.08–9.30, *P*<0.0001), as was c.1236G>A/HapB3 (1.59, 1.29–1.97, *P*<0.0001). The association between c.1601G>A and fluoropyrimidine-associated toxicity was not significant (adjusted RR 1.52, 95% CI 0.86–2.70, *P*=0.15). Analysis of individual types of toxicity showed consistent associations of c.1679T>G and c.1236G>A/HapB3 with gastrointestinal toxicity (adjusted RR 5.72, 95% CI 1.40–23.33, *P*=0.015; and 2.04, 1.49–2.78, *P*<0.0001, respectively) and hematological toxicity (adjusted RR 9.76, 95% CI 3.03–31.48, *P*=0.00014; and 2.07, 1.17–3.68, *P*=0.013, respectively), but not with hand-foot syndrome. *DPYD**2A and c.2846A>T were also significantly associated with severe fluoropyrimidine-associated toxicity (adjusted RR 2.85, 95% CI 1.75–4.62, *P*<0.0001; and 3.02, 2.22–4.10, *P*<0.0001, respectively).

Interpretation

DPYD variants c.1679T>G and c.1236G>A/HapB3 are clinically relevant predictors of fluoropyrimidine-associated toxicity. Upfront screening for these variants, in addition to the established variants *DPYD**2A and c.2846A>T, is recommended to improve the safety of patients with cancer treated with fluoropyrimidines.

INTRODUCTION

The fluoropyrimidines capecitabine, fluorouracil, and tegafur are the backbone of treatments for gastrointestinal, breast, and head and neck cancers. Of the patients treated with fluoropyrimidines, 10–30% have severe treatment-related toxicity, which is lethal in 0.5–1% of patients (with treatment-related mortality of up to 5% reported in elderly patients).[1–4] The most well known cause of intolerance to fluoropyrimidines is deficiency of the key enzyme for metabolism of fluorouracil, dihydropyrimidine dehydrogenase (DPD), encoded by the gene *DPYD*. DPD deficiency is detected in 39–61% of patients with severe toxicity, emphasizing its importance as a risk factor for severe toxicity.[5] The activity of DPD is regulated at the transcriptional level, including by transcription factors SP1 and SP3, and at the post-transcriptional level, for instance by microRNA 27-a (miR-27a) and microRNA 27-b.[5–8] A substantial proportion of the cases of DPD deficiency are, however, the result of deleterious polymorphisms in *DPYD*, which have therefore received widespread attention as predictors of fluoropyrimidine-associated toxicity.[9–18]

The most well established deleterious *DPYD* variants associated with fluoropyrimidine-associated toxicity are *DPYD**2A (IVS14+1G>A, c.1905+1G>A, or rs3918290) and c.2846A>T (D949V or rs67376798).[19,20] The results of several studies and a meta-analysis have shown strong associations between these variants—both with a frequency of heterozygotes of about 1% in white people—and fluoropyrimidine-associated toxicity.[9,10,12,13,21,22] Importantly, screening before treatment for *DPYD**2A, and a 50% reduction in starting dose given to patients who carry the variant allele heterozygously, results in therapeutic fluorouracil exposure and reduces the risk of severe toxicity, showing the clinical utility of upfront *DPYD* screening to prevent severe toxicity. Furthermore, this strategy of *DPYD* genotype-guided dosing in patients carrying *DPYD**2A was shown to be feasible in routine clinical practice and to be cost saving.[23]

Three other *DPYD* variants have been associated with altered DPD activity and fluoropyrimidine-associated toxicity—i.e., c.1679T>G, c.1236G>A, and c.1601G>A—but data on clinical validity are inconclusive. Conclusive evidence for clinical validity of *DPYD* variants is crucial before upfront screening and dose adjustments can be recommended as a strategy to improve safety of patients treated with fluoropyrimidines.

The variant c.1679T>G (I560S, *DPYD**13, or rs55886062) has a frequency of heterozygosity of about 0.2% in the white population,[10,12,24–26] and has been associated with reduced DPD activity in *in vitro* studies.[27] The Clinical Pharmacogenetics Implementation Consortium has recommended a 50% dose reduction for patients with this variant in heterozygous form.[28] However, because of the low frequency of c.1679T>G, the association between c.1679T>G and fluoropyrimidine-associated toxicity has not been shown definitively in any study.[10,12,14,16,29] More data on the clinical validity of this variant are therefore needed before advising upfront screening. For c.1236G>A (E412E or rs56038477), a synonymous variant that is in complete linkage with the deleterious deep intronic variant c.1129-5923C>G (rs75017182) in haplotype B3 (HapB3),[29,30] an association with fluoropyrimidine-associated toxicity has been shown in several studies,[14,29,30] but the results from other studies did not confirm these associations.[9,13,15,16,31] Data for the effect of c.1236G>A/HapB3 on DPD activity are inconclusive, and it therefore remains to be established whether a dose reduction should be recommended for patients with this variant.[28,30,32] A third variant, c.1601G>A (S534N, *DPYD**4, or rs1801158), has been associated with altered DPD activity,[27] and an increased risk of fluoropyrimidine-associated toxicity in one study,[16] but no significant association with toxicity was noted in other studies.[9,11,13,29,31,33]

Unlike the well-studied *DPYD* variants *DPYD**2A and c.2846A>T, data for clinical validity of c.1679T>G, c.1236G>A/HapB3, and c.1601G>A are inconsistent and no meta-analytic data are available. Therefore, we did a systematic review and meta-analysis using individual patient data from previous investigations to assess the clinical relevance of c.1679T>G, c.1236G>A/HapB3, and c.1601G>A as predictors of severe fluoropyrimidine-associated toxicity. The primary objective in this meta-analysis was to find out whether these *DPYD* variants are associated with severe (grade ≥3) fluoropyrimidine-associated toxicity, according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTC-AE), in patients treated with fluoropyrimidine-based anticancer regimens.

METHODS

Search strategy and selection criteria

We did a literature search of PubMed and Embase to identify studies reporting on associations between c.1679T>G, c.1236G>A/HapB3, and c.1601G>A and fluoropyrimidine-associated toxicity, published before Dec 17, 2014. Additionally, an unpublished pharmacogenetic analysis from our own institute, which investigated the association between *DPYD* variants and fluoropyrimidine-associated toxicity in 1606 patients, was also included in the analysis (Meulendijks, unpublished data). The following search terms were used for the literature search: "(DPYD OR DPD OR dihydropyrimidine dehydrogenase) AND (polymorphism OR Polymorphism, Single Nucleotide[mesh] OR Polymorphism, Genetic[mesh] OR pharmacogenet*[tiab] OR Pharmacogenetics[mesh] OR mutation/genetics OR genotype[mesh] OR polymorphisms OR variant OR variants OR SNP OR c.1236G>A OR E412E OR rs56038477 OR c.1129-5923C>G OR rs75017182 OR c.1601G>A OR S534N OR DPYD*4 OR rs1801158 OR c.1679T>G OR I560S OR rs55886062 OR DPYD*13) AND (toxicity OR adverse OR side-effects OR Antineoplastic Combined Chemotherapy Protocols/ adverse effects[mesh])".

All search results were screened by title and abstract, and full-text articles of potential relevance were retrieved and assessed. Reference lists were searched for additional relevant publications. Studies were eligible for inclusion if they met the following criteria: patients were treated with fluoropyrimidines (fluorouracil, capecitabine, or tegafur-uracil; as single agent or in combination with other anticancer drugs or with radiotherapy); patients were genotyped for c.1679T>G, c.1236G>A/HapB3, or c.1601G>A (for c.1236G>A/HapB3, both c.1236G>A and c.1129-5923C>G were a proxy for haplotype B3 and these variants were assumed to be in complete linkage based on published data[14,30] and our own unpublished data); the study had a cohort design (including secondary analyses of clinical trials) so as to allow appropriate estimation of the relative risk (RR); and toxicity was assessed and recorded according to the CTC-AE. If several studies reported on (or part of) the same patient population, patients were included in the analysis only once (i.e., the most extensive report was included). Studies were excluded from the primary analysis if any of the following was applicable: the patient population was selected on the basis of their toxicity phenotype or *DPYD* genotype status (if only some of the patients were selected on the basis of toxicity phenotype or *DPYD* status, these patients were excluded from the analysis), the study was reported in a language other than English, or none of the patients had any of the *DPYD* variants investigated. Review articles were excluded. For completeness, all identified case-control studies investigating the effect of *DPYD* variants on the risk of fluoropyrimidine-associated toxicity were selected for a secondary analysis (Supplement).

Data gathering

We aimed to gather all individual patient data from investigators who previously reported on associations between c.1679T>G, c.1236G>A/HapB3, or c.1601G>A and fluoropyrimidine-associated toxicity. The requested data consisted of the maximum toxicity per patient during the period studied by the investigators, patients' characteristics known to be relevant in relation to fluoropyrimidine-associated toxicity for use as covariables (preferably including age, sex, treatment regimen or concomitant chemotherapy, dose of the fluoropyrimidine, and renal function). If individual patient data could not be gathered, toxicity counts were extracted from the report. A descriptive analysis of the quality of the included studies was done independently by two investigators (LMH and DM) with the recommendations from Strengthening the Reporting of Genetic Association studies[34] and Human Genome Epidemiology Network[35] as guidelines. The reported results are based on consensus between the two investigators.

Statistical analysis

A summary of the statistical analysis is provided here (full details are provided in the Supplement). The primary endpoint was RR for any severe, CTC-AE grade 3 or greater fluoropyrimidine-associated toxicity in carriers of heterozygous or homozygous variant alleles compared with patients without the variant allele. A two-stage analysis approach was used. First, the endpoint was calculated for each individual study, based on individual patient data whenever available, with modified Poisson regression with adjustment for factors known to be associated with toxicity. Whenever available, the following covariables were included in the multivariable analysis: age, sex, fluoropyrimidine dose, renal function, and treatment regimen. If individual patient data could not be gathered, a crude RR was calculated using a 2 × 2 table, based on data extracted from the publication, and the crude RR was included in the analysis without correction for covariables. A zero-cell count continuity correction of 0.5 was applied if needed.[36] A dominant genetic model was applied because of the low frequency of homozygous variant genotypes.

In the second stage, RRs from the individual studies were combined by use of DerSimonian-Laird random-effects meta-analysis.[37] A random-effects model was chosen because true differences in effect size between patient populations, as a result of differences in patients' characteristics and treatment regimens, were assumed. Results were reported as RRs with their 95% CI and corresponding *P* values. Heterogeneity was assessed with Cochrane's *Q* test, with a threshold for the *P* value of less than 0.1 for significance, and the Higgins and Thompson I^2 statistic was assessed.[38] A Bonferroni correction for multiple testing of the three *DPYD* variants was applied—i.e., the threshold for significance for the primary endpoint was set at a *P* value of less than 0.0167. The same threshold for significance was used for analysis of subtypes of fluoropyrimidine-associated toxicity. The reported *P* values are unadjusted.

The effect of *DPYD* variants on risk of subtypes of fluoropyrimidine-associated toxicity—i.e., gastrointestinal toxicity, hematological toxicity, and hand-foot syndrome—was analyzed with a one-stage approach based on the retrieved individual patient data, with adjustment for age, sex, treatment regimen, and the study in which the patient was treated. To investigate the robustness of associations between *DPYD* variants and toxicity across patients' characteristics and treatment regimens, prespecified subgroups according to age, sex, and treatment regimen were assessed in the same pooled dataset. Statistical interaction terms between *DPYD* variants and patients' characteristics and treatment regimens were also assessed in this dataset.

Leave-one-out (leave-one-study-out) meta-analysis was done to assess robustness of findings in terms of the primary endpoint. Publication bias was assessed with Begg's funnel plots and Egger's regression test for funnel plot asymmetry. The effect of timeframe in which toxicity was assessed on the primary endpoint was investigated by comparison of the summary estimates from studies that assessed a short timeframe (shorter than the complete treatment duration) with studies that assessed a long timeframe (whole treatment duration) by use of metaregression.

Sensitivity and positive predictive value of the *DPYD* variants to predict severe fluoropyrimidine-associated toxicity were calculated for each individual study and subsequently combined using DerSimonian-Laird random-effects meta-analysis.[37] Frequencies of other established *DPYD* variants (*DPYD**2A and c.2846A>T) in groups of patients depending on c.1679T>G, c.1236G>A/HapB3, and c.1601G>A genotype were calculated whenever data for *DPYD**2A and c.2846A>T were available. Meta-analyses were repeated after excluding patients with either *DPYD**2A or c.2846A>T, to assess the potential effect of these variants on the results of the analysis. Additionally, meta-analysis was done for variants *DPYD**2A and c.2846A>T to compare effect sizes with those obtained for the investigated variants.

All statistical analyses were done in R (version 3.1.1). The PRISMA-individual patient data statement was used as a guideline for preparation of the final report.[39]

Role of the funding source

There was no funding source for this study. D Meulendijks, LM Henricks, and JHM Schellens had full access to the data and final responsibility to submit.

RESULTS

Figure 1 shows the selection process of studies investigating the associations of *DPYD* variants c.1679T>G, c.1236G>A/HapB3, and c.1601G>A with severe fluoropyrimidine-associated toxicity. Eight studies met the inclusion criteria (Table 1). These eight studies together included 7365 patients (Table 1). The c.1679T>G variant was measured in five studies (5616 patients), c.1236G>A/HapB3 in six studies (4261 patients), and c.1601G>A in five studies (3900 patients; Table 1). Individual patient data could be gathered from three (60%) of five studies for c.1679T>G (2535 patients), all six (100%) studies of c.1236G>A/HapB3 (4261 patients), and all five (100%) studies of c.1601G>A (3900 patients).

Three studies were prospective cohort studies, three were secondary analyses of randomized controlled trials, and two were retrospective cohort studies (Table 1). Patients were treated in Europe, the USA, and Australia, and ethnic origin, when stated, was predominantly white (Table 1). The median age of patients in the studies ranged between 58 years and 67 years, and slightly more men than women were enrolled in most studies (Table 1). Colorectal cancer was the most common type of tumor and patients most often received combination treatment including oxaliplatin (Table 1). The quality assessment of the included studies is summarized in the Supplement. Studies included in the main analysis scored positive on a mean of 8.5 of nine items. In all studies, the investigated endpoint was fluoropyrimidine-associated toxicity, although the toxicities that were scored varied between the studies, as did the timeframe in which toxicity was assessed (which varied between first cycle only and the full treatment duration; Supplement). The clinical data provided by the investigators and the covariables included in the multivariable analysis are also summarized in the Supplement.

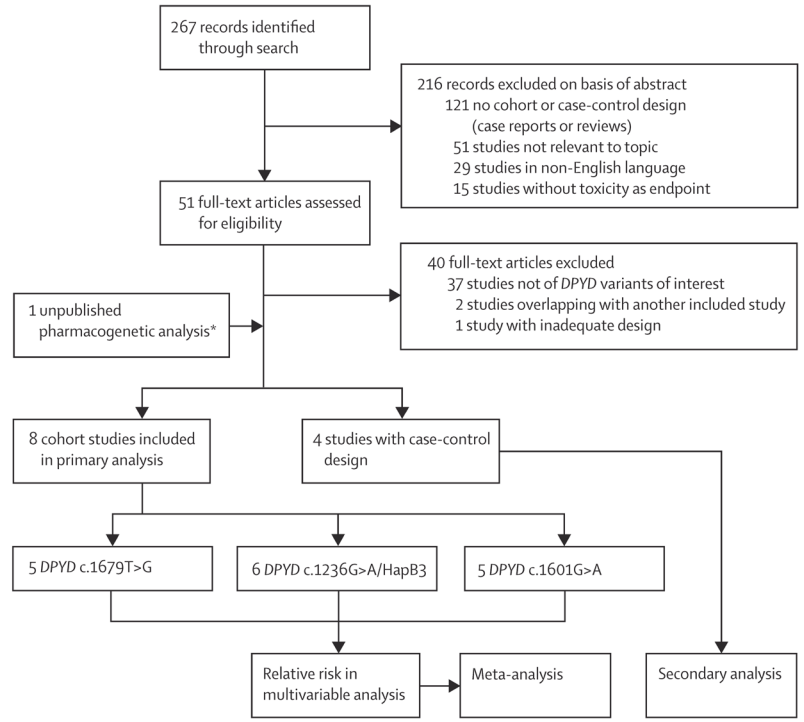

Figure 1. Flow diagram of study selection.
*A pharmacogenetic analysis was done in our own institute, the details of which will be reported separately.

Figure 2 shows the results of the primary analysis of the associations between *DPYD* variants c.1679T>G, c.1236G>A/HapB3, and c.1601G>A, and severe fluoropyrimidine-associated toxicity. Of 5616 patients included in the analysis of *DPYD* c.1679T>G, 11 (0.2%) were heterozygous. There was a significant association between c.1679T>G and global severe fluoropyrimidine-associated toxicity both before adjustment (RR 4.30, 95% CI 2.10–8.80, *P*<0.0001; Figure 2A).

Evidence of heterogeneity between the studies was substantial, possibly because of the small number of variant allele carriers. I^2 was 85%, and a *Q* test was significant (*Q* 26.67, *P*<0.0001). There was no indication of publication bias (Egger's regression test, *P*=0.16; Supplement). The leave-one-out sensitivity analysis showed that c.1679T>G remained associated with severe toxicity on exclusion of any of the studies (point estimates ranged from 3.20 to 6.01, with *P* values of less than 0.044; Supplement).

Analysis of the subtypes of fluoropyrimidine-associated toxicity showed a significant association between c.1679T>G and severe hematological toxicity (adjusted RR 9.76, 95% CI 3.03–31.48, *P*=0.00014), and also severe gastrointestinal toxicity was more frequent in individuals with the c.1679T>G variant allele (RR 5.72, 95% CI 1.40–23.33, *P*=0.015). None of the six individuals with the c.1679T>G variant allele in the pooled dataset had severe hand-foot syndrome, and therefore a RR for severe toxicity could not be calculated.

Table 1. Studies included in the primary analysis.

	Study design	Clinical data gathering	Ethnic origin; nationality	Number of patients	Age (years; median, range)	Men and women	Tumor type	Patients given capecitabine or fluorouracil	Treatment regimens	Investigated *DPYD* variants	Data used	Hardy-Weinberg equilibrium
Morel *et al.,* 2006 [10]	Cohort	Prospective	100% white; French	487	63 (23–88)	66% and 34%	Not reported (primarily colorectal cancer)	100% fluorouracil	35% fluorouracil + leucovorin or folinic acid, 20% FOLFIRI, 19% FOLFOX, 20% fluorouracil + platinum, and 6% FEC	c.1679T>G c.1601G>A (invariant and therefore not included)	Extracted from report	Not reported
Deenen *et al.,* 2011 [11]	RCT	Prospective	Not reported; Dutch	568[a]	63 (31–83)	61% and 39%	100% colorectal cancer	100% capecitabine	100% CAPOX-bevacizumab (with or without cetuximab)	c.1236G>A/ HapB3 c.1601G>A	Individual patient data	Yes
Jennings *et al.,* 2013 [15]	Cohort	Retrospective	Not reported; British	253	67 (23–88)	57% and 43%	100% colorectal cancer	63% capecitabine and 37% fluorouracil	23% capecitabine, 40% capecitabine + other drug, 25% fluorouracil, 12% fluorouracil + other drug	c.1236G>A/ HapB3	Individual patient data	Yes
Loganayagam *et al.,* 2013 [16]	Cohort	Retrospective	85% white, 12% African American or African Caribbean, and 4% Asian; British	430[b]	62 (20–83)	57% and 43%	85% colorectal cancer	57% capecitabine and 43% fluorouracil	36% CAPOX, 22% FOLFOX, 18% capecitabine, 24% capecitabine or fluorouracil + other	c.1679T>G c.1236G>A/ HapB3 c.1601G>A	Individual patient data	Yes
Rosmarin *et al.,* 2014 [13]	RCT	Prospective	Not reported; British, Australian, and Austrian	927[c]	65 (27–85)	57% and 43%	100% colorectal cancer	100% capecitabine	100% capecitabine with or without bevacizumab	c.1236G>A/ HapB3 c.1601G>A	Individual patient data	Yes
Lee *et al.,* 2014 [12]	RCT	Prospective	88% white, 7% African American or African Caribbean, and 5% Asian; North American	2594	58 (19–86)	53% and 47%	100% colorectal cancer	100% fluorouracil	100% FOLFOX (with or without cetuximab) or FOLFIRI (with or without cetuximab)	c.1679T>G	Extracted from report	Yes
Froehlich *et al.,* 2015 [14]	Cohort	Prospective	99% white, 1% Asian, African American, or African Caribbean; Swiss	500[d]	62 (18–99)	60% and 40%	55% colorectal cancer, 19% gastro-oesophageal cancer	21% capecitabine and 79% fluorouracil	35% FOLFOX, FOLFIRI, FOLFOXIRI, 20% fluorouracil (with or without leucovorin or folinic acid), 19% fluorouracil + platinum, 26% capecitabine or fluorouracil + other	c.1679T>G c.1236G>A/ HapB3 c.1601G>A[f]	Individual patient data	Yes
Meulendijks *et al.,* 2015 (unpublished)	Cohort	Prospective	96% white; Dutch	1606[e]	60 (21–89)	45% and 55%	53% colorectal cancer, 23% breast cancer, 14% gastric or gastro-oesophageal cancer	90% capecitabine and 10% fluorouracil	27% capecitabine + radiotherapy, 26% capecitabine, 24% capecitabine + platinum, 13% capecitabine + other, 10% fluorouracil-based	c.1679T>G c.1236G>A/ HapB3 c.1601G>A	Individual patient data	Yes

[a]*N*=568 for c.1236G>A/HapB3; *N*=481 for c.1601G>A. [b]*N*=425 for c.1236G>A/HapB3; *N*=430 for c.1601G>A and c.1679T>G. [c]*N*=909 for c.1236G>A/HapB3; *N*=888 for c.1601G>A. [d]500 patients were included prospectively in the analysis (15 patients selected on the basis of toxicity were excluded); data for 111 of 500 patients were reported by Amstutz and colleagues[29] in 2009 and are included only once. [e]*N*=1606 for c.1236G>A/HapB3, *N*=1601 for c.1601G>A, and *N*=1605 for c.1679T>G. [f]Data for c.1601G>A were not reported in original report.[14]
Abbreviations: CAPOX: capecitabine plus oxaliplatin; FEC: fluorouracil, epirubicin, and cyclophosphamide; FOLFIRI: fluorouracil, leucovorin, and irinotecan; FOLFOX: fluorouracil, leucovorin, and oxaliplatin; FOLFOXIRI: fluorouracil, leucovorin, oxaliplatin, and irinotecan; RCT: randomised controlled trial.

In the metaregression analysis to investigate the effect of timeframe, the effect of c.1679T>G on risk of severe toxicity seemed similar in studies with long and short timeframes (model coefficient for long vs short timeframe –0.76, 95% CI –2.28 to 0.76, *P*=0.33; Supplement).

Of 4261 patients who were included in the analysis of c.1236G>A/HapB3, 174 (4.1%) patients were heterozygous, and three (0.1%) patients were homozygous polymorphic. There was a significant association between c.1236G>A/HapB3 and global severe fluoropyrimidine-associated toxicity (unadjusted RR 1.72, 95% CI 1.22–2.42, *P*=0.0018; adjusted RR 1.59, 95% CI 1.29–1.97, *P*<0.0001; Figure 2B). Leave-one-out sensitivity analysis showed that the association was consistent on exclusion of the individual studies (*P*<0.006; Supplement). The point estimate ranged from 1.50 (with exclusion of Froehlich and colleagues' study[14]) to 1.72 (with exclusion of Rosmarin and colleagues' study[13]). There was little evidence for heterogeneity (I^2 23% and *Q* 6.52, *P*=0.26) and no indication of publication bias (Egger's regression test, *P*=0.99; Supplement). In terms of the subtypes of toxicity, c.1236G>A/HapB3 was most strongly associated with gastrointestinal toxicity (adjusted RR 2.04, 95% CI 1.49 to 2.78, *P*<0.0001) and hematological toxicity (2.07, 1.17 to 3.68, *P*=0.013). Like c.1679T> G, an association was not found between c.1236G>A/HapB3 and hand-foot syndrome (RR 1.11, 95% CI 0.70 to 1.77, *P*=0.65). The risk of severe hand-foot syndrome was also not increased in the subgroup of patients treated with capecitabine-based chemotherapy (RR 1.14, 95% CI 0.53 to 2.44; *P*=0.74). The effect of c.1236G>A/HapB3 on risk of toxicity seemed similar for studies assessing a long timeframe versus a short timeframe (model coefficient for long vs short timeframe –0.19, 95% CI –0.64 to 0.26; *P*=0.41; Supplement).

Of 3900 patients included in the analysis of c.1601G>A, 182 (4.7%) patients were heterozygous and two (0.1%) patients were homozygous. The primary analysis showed no significant association between c.1601G>A and global severe fluoropyrimidine-associated toxicity (unadjusted RR 1.69, 95% CI 0.78–3.65, *P*=0.15; adjusted RR 1.52, 95% CI 0.86–2.70, *P*=0.15; Figure 2C). We noted substantial between-study heterogeneity (I^2 91% and *Q* 42.48; *P*<0.0001), and a stronger effect size was noted in the study by Loganayagam and colleagues[16] than in the remaining studies (Figure 2C). Leave-one-out sensitivity analysis showed that heterogeneity dropped from 91% to 0% on exclusion of the study by Loganayagam and colleagues (Supplement). The calculated RR thereby dropped from 1.52 to 1.20 (*P*=0.11; Figure 2C; Supplement). There was no statistical evidence of publication bias (Egger's regression test, *P*=0.35) but Loganayagam and colleagues' study seemed to be an outlier in the funnel plot (Supplementary Figure 1). A mixed-effect meta-analysis incorporating between-study heterogeneity showed no significant effect of c.1601G>A (RR 1.13, 95% CI 0.79–1.60, *P*=0.50). Two (12.5%) of 16 patients with c.1601G>A in Loganayagam and colleagues' study[16] also had *DPYD**2A or c.2846A>T. Addition of the *DPYD**2A or c.2846A>T genotype to the regression model for Loganayagam and colleagues' study[16] slightly reduced the effect estimate for c.1601G>A, but it remained significant (RR 2.89, 95% CI 2.26–3.71, *P*<0.0001; Supplement). The effect of c.1601G>A on risk of toxicity seemed similar for studies with a long timeframe versus a short timeframe (log RR –0.44, –1.36 to 0.47; *P*=0.34; Supplement).

In the pooled dataset, a statistical interaction term between the study in which patients were treated and the effect of c.1601G>A was highly significant for Loganayagam and colleagues' study (*P*<0.0001), and on exclusion of the data from this study the association did not remain significant (*P*=0.13). Analysis of individual types of toxicity showed a strong association between c.1601G>A and severe gastrointestinal toxicity (RR 2.00, 95% CI 1.45–2.77, *P*<0.0001) and hematological toxicity (1.94, 1.16–3.27; *P*=0.12), but not hand-foot syndrome (0.86, 0.50–1.47; *P*=0.59). However,

Figure 2. Meta-analyses of studies investigating associations between *DPYD* variants and severe fluoropyrimidine-associated toxicity.
Abbreviations: RR: relative risk.

also in this analysis, there was a strong effect of Loganayagam and colleagues' study[16] and, on exclusion, none of the associations remained significant (RR 1.44, 95% CI 0.96–2.17, *P*=0.078 for gastrointestinal toxicity; 1.40, 0.86–2.17, *P*=0.31 for hematological toxicity; and 0.83, 0.48–1.45, *P*=0.50 for hand-foot syndrome).

We investigated the effects of patients' characteristics and treatment regimens on risk of severe fluoropyrimidine-associated toxicity in patients carrying c.1679T>G or c.1236G>A/HapB3 within the pooled dataset. No significant interaction was noted between c.1679T>G and age or c.1236G>A/HapB3 and age (*P*=0.38 and *P*=0.33, respectively) or between sex and c.1679T>G or sex and c.1236G>A/HapB3 (*P*=0.35 and *P*=0.33, respectively). Similarly, no significant interactions between the *DPYD* variants and treatment regimens were noted (data not shown). In a further subgroup analysis by patients' characteristics and treatment regimens, using the pooled dataset that included all data received from the investigators, the effect of *DPYD* variants c.1679T>G and c.1236G>A/HapB3 on risk of severe toxicity seemed to be fairly homogeneous (Figure 3). Carrier frequencies of *DPYD**2A and c.2846A>T were low among patients with c.1679T>G or c.1236G>A/HapB3 (0% and 0.6%, respectively), and somewhat higher in patients with c.1601G>A (2.7%; Supplement). Results of the meta-analysis after exclusion of patients with D*PYD**2A or c.2846A>T showed similar summary

estimates for the investigated variants, indicating that the overall effect of *DPYD**2A and c.2846A>T on the outcome of the analysis was small (Supplement). *DPYD**2A and c.2846A>T were both significantly associated with severe fluoropyrimidine-associated toxicity in the meta-analysis (RR 2.85, 95% CI 1.75–4.62, *P*<0.0001; and 3.02, 2.22–4.10, *P*<0.0001, respectively; Figure 4). For *DPYD**2A, the evidence for heterogeneity between the studies was strong: I^2 was 73%, and a *Q* test was significant (*Q* 21.8, *P*=0.0013). The evidence for heterogeneity between studies for c.2846A>T was also strong: I^2 was 80%, and a *Q* test was significant (*Q* 34.2, *P*<0.0001). The findings did not indicate publication bias for *DPYD**2A and c.2846A> T (Egger's regression test, *P*=0.49 and *P*=0.51, respectively).

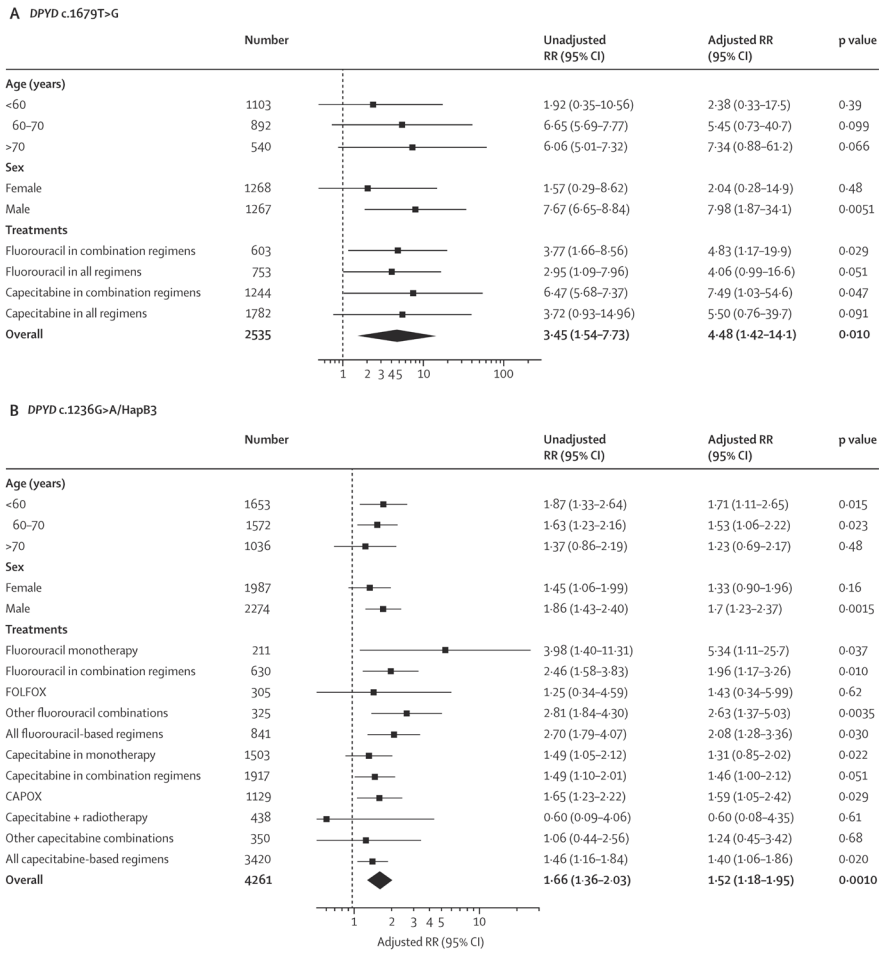

Figure 3. Effect of *DPYD* c.1679T>G (A) and c.1236G>A/HapB3 (B) in subgroups of patients.
One patient with c.1679T>G was treated with fluorouracil monotherapy and did not have severe toxicity (not shown in the figure because a RR could not be calculated). Similarly, one patient with c.1679T>G was treated with capecitabine monotherapy and did not have severe toxicity.
Abbreviations: RR: relative risk.

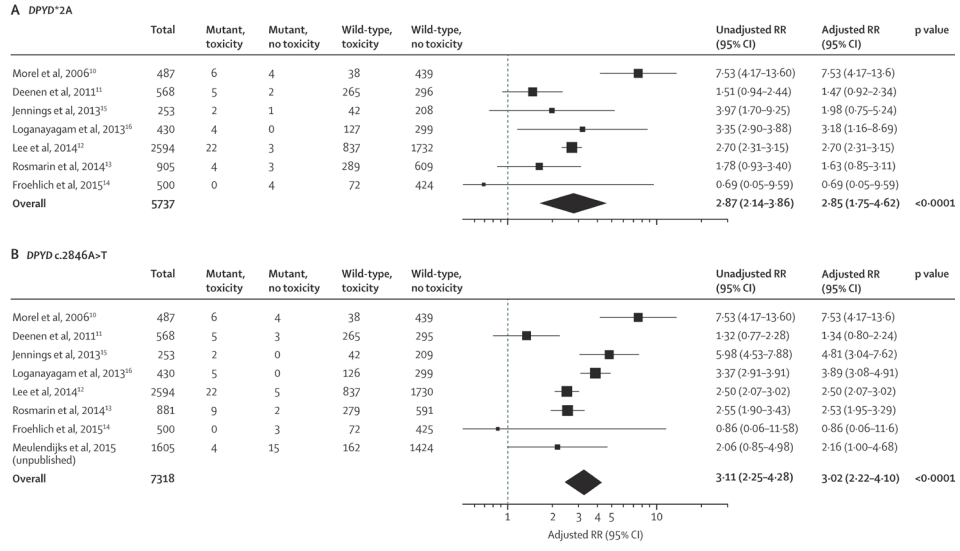

Figure 4. Meta-analysis of variants *DPYD2A (A) and c.2846A>T (B).**
Abbreviations: RR: relative risk.

The sensitivity of c.1679T>G in prediction of fluoropyrimidine-associated toxicity was estimated by meta-analysis as 0.3% (95% CI 0.0–0.6), whereas the sensitivity of c.1236G>A/HapB3 was 6.4% (4.2–8.6). The positive predictive value of c.1679T>G for severe toxicity was 46% (95% CI 5–87), and the positive predictive value of c.1236G>A/HapB3 was 41% (18–64).
In the secondary analysis of the four case-control studies (799 patients; Supplement), the summary effect estimates were similar to those from the primary analysis, but associations between the *DPYD* variants and global severe toxicity were not significant (Supplement).

DISCUSSION

The results of this analysis show that in addition to patients who are carriers of *DPYD**2A or c.2846A>T, patients who have the *DPYD* c.1679T>G or c.1236G>A/HapB3 variant alleles are at significantly increased risk of severe fluoropyrimidine-associated toxicity, confirming the clinical validity of these *DPYD* variants.
Substantial evidence exists of the clinical validity of *DPYD**2A and c.2846A>T, and current guidelines recommend a dose reduction of fluoropyrimidines in patients with these variants.[21,28] For c.1679T>G, until now only eight patients with this mutation had been described in a clinical setting (now 11 including this analysis).[10,12,14,16] The results of this meta-analysis show that the risk of global severe toxicity was increased about four times in patients with c.1679T>G. Risk of hematological and gastrointestinal toxicities were increased 9.8 and 5.7 times, respectively. Based on the available functional data for c.1679T>G, a heterozygous genotype is expected to result in a 40–50% decrease in DPD activity, similar to the effect of *DPYD**2A.[27,40,41] In view of DPD accounting for 80–90% of fluorouracil metabolism,[42] the 40–50% decrease in DPD activity is expected to result in a 50–100% increase in tissue exposure to fluorouracil. Indeed, systemic fluorouracil exposure

was shown to be 50% higher in *DPYD**2A carriers.[41] Based on the available functional data, and the clinical data presented here, we recommend a dose reduction of 50% in patients with c.1679T>G, in line with the recommendation by the Clinical Pharmacogenetics Implementation Consortium.[43]

Clinical validity of c.1236G>A/HapB3 has remained uncertain until now.[9,13–16,29–31] We found that c.1236G>A/HapB3, for which about 4% of the white patients are heterozygous, was significantly associated with risk of severe toxicity. The magnitude of the effect was smaller than that for c.1679T>G, which is what was expected based on the functional consequences of this variant.[30] Because c.1236G>A/HapB3 has a fairly high frequency, it provides fairly high sensitivity to identify patients at risk of severe toxicity. c.1236G>A is in complete linkage with the deleterious polymorphism c.1129-5923C>G in intron 10 (rs75017182), and both variants occur within haplotype B3.[14,30] c.1129-5923C>G results in aberrant pre-mRNA splicing—i.e., a 44-bp fragment is inadvertently inserted into mature mRNA, resulting in a premature stop codon.[30] Van Kuilenburg and colleagues[30] showed that although c.1129-5923C>G resulted in the formation of corrupt mRNA in a patient homozygous for c.1236G>A/HapB3, wild-type mRNA could still be detected in this patient. The production of normal mRNA was not completely abolished by c.1129-5923C>G in a homozygous patient, indicating that splicing efficiency to produce wild-type mRNA is reduced but not completely abolished. In agreement with this finding, we previously noted that DPD activity in two patients with c.1236G>A/HapB3 in homozygous form was reduced by about 50%, and not completely impaired.[47] A homozygous genotype of *DPYD**2A, by contrast, results in complete DPD deficiency (about 0% activity).[20] These data show that c.1236G>A/HapB3 results in about half the reduction in DPD activity compared with *DPYD**2A (or c.1679T>G). This finding, combined with the presented data for the association between c.1236G>A/HapB3 and fluoropyrimidine-associated toxicity, lends support to an upfront dose reduction of 25% in patients with this variant in heterozygous form, which we expect normalizes fluorouracil exposure and risk of fluoropyrimidine-associated toxicity.[43] Few data exist about the safety of fluoropyrimidine treatment in patients homozygous for c.1236G>A/HapB3 and great caution should be used when administering fluoropyrimidines to these patients. We expect that a 50% reduced dose will usually be tolerated because we previously treated three patients homozygous for c.1236G>A/HapB3 safely with low doses of capecitabine (825 mg/m² twice a day).[47] Importantly, after *DPYD* genotype-guided dose reduction, subsequent dose-titration upward (starting in cycle two or three) is strongly recommended if deemed safe based on tolerability or therapeutic drug monitoring, to avoid underdosing of patients who might be able to tolerate higher doses.

In the secondary analysis of case-control studies, the effect estimates for c.1236G>A/HapB3 and c.1601G>A were similar to those in the primary analysis. For c.1601G>A, both the primary and the secondary analyses showed no significant association with severe toxicity. Unlike the results of the primary analysis, the association between c.1236G>A/HapB3 and severe toxicity was not significant in the analysis of case-control studies. This non-significance is most likely explained by a much smaller number of patients being included in the secondary analysis (799 vs 4261 patients in the primary analysis).

Although the risks of severe gastrointestinal and hematological toxicity were increased in c.1679T>G and c.1236G>A/HapB3 carriers, the risk of hand-foot syndrome was not. This finding could indicate that there is a weaker association between *DPYD* variants and occurrence of hand-foot syndrome, but could also be the result of severe hand-foot syndrome generally occurring at later cycles of fluoropyrimidine treatment than do severe gastrointestinal and hematological toxicities (cycle three vs cycle one or two, respectively; Meulendijks, unpublished data), and the timeframe in which toxicity was monitored was short for some of the studies. Additionally, treatment modifications for gastrointestinal or hematological toxicity might affect the risk of severe hand-foot syndrome in later cycles.

For c.1601G>A, little evidence exists for an association with toxicity, and strong evidence exists for between-study heterogeneity. The results of most larger studies of patients with c.1601G>A have shown small, non-significant, increases in risk of fluoropyrimidine-associated toxicity. Although c.1601G>A has been detected in patients with DPD deficiency,[26] a functional analysis with an established *in vitro* cellular system showed that c.1601G>A was associated with an increase in DPD activity instead of a decrease.[27] The investigators therefore proposed that c.1601G>A could have a protective effect on fluoropyrimidine-associated toxicity. Our results do not suggest, however, a protective effect. The RR (1.52, 95% CI 0.86–2.70) indicates that a protective effect with a RR of less than 0.86 is unlikely. The stronger effect for c.1601G>A in the study by Loganayagam and colleagues[16] could partly—but not completely—be explained by the presence of other *DPYD* variants. Other possible confounding factors related to risk of toxicity, including patient and treatment-related factors, or the concomitant presence of other genetic polymorphisms associated with toxicity, or which interact with *DPYD*, contributed to the large effect size in this study.[6,16] Of interest in this respect are polymorphisms in *MIR27A*, the gene encoding miR-27a, which has been shown to regulate DPD activity in human beings.[7] Amstutz and colleagues[6,7] showed that rs895819, a polymorphism known to increase miR-27a expression and reduce DPD activity, strongly increased patients' risk of fluoropyrimidine-associated toxicity when present in combination with *DPYD* variants. The results of their study showed that in patients who had both a *DPYD* variant and rs895819, incidence of severe fluoropyrimidine-associated toxicity was strongly increased (12 [71%] of 17 patients), whereas in patients who were carriers of a *DPYD* variant but not rs895819, incidence of severe fluoropyrimidine-associated toxicity was average (five [25%] of 20 patients). These findings, which suggest that genotyping of *MIR27A* in conjunction with *DPYD* variants can lead to a substantially higher positive predictive value for identifying patients at risk of severe toxicity, were confirmed in a second cohort of 1592 patients (Meulendijks, unpublished data). We believe it is therefore likely that the diagnostic accuracy of *DPYD* genotyping could be further improved by combining *DPYD* genotyping with *MIR27A* genotyping. Although definitive evidence of clinical validity is needed before clinical implementation, *MIR27A* genotyping should be included in future studies of the clinical validity and clinical utility of *DPYD* genotype-guided dosing of fluoropyrimidines.

A strength of the current analysis is that we were able to retrieve most of the available individual patient data and analyze the data in a multivariable analysis, thereby adjusting for other relevant factors associated with toxicity. The risk estimates obtained from the analysis with a random-effects model indicate the mean risk ratios that are likely to occur in other patient populations treated with fluoropyrimidines, and the results of this analysis therefore can most likely be extrapolated to other clinical settings. However, the frequency of variants c.1679T>G and c.1236G>A/HapB3 might differ depending on ethnic origin. For instance, c.1236G>A/HapB3 was absent in Japanese and Korean populations, indicating that clinical utility might be lower in non-white populations.[18] Reliable frequency data for c.1679T>G in non-white populations are not available. Further research needs to be done in patient populations of other ethnic origins to

establish the clinical value of *DPYD* genotypes as predictors of fluoropyrimidine-associated toxicity in these populations. The dosing recommendations proposed for c.1679T>G and c.1236G>A/HapB3 are based on a small amount of functional data, in addition to the clinical data reported here. To establish more definitively the optimum starting doses, a comprehensive pharmacokinetic–pharmacodynamic modelling approach in a sufficiently large number of patients is needed.

We investigated the effect of timeframe in which toxicity was assessed on the primary endpoint (Supplement). This analysis showed that with both long and short timeframes, an effect of c.1679T>G and c.1236G>A/HapB3 on risk of severe toxicity was notable. Effect estimates for all three *DPYD* variants were non-significantly lower for long timeframes than with short timeframes, most likely as a result of the ability to detect an increased risk of toxicity in variant allele carriers decreases with an increasing proportion of patients in the control group having at least one severe adverse event (this rate will increase with longer treatment). The relative risk will gradually trend towards 1 (no difference) as a result. This effect can, therefore, only result in an underestimation of the effect of the *DPYD* risk variants. The results of the analysis show, however, that the impact of this effect on the overall conclusions was small. Although our data show that *DPYD* variants can be used to identify patients with DPD deficiency at risk of fluoropyrimidine-associated toxicity, a negative test for specific *DPYD* variants does not guarantee that a patient is DPD proficient. That is, DPD deficiency cannot always be traced back to a (currently known) genetic alteration in *DPYD* associated with reduced enzyme activity. An upfront screening strategy with *DPYD* genotyping alone therefore has little sensitivity to identify patients at risk. An estimated half of patients with DPD deficiency can be identified by screening for the four *DPYD* variants for which clinical validity has now been established, although a reliable estimate is not available.[17,18] A combined *DPYD* genotyping and DPD phenotyping approach is likely to substantially improve sensitivity of the upfront test.[44] Definitive evidence on clinical validity of phenotyping tests is not yet available, however. The value of DPD phenotyping is being investigated in two ongoing prospective clinical studies (NCT01547923 and NCT02324452). Additional screening approaches might be useful, including *MIR27A* genotyping, as described, or possibly screening of mutations in *TYMS*.[13,45]

One of the common concerns in meta-analysis is the issue of publication bias.[46] However, we assessed this in our study, and there was little indication for an effect of publication bias on the conclusions drawn from this analysis.

In conclusion, our analysis confirms the clinical validity of *DPYD* variants c.1679T>G and c.1236G>A/HapB3, in addition to *DPYD**2A and c.2846A>T, as predictors of fluoropyrimidine-associated toxicity. The magnitude of effect of c.1679T>G is in the same range as that of *DPYD**2A, and a dose reduction of 50% is advised for individuals with variant alleles.[28] The effect of c.1236G>A/HapB3 on risk of toxicity is smaller than for *DPYD**2A or c.1679T>G, in accordance with the functional effect of this variant.[30] A dose reduction of 25% is rational in heterozygous carriers of c.1236G>A/HapB3, occurring in about 4% of white patients, and we recommend adding c.1236G>A/HapB3 to the guideline on dosing recommendations for *DPYD* variants.[28] Clinical validity has now been established for four *DPYD* variants—*DPYD**2A, c.2846A>T, c.1679T>G, and c.1236G>A/HapB3—and upfront screening for these mutations with dose adaptation in variant allele carriers is advised to improve safety of patients treated with fluoropyrimidines. As upfront screening for one *DPYD* variant has been shown to be feasible and cost saving in routine clinical practice, with improved safety, it is likely that upfront screening for an extended panel of *DPYD* variants will further improve the safety of the large group of patients treated with fluoropyrimidines.

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DECLARATION OF INTERESTS

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SUPPLEMENT

SUPPLEMENTARY METHODS

Statistical analysis (detailed)

The primary endpoint of the analysis was relative risk (RR) for global (any) severe, CTC-AE grade ≥3, fluoropyrimidine-associated toxicity in heterozygous or homozygous variant allele carriers compared to wild type patients, after adjustment for other factors known to be associated with toxicity. Toxicity was dichotomized as none to moderate (grade 0–2) versus severe (grade ≥3). Global toxicity was defined as the maximum toxicity score derived from the fluoropyrimidine-associated toxicity data provided by the investigators. Global toxicity included a selection of fluoropyrimidine-associated toxicities, as detailed in Supplementary Table 1.

A two-stage analysis approach was used. First, the endpoint was calculated for each individual study, based on individual patient data (IPD) whenever available, using modified Poisson regression, in multivariable analysis with adjustment for other factors known to be associated with toxicity. The following covariates were included in the models, whenever available: age (continuous), sex, dose of the fluoropyrimidine (expressed as the fraction of the maximum dose, range: 0–1), treatment regimen (grouped as 5-FU monotherapy, 5-FU plus cisplatin, 5-FU plus oxaliplatin, 5-FU plus irinotecan, 5-FU in triplet combination, 5-FU plus other, capecitabine monotherapy, capecitabine plus oxaliplatin, capecitabine in triplet combination, capecitabine plus other, and 5-FU/capecitabine plus radiotherapy), and renal function (expressed as MDRD-4).[1–3] Supplementary Table 1 lists the covariates that were available for each study and included in the analysis. A modified Poisson regression approach was used to calculate the RR in multivariable analysis, as described by Zou.[4] Standard errors were calculated using robust variance estimation using the 'coeftest' function from package 'lmtest' in R v3.1.1.

If IPD could not be collected, a crude RR was calculated using a 2x2 table based on data extracted from the publication, and this RR was included in the primary analysis without correction for covariates. Data were extracted from the report independently by two investigators (LMH and DM), and data were compared and discrepancies resolved. A zero-cell count continuity correction of 0.5 was applied if required.[5]

A dominant genetic model was applied for all investigated *DPYD* variants, in view of the low frequency of homozygous variant genotypes.

In the second stage, RRs derived from the individual studies were combined using DerSimonian and Laird random-effects meta-analysis.[6] 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. Results were reported as RRs with their 95% confidence interval (CI) and corresponding *P* values. Heterogeneity was assessed using Cochrane's *Q* test, with a threshold of $P<0.1$ for significance, and the Higgins and Thompson I^2 statistic was assessed in addition.[7] A Bonferroni correction for multiple testing for three *DPYD* variants was applied, *i.e.* the formal significance level for the primary endpoint was set at $P<0.0167$. The same threshold for significance was used for analysis of subtypes of fluoropyrimidine-associated toxicity. The reported *P* values are unadjusted.

The effect of *DPYD* variants on risk of subtypes of fluoropyrimidine-associated toxicity, *i.e.* gastrointestinal toxicity, hematological toxicity, and hand-foot syndrome (HFS), was analyzed in the subset of data which had been provided by the investigators. A one-stage approach, in which

Supplementary Table 1. Endpoints used in individual studies and covariates used in current analysis.

Study	Endpoint	Toxicity window	Covariates available	Covariates included in analysis
Cohort studies included in primary analysis				
Morel *et al.* 2006	grade ≥3 toxicity of selected fluoropyrimidine-associated toxicities: diarrhea, mucositis, neutropenia, thrombocytopenia, neurotoxicity, cardiac toxicity, and HFS	First two cycles	None (data extracted from publication)	None (data extracted from publication)
Deenen *et al.* 2011	grade ≥3 toxicity of selected fluoropyrimidine-associated toxicities: diarrhea, neutropenia, mucositis, HFS, nausea/vomiting [a]	Whole treatment duration	Age, gender, MDRD	Age, gender, MDRD
Jennings *et al.* 2013	grade ≥3 toxicity of selected fluoropyrimidine-associated toxicities: gastrointestinal symptoms, mucositis/stomatitis, HFS, cardiac toxicity, neutropenia, anemia, thrombocytopenia, abnormal liver function tests	First 12 weeks	Age, gender, treatment regimen	Age, gender, treatment regimen
Loganayagam *et al.* 2013	grade ≥3 toxicity of selected fluoropyrimidine-associated toxicities: diarrhea, neutropenia, mucositis, HFS	First four cycles	Age, gender, MDRD, starting dose capecitabine, treatment regimen	Age, gender, MDRD, starting dose capecitabine, treatment regimen [b]
Rosmarin *et al.* 2014	grade ≥3 toxicity of selected fluoropyrimidine-associated toxicities: diarrhea, nausea/vomiting, mucositis/stomatitis, neutropenia, thrombocytopenia, HFS	Whole treatment duration	Age, gender	Age, gender
Lee *et al.* 2014	grade ≥3 toxicity of selected fluoropyrimidine-associated toxicities: fatigue, anorexia, dehydration, diarrhea, stomatitis/mucositis, nausea/vomiting, leukocytopenia, neutropenia, febrile neutropenia, thrombocytopenia, pain	Whole treatment duration	None (data extracted from publication)	None (data extracted from publication)
Froehlich et al. 2015 (Amstutz et al. 2009) [c]	grade ≥3 toxicity of selected (fluoropyrimidine-associated) toxicities: febrile neutropenia, leukocytopenia, thrombocytopenia, anemia, diarrhea, mucositis, nausea/vomiting, localised infection, dehydration, HFS, hair loss, dry skin	First two cycles	Age, gender, treatment regimen	Age, gender, treatment regimen
Meulendijks *et al.* 2015	grade ≥3 toxicity of selected fluoropyrimidine-associated toxicities: neutropenia, leukocytopenia, thrombocytopenia, diarrhea, mucositis, HFS, cardiological toxicity	First cycle	Age, gender, treatment regimen	Age, gender, treatment regimen
Case-control studies included in secondary analysis				
Kuilenburg *et al.* 2010	grade ≥3 toxicity of selected fluoropyrimidine-associated toxicities: neutropenia, thrombocytopenia, mucositis, diarrhea, nausea/vomiting, neurotoxicity, cardiac toxicity, alopecia, HFS	First three cycles	Age, gender, treatment regimen	Age, gender, treatment regimen
Schwab *et al.* 2008	grade ≥3 toxicity of selected fluoropyrimidine-associated toxicities: leukocytopenia, diarrhea, and mucositis	Whole treatment duration	Gender, treatment regimen	Gender (regimen not included because all regimens were monotherapy 5-FU)
Gross *et al.* 2008	grade ≥3 toxicity of selected fluoropyrimidine-associated toxicities: neutropenia, thrombocytopenia, mucositis, diarrhea, nausea/vomiting, neurotoxicity, cardiac toxicity, alopecia, HFS	First three cycles	Age, gender, treatment regimen	Age, gender, treatment regimen
Kleibl *et al.* 2009	grade ≥3 toxicity of selected fluoropyrimidine-associated toxicities: mucositis, diarrhea, nausea/vomiting, leukocytopenia, neutropenia, thrombocytopenia	First two cycles	Age, gender, treatment regimen	Age, gender, treatment regimen

Abbreviations: HFS: hand-foot syndrome; MDRD: Modification of Diet in Renal Disease (estimated glomerular filtration rate).
[a] Original study had diarrhea as endpoint, as well as overall toxicity (which included all toxicities, irrespective of relation to study treatment). The fluoropyrimidine-associated toxicities that are listed in the table were used as the endpoint in this study.
[b] Regimen not included for c.1679T>G, due to low number of patients.
[c] The data included in the Froehlich *et al.* dataset was an extension of the dataset used by Amstutz *et al.* and therefore the Froehlich *et al.* dataset was used for this analysis.

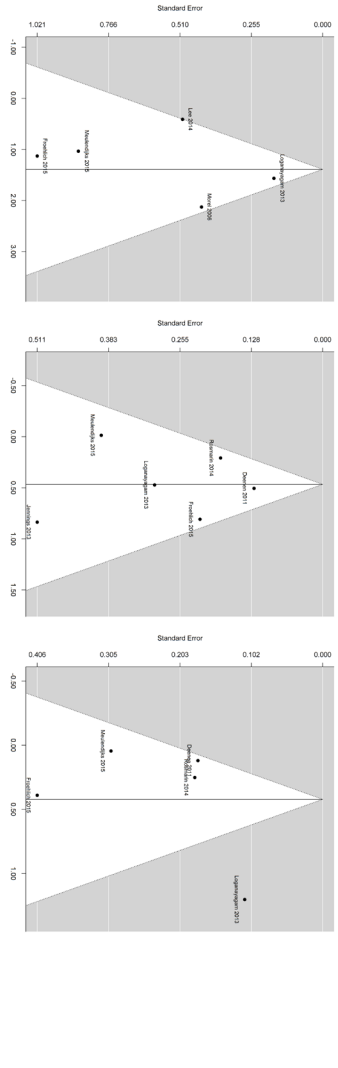
the retrieved data were pooled into one dataset, was used for this analysis. Individual types of fluoropyrimidine-associated toxicity (gastrointestinal toxicity, hematological toxicity, and hand-foot syndrome) included the following toxicity items, whenever available (and as shown in Supplementary Table 1): gastrointestinal toxicity included diarrhea, mucositis/stomatitis, and nausea/vomiting; hematological toxicity included thrombocytopenia, neutropenia, leukocytopenia, and anemia. Hand-foot syndrome was considered separately.

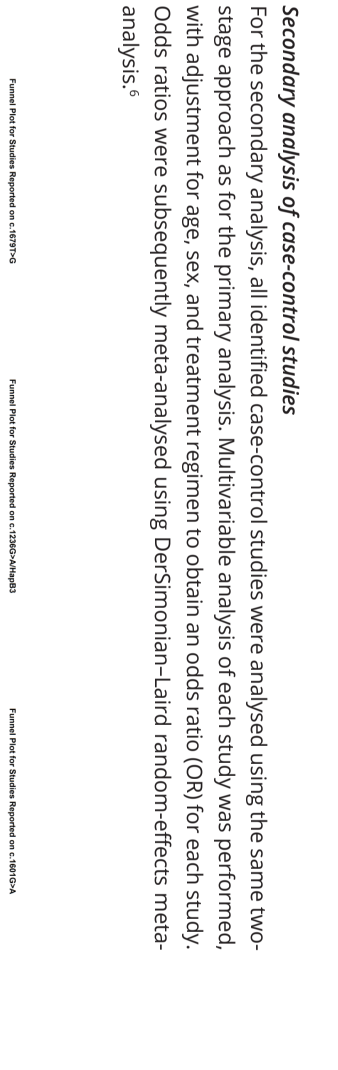
Modified Poisson regression was used to obtain RRs from multivariable analysis, with adjustment for age, sex, treatment regimen (being the covariates that were available for every study included in the analysis), as well as for the study in which the patient was treated. To allow merging of the dataset for analysis of the pooled data, treatment regimens were re-categorized, as follows: capecitabine monotherapy, capecitabine plus cisplatin/carboplatin, capecitabine plus oxaliplatin, capecitabine-based triplet combination, capecitabine plus radiotherapy, capecitabine plus other, 5-fluorouracil monotherapy, 5-fluorouracil plus cisplatin/carboplatin, 5-fluorouracil plus oxaliplatin, 5-fluorouracil plus irinotecan, 5-fluorouracil-based triplet combination, and 5-fluorouracil plus other. To investigate the robustness of associations between *DPYD* variants and toxicity across different treatment regimens, pre-specified subgroups according to age, sex, and the treatment regimen administered were investigated in the same pooled dataset. Statistical interaction terms between *DPYD* variants and patient and treatment characteristics were also assessed in the pooled dataset.

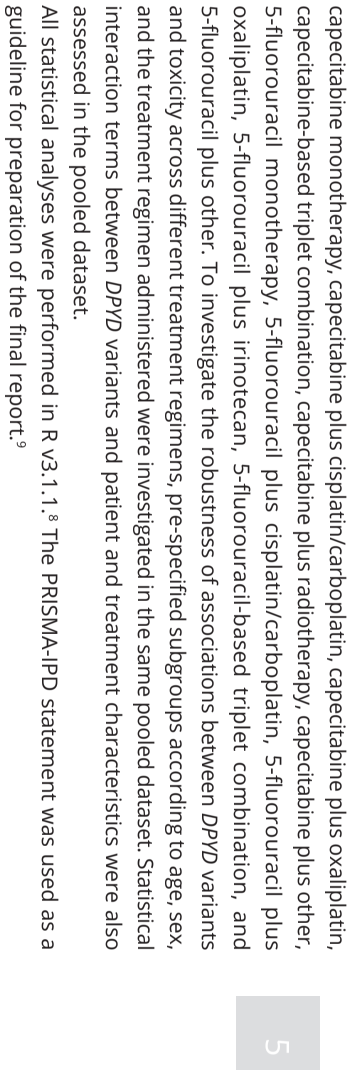
All statistical analyses were performed in R v3.1.1.[8] The PRISMA-IPD statement was used as a guideline for preparation of the final report.[9]

Secondary analysis of case-control studies

For the secondary analysis, all identified case-control studies were analysed using the same two-stage approach as for the primary analysis. Multivariable analysis of each study was performed, with adjustment for age, sex, and treatment regimen to obtain an odds ratio (OR) for each study. Odds ratios were subsequently meta-analysed using DerSimonian–Laird random-effects meta-analysis.[6]







Supplementary Figure 1. Funnel plots to assess publication bias.

Supplementary Table 2. Quality assessment of studies included in the meta-analysis.

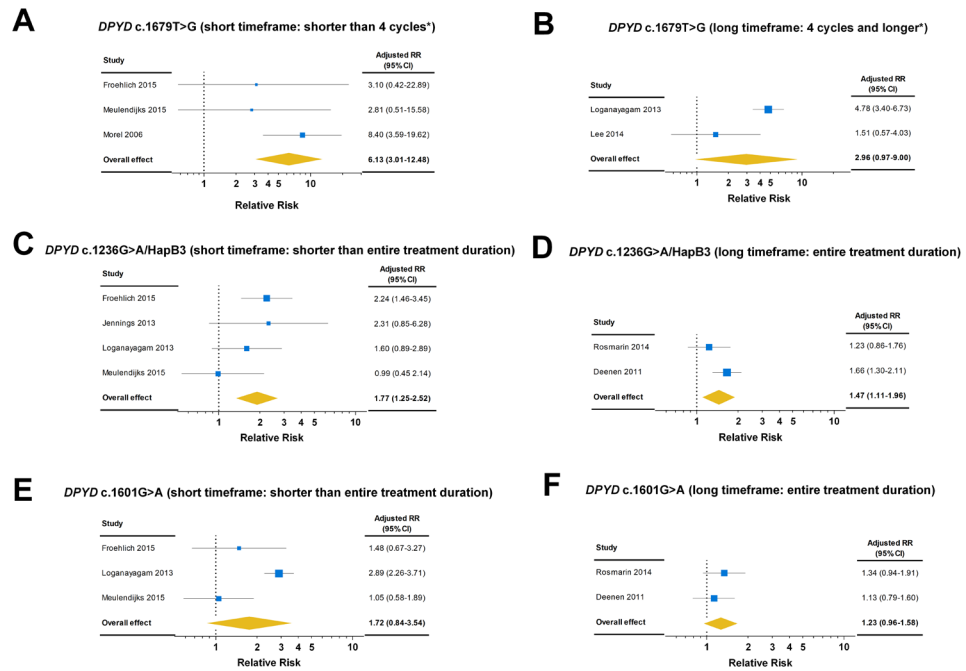
Study	Objectives and hypothesis stated	Study design clear	Eligibility criteria for participants stated	Ethnicity stated	Clear definition of endpoint	Assessment of HWE	Sufficient data on patient/ treatment characteristics [a]	Genotype frequencies stated	Endpoints clearly stated
Cohort studies included in primary analysis									
Morel *et al.* 2006	+	+	+	+	+	-	+	+	+
Deenen *et al.* 2011	+	+	+	-	+	+	+	+	+
Jennings *et al.* 2013	+	+	+	-	+	+	+	+	+
Loganayagam *et al.* 2013	+	+	+	+	+	+	+	+	+
Rosmarin *et al.* 2014	+	+	+	-	+	+	+	+	+
Lee *et al.* 2014	+	+	+	+	+	+	+	+	+
Froehlich *et al.* 2015	+	+	+	+	+	+	+	+	+
Meulendijks *et al.* 2015	+	+	+	+	+	+	+	+	+
Case-control studies included in secondary analysis									
Kuilenburg *et al.* 2010	+	+/-	-	-	+/-	-	+	+	+
Schwab *et al.* 2008	+	+	+	-	+	+	+	+	+
Gross *et al.* 2008	+	+	+	-	+	-	+	+	+
Kleibl *et al.* 2009	+	+	+	+	+	+	+	+	+

Abbreviations: HWE: Hardy-Weinberg equilibrium.
[a] *i.e.* factors known to be associated with toxicity: age, gender, other concomitant chemotherapy.

SUPPLEMENTARY RESULTS

Effect of timeframe in which toxicity was assessed on the primary endpoint

Additional exploratory analyses were performed to investigate the effect of timeframe in which toxicity was assessed on the primary endpoint. This was done by comparing summary estimates from studies that assessed a short timeframe (shorter than the complete treatment duration) with studies that assessed a long timeframe (whole treatment duration). This exploratory analysis showed that for the *DPYD* variants identified as clinically relevant (c.1679T>G and c.1236G>A/HapB3), an effect on risk of severe toxicity was notable both when considering a short, and when considering a long timeframe (Supplementary Figure 2). Meta-regression showed that effect estimates were reduced non-significantly for long versus short timeframe: -0.52, *P*=0.46 (for c.1679T>G); -0.19, *P*=0.41 (for c.1236G>A/HapB3); -0.44, *P*=0.34 (for c.1601G>A).



Supplementary Figure 2. Results of analysis to determine the effect of timeframe in which toxicity was assessed.
* For c.1679T>G, the studies had to be grouped differently in order to have at least two studies per group.

Analysis of the effect of DPYD*2A and c.2846A>T genotypes in the Loganayagam 2013 study

As can be seen in Supplementary Table 4, 13% of the patients with the c.1601G>A variant in the Loganayagam 2013 study carried *DPYD**2A or c.2846A>T (2/16 patients; one carried *DPYD**2A, one carried c.2846A>T). This could explain the effect estimate from this study that appeared to deviate from the overall estimate. To investigate this, *DPYD**2A/c.2846A>T genotype was added to the regression model for the Loganayagam study. The adjusted RR that was calculated originally was 3.33 (95%CI: 2.68–4.14, *P*<0.0001). After addition of *DPYD**2A/c.2846A>T genotype (mutant *vs.* wild type), the RR for c.1601G>A was 2.89 (95%CI: 2.26–3.71, *P*<0.0001), while the estimate for *DPYD**2A/c.2846A>T genotype was 2.75 (95%CI: 1.89–4.02, *P*<0.0001). This analysis shows that although the strong effect of c.1601G>A was slightly modified by other *DPYD* genotypes, this did not completely explain the greater effect size observed for the Loganayagam study.

Results of the meta-analyses when patients carrying DPYD*2A or c.2846A>T were excluded

When patients identified as carrying *DPYD**2A or c.2846A>T were excluded from the analysis, the following summary estimates were obtained:

- RR 4.63 (95%CI: 3.33–6.45, *P*<0.0001) for c.1679T>G
- RR 1.61 (95%CI: 1.32–1.96, *P*<0.0001) for c.1236G>A/HapB3
- RR 1.50 (95%CI: 0.93–2.40, *P*=0.094) for c.1601G>A

Supplementary Table 3. Leave-one-out sensitivity analysis.

***DPYD* c.1679T>G**

	RR	95%CI	SE	*z* value	*P* value	Q	*P* value *Q*-test	tau²	I²	H²
Morel 2006	3.20	1.66–6.17	0.3352	3.4677	0.0005	5.0092	0.1711	0.1819	40.1098	1.6697
Loganayagam 2013	3.42	1.30–8.95	0.4913	2.5001	0.0124	6.9000	0.0752	0.5174	56.5219	2.3000
Lee 2014	5.01	3.68–6.81	0.1571	10.2591	<0.0001	2.1523	0.5414	<0.0001	<0.0001	1.0000
Froehlich 2015	4.03	2.10–7.72	0.3321	4.1942	<0.0001	7.2292	0.0649	0.2402	58.5014	2.4097
Meulendijks 2015	4.10	2.12–7.91	0.3353	4.2058	<0.0001	7.0687	0.0697	0.2384	57.5594	2.3562

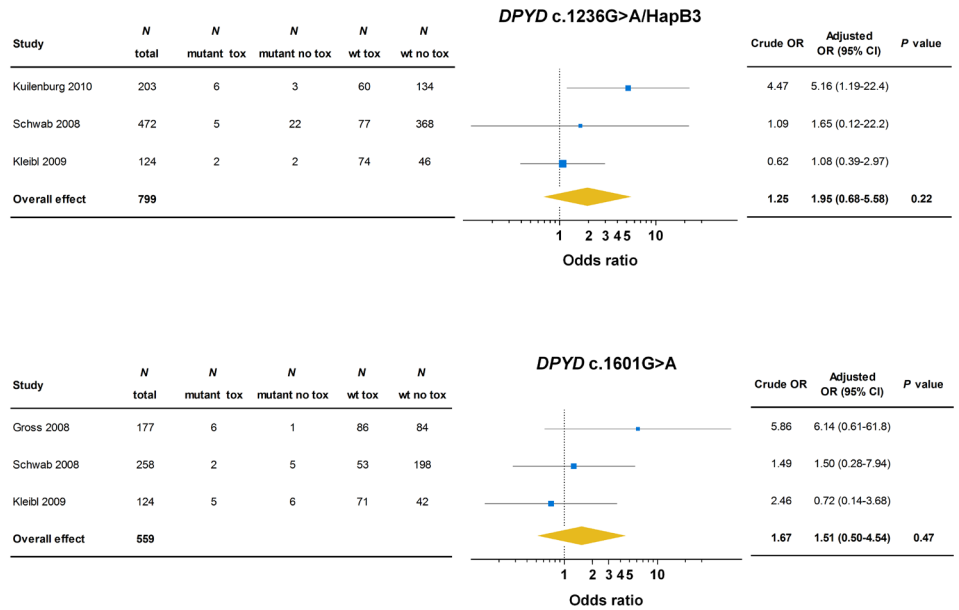
***DPYD* c.1236G>A/HapB3**

	RR	95%CI	SE	*z* value	*P* value	Q	*P* value *Q*-test	tau²	I²	H²
Deenen 2011	1.56	1.00–2.14	0.1607	2.7818	0.0054	6.3549	0.1742	0.0461	37.0561	1.5887
Jennings 2013	1.56	1.23–1.96	0.1163	3.8467	0.0001	5.9887	0.2000	0.0219	33.2076	1.4972
Loganayagam 2013	1.59	1.34–2.05	0.1304	3.5571	0.0004	6.5218	0.1634	0.0308	38.6668	1.6304
Rosmarin 2014	1.72	1.26–2.07	0.0961	5.6281	<0.0001	3.9256	0.4162	<0.0001	<0.0001	1.0000
Froehlich 2015	1.50	1.25–1.80	0.0922	4.4315	<0.0001	3.7042	0.4475	<0.0001	<0.0001	1.0000
Meulendijks 2015	1.64	1.14–2.02	0.1046	4.7564	<0.0001	4.9610	0.2913	0.0110	19.3704	1.2402

***DPYD* c.1601G>A**

	RR	95%CI	SE	*z* value	*P* value	Q	*P* value *Q*-test	tau²	I²	H²
Deenen 2011	1.65	0.86–3.19	0.3357	1.4959	0.1347	29.5109	<0.0001	0.3829	89.8343	9.8370
Loganayagam 2013	1.20	0.96–1.50	0.1127	1.6101	0.1074	0.7308	0.8659	<0.0001	<0.0001	1.0000
Rosmarin 2014	1.59	0.78–3.23	0.3626	1.2757	0.2021	34.8385	<0.0001	0.4570	91.3888	11.6128
Froehlich 2015	1.53	0.80–2.93	0.3306	1.2886	0.1976	41.8705	<0.0001	0.3971	92.8350	13.9568
Meulendijks 2015	1.66	0.87–3.15	0.3273	1.5499	0.1212	37.4024	<0.0001	0.3754	91.9791	12.4675

Abbreviations: RR: relative risk; 95%CI: 95% confidence interval; SE: standard error.



Supplementary Figure 3. Results of secondary analysis of case-control studies.

Findings from secondary analysis of case-control studies

Four studies with a case-control design were identified and included in the secondary analysis, in which c.1236G>A/HapB3 and c.1601G>A, but not c.1679T>G had been measured (Supplementary Table 5). A subset of the patients analyzed in Van Kuilenburg *et al.*[11] for c.1236G>A/HapB3 were reported previously in Gross *et al*.[10] The larger subset (Van Kuilenburg *et al.*[11]) was used to analyze c.1236G>A/HapB3. This analysis included a total of 799 patients for c.1236G>A/HapB3, and 558 patients for c.1601G>A. No significant associations between the *DPYD* variants and global severe toxicity were evident (Supplementary Figure 3). However, the summary effect sizes were in the same range as determined in the primary analysis: the RR was 1.89 for c.1236G>A/HapB3 (95%CI: 0.67–5.28, *P*=0.2260), and RR was 1.40 for c.1601G>A (95%CI: 0.48–4.08, *P*=0.5374). There was little indication for heterogeneity among the case-control studies for c.1236G>A/HapB3 (*Q*=3.20, *P*=0.2022, I^2=37%) and c.1601G>A (*Q*=2.24, *P*=0.3271, I^2=11%).

Supplementary Table 4. Carrier frequencies of *DPYD2A and c.2846A>T.**
The table below shows the frequencies of *DPYD**2A and c.2846A>T carriership (heterozygous or homozygous) according to c.1679T>G, c.1236G>A/HapB3 and c.1601G>A genotypes, in the patients for whom *DPYD**2A and c.2846A>T were measured.

Study	Patients carrying c.1236G>A/HapB3		Patients carrying c.1679T>G		Patients carrying c.1601G>A		Overall	
	N=173		N=6		N=184		*N*=2656	*N*=4237
	*DPYD**2A	c.2846A>T	*DPYD**2A	c.2846A>T	*DPYD**2A	c.2846A>T	*DPYD**2A	c.2846A>T
Deenen 2011	0%	0%	-	-	0%	0%	1.2% (7/568)	1.4% (8/568)
Jennings 2013	0%	0%	-	-	-	-	1.2% (3/253)	0.8% (2/253)
Loganayagam 2013	0%	0%	0%	0%	13% (2/16)	-	0.9% (4/430)	1.2% (5/430)
Rosmarin 2014	2.3% (1/43)	0%	-	-	7.3% (3/41)	-	0.8% (7/905)	1.2% (11/881)
Froehlich 2015	0%	0%	0%	0%	0%	0%	0.8% (4/500)	0.6% (3/500)
Meulendijks 2015	0%	0%	0%	0%	0%	0%	0%[a]	1.2% (19/1605)
Overall	**0.6%**		**0%**		**2.7%**			**1.7%**

[a] Patients carrying *DPYD**2A were excluded from the respective analysis.

Supplementary Table 5. Studies included in secondary analysis of case-control studies.

Study	Study design	Clinical data collection	Race (Nationality)	N patients	Age, median (range)	Male / female (%)	Capecitabine/ 5-FU	Treatment regimens	Investigated DPYD variants	Data used	Reference
Van Kuilenburg *et al.* 2010	Case-control	Retrospective	NR (German)	203	60 (29-81)	47 / 53	99% 5-FU 1% CAP	30% 5-FU + PT (± PAC) 12% FOLFOX 21% 5-FU ((± LV) 36% 5-FU + other 1% CAP	c.1236G>A	IPD	[11]
Kleibl *et al.* 2009	Case-control	Retrospective	100% CAU (Czech)	124	61 (30-79)	49 / 51	86% 5-FU 14% CAP	29% 5-FU bolus 42% 5-FU continuous or CAP 24% FOLFOX, FOLFIRI 5% CAP/5-FU + other	c.1236G>A c.1601G>A	IPD	[12]
Gross *et al.* 2008	Case-control	Retrospective	NR (German)	181 [b]	63 (29-81)	55 / 45	92% 5-FU 8% CAP	27% 5-FU + PT (± PAC) 18% FOLFOX 4% FOLFIRI 4% 5-FU (+ LV) 40% 5-FU + other 8% CAP (± PT)	c.1236G>A c.1601G>A	IPD	[10]
Schwab *et al.* 2008 [a]	Case-control	Prospective	NR (German)	683 [c]	64 (25-89)	56 / 44	100% 5-FU	100% 5FU ± LV	c.1236G>A c.1601G>A	IPD	[13]

Abbreviations: 5-FU: 5-fluorouracil; CAP: capecitabine; CAU: Caucasian; FOLFIRI: 5-fluorouracil, leucovorin, and irinotecan; FOLFOX: 5-fluorouracil, leucovorin, and oxaliplatin; IPD: individual patient data; LV: leucovorin/folinic acid; MONO: monotherapy; NR: not reported; PAC: paclitaxel; PT: platinum.
[a] This study was analyzed as a case-control study since part of the patients that were measured for c.1236G>A/HapB3 and c.1601G>A were selected based on toxicity phenotype.
[b] *N*=128 for c.1236G>A/HapB3; *N*=177 for c.1601G>A.
[c] *N*=472 for c.1236G>A/HapB3; *N*=258 for c.1601G>A.

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6

Patients homozygous for *DPYD* c.1129-5923C>G/haplotype B3 have partial DPD deficiency and require a dose reduction when treated with fluoropyrimidines

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SUMMARY

Purpose

Dihydropyrimidine dehydrogenase (DPD) is a critical determinant of 5-fluorouracil pharmacology, and reduced activity of DPD as a result of deleterious polymorphisms in the gene encoding DPD (*DPYD*) can result in severe treatment-related toxicity. Dosing recommendations to individualize treatment have been provided for three *DPYD* variants (*DPYD**2A, c.2846A>T, and c.1679T>G). A fourth variant, c.1129-5923C>G/HapB3, has been shown to increase the risk of fluoropyrimidine-associated toxicity, but little is known about the functional effects of this variant.

Methods

By performing a large retrospective screen for *DPYD* variants, we identified three patients who were homozygous for c.1129-5923C>G/HapB3. We describe their clinical course of treatment and analyzed DPD activity and *DPYD* gene expression, to provide insight into the phenotypic effects of c.1129-5923C>G/HapB3.

Results

DPD activity could be measured in two patients and was 4.1 and 5.4 nmol/(mg*h), (DPD activity 41% and 55% compared to controls, respectively). The fluoropyrimidine dose had to be reduced during treatment in both patients. In line with partial DPD deficiency in both patients, sequence analysis of DPD cDNA demonstrated a normal-sized (wild type) cDNA fragment of 486 bp, as well as a larger-sized (mutant) 530-bp fragment containing an aberrant 44-bp insertion in intron 10. Patient three tolerated treatment well, but DPD activity measurement was not possible as the patient had deceased at the time of performing the study.

Conclusions

The presented functional and clinical data indicate that the c.1129-5923C>G variant is both functionally and clinically relevant, and support an upfront dose reduction of the fluoropyrimidine starting dose in patients carrying c.1129-5923C>G homozygously.

INTRODUCTION

The fluoropyrimidine anticancer drugs 5-fluorouracil (5-FU), capecitabine, and tegafur are used by approximately two million patients per year worldwide for colorectal, gastric, and breast cancer.[1–5] Of these patients, 10–30% experience severe, sometimes lethal, fluoropyrimidine-associated toxicity, creating a substantial clinical problem.[6] A well-known cause of intolerance to fluoropyrimidines is deficiency of the main 5-FU metabolic enzyme, dihydropyrimidine dehydrogenase (DPD), which occurs in 3–8% of all patients.[7]

DPD deficiency results from deleterious polymorphisms in the gene encoding DPD (*DPYD*) in the majority of cases, although other mechanisms (including posttranscriptional regulation, e.g., by microRNAs) might affect DPD activity as well.[8] Three *DPYD* variants are established predictors of fluoropyrimidine-associated toxicity—i.e., *DPYD**2A, c.2846A>T, and c.1679T>G (*DPYD**13).[9] For heterozygous carriers of these variants, a 50% dose reduction is recommended by the Clinical Pharmacogenetics Implementation Consortium (CPIC), based on the fact that a dysfunctional *DPYD* allele results in ~50% reduction of DPD enzyme activity and a 1.5–2-fold increase in 5-FU exposure when patients are treated with full-dose fluoropyrimidines.[9–11] Additional *DPYD* variants have previously been associated with fluoropyrimidine-related toxicity, including c.1236G>A.[12,13] Importantly, it was recently shown that upfront *DPYD* screening and dose reduction in variant allele carriers improved safety and was feasible in routine clinical practice, underscoring the clinical utility of *DPYD* screening.[14]

It was recently shown in a meta-analysis that c.1129-5923C>G, an intronic polymorphism occurring in intron 10 of *DPYD*, significantly increases the risk of severe fluoropyrimidine-associated toxicity.[15] However, a dosing recommendation has thus far not been proposed by the CPIC, and the effect of c.1129-5923C>G/HapB3 on DPD enzyme activity in patients is not well described. c.1129-5923C>G is located in intron 10 and occurs in a haplotype termed haplotype B3 (HapB3), with an allele frequency of ~0.02 in Caucasians.[16,17] c.1129-5923C>G creates a cryptic splice donor site, which leads to insertion of an aberrant 44-bp fragment into mature *DPYD* mRNA, with a premature stop codon as a result.[18] The fact that the phenotypic effects of c.1129-5923C>G/HapB3 on DPD enzyme activity in patients are not well described, hampers formulation of a rational dosing recommendation. Previously, we showed that four patients carrying c.1129-5923C>G/HapB3 were suffering from partial DPD deficiency.[18] However, DPD activity was measured in these patients because they had experienced severe (grade ≥3) treatment-related toxicity, and the observations might therefore have been biased toward lower values for DPD activity (since the patients were selected for DPD activity measurement based on their toxicity phenotype).[18] Because little or no other data are available on the phenotypic consequences of c.1129-5923C>G, it remains uncertain to what extent c.1129-5923C>G reduces DPD activity.

The full phenotypic consequences of *DPYD* variants become evident in patients with homozygous genotypes, and this provides an opportunity to study the variant's effect on enzyme activity. However, the frequency of the homozygous genotype of c.1129-5923C>G/HapB3 is very low and is anticipated to be only ~0.04% based on the low allele frequency of 2%.[17] Using a large retrospective screen for *DPYD* variants, we identified three patients who were homozygous for c.1129-5923C>G/HapB3 and treated with fluoropyrimidines. We describe the clinical course of treatment of these patients and analyzed DPD activity and *DPYD* gene expression, to provide insight into the phenotypic effects of c.1129-5923C>G.

PATIENTS AND METHODS

The patients were identified as homozygous carriers of c.1129-5923C>G/HapB3 during two pharmacogenetic analyses, which were performed as a secondary endpoint of two clinical studies (NCT00838370 and NCT01359397).[14,19] The studies were approved by the Medical Ethics Committees of the Netherlands Cancer Institute and the local study sites. Patients provided written informed consent for the respective studies and for the additional analyses described here.

Toxicity was monitored and recorded during treatment according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTC-AE) v3.0. Genomic DNA for determination of *DPYD* genotypes was collected prior to treatment, and c.1129-5923C>G was genotyped as described previously.[18] The presence of haplotype B3 was confirmed by genotyping of the haplotype B3 tagging variants c.959-51T>G, c.1129-5923C>G, and c.1236G>A.[16] Other known deleterious *DPYD* variants (*DPYD**2A, c.2846A>T, and c.1679T>G) were genotyped according to previously described methods and were not found to be present in the described patients.[12] *DPYD* genotype status was unknown at the time of treatment. In two patients, DPD enzyme activity in peripheral blood mononuclear cells (PMBCs) was determined, as described previously.[18] In order to determine the functional consequences of c.1129-5923C>G on mRNA expression, sequence analysis of cDNA of intron 10 was performed as reported previously.[20] The latter two assays were performed >4 weeks after the last treatment with fluoropyrimidines, to avoid possible interference between 5-FU treatment and DPD activity measurement.

RESULTS

Clinical course of treatment of patients homozygous for c.1129-5923C>G/HapB3

The clinical characteristics of the patients are summarized in Table 1.

The first patient was a female, aged 47, treated with neoadjuvant chemoradiotherapy for locally advanced rectal cancer (T3N2M0). She received capecitabine 825 mg/m^2 b.i.d. (2 × 1500 mg) for 33 days, combined with 25 fractions of radiotherapy (2 Gy each) on weekdays, to a total dose of 50 Gy. On day 9 of treatment she developed leukocytopenia grade 2 (2.3×10^9/L), neutropenia grade 2 (1.3×10^9/L), hand-foot syndrome (grade 1), diarrhea (grade 1), and fatigue (grade 1). These symptoms intensified, until it was decided on day 15 to reduce the dose of capecitabine by 40% (capecitabine in the weekend was omitted, and on weekdays the evening dose was reduced to 1000 mg). After dose reduction, treatment was well tolerated. On day 23, the dose of capecitabine was increased slightly (by 10%). Five days later she again developed leukocytopenia (2.5×10^9/L, grade 2) and neutropenia (1.5x10^9/L, grade 1). Despite these symptoms, treatment could be finished at reduced dose. The patient subsequently received surgery and is currently disease-free, four years after treatment.

Patient 2 was a male, aged 67, diagnosed with a metastasized adenocarcinoma of the distal esophagus (T3N1M1). He received capecitabine 850 mg/m^2 b.i.d. (days 1–14), combined with docetaxel (50 mg/m^2), oxaliplatin (100 mg/m^2), and bevacizumab (7.5 mg/kg) on day 1 in three-week cycles. On day 7 of treatment, he experienced fatigue (grade 2). He self-reported the symptoms to be 'intolerable, and related to capecitabine use.' On day 11, the patient was hospitalized with fever (38.7°C; grade 1, without apparent focus) and neutropenia grade 2 (1.3×10^9/L). He was released after a brief period of hospitalization, at which point he refused further treatment with capecitabine.

Table 1. Characteristics of the patients carrying homozygous variant genotypes for c.1129-5923C>G/HapB3.

Characteristic	Patient 1	Patient 2	Patient 3
Sex	F	M	M
Age	47	67	69
Body surface area (m^2)	1.76	1.86	1.88
Race	Caucasian	Caucasian	Caucasian
Primary tumor	CRC	GEJ	CRC
WHO performance status	0	0	0
Estimated glomerular filtration rate	>80 mL/min	62 mL/min	57 mL/min
Hematology/blood chemistry prior to treatment	Normal	Normal	Leukocytes increased
DPD activity (nmol/[mg*h]) (% of normal)[a]	4.1 (41%)	5.4 (55%)	NA [b]
Treatment	CAP + RT	DOC + B	CAP + RT
Capecitabine dose (mg/m^2 b.i.d.)	825	850	825
Capecitabine schedule	Days 1–33 (continuous)	Days 1–14 (Q3W)	Days 1–26 (continuous)
Toxicity during treatment (maximum grade)	LEU 2 NEU 2 HFS 1 DIA 1 FAT 1	MAL 2 FAT 2 FEV 1 NEU 2	No toxicity
Dose adaption required during treatment	A 40% dose reduction was required on day 15, after which treatment could be finished	Treatment 1: had to be discontinued on day 11 of cycle 1 Treatment 2: an *a priori* 36% reduced dose was not tolerated	No dose reduction was required to finish treatment

[a] Normal range 5.9–14.0 nmol/(mg*h) (median 9.9 nmol/[mg*h])

[b] Patient 3 had deceased at the time of this study, and a DPD activity measurement could therefore not be performed.

Abbreviations: CAP + RT: capecitabine combined with radiotherapy; CRC: colorectal cancer; DIA: diarrhea; DOC + B: docetaxel, oxaliplatin, and capecitabine plus bevacizumab; DPD: dihydropyrimidine dehydrogenase; GEJ: adenocarcinoma of the gastroesophageal junction; FAT: fatigue; FEV: fever; HFS: hand–foot syndrome; LEU: leukocytes; MAL: malaise; NA: not available (not measured); NEU: neutropenia; Q3W: every 3 weeks; WHO World Health Organization.

Four months later, when his disease had further progressed, a second course of treatment with capecitabine was initiated, as monotherapy (for which the standard dose is 1250 mg/m^2). In view of the side effects experienced during the first treatment, the dose of capecitabine was reduced *a priori* from 1250 to 800 mg/m^2 b.i.d. During the first cycle, the patient again reported fatigue (grade 2), thought to be capecitabine-related. After one cycle the patient decided not to receive any further treatment.

Patient 3 was a male, aged 69, treated for locally recurrent rectal cancer. He received neoadjuvant chemoradiotherapy, with capecitabine 825 mg/m^2 b.i.d. and concomitant radiotherapy on weekdays, in 20 fractions of 1.8 Gy to a total dose of 36 Gy. The treatment was well tolerated and could be completed without dose reductions or delays. No adverse events were reported during the 4 weeks of treatment, and hematology after treatment was similar to prior to treatment. After neoadjuvant chemoradiotherapy, the patient's tumor was resected. One year after surgery a relapse was diagnosed, and the patient eventually deceased as a result of progressive disease.

Analysis of DPD activity and intron 10 cDNA

As given in Table 1, patients 1 and 2 were both found to have partial DPD deficiency, with 41% and 55% activity remaining when compared to normal. Patient 3 had deceased at the time of this study, and a DPD activity measurement could therefore not be performed.

In line with the presence of a partial DPD deficiency in the two patients, analysis of the coding sequence of DPD cDNA demonstrated the presence of a normal-sized (wild type) cDNA fragment, of 486 bp, as well as a larger-sized (mutant) 530-bp fragment (Figure 1). Sequence analysis revealed that the 486-bp fragment was indeed wild type and that the 530-bp fragment contained the aberrant 44-bp insertion, corresponding to nucleotides c.1129-5967_1129-5924 in intron 10.[18]

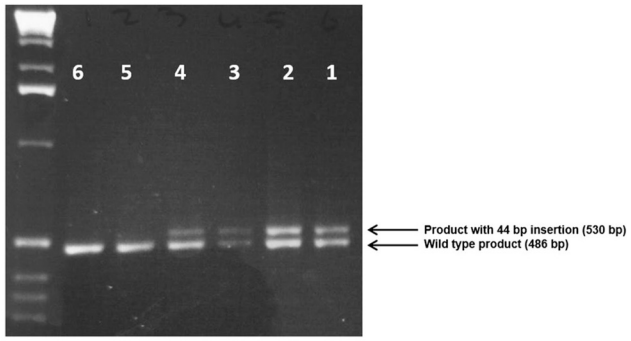

Figure 1 Fragment analysis of amplified cDNA from intron 10 of *DPYD* in patients with homozygous variant genotypes for c.1129-5923C>G/HapB3.
The figure shows the results of the intron 10 cDNA fragment analysis using gel electrophoresis. The 486- and 530-bp fragments correspond to wild-type and mutant cDNA fragments, respectively. *Lanes 1* and *2* contain cDNA of patients 1 and 2, respectively. *Lanes 3* and *4* contain cDNA from two patients reported previously, a patient homozygous for c.1129-5923C>G and a patient heterozygous for c.1129-5923C>G, respectively.[18] *Lanes 5* and *6* contain cDNA of a c.1129-5923C>G wild-type control.

DISCUSSION

DPD deficiency as a result of deleterious polymorphisms in *DPYD* is a well-established risk factor for fluoropyrimidine-associated toxicity.[9] The clinical validity of *DPYD* c.1129-5923C>G/HapB3 is only recently being recognized. A meta-analysis summarizing all evidence on the clinical validity of c.1129-5923C>G/HapB3 demonstrated that risk of fluoropyrimidine-associated toxicity was increased 1.6-fold (RR 1.6, 95% CI 1.29–1.97, *P*<0.0001) in variant allele carriers.[15] However, little is yet known about the functional effects of this variant, and there is no consensus on the dosing recommendation for patients carrying this variant.

We described three patients homozygous for c.1129-5923C>G/HapB3 who were treated with fluoropyrimidines, and determined the effect of c.1129-5923C>G on DPD enzyme activity in two patients. An approximately 50% reduction of DPD activity was found in these patients, which was shown to be associated with aberrant mRNA processing, thereby confirming the functional relevance of c.1129-5923C>G, as proposed previously.[18] Given the presence of residual DPD activity in both our patients with homozygous genotypes for whom DPD activity was measured, it is evident that c.1129-5923C>G is not a fully non-functional (i.e., catalytically inactive) variant, such as *DPYD**2A which results in ~0% DPD activity in homozygous individuals.[21,22] In fact, the presented data indicate that the magnitude of effect of c.1129-5923C>G on DPD activity may be approximately half that of a fully non-functional variant such as *DPYD**2A, since ~50% DPD activity remained in the two homozygous individuals. These results are in line with a recent proposal to differentiate between fully non-functional and partially functional *DPYD* variants when reducing the fluoropyrimidine starting dose.[23]

In line with the presence of residual DPD activity, the presented clinical data show that the patients homozygous for c.1129-5923C>G/HapB3 were able to tolerate low doses of fluoropyrimidines (note that the starting dose of capecitabine which all three patients received was relatively low compared to the approved dose for monotherapy, i.e., 1250 mg/m^2 b.i.d.). Nevertheless, the administered dose was not tolerated in two out of three patients, since the dose had to be reduced. Since the starting dose of capecitabine was relatively low, it is conceivable that higher doses of capecitabine would have resulted in more pronounced toxicity. In line with this, Amstutz *et al.* described a patient with a homozygous genotype for c.1129-5923C>G/HapB3 who was treated with full-dose 5-FU plus cisplatin, who experienced fatal toxicity during the first cycle.[16]

The available data thus far indicate that full-dose treatment with fluoropyrimidines in patients homozygous for c.1129-5923C>G/HapB3 should be avoided. The degree of dose reduction required to allow safe treatment cannot be determined based on the currently available data, since only a small number of patients with homozygous genotypes of c.1129-5923C>G/HapB3 have been described so far. Considering the presented clinical data and the DPD activity measurements in this study, which showed an approximately 50% reduction of DPD activity, it seems that a 50% dose reduction of the fluoropyrimidine dose might be feasible in patients homozygous for c.1129-5923C>G/HapB3. However, additional data are required before a definitive dosing recommendation can be provided. In our view, until more data are available, patients homozygous for c.1129-5923C>G/HapB3 should not be treated with full-dose fluoropyrimidines.

In conclusion, the presented clinical and functional data demonstrate that the c.1129-5923C>G variant is both functionally and clinically relevant. This report confirms the functional relevance of c.1129-5923C>G and adds to the few data available on the effect of c.1129-5923C>G on DPD enzyme activity in patients. The presented data, combined with the functional data reported previously and the available evidence on the clinical validity of c.1129-5923C>G,[15,18] support an upfront dose reduction of the fluoropyrimidine starting dose of approximately 50% in patients carrying c.1129-5923C>G homozygously, although the exact degree of dose reduction required for patients carrying c.1129-5923C>G should be determined in larger patient populations.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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7

Treatment algorithm for homozygous or compound heterozygous *DPYD* variant allele carriers with low-dose capecitabine

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SUMMARY

Background

Deficiency of the enzyme dihydropyrimidine dehydrogenase (DPD), caused by pathogenic *DPYD* polymorphisms, results in a highly increased risk of severe treatment-related toxicity for the fluoropyrimidine anti-cancer drugs 5-fluorouracil (5-FU) and capecitabine. *DPYD* screening and dose reduction in heterozygous *DPYD* variant carriers are known to improve patient safety. However, no treatment algorithms for homozygous or compound heterozygous *DPYD* genotypes are available, as information about the effect of these genotypes on DPD phenotype is scarce. Therefore potentially effective anti-cancer treatment may then be withheld.

Methods

In this case series, six unique patients with a homozygous or compound heterozygous *DPYD* genotype were identified before start of fluoropyrimidine-based treatment. DPD enzyme activity was measured to determine starting dose, pharmacokinetic analyses were performed to investigate if exposure was adequate and treatment-related toxicity was monitored to determine tolerability of dose.

Results

For a patient with a homozygous *DPYD**2A genotype, it was shown that DPD enzyme activity was absent, and dose had to be extremely reduced to allow safe and effective treatment. In the other patients, carrying the *DPYD* variants c.1236G>A or c.2846A>T homozygously or simultaneously, DPD enzyme activity was significantly reduced but not absent. These patients were safely treated with a tailored fluoropyrimidine dose.

Conclusions

This case series provided new insights in the functional and clinical effects of homozygous and compound heterozygous *DPYD* genotypes. It is shown that it is safe to treat these patients with tailored fluoropyrimidine doses, leading to adequate exposure without occurrence of severe treatment-related toxicity. These findings can be used for clinical decision making when encountering future patients with these *DPYD* genotypes before start of fluoropyrimidine treatment.

INTRODUCTION

Fluoropyrimidines, including 5-fluorouracil (5-FU) and its oral prodrug capecitabine, are widely used in the treatment of several types of cancer. The enzyme dihydropyrimidine dehydrogenase (DPD) is responsible for over 80% of 5-FU conversion into inactive metabolites.[1] Pathogenic single nucleotide polymorphisms (SNPs) in *DPYD,* the gene encoding DPD, can result in decreased function of DPD and are associated with a strongly increased risk of severe and potentially fatal fluoropyrimidine-induced toxicity.[2] Pretreatment screening for *DPYD* SNPs and reduction of the starting fluoropyrimidine dose in *DPYD* variant carriers has significantly improved patient safety.[3] In a recent perspective, we recommended reduction of the starting fluoropyrimidine dose in heterozygous carriers of one of four clinically relevant *DPYD* variants by 25% (c.2846A>T or c.1236G>A/haplotypeB3) or 50% (*DPYD**2A or c.1679T>G).[4]

However, for homozygous *DPYD* variant carriers, or for patients who carry multiple variants simultaneously, no dosing guidelines are available yet, because experimental data about the magnitude of the effect of these genotypes on DPD activity are scarce. In the Clinical Pharmacogenetics Implementation Consortium (CPIC) guideline, fluoropyrimidine-treatment in homozygous *DPYD* variant carriers is discouraged, which implies that a potentially effective anticancer therapy is withheld from these patients.[5]

In this article, six unique patients with a homozygous or compound heterozygous *DPYD* variant allele genotype who were treated with tailored fluoropyrimidine treatment are described. For three patients, pharmacokinetics, DPD-phenotyping and clinical course are included. Data about the other three homozygous *DPYD* variant carriers are provided in the Supplement.

METHODS

Detailed methods are in the Supplement. Before the start of fluoropyrimidine treatment, genotyping for four *DPYD* SNPs (*DPYD**2A, c.2846A>T, c.1679T>G, c.1236G>A/haplotypeB3) was performed as part of routine clinical care. Written informed consent for additional sample collection and use of clinical data was obtained for all patients. Analyses were part of individual patient care, so institutional review board approval was not applicable.

Genotyping results showed a homozygous or compound heterozygous *DPYD* genotype, and the functional effects of these genotypes were uncertain. Therefore pretreatment DPD activity was measured in peripheral blood mononuclear cells (PBMCs). DPD activity was used to reach an individualized dose, in which the percentage of remaining DPD activity was used as guideline for the starting dose (expressed as percentage of the originally planned dose).

Pharmacokinetic analyses in three of six patients were performed to investigate whether applied dose reductions were adequate. Pretreatment plasma uracil, the endogenous DPD substrate, and dihydrouracil levels were quantified; results were unknown before the start of treatment.

RESULTS

Clinical course of treatment

Patient characteristics are listed in Table 1.

Patient 1: A male patient with metastatic colorectal carcinoma was scheduled for palliative chemotherapy (capecitabine, oxaliplatin and bevacizumab). *DPYD* screening showed that the patient was homozygous for *DPYD**2A; measurement of DPD activity in PBMCs of this patient indicated absence of DPD activity. Therefore, it was decided to drastically reduce the capecitabine

dose from 2,300 mg (1,000 mg/m^2) twice daily to 150 mg twice daily (6.5% of planned dose) and to start with capecitabine monotherapy. Seven days after the start of treatment, the patient experienced severe toxicity (grade 3 diarrhea, grade 3 oral mucositis, and grade 4 neutropenia). Capecitabine was discontinued immediately. The adverse events resolved within 1 week (neutropenia) to 2 months (diarrhea, mucositis).

After a 2-month period without any anticancer therapy, the patient had fully recovered, and monotherapy with capecitabine was restarted. On the basis of the severe toxicity and the pharmacokinetic results of the first cycle, the dose was further reduced to 150 mg once every 5 days (ie, 0.65% of originally planned dose). This was tolerated well for 1 month, but the patient then experienced diarrhea (grade 2), after which capecitabine was stopped for 3 weeks. The capecitabine schedule was then adjusted again to introduce a rest period of 5 days after every two intakes (every third intake was skipped). This schedule was tolerated well; thus, it was decided to add bevacizumab and oxaliplatin. This addition was well tolerated and resulted in stable disease as the best treatment response.

Patient 2: This female patient with locally advanced colorectal carcinoma had a planned treatment that consisted of neoadjuvant chemotherapy (capecitabine 825 mg/m^2 twice daily, or 1,500 mg) combined with radiotherapy (5-week schedule). Pretreatment *DPYD*-screening revealed that the patient was homozygous for c.2846A>T; DPD activity was reduced to 29%. It was decided to reduce the capecitabine dose to 500 mg once daily, (ie, 17% of planned dose, slightly lower than recommended dose of 29% on the basis of DPD activity, as decided by physician and patient). DPD activity was not immediately known, so chemotherapy started on day 7 of the radiotherapy schedule. Treatment was completed and tolerated well without occurrence of severe toxicity. After treatment, surgery was performed.

Patient 3: A male patient with metastatic colorectal cancer had a treatment plan to start capecitabine and oxaliplatin. The patient carried both c.2846A>T and c.1236G>A variants heterozygously. DPD activity was reduced to 45%. Remaining DPD activity was more than 50% reduced, so it was considered likely that this patient was a compound heterozygous carrier (variants present on different alleles). In the first cycle, capecitabine was reduced to 1,800 mg daily (51% of planned daily dose of 3,500 mg; 1,750 mg/m^2), which was tolerated without toxicity. When the dose in cycle 2 was increased to 71% of planned dose (2,500 mg), grade 3 thrombocytopenia occurred. Therefore the dose was reduced again, to 57% of the planned dose (2,000 mg). This dose was continued during the third cycle. However, the patient developed grade 2 thrombocytopenia after 8 days, and the daily capecitabine dose was adjusted to 1,000 mg for the rest of the cycle. Platelets increased again until normal values were reached. After three cycles, disease progression was established, and capecitabine treatment was discontinued.

Pharmacokinetic results

In all three patients, additional pharmacokinetic measurements were performed (Figure 1). For patient 1, only levels of capecitabine and of the metabolites 5′-deoxy-5-fluorocytidine, 5′-deoxy-5-fluorouridine and 5-FU could be quantified; other 5-FU metabolites were not detectable. For patients 2 and 3, all metabolites were quantifiable. Results of noncompartmental analysis of the pharmacokinetic results in plasma are shown in Table 2 and include values normalized to control values.[6] In patient 1, 5-FU exposure was highly increased: the mean area under the plasma concentration-time curve (AUC) of 5-FU was 4,024 ng*h/ml, which is 10 times higher than in other

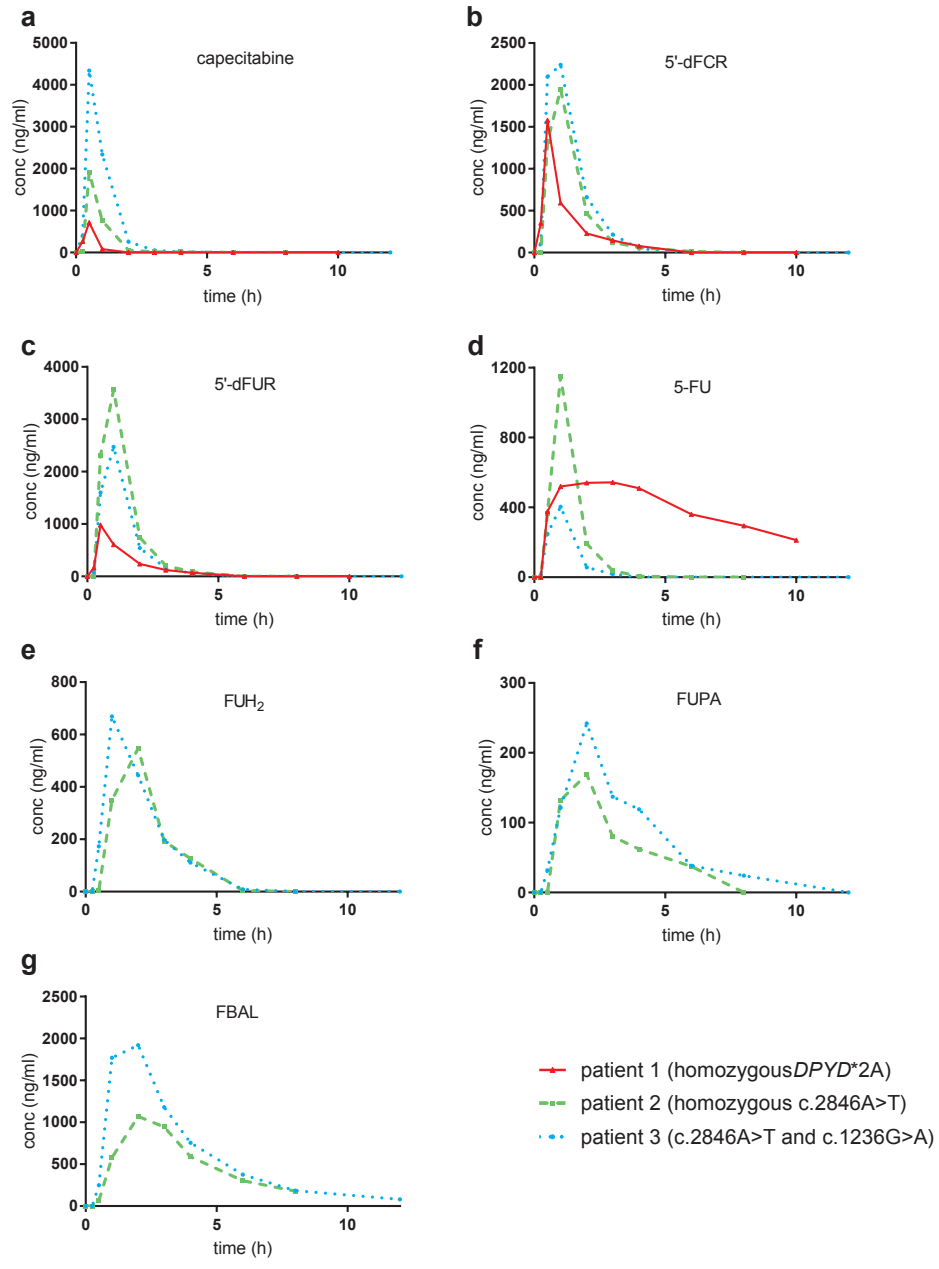

Figure 1. Pharmacokinetic results in plasma of patients 1, 2, and 3.
Results of plasma levels of capecitabine (A) and the metabolites 5′-deoxy-5-fluorocytidine (5′-dFCR, B), 5′-deoxy-5-fluorouridine (5′-dFUR, C), 5-fluorouracil (5-FU, D), dihydro-5-fluorouracil (FUH_2, E), α-fluoro-ureidopropionic acid (FUPA, F) and fluoro-β-alanine (FBAL, G). For all three patients, the results after the first intake of capecitabine are depicted.

Table 1. Clinical characteristics of patients 1 through 6.

Characteristic	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
DPYD genotype	homozygous *DPYD**2A	homozygous c.2846A>T	heterozygous for c.2846A>T & c.1236G>A	homozygous c.2846A>T	homozygous c.1236G>A	homozygous c.1236G>A
Sex	Male	Female	Male	Female	Female	Female
Age (years)	47	52	61	69	75	58
Body surface area (m^2)	2.3	1.8	2.0	1.5	1.8	1.5
Ethnicity	White	White	White	White	White	White
Tumor type	CRC (metastatic)	CRC (locally advanced)	CRC (metastatic)	CRC (local)	Vulva carcinoma (locally advanced)	Breast cancer (local)
WHO performance status	0	0	1	0	1	0
Baseline DPD activity in PBMCs (nmol/(mg*h), % of reference activity	<LLOQ [a] (0%)	2.9 [b] (29%)	4.5 [b] (45%)	0.97 [a] (10%)	7.8 [b] (79%)	4.2 [b] (42%)
Baseline uracil level (ng/ml) [c]	1,920	28.7	35.6	ND	14.5	26.8
Baseline dihydrouracil level (ng/ml) [c]	<LLOQ	71.7	114	ND	104	164
Treated with fluoropyrimidines [d]	Yes: C1 6.5% of planned dose (severe toxicity), then 10-fold reduction to 0.65% of planned dose	Yes: 17% of planned dose	Yes: C1 51% of planned dose, C2 72%, C3 57%	No: disease free after surgery	Yes: C1 75% of planned dose; C2 100%	Yes: 50% of planned dose (5-FU)

[a] Measured according to method by Pluim *et al.*[25] Reference DPD activity: 9.6 ± 2.2 nmol/(mg*h).[25]
[b] Measured according to method by Van Kuilenburg *et al.*[26] Reference DPD activity: 9.9 ± 2.8 nmol/(mg*h).[14]
[c] Reference baseline uracil level: median 8.32 ng/ml, range 3.2 to 38.2 ng/ml, reference baseline dihydrouracil level: median 91.9 ng/ml, range 31.9 to 189.0 ng/ml. Both were determined in a cohort of 550 patients.[27]
[d] The amount of dose reduction and clinical course for patients 1, 2, and 3 are described in detail in the main article. Details for patients 4, 5, and 6 are described in the Supplementary Results.
Abbreviations: C1: cycle 1; C2: cycle 2; C3: cycle 3; CRC: colorectal carcinoma; DPD: dihydropyrimidine dehydrogenase; LLOQ: lower limit of quantification; ND: not determined; PBMCs: peripheral blood mononuclear cells; 5-FU: 5-fluorouracil.

pharmacokinetic studies with capecitabine.[6–9] These results were used for the decision to lower the dose 10-fold in the second cycle.
Baseline uracil and dihydrouracil levels are listed in Table 1. Results of urine analysis for patient 1 are shown in Supplementary Figure 1.

DISCUSSION

To our knowledge, this is the first report to describe prospectively identified patients, who are homozygous or compound heterozygous for *DPYD* variants, who could be treated safely with fluoropyrimidines. Multiple occurrences of fatal or life-threatening toxicity after fluoropyrimidine treatment have been described and, retrospectively, the patients who experienced these toxicities were identified as homozygous *DPYD* variant carriers who had complete DPD deficiency.[10–15]
Pretreatment identification of the patient homozygous for *DPYD**2A with complete DPD deficiency saved this patient from receipt of a full fluoropyrimidine dose, which most likely would have been fatal. We hypothesized that a dose of 5 to 10% would be well tolerated by this patient, because this percentage is usually excreted unchanged in urine.[16] However, this dose still resulted in severe toxicity. We showed, though, that treatment with an extremely low dose of capecitabine (0.65% of standard dose) was safe and feasible. Baseline uracil levels were extremely high, which confirmed the expected absent enzyme activity. Also, pharmacokinetic results showed that 5-FU could not be metabolized further, because the 5-FU half-life and exposure were highly increased. Urine results of this patient differed from results described elsewhere. In patients who are not DPD deficient, FBAL is the major urinary metabolite,[9,17] whereas this metabolite was not present in the urine analyzed in this paper. Evidence about pharmacokinetic-based dosing for capecitabine is limited. However, for 5-FU, dosing based on plasma levels is described more extensively.[18,19]
The two patients who had a homozygous c.2846A>T genotype and a c.2846A>T/c.1236G>A genotype had a partial remaining DPD activity. Pharmacokinetic results showed that administration of a moderately reduced dose of capecitabine resulted in adequate exposure.
A variation in retained DPD activity in the two carriers of the homozygous c.1236G>A variant was determined. This is in contrast to results by Meulendijks *et al.* in which DPD activity was reduced approximately 50% in two patients.[20] The c.1236G>A variant is part of haplotype B3, of which the intronic variant c.1129-5923C>G is expected to be responsible for the effect on DPD activity.[21] Nie *et al.* showed that this intronic variant resulted in a 35% reduction of DPD enzyme function.[22]
Because patients only underwent genotyping for four *DPYD* variants, the effects of additional deleterious *DPYD* variants cannot be ruled out. For example, *MIR27A* polymorphisms could play a role in variation of DPD activity, because these polymorphisms reduce DPD activity.[23,24]
In conclusion, we showed that fluoropyrimidine treatment in homozygous or compound heterozygous *DPYD* variant allele carriers is feasible and that therapy does not have to be withheld. Additional DPD phenotyping tests, such as measurement of DPD activity in PBMCs, are recommended to compose an individualized treatment. After an initial dose reduction, tolerability in patients should be monitored closely, and the dose should be individually titrated according to tolerance.

Table 2. Pharmacokinetic parameters of capecitabine and metabolites in plasma of patients 1 through 3.

	$AUC_{0\text{-last time point}}$ (ng*h/ml) [a]							$T_{1/2}$ (h) [a]			
Metabolite	**Patient 1 [b]**	**Patient 1 normalized**	**Patient 2 [c]**	**Patient 2 normalized**	**Patient 3 [d]**	**Patient 3 normalized**	**Mean (CV%) control value [e]**	**Patient 1 [b]**	**Patient 2 [c]**	**Patient 3 [d]**	**Mean (CV%) control value [e]**
Capecitabine	296 (195 – 387)	3,871	1,186	3,626	3,357	7,134	4,281 (31%)	0.41 (0.24 – 0.61)	0.51	0.41	0.76 (55%)
5'-dFCR	1,310 (960 – 1,495)	17,131	2,445	7,476	3,198	6,796	8,192 (30%)	0.83 (0.70 – 1.04)	0.84	0.44	1.0 (35%)
5'-dFUR	1,257 (1,224 – 1,308)	16,438	4,139	12,655	2,912	6,188	7,673 (29%)	0.99 (0.76 – 1.26)	0.55	0.55	0.9 (34%)
5-FU	4,024 (3,795 – 4,351)	52,622	1,079	3,299	407	865	381 (40%)	5.31 (4.91 – 6.07)	0.39	0.57	1.0 (57%)
5-FU relative exposure [g]	1.1	-	1.4	-	1.1	-	1 (reference)	-	-	-	-
FUH_2	<LLOQ	<LLOQ	1,112	3,303	1,318	2,718	ND	<LLOQ	0.59	0.72	ND
FUPA	<LLOQ	<LLOQ	476	1,414	740	1,526	ND	<LLOQ	2.01	1.84	ND
FBAL	<LLOQ	<LLOQ	4,106	12,195	6,979	14,394	14,177 (31%)	<LLOQ	2.12	2.49	2.6 (33%)

[a] For patient 1, the mean values of three intakes (C1D1, C2D1, and C2D16) are reported, including range. The ingested dose was the same (150 mg) for all three days. For patients 2 and 3, the values of the first intake are reported.
[b] Patient 1 was a homozygous *DPYD**2A variant allele carrier. Results depicted are after an intake of 150 mg capecitabine (65 mg/m^2).
[c] Patient 2 was a homozygous c.2846A>T variant allele carrier. Results depicted are after an intake of 500 mg capecitabine (278 mg/m^2).
[d] Patient 3 was a heterozygous carrier of both c.2846A>T and c.1236G>A. Results depicted are after an intake of 800 mg (400 mg/m^2).
[e] Control values are derived from Deenen *et al.*[6] and are the mean values and CV% for patients with advanced cancer of the stomach or gastroesophageal junction (*N*=22), after administration of 850 mg/m^2 capecitabine (dose level 2 of the study). Capecitabine and metabolite control values are measured using the same assay as for patients 1, 2, and 3.
[f] Normalized AUC values (normalized AUC = AUC * (850 mg/m^2 / administered dose in mg/m^2)
[g] For the 5-FU AUC, the relative exposure is depicted, corrected for the dosing interval. 5-FU relative exposure =5-FU AUC / (factor * 5-FU AUC from Deenen *et al.*[6]). Factor patient 1 = 10 (as dosing 1x in the 5 days), factor patient 2 = 2 (as dosing once daily), factor patient 3 = 1 (as dosing twice daily).
Abbreviations: 5'-dFCR: 5'-deoxy-5-fluorocytidine; 5'-dFUR: 5'-deoxy-5-fluorouridine; AUC: area under the curve; CV%: coefficient of variation; C1D1: cycle 1 day 1; C2D1: cycle 2 day 1; C2D16: cycle 2 day 16; FBAL: fluoro-β-alanine; FUH_2: dihydro-5-fluorouracil; FUPA: α-fluoro-ureidopropionic acid; LLOQ: lower limit of quantification; ND: not determined; $T_{1/2}$: half-life; 5-FU: 5-fluorouracil.

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SUPPLEMENT

SUPPLEMENTARY METHODS

Patients

Patients were treated at three different institutes in the Netherlands (the Netherlands Cancer Institute, Amsterdam; Erasmus Medical Center, Rotterdam; Fransciscus Gasthuis & Vlietland, Rotterdam). Toxicity was scored according to the Common Terminology Criteria for Adverse Events (CTC-AE) version 4.03.

DPYD genotyping

Genomic DNA was isolated from peripheral blood cells and screening for the *DPYD* variants *DPYD**2A (IVS14+1G>A, rs3918290), c.2846A>T (rs67376798), c.1679T>G (*DPYD**13, rs55886062), and c.1236G>A (rs56038477) was performed using standard operating procedures in two different institutes (the Netherlands Cancer Institute, Amsterdam and Erasmus Medical Center, Rotterdam). This screening was performed before treatment as part of routine clinical care. In the Netherlands Cancer Institute, screening for *DPYD* variants was performed with the Roche LightCycler® 480II platform (Roche Diagnostics, Almere, the Netherlands) by using commercially available probes and primers (TIB Molbiol, Berlin, Germany), and results were confirmed by direct sequencing. At the Erasmus Medical Center, each sample was genotyped on two different platforms (Taqman (with predefined Drug metabolizing Enzyme (DME) assays) and PCR-restriction fragment length polymorphism (RFLP) assays) to allow checks for potential wrong genotyping. Details about the assays are included in Supplementary Table 1. Wild type, heterozygous, and no template controls were included in each run. Both laboratories participated during the study in the Dutch national quality control program for *DPYD* proficiency testing (SKML), in which all four *DPYD* variants were included.

DPD activity in PBMCs

Dihydropyrimidine dehydrogenase (DPD) activity was measured in peripheral blood mononuclear cells (PBMCs), isolated from a baseline (pretreatment) peripheral blood sample. One of two comparable validated methods by Van Kuilenburg *et al.* or Pluim *et al.* was used; both used radio-labeled thymine (^{14}C-labeled thymine or ^{3}H-labeled thymine) as a substrate and consisting of high performance liquid chromatography (HPLC) with online radioisotope detection and with liquid scintillation counting.[1,2] Reference values for both assays were highly comparable, respectively 9.9 ± 2.8 nmol/(mg*h) for the method of Van Kuilenburg *et al*.[2] and 9.6 ± 2.2 nmol/(mg*h) for the method of Pluim *et al.*[1]

Uracil and dihydrouracil plasma levels

Endogenous uracil and dihydrouracil levels were quantified in a baseline (pretreatment) plasma sample by using a validated ultra-performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS) method.[3] Analytes were extracted by protein precipitation; chromatographic separation was performed on an Acquity UPLC HSS T3 column (Acquity Waters, Milford, MA) and were analyzed with MS/MS with an electrospray ionization source.[3] All samples were measured at one institute (The Netherlands Cancer Institute).

Pharmacokinetic measurements

Peripheral blood samples for patient 1 were obtained for pharmacokinetic analysis on cycle 1, day 1 (C1D1); cycle 2, day 1 (C2D1); and cycle 2, day 16 (C2D16). Samples on those 3 days were collected at 10 time points up to 10 hours after capecitabine intake (at predose, and at 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 6 hours, 8 hours, and 10 hours after capecitabine intake). For patients 2 and 3, samples were collected only on C1D1, on the same time points as for patient one, except for the latest time point (the sample 10 hours after capecitabine intake was not collected for patient 2 and the last sample was collected at 12 hours instead of 10 hours for patient 3). For patient 1, pharmacokinetic results of the cycle one were known before the start of cycle two. For patients 2 and 3, pharmacokinetic results were not known during treatment, because samples were analyzed after treatment had finished.

Capecitabine and its metabolites 5'-deoxy-5-fluorocytidine (5'-dFCR), 5'-deoxy-5-fluorouridine (5'-dFUR), 5-fluorouracil (5-FU), dihydro-5-fluorouracil (FUH_2), α-fluoro-ureidopropionic acid (FUPA) and fluoro-β-alanine (FBAL), were quantified by using HPLC coupled to electrospray MS/MS. Two individual validated assays were used, one for the simultaneous quantification of capecitabine, 5'-dFCR and 5'-dFUR, and another for 5-FU, FUH_2, FUPA and FBAL.[4] In addition, for patient 1, urine samples were collected for additional analysis on C2D1 and C2D16. Urine was collected per portion from predose up to 10 hours after capecitabine intake on both days. The same HPLC-MS/MS method as for plasma samples was used. Urine samples were diluted 20 times in blank plasma before additional sample pretreatment. All pharmacokinetic samples were measured at the same institute (The Netherlands Cancer Institute).

SUPPLEMENTARY RESULTS

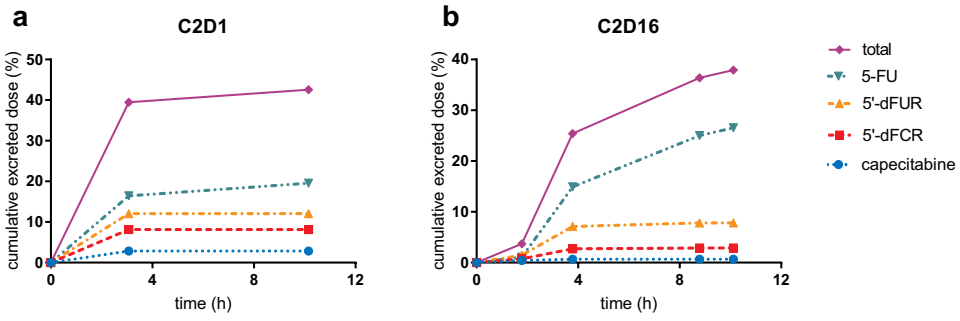
Dihydrouracil-uracil levels

Together with the sample for DPD activity in PBMCs, a pretreatment plasma sample was taken to measure uracil and dihydrouracil levels (Table 1). For patient 1, the baseline uracil plasma level was extremely high (1,920 ng/ml) compared with reference levels (median 8.32 ng/ml; range 3.2 to 38.2 ng/ml, *N*=550),[5] and no dihydrouracil could be detected. For patients 2 and 3, uracil levels increased compared with reference levels (a value of 28.7 ng/ml for patient 2 and of 35.6 ng/ml for patient 3), and both values were within the top 1% of reference values. Dihydrouracil levels were in the normal range for patients 2 and 3, compared to reference levels.

Supplementary Table 1. *DPYD* genotyping assays.

***DPYD* SNP**	**Rs-number**	**PCR-RFLP forward primer**	**PCR-RFLP reverse primer**	**Enzyme**	**Taqman DME assay**
*2A (IVS14+1G>A)	rs3918290	5'-CTTGTTTTAGATGTTA AATCACACATA - 3'	5'- CTTGTTTTAGATGTTAAAT CACACATA - 3'	NdeI	C__30633851_20
c.1679T>G	rs55886062	5'- CCAGCTTCAAAAGCT CTTC- 3'	5'- CTTCCGTTTCTGCCAAG C -3'	TFiI	C_11985548
c.1236G>A	rs56038477	5'- CACTGTACCTTTAGGA TCAC - 3'	5'- ATGCAGTTTGTTCGGAC TGA -3'	DdeI	C_25596099
c.2846A>T	rs67376798	5'- CATAGCATTCTAATTC CAGC - 3'	5'- CAAGTTGTGGCTATGAT CG -3'	Taqa1	C_27530948

Abbreviations: DME: drug metabolizing enzyme; PCR-RFLP: polymerase chain reaction - restriction fragment length polymorphism; Rs: reference SNP number; SNP: single nucleotide polymorphism.



Supplementary Figure 1. Pharmacokinetic results in urine of patient 1.
Patient 1 was a homozygous *DPYD**2A carrier. Results of urine excretion on cycle 2 day 1 (C2D1, A) and cycle 2 day 16 (C2D16, B) of capecitabine and the metabolites 5'-deoxy-5-fluorocytidine (5'-dFCR), 5'-deoxy-5-fluorouridine (5'-dFUR), 5-fluorouracil (5-FU) and of the total excretion, after intake of 150 mg capecitabine. Excretion was calculated as a percentage of the administered dose of capecitabine.

Other homozygous DPYD variant allele carriers

Three additional patients with a homozygous *DPYD* variant genotype were identified during routine *DPYD*-screening (Table 1). In these patients, DPD activity in PBMCs was measured pretreatment also and was used to determine the level of dose reduction.

Two out of three patients (patients 5 and 6) were homozygous for the c.1236G>A variant. DPD activity varied between these two patients, from approximately 42% to 79% residual activity. The two patients both received reduced fluoropyrimidine doses without occurrence of severe toxicity. It is unclear why there is a relatively high variation of the effect of this genotype on DPD phenotype in patients, and more research on this variant is advised.

In addition, one patient with a homozygous c.2846A>T genotype (patient 4) was identified. The residual DPD activity in this patient was approximately 10%. This patient was disease free after surgery, so it was decided not to treat this patient with adjuvant chemotherapy. The remaining activity of patient 4 was lower than of patient 2, who had the same *DPYD* genotype and who had a residual activity of 29%. According to the calculated DPD activity score, as described by Henricks *et al*.,[6] it could be concluded that a 50% dose reduction would be appropriate for homozygous c.2846A>T carriers, because a 25% dose reduction is recommended for heterozygous carriers of this variant. However, on the basis of the DPD activity results in these two patients, this amount of dose reduction seems insufficient for homozygous c.2846A>T carriers. Pharmacokinetic results in patient 2 showed that the normalized area under the plasma concentration-time curve (AUC) of 5-FU was 3,299 ng*h/ml, which is nine-fold higher than the mean control value of Deenen *et al.*,[7] which showed that 5-FU clearance might be impaired more than expected on the basis of the value of 29% remaining DPD activity in PBMCs.

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8

Capecitabine-based treatment of a patient with a novel *DPYD* genotype and complete dihydropyrimidine dehydrogenase deficiency

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SUMMARY

Fluoropyrimidines are frequently used anti-cancer drugs. It is known that patients with reduced activity of dihydropyrimidine dehydrogenase (DPD), the key metabolic enzyme in fluoropyrimidine inactivation, are at increased risk of developing severe fluoropyrimidine-related toxicity. Upfront screening for DPD deficiency and dose reduction in patients with partial DPD deficiency is recommended and improves patient safety. For patients with complete DPD deficiency, fluoropyrimidine-treatment has generally been discouraged. During routine pretreatment screening, we identified a 59-year old patient with a sigmoid adenocarcinoma who proved to have a complete DPD deficiency. Genetic analyses showed that this complete absence of DPD activity was likely to be caused by a novel *DPYD* genotype, consisting of a combination of amplification of exon 17 and 18 of *DPYD* and heterozygosity for *DPYD**2A. Despite absence of DPD activity, the patient was treated with capecitabine-based chemotherapy, but capecitabine dose was drastically reduced to 150 mg once every five days (0.8% of original dose). Pharmacokinetic analyses showed that the area under the concentration-time curve (AUC) and half-life of 5-fluorouracil were respectively tenfold and fourfold higher than control values of patients receiving capecitabine 850 mg/m^2. When extrapolating from the dosing schedule of once every 5 days to twice daily, the AUC of 5-fluorouracil was comparable to controls. Treatment was tolerated well for eight cycles by the patient without occurrence of capecitabine-related toxicity. This case report demonstrates that a more comprehensive genotyping and phenotyping approach, combined with pharmacokinetically-guided dose administration, enables save fluoropyrimidine-treatment with adequate drug exposure in completely DPD deficient patients.

INTRODUCTION

The fluoropyrimidine anti-cancer drugs 5-fluorouracil (5-FU) and its oral prodrug capecitabine are widely used for the treatment of several solid tumor types. After oral administration, capecitabine is rapidly converted into 5-FU through a three-step conversion. Approximately 80-90 % of 5-FU is inactivated in the liver by the enzyme dihydropyrimidine dehydrogenase (DPD) and DPD is, therefore, considered to be the key enzyme in the catabolism of 5-FU.[1] DPD activity has shown to be highly variable in the population, with an estimated 3 to 5% of the population being partially DPD deficient.[2,3] Patients with reduced DPD activity have an increased risk of developing severe and potentially fatal fluoropyrimidine-associated toxicity, when treated with a full dose of capecitabine or 5-FU.[4] Reduced DPD activity can often be attributed to the presence of pathogenic single nucleotide polymorphisms (SNPs) in *DPYD*, the gene encoding for the DPD enzyme. Four *DPYD* SNPs that are currently considered clinically relevant are *DPYD**2A (c.1905+1G>A, IVS14+1G>A), c.1679T>G, c.2846A>T and c.1236G>A/HaplotypeB3.[4] Dose reduction of capecitabine and 5-FU is recommended in heterozygous carriers of these variants.[5] Upfront screening for *DPYD**2A and dose reduction in heterozygous carriers has shown to improve patient safety.[6] For patients with complete DPD deficiency, such as patients homozygous for *DPYD**2A, fluoropyrimidine-containing regimens have been discouraged and, therefore, potentially effective anti-cancer treatment is withheld.

The combined sensitivity of these four risk variants to predict severe fluoropyrimidine-associated toxicity remains low and there is increasing awareness that additional rare variants may collectively explain an appreciable fraction of DPD deficient patients.[7] Therefore, other approaches to detect DPD deficiency, including more extensive *DPYD* genotyping or DPD phenotyping methods are gaining attention. A DPD phenotyping approach that is often used is *ex vivo* quantification of DPD activity in peripheral blood mononuclear cells (PBMCs).[8]

Here we describe a patient with a novel *DPYD* genotype and complete DPD deficiency, that was safely treated with a pharmacokinetically-guided administration of capecitabine. Our study demonstrates that a more comprehensive genotyping and phenotyping approach, combined with pharmacokinetically-guided dose administration, enables the save treatment of completely DPD deficient patients with fluoropyrimidines.

MATERIALS AND METHODS

Patient

The patient was identified during routine pretreatment screening and was treated as part of individualized standard medical care, not part of a clinical trial. Toxicity was scored according to the Common Terminology Criteria for Adverse Events (CTC-AE) version 4.03. Blood and urine samples for genetic, DPD phenotyping and pharmacokinetic analyses were collected with the aim of supporting clinical decision making. The patient gave written informed consent for use of data for scientific publication.

DPD enzyme activity assay and pyrimidine metabolites

PBMCs were isolated as described before from peripheral blood collected in an EDTA tube.[8] The activity of DPD was determined in a reaction mixture containing 35 mM potassium phosphate (pH 7.4), 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 250 µM NADPH and 25 µM [4-^{14}C]-thymine. Separation of radiolabeled thymine from radiolabeled dihydrothymine was performed by reversed-phase

high performance liquid chromatography (HPLC) with online detection of the radioactivity.[8] Concentrations of uracil and thymine (endogenous substrates of DPD) in plasma and urine were determined using reversed-phase HPLC hyphenated with electrospray tandem mass spectrometry (MS/MS).[9,10]

PCR amplification and sequence analysis of coding exons of DPYD

DNA was isolated from whole blood using the Nucleospin Tissue kit (Machery-Nagel, Dünen, Germany). Polymerase chain reaction (PCR) amplification of all 23 coding exons and flanking intronic regions of *DPYD* was carried out using intronic primer sets, as described before.[7] Sequence analysis of genomic fragments amplified by PCR was carried out on an Applied Biosystems model 3730 automated DNA sequencer using the dye-terminator method (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The *DPYD* sequence of the DPD deficient patient was compared to those observed in controls and the reference sequence of *DPYD* (Ref Seq NM_000110.3; Ensembl ENST00000370192).

MLPA and SNP array analysis

The multiplex ligation-dependent probe amplification (MLPA) test for *DPYD* (P103, MRC-Holland, Amsterdam, The Netherlands) contains 38 probes for *DPYD*, including one probe to detect the *DPYD**2A variant, and nine control probes specific for DNA sequences outside *DPYD*. MLPA was performed as described before.[7,11] Data analysis was performed using Gene Mapper software (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).[7,11] An Affymetrix Cytoscan HD SNP array was performed using standard protocols. The relative DNA copy numbers at the copy number variation (CNV) loci were determined by comparison of the normalized array signal intensity data for the DNA sample of the DPD deficient patient against the HapMap270 reference file provided by Affymetrix, using ChAS software (v 3.1.0.15, Affymetrix, Thermo Scientific, Waltham, MA USA).

Pharmacokinetic analyses

At the first intake of capecitabine, peripheral blood samples were collected in heparin tubes on ten pre-defined time points, up to ten hours after capecitabine intake; isolated plasma was stored at -80°C until analysis. Urine was collected as well during these ten hours, and was collected per portion and stored at -80°C. Plasma and urine samples were used for measurement of capecitabine and its metabolites 5'-deoxy-5-fluorocytidine (5'-dFCR), 5'-deoxy-5-fluorouridine (5'-dFUR), 5-fluorouracil (5-FU), dihydro-5-fluorouracil (FUH_2), α-fluoro-ureidopropionic acid (FUPA) and fluoro-β-alanine (FBAL). Levels were quantified with validated methods using HPLC-MS/MS.[12]

RESULTS

Clinical course

In December 2016, a 59-year old female patient was diagnosed with a sigmoid adenocarcinoma and underwent a sigmoid resection (pT4N2M0). She was subsequently scheduled for adjuvant chemotherapy treatment (capecitabine 1,000 mg/m^2 twice daily for 14 days and oxaliplatin 130 mg/m^2 on Day 1, given in a three-weekly cycle, eight cycles in total). Before start of this fluoropyrimidine-containing chemotherapy, *DPYD* screening for four *DPYD* variants was performed (*DPYD**2A, c.1679T>G, c.2846A>T, c.1236G>A), which is standard procedure in the hospital. The

patient was then found to be heterozygous for the *DPYD**2A variant and as an additional investigation, analysis of the DPD activity in PBMCs was performed, before determining the individualized starting dose of capecitabine. This revealed a complete DPD deficiency [DPD enzyme activity in PBMCs = 0.05 nmol/(mg*h), reference activity: 9.9 ± 2.8 nmol/(mg*h)[13]]. Based on these DPD phenotyping results and on previous experience with another patient with complete DPD deficiency,[14] it was decided to start with capecitabine- and oxaliplatin-based treatment with a drastically lowered capecitabine dose. An absolute dose of 150 mg (77 mg/m^2) on Days 1 and 6 for the first two cycles was chosen (approximately 0.8% of originally planned dose). Oxaliplatin was given in the originally planned dose. After the first intake of capecitabine (Day 1), pharmacokinetic results were awaited before continuing with the second dose (Day 6) as a safety precaution. From the third cycle onwards, capecitabine was administered on Days 1, 6 and 11, as treatment during the first two cycles was considered safe. The capecitabine treatment was tolerated well, without occurrence of capecitabine-related toxicity (e.g. no diarrhea, hand-foot syndrome or leukopenia occurred), and eight cycles were completed as planned. However, the patient experienced severe neurological toxicity, most likely caused by the oxaliplatin. Sensory neuropathy developed during the first cycle, and became more severe (grade 3) during the second cycle. Therefore, the oxaliplatin dose was decreased to 75% from the third cycle onwards and discontinued after cycle six.

DPYD genetic results

Since initial upfront screening for four *DPYD* variants (*DPYD**2A, c.1679T>G, c.2846A>T, c.1236G>A) revealed heterozygosity for *DPYD**2A (thus expecting only a partial DPD deficiency), whereas analysis of the DPD activity in PBMCs showed the presence of a complete DPD deficiency, additional genetic *DPYD* analyses were performed. Sequence analysis of all 23 coding exons and flanking intronic regions of *DPYD* showed that the patient was heterozygous for the *DPYD**2A variant only. However, subsequent MLPA analysis showed amplification of exons 17 and 18 of *DPYD* (Figure 1A). To delineate the boundaries and size of the amplification, SNP array analysis was performed. Detailed analysis of the chromosome 1p21.3 region showed a minimal amplified region of 31kB ranging from base pair 97757459 to 97788493 (hg19) encompassing exons 17 and 18 of *DPYD* (Figure 1B).

Pharmacokinetic and pyrimidine metabolite results

Strongly elevated concentrations of endogenous uracil and thymine were detected in plasma and urine of the patient which is in line with the presence of a complete DPD deficiency. (Table 1). When calculating a patient/control ratio, it was noted that the ratio for thymine was markedly higher than the ratio for uracil, both in plasma and urine. Pharmacokinetic analyses showed that only capecitabine, 5'-dFCR and 5'-dFUR and 5-FU could be quantified in plasma, the metabolites FUH_2, FUPA and FBAL were below the lower limit of quantification (Table 1 and Figure 2A-D). 5-FU exposure (area under the concentration-time curve; AUC) and half-life were respectively tenfold and fourfold higher than control values.[15] When extrapolating from the dosing schedule of once every 5 days to twice daily (tenfold difference), the AUC of 5-FU was comparable to the control value. When calculating a patient/control ratio for which values were normalized for the administered dose in mg/m^2, the 5-FU AUC of the patient is around 113 times higher than observed in patients receiving capecitabine 850 mg/m^2 (42,942 ng*h/ml vs 381 ng*h/ml).

In urine the same metabolites as in plasma were detectable, and additionally, a very small proportion was detected as FBAL (Figure 2E). Approximately 70% of the administered dose was recovered in the urine after 10 hours, of which approximately half as 5-FU.

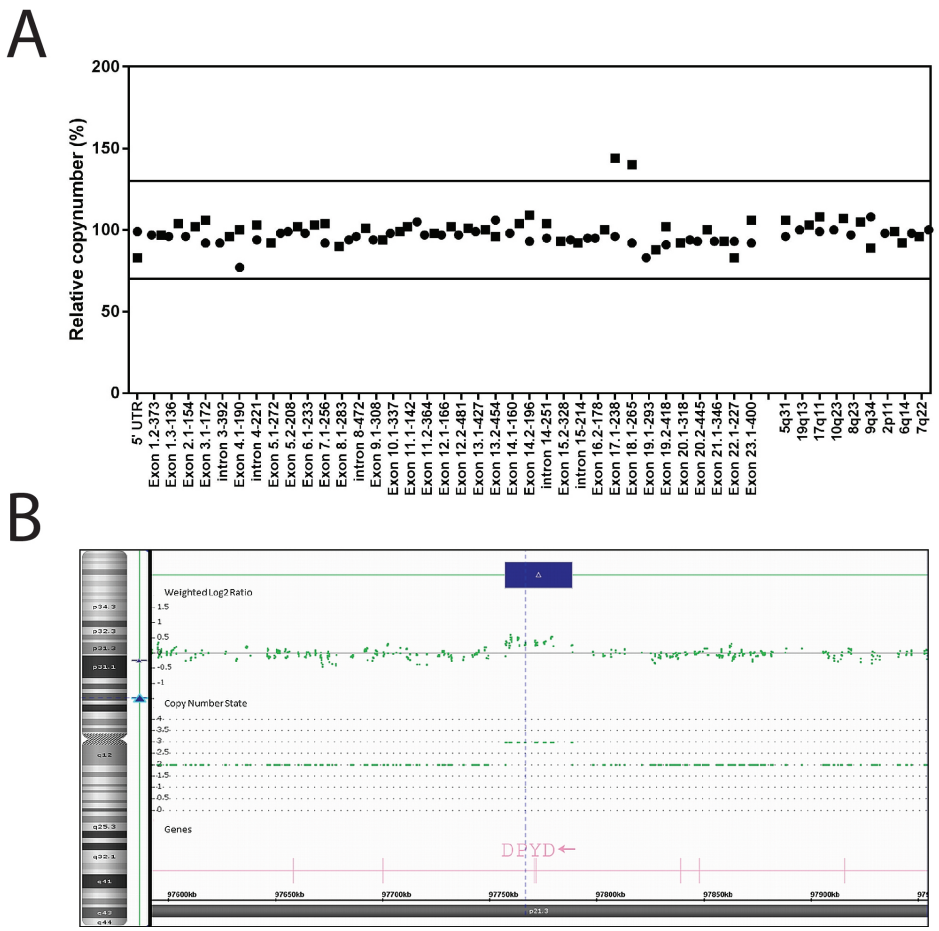
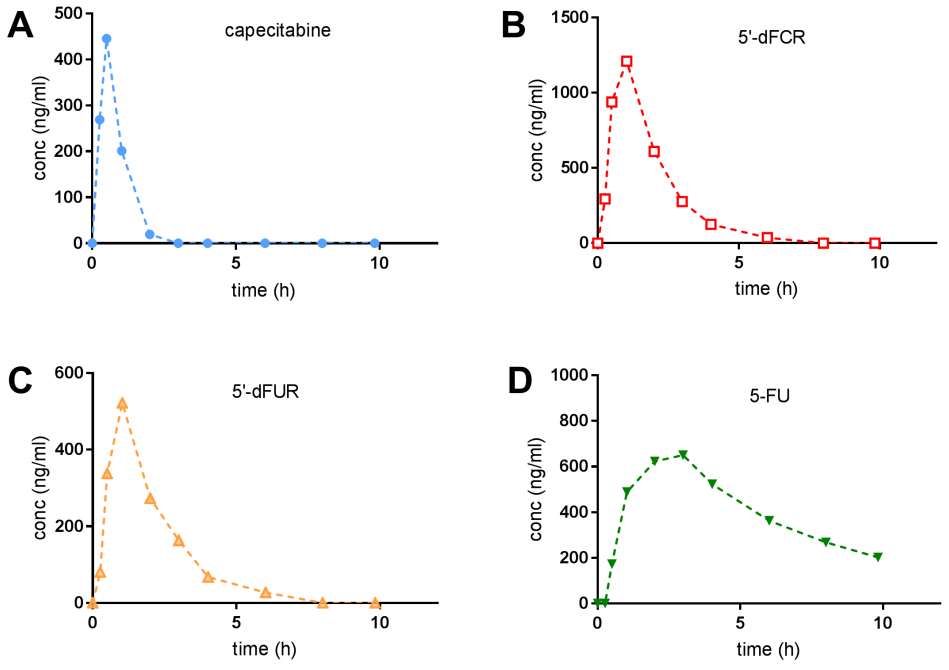


Figure 1. Analysis of copy number changes in *DPYD* using MLPA.
Panel A shows the MLPA analysis of the patient (■) and a control (●). The solid lines represent the cut-off values indicative for amplification (relative copy number >1.3) or deletion (relative copy number <0.7) of that particular sequence. Panel B shows detection of copy number changes by SNP array for the patient. The *y*-axis represents the weighted log2 ratio of the intensities of patient and the copy number state. On the *x*-axis SNPs are ordered by kB position. The panel shows a view for the probes located in the *DPYD* region (hg19). The box represents the minimal amplified region for the patient.

Table 1. Endogenous and pharmacokinetic parameters in plasma and urine and comparison to control values.

Endogenous parameters	**Patient values**	**Control values (mean ± SD)**	**Patient/control ratio**
DPD activity (nmol/(mg*h))	0.05	9.9 ± 2.8 [a]	0.0050
Plasma uracil level (μM)	15.5	0.3 ± 1.0 [b]	52
Plasma thymine level (μM)	7.9	0.01 ± 0.03 [b]	790
Urine uracil level (μmol/mmol creatinine)	124	7.1 ± 5.5 [c]	17
Urine thymine level (μmol/mmol creatinine)	66	0.1 ± 0.3 [c]	660
Metabolites in plasma: $AUC_{0\text{-last time point}}$ (ng*h/ml) [d]	**Patient values**	**Control values (mean, CV%) [e]**	**Patient values normalized for administered dose [h]**
Capecitabine	358	4,281 (31%)	3,952
5′-dFCR	2,364	8,192 (30%)	29,077
5′-dFUR	1,072	7,673 (29%)	11,834
5-FU	3,890	381 (40%)	42,942
5-FU relative exposure [f]	*1.02*	*1 (reference value)*	-
FUPA	<LLOQ [g]	ND	NA
FUH_2	<LLOQ [g]	ND	NA
FBAL	<LLOQ [g]	14,177 (31%)	NA
Metabolites in plasma: $T_{1/2}$ (h) [d]	**Patient values**	**Control values (mean, CV%) [e]**	**Patient/control ratio**
Capecitabine	0.41	0.76 (55%)	0.54
5′-dFCR	1.00	1.0 (35%)	1.0
5′-dFUR	1.18	0.9 (34%)	1.3
5-FU	4.26	1.0 (57%)	4.3
FUPA	<LLOQ [g]	ND	NA
FUH_2	<LLOQ [g]	ND	NA
FBAL	<LLOQ [g]	2.6 (33%)	NA

[a] Control values are derived from Van Kuilenburg *et al.*[13] (*N*=54).
[b] Control values are determined in a group of *N*=57 patients.
[c] Control values are determined in a group of *N*=112 patients.
[d] $AUC_{0\text{-last time point}}$ and $T_{1/2}$ are calculated using non-compartmental analysis based on plasma levels measured up to 10 hr after the first capecitabine intake (150 mg, 77 mg/m^2).
[e] Control values are derived from Deenen *et al.*[15] and are the mean values for 22 patients, after administration of 850 mg/m^2 capecitabine.
[f] For the 5-FU AUC, the relative exposure after extrapolation for the dosing interval is depicted. 5-FU relative exposure = 5-FU AUC patient value / (factor * 5-FU AUC from Deenen *et al.*[15]). Factor = 10 (as dosing 1x in the 5 days, compared to twice daily in Deenen *et al.*[15]).
[g] LLOQ of FUPA, FUH_2 and FBAL is 50 ng/ml.
[h] Patient values for AUC, normalized for the administered dose. Dose for Deenen *et al.*[15] was 850 mg/m^2, the patient received a dose of 77 mg/m^2, so normalized AUC = 5-FU AUC patient value * (850/77).
Abbreviations: 5′-dFCR: 5′-deoxy-5-fluorocytidine; 5′-dFUR: 5′-deoxy-5-fluorouridine; 5-FU: 5-fluorouracil; AUC: area under the concentration-time curve; FBAL: fluoro-β-alanine; FUH_2: dihydro-5-fluorouracil; FUPA: α-fluoro-ureidopropionic acid; LLOQ: lower limit of quantification; NA: not applicable; ND: not determined; SD: standard deviation; $T_{1/2}$: half-life.



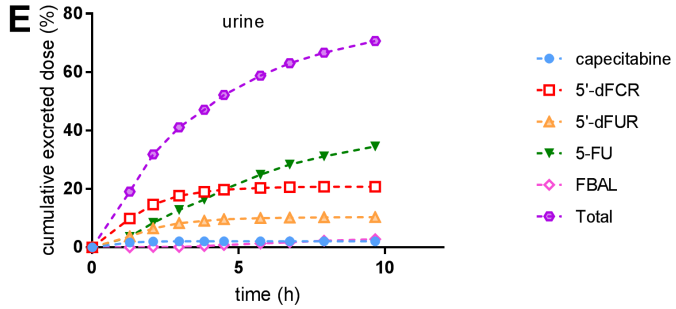


Figure 2. Plasma and urine levels of capecitabine and metabolites.
Results of plasma levels of capecitabine (A) and the metabolites 5'-deoxy-5-fluorocytidine (5'-dFCR, B), 5'-deoxy-5-fluorouridine (5'-dFUR, C) and 5-fluorouracil (5-FU, D), after the first intake of capecitabine. Panel E depicts results of urine excretion of capecitabine and 5'-dFCR, 5'-dFUR, 5-FU, fluoro-β-alanine (FBAL) and the total excretion, after the first intake of capecitabine. Excretion is calculated as percentage of the administered dose of capecitabine (150 mg).

DISCUSSION

DPD deficiency is now generally accepted as a major determinant of severe fluoropyrimidine-associated toxicity. This case report describes a patient who, if not identified before treatment as being completely DPD deficient and treated with a full capecitabine dose, may well have experienced fatal fluoropyrimidine-related toxicity. This emphasizes the importance of prospective screening for DPD deficiency. Ample evidence has been provided that carriers of the *DPYD**2A, c.1679T>G, c.2846A>T and c.1236G>A/HaplotypeB3 variants have an increased risk of developing toxicity.[4] In addition, dose adaptation for these *DPYD* variants is recommended by the Clinical Pharmacogenetics Implementation Consortium (CPIC).[5] However, standard screening for these four *DPYD* variants only, as is most often performed, would not have been sufficient to prevent severe and most likely fatal toxicity for this patient, as she would have received a 50% dose reduction only. Implementation of a more extensive genetic *DPYD* screening and/or a DPD phenotyping approach, is expected to identify a larger proportion of the patients with DPD deficiency who are at risk of severe fluoropyrimidine-related toxicity.[7]

Genetic analysis of this patient showed an amplification of exon 17 and 18 of the *DPYD* gene. In addition to the observed heterozygosity for the *DPYD**2A variant, a conclusive genotype was obtained that was likely to underlie the complete DPD deficiency. To our knowledge, amplification of exon 17 and 18 of the *DPYD* gene has not been described before. Recently, heterozygosity of an amplification of exon 9-12 in *DPYD* was shown to result in a profoundly decreased DPD activity.[7] Previously, we have shown that large deletions in *DPYD* occurred in 7% of pediatric patients with a complete DPD deficiency.[11] Thus, genomic rearrangements in *DPYD* can provide a molecular basis for a DPD deficiency in patients with a phenotypically-established reduced DPD activity.

In literature several examples are described of patients experiencing fatal toxicity who were retrospectively identified as completely DPD deficient.[16,17] Therefore, fluoropyrimidine treatment in completely DPD deficient patients has been generally discouraged. As there was a high medical need to treat this patient, based on poor tumor characteristics and no appropriate alternative chemotherapeutic regimens, it was still decided to start with a capecitabine-containing treatment. A dose of 150 mg once every 5 days was chosen, based on previous experience in our institute, where another patient with complete DPD deficiency (due to homozygosity for *DPYD**2A) tolerated this dose well and resulted in adequate drug exposure.[14] Applying very low doses of capecitabine is hampered by the available formulations of capecitabine (i.e. 150 and 500 mg), which we resolved by dosing intermittently once every five days.

One tablet of 150 mg resulted in a very high plasma exposure of 5-FU, with an AUC value around ten times higher than in pharmacokinetic studies with capecitabine in standard dosage in non-DPD deficient patients.[15,18–20] When correcting for the dosing interval of once every 5 days, which is ten times less than standard twice-daily dosing, 5-FU exposure in our patient was comparable to reference levels associated with efficacy and acceptable toxicity.[15,18–20]

Complete DPD deficiency has not only be linked with severely increased risk for fluoropyrimidine-related toxicity, but also with neurological or developmental abnormalities in several cases.[21–25] Our patient, however, did not present with any physical or psychomotor abnormalities.[21–25]

In conclusion, this case report shows the clinical need of an appropriate prospective screening approach for DPD deficiency. Since screening for the most common *DPYD* variants will not identify all patients at risk of severe toxicity; it is recommended to investigate the feasibility of more extensive genetic screening and/or DPD phenotyping methods. Furthermore, we showed that a patient with a complete DPD deficiency can be safely treated with a very low dose of a fluoropyrimidine drug.

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9

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

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SUMMARY

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.

INTRODUCTION

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 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.*[21]
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 (details in Supplement). 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 ≥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 Supplement). The genomic region containing *MIR27A* was amplified using PCR, followed by sequencing (Supplement).

Endpoints and data analysis

Endpoints: The primary endpoint of the study was severe, CTC-AE grade ≥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. ≥1 *DPYD* variant alleles). A statistical interaction term between *MIR27A* variants (zero vs. ≥1 variant alleles) and *DPYD* variants (zero vs. ≥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 Der-Simonian 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 ($D'=0.991$, $X^2=531.2$, $P<0.00001$; $r=-0.099$; Supplementary Table 2). The characteristics of the overall population are summarized in Table 1.

Table 1. Patient characteristics according to *DPYD* genotype.

	Overall population (*N*=1,592)	Patients without *DPYD* variants (*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** [a]			
0	665 (42%)	603 (42%)	62 (38%)
1	738 (46%)	658 (46%)	80 (49%)
2	189 (12%)	168 (12%)	21 (13%)
***DPYD* variants** [b]			
Wild-type	1,429 (90%)	1,429 (100%)	-
c.2846A>T	19 (1%)	-	19 (12%)
c.1679T>G	3 (0.2%)	-	3 (2%)
c.1129-5923C>G (C.1236G>A) [c]	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%)

[a] Sum of the no. of risk alleles present for rs895819 and rs11671784.

[b] 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.

[c] 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.

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 ≥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 (≥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) (Figure 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 (Figure 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 *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; Supplementary Figure 1).

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 Figure 1) was repeated taking into account the

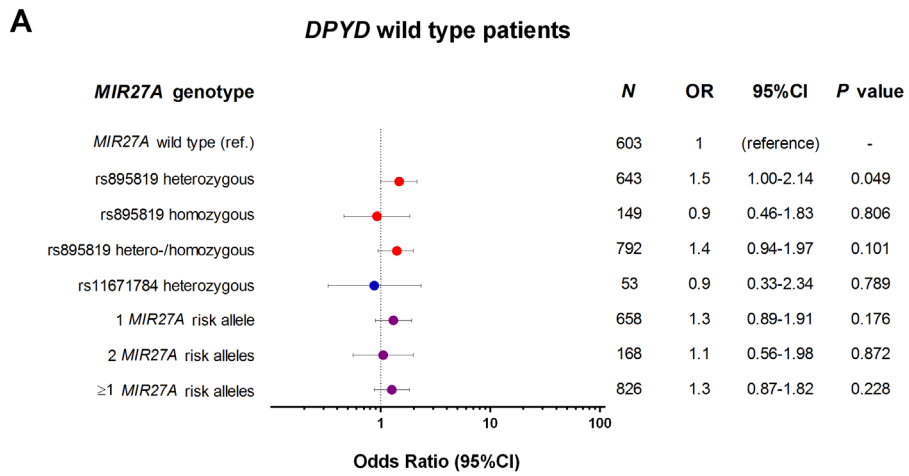


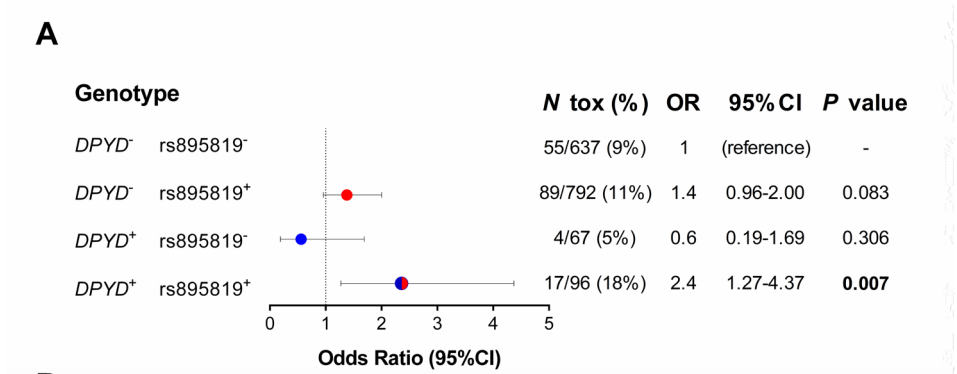
Figure 1. Associations between *MIR27A* variants and fluoropyrimidine-associated toxicity according to *DPYD* status.

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).

Abbreviations: CI: confidence interval; *DPYD*: dihydropyrimidine dehydrogenase (gene); *MIR27A:* microRNA 27a (gene); *N* tox: number of patients experiencing severe toxicity/total number of patients; OR: odds ratio.

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 (Figure 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 (Figure 2C). This analysis confirmed the increased risk of toxicity for *DPYD*$^{+}$/rs895819^{+} patients.
As shown in Supplementary Table 3, 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).

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 (Figure 3A), and to investigate the robustness of associations between *MIR27A* variants and risk of toxicity depending on the type of *DPYD* variant present (Figure 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.

◀ 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.
Abbreviations: CI: confidence interval; *DPYD*: dihydropyrimidine dehydrogenase (gene); *MIR27A:* microRNA 27a (gene); NA: not available (could not be calculated because 0/5 patients experienced severe toxicity); *N* tox: number of patients experiencing severe toxicity/total number of patients; OR: odds ratio.

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.

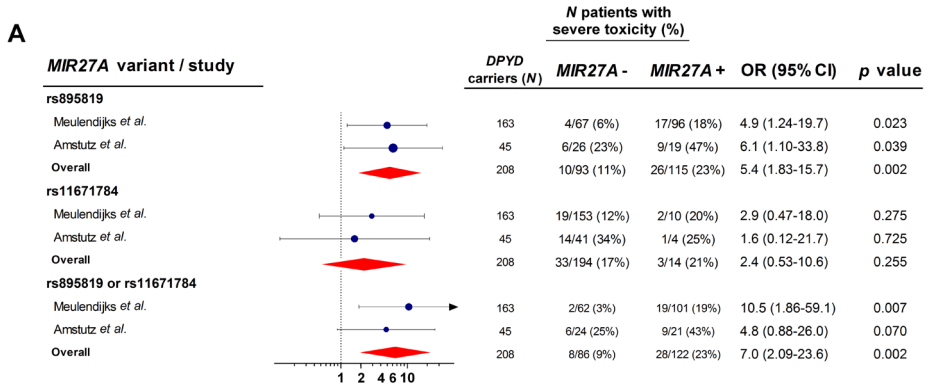


Figure 3. Results of meta-analysis to determine the effect of *MIR27A* variants in patients with *DPYD* variants, and pooled dataset analysis to determine the effect of individual *DPYD* variants.
Panel (a) shows the results of the meta-analysis to determine the overall effect of *MIR27A* variants in 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 ≥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).
Abbreviations: CI: confidence interval; *DPYD:* dihydropyrimidine dehydrogenase (gene); *MIR27A*: microRNA 27a (gene); OR: odds ratio.

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 (Figure 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 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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SUPPLEMENT

SUPPLEMENTARY METHODS

Determination of DPYD variants

The *DPYD* variants c.2846A>T (rs67376798), c.1679T>G (rs55886062), c.1129-5923C>G (rs75017182), and c.1601G>A (rs1801158) had been determined for the purpose of a previous study in which the clinical relevance of these variants as predictors of severe fluoropyrimidine-associated toxicity was investigated (Meulendijks *et al., submitted*). All 1613 patients who screened wild-type for *DPYD**2A in study NCT00838370 were included in the respective pharmacogenetic analysis. The four candidate pharmacogenetic variants in *DPYD,* c.2846A>T, c.1679T>G, c.1129-5923C>G, and c.1601G>A (Supplementary Table 1), were identified and selected based on a literature search, and were selected to investigate clinical validity.

DPYD variants were determined using standard PCR methods. Briefly, germline genomic DNA was isolated from peripheral blood cells using the MagNA Pure Total Nucleic Acid Isolation Kit I on MagNA Pure LC (Roche Diagnostics, Almere, the Netherlands), and c.2846A>T, c.1679T>G, and c.1601G>A were determined using real-time PCR assays with allele-specific TaqMan probes (Applied Biosystems, Foster City, CA). The c.1129-5923C>G variant was determined by genotyping the c.1236G>A variant as a proxy (c.1236G>A is in the same haplotype as c.1129-5923C>G, haplotype B3, and these variants have thus far been reported to be in complete linkage),[1,2] using a TaqMan assay (Applied Biosystems). The presence of haplotype B3 was confirmed by genotyping the haplotype B3 tagging variants c.959-51T>G and c.1129-5923C>G. In each PCR run sequenced wild-type and heterozygous controls were included, as well as homozygous controls, if available, and two negative controls.

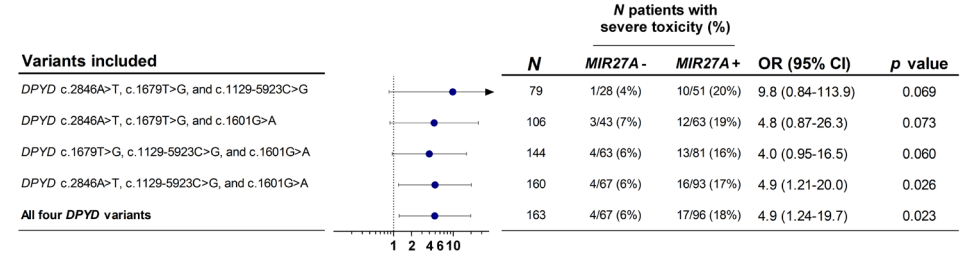
Genotype data of all four *DPYD* variants were complete for a total of 1600/1613 patients (99.2%). The data from these 1600 patients was the basis for the current study.

Determination of MIR27A variants

Genotyping for *MIR27A* variants was successfully performed in 1592/1600 patients (99.5%). The genomic region containing *MIR27A* was amplified using PCR, followed by sequencing. For amplification of *MIR27A*, the following primers were used: 5'-GTCCCCAAATCTCATTACCTCCTT-3' (forward) and 5'-GGTCTGATTCTGAGTCCTCATCTC-3' (reverse). PCR reactions were performed in a 25 µL volume, containing 2.5 µL 10X PCR Buffer II for AmpliTaq Gold (Applied Biosystems), 200 µM of each DNTP, 0.2 µM of forward and reverse primer, and 0.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR conditions were as follows: 5 min at 95°C; followed by 40 cycles of 30 sec at 95°C, 30 sec at 58°C, and 1 min at 72°C; followed by 10 min at 72°C. PCR products were sequenced using the BigDye® Terminator Cycle sequencing v3.1 kit (Applied Biosystems). 5 µL of PCR product was purified using 2 µL Illustra ExoProStar 1-step (GE Healthcare, Hoevelaken, the Netherlands) 1:5 dilution, incubated for 75 min at 37°C, followed by 15 min at 80°C. Subsequently, 7 µL purified PCR product was sequenced using 1 µL BigDye® Terminator v3.1 and 0.4 µM forward or reverse primer in a total reaction volume of 20 µL, under the following conditions: 96°C for 10 sec followed by 50°C for 5 sec and 60°C for 4 min, for 24 cycles. The sequence fragments were analyzed using an automated sequencer (ABI3730, Applied Biosystems). Two negative controls were included in each run. Calling of genotypes based on the sequencing results was performed manually, independently by two investigators (D Meulendijks, LM Henricks) and results were compared. Concordance between the two investigators was 100%.

Supplementary Table 1. Overview of *DPYD* variants analyzed.

Pharmacogenetic variant	RefSNP number	Star allele designation	Amino acid change	Biochemical consequences	Clinical findings
***DPYD* c.2846A>T**	rs67376798	-	D949V	Interferes with cofactor binding and/or electron transport.[3] Homozygous *in vitro* expression of this variant in HEK293 cells results in significantly decreased enzyme activity (rest activity 59% compared to wild-type *DPYD*, *P*=0.0031).[4]	Different high-quality studies and a meta-analysis have demonstrated an association between this variant and increased risk of fluoropyrimidine-associated toxicity.[5–12]
***DPYD* c.1679T>G**	rs55886062	*DPYD**13	I560S	Change of hydrophobic into hydrophilic residue; thought to result in destabilization of the protein. Homozygous *in vitro* expression of this variant in HEK293 cells results in significantly decreased enzyme activity (rest activity 25% compared to wild-type *DPYD*, *P*=5.2 x 10^{-9}).[13]	An association between this variant and increased risk of severe toxicity was found in different studies.[5,9] No significant effect was found in some other studies, possibly due to a low frequency.[2,10,14]
***DPYD* c.1129-5923C>G**	rs75017182	-	-	Deep intronic mutation that leads to aberrant pre-mRNA splicing. In complete linkage with the synonymous mutation c.1236G>A, and three intronic polymorphisms, c.483+18G>A, c.959-51T>G, and c.680+139G>A. Together these polymorphisms constitute a haplotype that has been termed haplotype B3.[1,14]	Several studies have demonstrated an association between this variant and the risk of fluoropyrimidine-associated toxicity.[1,2,8,14] No significant effect on risk of severe toxicity was found in other studies.[7,9,12,15–17]
***DPYD* c.1601G>A**	rs1801158	*DPYD**4	S534N	Has been associated with a significant decrease in DPD activity, as measured in PBMCs.[18] *In vitro* expression of this variant in HEK293 cells resulted in significantly increased enzyme function (36% more active than wild-type *DPYD*, *P*=3.4 x 10^{-7}).[13]	One study found an association between this variant and increased risk of severe toxicity.[9] Multiple other studies did not find a relationship.[7,8,12,14,15,17]



Supplementary Figure 1. Sensitivity analysis to determine associations between rs895819 and fluoropyrimidine-associated toxicity in patients carrying *DPYD* variants.

Supplementary Table 2. Frequencies of combinations of *MIR27A* genotypes.
In line with the almost complete linkage disequilibrium that we observed (*D'*=0.99), it can be seen in the table that none of the patients with a homozygous variant genotype for rs895819 carried the rs11671784 variant. The percentages correspond to the frequency of rs11671784 in patients carrying the rs895819 genotype presented in that row.

		rs11671784	
		C/C	C/T
rs895819	**A/A**	665 (94.5%)	39 (5.5%)
	A/G	699 (96.7%)	24 (3.3%)
	G/G	165 (100%)	0 (0%)

Supplementary Table 3. Performance of combined *DPYD* and *MIR27A* screening to identify patients at risk of first cycle severe toxicity.

Screening result	*N*	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Screening strategy including only *DPYD* variants					
DPYD+	163	13%	90%	13%	90%
Screening strategy including *DPYD* and *MIR27A* variants					
DPYD+ / MIR27A–	62	1%	96%	3%	89%
DPYD+ / rs895819+	91	10%	95%	19%	90%
DPYD+ / rs11671784+	5	1%	100%	40%	90%
DPYD+ / rs895819+ or DPYD+ / rs11671784+	96	12%	95%	20%	90%
DPYD+ / rs11671784+ and rs895819+	5	0%	100%	0%	90%

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10

Effectiveness and safety of reduced-dose fluoropyrimidine therapy in patients carrying the *DPYD**2A variant: a matched pair analysis

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SUMMARY

Background

Carriers of the genetic variant *DPYD**2A, resulting in dihydropyrimidine dehydrogenase deficiency, are at significantly increased risk of developing severe fluoropyrimidine-associated toxicity. Upfront *DPYD**2A genotype-based dose reductions improve patient safety, but uncertainty exists whether this has a negative impact on treatment effectiveness. Therefore, this study investigated the effectiveness and safety of *DPYD**2A genotype-guided dosing.

Methods

Patients planned for fluoropyrimidine-based chemotherapy treatment were prospectively genotyped for *DPYD**2A. A cohort of 40 prospectively identified *DPYD**2A carriers, treated with a ~50% reduced fluoropyrimidine dose, was identified. For effectiveness analysis, a matched pair-analysis was performed where for each *DPYD**2A carrier a matched *DPYD**2A wild-type patient was identified. Matching was performed on tumor type, disease stage, sex, age, WHO status, line of treatment and treatment regimen. Overall survival and progression-free survival were compared between *DPYD**2A carriers and matched wild-type patients. The frequency of severe (grade ≥3) treatment-related toxicity was compared to 1] a cohort of wild-type patients treated with full dose and 2] historical controls derived from literature, i.e. *DPYD**2A variant carriers who received a full fluoropyrimidine dose.

Results

40 *DPYD**2A carriers were treated with a mean fluoropyrimidine dose intensity of 53%. For 37 out of 40 *DPYD**2A carriers a matched control could be identified. Compared to matched controls, reduced doses did not negatively affect overall survival (median 27 months versus 24 months for matched controls, P=0.47) nor progression-free survival (median of 14 months versus 10 months, P=0.54). Risk of severe fluoropyrimidine-related toxicity in the *DPYD**2A carriers treated with reduced dose was 18%, which was comparable to wild-type patients (23%, P=0.57) and significantly lower than the risk of 77% in historical controls, *DPYD**2A carriers treated with full dose (P<0.001).

Conclusions

This study is the first to show that *DPYD**2A genotype-guided dosing appears to have no negative effect on effectiveness of fluoropyrimidine-based chemotherapy, while resulting in significantly improved patient safety.

INTRODUCTION

Fluoropyrimidine drugs, including 5-fluorouracil (5-FU) and its oral prodrug capecitabine, are the cornerstone of chemotherapeutic treatment for multiple solid tumor types, including colorectal, breast and gastric cancer. An estimated two million patients worldwide are treated yearly with this class of anti-cancer drugs.[1] However, these drugs are associated with substantial treatment-related toxicity, with around 30% of treated patients experiencing severe toxicity, (grade 3 or higher according to the Common Terminology Criteria for Adverse Events (CTC-AE)), often leading to hospitalization and interruption or discontinuation of therapy. The most common adverse events include diarrhea, mucositis, hand-foot syndrome and myelosuppression.[2]

In recent years, it has become clear that fluoropyrimidine-related toxicity is often related to deficiency of the enzyme dihydropyrimidine dehydrogenase (DPD), the main metabolic enzyme of fluoropyrimidines.[2,3] An estimated 3-8% of the population is subject to reduced DPD activity, and when treated at a full dose of 5-FU or capecitabine, exposure to 5-FU is increased, resulting in a higher risk of developing severe toxicity.[3,4] Most often, DPD deficiency is the result of single nucleotide polymorphisms (SNPs) in *DPYD*, the gene encoding DPD. The first *DPYD* variant that was discovered, also considered to be one of the most clinically relevant variants, is the *DPYD**2A variant (IVS14+1G>A, c.1905+1G>A, rs3918290), which results in skipping of exon 14, and hence a non-functional enzyme.[5,6] Heterozygous *DPYD**2A variant allele carriers, with a frequency of ~1% in the Western population, carry one functional allele and one non-functional allele and therefore have approximately 50% DPD enzyme function compared to normal.[7]

In a previously performed large clinical trial we showed that by reducing the fluoropyrimidine starting dose by 50% in heterozygous *DPYD**2A carriers, these patients can be safely treated. The frequency of severe treatment-related toxicity was reduced from 73% in a historical cohort of *DPYD**2A carriers treated with full dose, to 28% by reducing the starting fluoropyrimidine dose by ~50% in *DPYD**2A carriers. The risk of toxicity in these DPD deficient patients was thus found to be reduced to the background risk of toxicity in patients without DPD deficiency, which was 23% in the same study, in the cohort of *DPYD* wild-type patients treated at full dose.[8] Furthermore, pharmacokinetic analyses showed that drug exposure in the heterozygous *DPYD**2A carriers treated at a reduced dose was comparable to control values of wild-type patients treated with standard dose, suggesting that exposure was adequate.

However, upfront screening for DPD deficiency and dose reduction in patients carrying *DPYD* variant alleles is still not standard practice in all treatment centers where patients are treated with fluoropyrimidines. The most critical uncertainty related to dose reduction in DPD deficient patients, as argued by those who are critical in relation to *DPYD* screening, is whether fluoropyrimidine treatment will still be efficacious when doses are reduced. For this reason, we undertook a study to investigate effectiveness of fluoropyrimidine therapy after dose reduction in DPD deficient patients carrying the *DPYD**2A allele. In the largest study performed in this respect, we investigated a cohort of 40 *DPYD**2A variant allele carriers treated with a reduced dose and determined effectiveness of treatment compared to matched controls of *DPYD**2A wild-type patients treated with a full dose.

PATIENTS AND METHODS

Patient selection

This study was performed in a single center in which all patients who were treated with

fluoropyrimidine-based therapy as part of routine clinical care, were screened prospectively for the *DPYD**2A variant prior to start of therapy. If patients were identified as heterozygous carriers of *DPYD**2A, the fluoropyrimidine starting dose was reduced by approximately 50%. It was allowed to titrate the dose upwards during treatment after two cycles based on tolerance, as decided by the treating physician.

Patients who were heterozygous carriers of *DPYD**2A were included in this analysis, comprising all *DPYD**2A carriers who were screened between May 2007 and April 2015, who started with fluoropyrimidine-based chemotherapy, either as monotherapy or in combination with other chemotherapeutic agents or radiotherapy. The first 18 patients were identified during a prospective study that enrolled patients from May 2007 to October 2011 (NCT00838370). Safety data of these patients have been published by Deenen *et al.*[8] After closing of the trial, prospective *DPYD**2A screening was continued in our institute (The Netherland Cancer Institute, Amsterdam, The Netherlands), as part of routine clinical care. Patients identified as *DPYD**2A carriers in this second period, taken together with the first identified *DPYD**2A carriers were considered group 1.

Results on effectiveness of fluoropyrimidine treatment and risk of severe toxicity in this group were compared to group 2, which consisted of all *DPYD**2A wild-type patients from the study of Deenen *et al.*[8] screened between May 2007 and October 2011. For effectiveness analyses a selection of patients in group 2 was made, based on identified matched controls for the patients in group 1. For toxicity analyses, a comparison was made between group 1 and the entire cohort of group 2, and also between group 1 and a literature cohort (group 3). This historical literature cohort consisted of *DPYD**2A carriers who were treated with a full dose of fluoropyrimidines. For this historical cohort, the same publications as used for the previous clinical trial were included, describing unselected cohort studies of patients genotyped for *DPYD**2A and treated with fluoropyrimidine-based chemotherapy. Furthermore, using the same search terms, the historical cohort was expanded with publications after February 1, 2014 (end date of search by Deenen *et al.*).[8]

Patients of whom data were included, were treated according to routine clinical care, and data was collected retrospectively, thus institutional review board (IRB) approval was not required. Data from wild-type control patients and a subset of *DPYD**2A carriers were derived from the study of Deenen *et al.*,[8] for which IRB approval was granted by The Netherlands Cancer Institute, Amsterdam, The Netherlands.

Study design

This study investigated both effectiveness and toxicity. The primary endpoint for effectiveness was overall survival (defined as the time between initiation of treatment and death, by any cause). Secondary endpoints for effectiveness were progression-free survival (defined as the time between initiation of treatment and first signs of disease progression by either radiology or clinical signs, or death, whichever came first) and objective tumor response (according to RECIST 1.1 criteria).

A secondary aim of the study was investigating the incidence of severe (CTC-AE grade ≥3) fluoropyrimidine-associated toxicity. Overall fluoropyrimidine-associated toxicity and several subtypes of toxicity such as hematological toxicity (including neutropenia, leukopenia, thrombocytopenia), gastrointestinal toxicity (including diarrhea and mucositis) and hand-foot syndrome were investigated. Other parameters associated with toxicity that were investigated, included hospitalization for treatment-related toxicity, treatment interruptions due to toxicity and incidence of treatment-related death.

Matching

For all *DPYD**2A carriers (group 1) a matched control was identified from the *DPYD**2A wild-type cohort (group 2) for the primary effectiveness analyses. A one-to-one matching procedure was performed. Patients were matched on covariables that were known to have a relevant influence on treatment outcome.

First, automatic matching in the database was performed based on the following criteria: treatment at the same institute, tumor type (colorectal cancer, gastric or esophageal cancer, breast cancer, pancreatic cancer, head and neck cancer or other), disease stage (local and locally advanced or metastatic), sex, treatment received (capecitabine/5-FU, radiotherapy yes/no, monotherapy/ combination therapy) and age at first administration of fluoropyrimidine treatment (±5 years).

After automatic matching, a manual selection to identify the best matching control was performed (as automatic matching in the database was not possible for the remaining criteria). The following criteria were used for manual selection: if tumor type was defined as "other", a similar tumor type was selected, the line of treatment, specification of concomitant chemotherapy and WHO-status at baseline). If more than one wild-type patient was available fulfilling all matching criteria, the paired match was chosen at random. If there was no exact match available fulfilling all matching criteria, a discrepancy on one matching variable was allowed, but this excluded tumor type and disease stage, as those variables were expected to have the largest impact on treatment outcome.

The *DPYD**2A wild-type cohort had retrospectively been genotyped for three other *DPYD* variants (c.2846A>T, c.1679T>G, c.1236G>A) for another study.[9] Carriers of these variants were excluded from the matching process.

Statistical analysis

Patient and treatment characteristics were analyzed by group using descriptive statistics. Overall survival and progression-free survival were compared between the matched groups 1 and 2 using Kaplan-Meier estimates and the log-rank test for equality of survival curves. A log-rank hazard ratio was calculated as well. Patients alive at last follow-up were censored. Objective tumor response was compared using the McNemar's test, where the proportions of patients with disease control (complete response, partial response, stable disease) and disease progression were compared.

For toxicity analyses, the Fisher's exact test was used to compare frequencies of severe toxicities, hospitalization, treatment interruptions and treatment-related death between groups (group 1 vs group 2 and group 1 vs group 3).

For all analyses, *P* values <0.05 were considered statistically significant. Statistical analyses were performed using SPSS Version 22.0 (IBM SPSS Statistics.).

RESULTS

Overall patient characteristics

For the current analysis, 16 out of 18 patients that were identified during the prospective study were included, as the two other patients were treated at another hospital, and survival data for these patients could not be retrieved. An additional 24 heterozygous *DPYD**2A carriers were identified during routine screening and these patients were included in this study as well. This resulted in a total of 40 identified *DPYD**2A carriers who were treated with fluoropyrimidines at

a reduced starting dose (Figure 1). The cohort of *DPYD**2A wild-type patients from the previous prospective clinical trial was used as control group. This cohort included 1613 fluoropyrimidine-treated patients that were prospectively screened as wild-type for *DPYD**2A. As clinical data were incomplete for 7 of these patients, 1606 patients were included in the current analysis.
Baseline characteristics for *DPYD**2A carriers (group 1) and *DPYD**2A wild-type patients (group 2) are depicted in Table 1. The 40 *DPYD**2A carriers were treated with a mean dose intensity of 53.0% (mean dose of the entire treatment duration). The mean dose intensity for the first cycle was 51.6%. In eleven patients, doses were titrated upwards during treatment, in seven patients doses had to be further reduced after the initial dose reduction of 50%.
For the effectiveness analysis, matched controls for the *DPYD**2A carriers were identified in the wild-type cohort. For three *DPYD**2A carriers no suitable match could be identified, thus those three patients were excluded from effectiveness analyses, leaving 37 evaluable patients. Perfect matching was not possible for all remaining patients, mostly as the WHO status was often unknown (which was caused by the retrospective nature of data collection and often incomplete patient files). Small discrepancies on matching factors were then allowed. In Supplementary Table 1 an overview of these discrepancies is given.
For the literature control cohort, used for comparison of toxicity, a total of 17 published studies were selected, describing clinical data on 86 *DPYD**2A variant allele carriers.[10–26]

Effectiveness of genotype-guided dosing

Overall survival was compared between the 37 *DPYD**2A carriers receiving genotype-guided dosing and 37 matched wild-type controls (Figure 2). Median survival of *DPYD**2A carriers was 27 months (2.3 years), with a range of 1 months to 83 months (6.9 years). Median survival of wild-type patients was 24 months (2.0 years) with a range of 0.7 months to 97 months (8.1 years). The log-rank test showed that overall survival was not significantly different between both groups (*P*=.47). The hazard ratio comparing *DPYD**2A carriers to wild-type patients was 0.82 (95% confidence interval (95%CI): 0.47-1.43).
Also progression-free survival curves were similar for both groups (Supplementary Figure 1). Median progression-free survival for *DPYD**2A carriers was 14 months (1.2 years) with a range of 0.7 months to 83 months (6.9 years), and median progression-free survival for wild-type patients was 10 months with a range of 0.2 months to 97 months (8.1 years). Progression-free survival curves were not statistically significantly different (*P*=0.54). When comparing *DPYD**2A carriers to wild-type patients, the hazard ratio was 0.83 (95%CI: 0.47-1.50).
There was no statistically significant difference for the proportions of patients with disease control for both groups either (*P*>0.99, Supplementary Table 2). 12 out of 37 *DPYD**2A carriers had controlled disease (of whom four had a partial response and eight stable disease), and 10 out of 37 wild-type patients (of whom one with complete response, six patients with partial response and three with stable disease).

Toxicity of genotype-guided dosing

Genotype-guided dosing resulted in 7 out of 40 patients (18%) in group 1 experiencing grade ≥3 overall fluoropyrimidine-related toxicity. The incidence of gastrointestinal toxicity, hematological toxicity and hand-foot syndrome was respectively 10%, 10% and 5% (Table 2). Both for overall toxicity and the subtypes of toxicity, frequencies were highly comparable to the cohort of *DPYD**2A

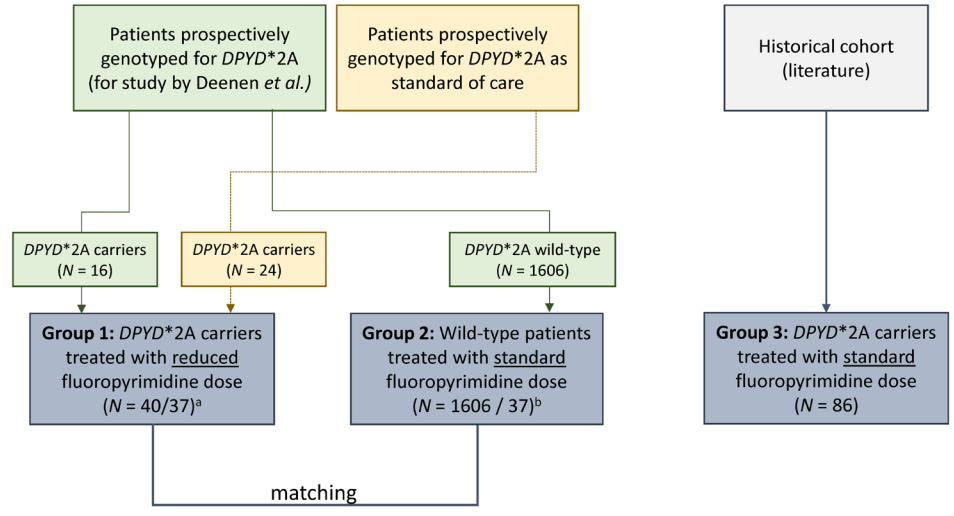

Figure 1. Selection of patients.
[a] For toxicity analyses all patients from group 1 were included (*N*=40). As no appropriate matches could be identified for 3 patients, 37 patients were included for effectiveness analyses.
[b] For toxicity analyses all patients from group 2 were included (*N*=1606). For effectiveness analyses a subgroup was included (*N*=37) which consisted of patients that were matched to the patients of group 1.

wild-type patients (Table 2). The same accounted for incidence of treatment-related hospitalization, treatment interruptions and treatment-related death. None of these outcomes were significantly different between group 1 and group 2 (Table 2).
Toxicity risk was also compared to the historical literature cohort (group 3). This showed that genotype-guided dosing resulted in a significantly lower risk of severe toxicity, i.e. 77% in group 3 versus 18% in group 1 (*P*<0.001). Individual patient characteristics of *DPYD**2A carriers in the historical cohort are depicted in Supplementary Table 3. Treatment-related death was not present in the genotype-guided dosing *DPYD**2A carriers, whereas this was 8% in the historical cohort (7 out of 86 patients).

DISCUSSION

This is to our knowledge the largest study so far determining whether effectiveness of fluoropyrimidine chemotherapy is affected by dose reduction in DPD deficient patients who are carriers of the *DPYD**2A variant. Due to the heterogeneous patient population receiving fluoropyrimidine therapy and the low frequency of *DPYD**2A (approximately 1%), trials investigating the effectiveness of genotype-guided dosing are difficult to perform, as these require a very large sample size. We prospectively screened over 4000 patients to identify 40 patients with the *DPYD**2A variant, of which efficacy and safety data of fluoropyrimidine-based treatment were collected retrospectively. Subsequently, we performed a matched pair analysis using control patients from the same institute. By choosing matching factors known to be associated with effectiveness of fluoropyrimidine chemotherapy, we aimed to make the comparison between *DPYD**2A carriers and wild-type patients as reliable as possible.

Table 1. Baseline and treatment characteristics of patients (group 1 and 2).

	Group 1: *DPYD2A carriers treated with reduced dose (*N*=40)**	**Group 2: Wild-type patients treated with standard dose (*N*=1606)**
Sex		
Male	14 (35%)	720 (45%)
Female	26 (65%)	886 (55%)
Age, median [range]	61.7 [33.8 – 90.8]	61.2 [20.8 – 88.8]
Ethnic origin		
Caucasian	39 (98%)	1540 (96%)
Southeast Asian	1 (2%)	14 (1%)
African	0 (0%)	21 (1%)
Other	0 (0%)	31 (2%)
BSA, median [range]	1.8 [1.5 – 2.2]	1.9 [1.1 – 2.7]
Disease status		
Locally advanced CRC	9 (23%)	534 (33%)
Metastatic CRC	4 (10%)	320 (20%)
Locally advanced BC	3 (8%)	119 (7%)
Metastatic BC	12 (30%)	250 (16%)
GC	2 (5%)	227 (14%)
Other	10 (25%)	156 (10%)
Previously treated with chemotherapy		
Yes	17 (43%)	359 (22%)
No	23 (58%)	1247 (78%)
Treatment regimen		
CAP mono	15 (38%)	424 (26%)
CAP + Pt	2 (5%)	378 (24%)
CAP triplet	0 (0%)	114 (7%)
CAP + RT	12 (30%)	436 (27%)
CAP + other	9 (23%)	86 (5%)
5-FU mono	0 (0%)	16 (1%)
5-FU + RT	0 (0%)	54 (3%)
5-FU + other	2 (5%)	98 (6%)

Abbreviations: 5-FU mono: 5-fluorouracil monotherapy; 5-FU + other: 5-FU combined with other chemotherapeutics; 5-FU + RT; 5-fluorouracil combined with radiotherapy; BC: breast cancer; CAP mono: capecitabine monotherapy; CAP + other; capecitabine combined with other chemotherapeutics; CAP + Pt: capecitabine plus platinum agent; CAP triplet: capecitabine combined with platinum agent and taxane; CAP + RT: capecitabine combined with radiotherapy; CRC: colorectal cancer; GC: gastric or gastroesophageal cancer.

The study shows that both overall and progression-free survival were comparable between *DPYD**2A variant allele carriers receiving reduced dose and wild-type patients receiving standard dose fluoropyrimidines. These results endorse the assumption that dose reductions do not result in inferior treatment outcome in these DPD deficient patients. This assumption has previously been made on the basis of DPD activity which is approximately 50% reduced in *DPYD**2A variant allele carriers, and a 50% fluoropyrimidine reduced dose is therefore expected to result in exposure that is comparable to exposure in *DPYD* wild-type patients receiving standard dose. This has also been shown by the fact that pharmacokinetic analyses in the previous prospective study confirmed that 5-FU exposure was equal between *DPYD**2A genotype-dosed patients and wild-

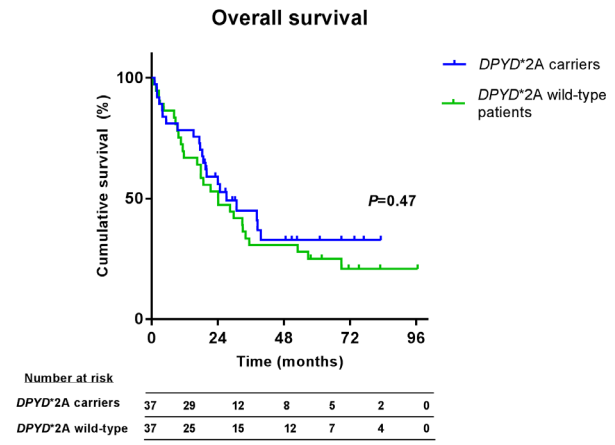

Figure 2. Overall survival analysis of *DPYD2A carriers with reduced dose versus wild-type patients with standard dose.**
Shown is the Kaplan-Meier survival curve for overall survival. The *P*-value was calculated using the log-rank test for equality of survival curves. Patients alive at last follow-up were censored.

type patients receiving full dose.[8] Furthermore, we found that toxicity risk in the *DPYD**2A carriers receiving a reduced dose was similar to toxicity risk in the cohort of wild-type patients, which further endorses this assumption.

This study also confirms that upfront genotyping for *DPYD**2A improves patient safety of fluoropyrimidine therapy, in line with what was previously shown in a large prospective trial.[8] In the previous prospective study, grade ≥3 toxicity was found to be decreased from 73% to 28% by genotype-guided dosing, and in our current analysis this risk dropped from 77% to 18%, which is of the same order of magnitude. Treatment-related death decreased from 8% to 0% in our study. Our results are derived from a real-world population, which strengthens the implications of these findings for clinical practice. Importantly, Deenen *et al*. previously showed that genotype-guided dosing is also cost saving, as costs for treatment of severe adverse events and hospitalization are decreased, and outweigh costs of screening of the entire population.[8]

A few other small studies have been performed, that did investigate the effect of fluoropyrimidine dose individualization on effectiveness. In the study by Launay *et al.*[27] 5-FU individualized dosing was based on DPD phenotype (measured as the ratio between uracil, the endogenous substrate of DPD and its product dihydrouracil). Of the 59 included patients with digestive cancer, 15 (25%) were identified as DPD deficient and received a reduced dose of 5-FU (average dose reduction of 35%). These dose reductions did not result in lower effectiveness in this small group of patients compared to the non-DPD deficient patients (*P*=0.89 when comparing the number of patients with clinical benefit, stable disease and progressive disease).[27] A drawback of this study is the low sample size.

Our current study focused only on *DPYD**2A genotyping, while it has become clear in recent years that other *DPYD* variants result in DPD deficiency as well. Currently, the three additional variants c.2846A>T, c.1236G>A and c.1679T>G are considered clinically relevant and upfront genotyping for this panel is recommended.[28–30] For these polymorphisms it is expected that similar to *DPYD**2A,

genotype-guided dosing does not negatively affect treatment outcome, while safety is significantly improved.

Due to the retrospective design of this study, patient data was not always complete, which hampered the matching of *DPYD**2A carriers to wild-type patients to some extent, as matching factors could not always be retrieved. Ideally additional matching factors which are thought to be relevant would be included, e.g. molecular subtypes of cancer which affect prognosis as well. However, this was not feasible with our current study design, as these data were not available. Due to the large control group of wild-type patients of 1606 patients that was available for matching, all available data were used in the best way possible to make the matching as adequate as possible.

In conclusion, this retrospective and matched-pair analysis supports that dose reductions in *DPYD**2A carriers do not result in inferior effectiveness of fluoropyrimidine-based chemotherapy, and that toxicity risk normalizes to the background toxicity risk in wild-type patients. Although these findings are preferably replicated, they support current clinical guidelines which recommend a 50% upfront fluoropyrimidine dose reduction in *DPYD**2A variant allele carriers.[30]

Table 2. Treatment outcome of patients (group 1, 2 and 3).

	Group 1: *DPYD**2A carriers treated with reduced dose (N=40)	Group 2: Wild-type patients treated with standard dose (N=1606)	Group 3: *DPYD**2A carriers treated with standard dose from literature (N=86)	P value group 1 vs 2	P value group 1 vs 3
Mean dose intensity (% of standard dose)	53%	92%	Unknown [a]	NA	NA
Hand-foot syndrome Grade 3	2 (5%)	84 (5%)	Unknown	>0.99	NA
Hematological toxicity Grade ≥3	4 (10%)	158 (10%)	48 (56%)	>0.99	**<0.001**
Gastrointestinal toxicity Grade ≥3	4 (10%)	150 (9%)	33 (38%)	0.78	**0.001**
Overall toxicity Grade ≥3	7 (18%)	372 (23%)	66 (77%)	0.57	**<0.001**
Treatment interruptions	5 (13%)	309 (19%)	Unknown	0.41	NA
Hospitalization	6 (15%)	179 (11%)	Unknown	0.44	NA
Treatment-related death	0 (0%)	2 (0%)	7 (8%)	>0.99	0.096

[a] Dose intensity is unknown, but patients started with standard dose (~100%).
[b] *P* value calculated using Fisher's Exact test.
Abbreviations: NA: not applicable.

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SUPPLEMENT

Supplementary Table 1. Overview of matched pairs.
Matched pairs between patients from group 1 (*DPYD**2A carriers treated with reduced dose) and group 2 (wild-type patients treated with standard dose) were made. Group 1 consisted of 40 patients. For 3 patients of group 1 no appropriate match could be identified, those patients were excluded from the effectiveness analyses. For 20 patients of group 1 matching could not be performed for all predetermined factors. The data below show for which factors there was a discrepancy. These factors were thought to have no major effect on outcome, therefore those patients were included in the effectiveness analyses. For the 17 remaining patients matching on all predetermined factors could be performed, resulting in a total of 37 patients evaluable for effectiveness analyses.

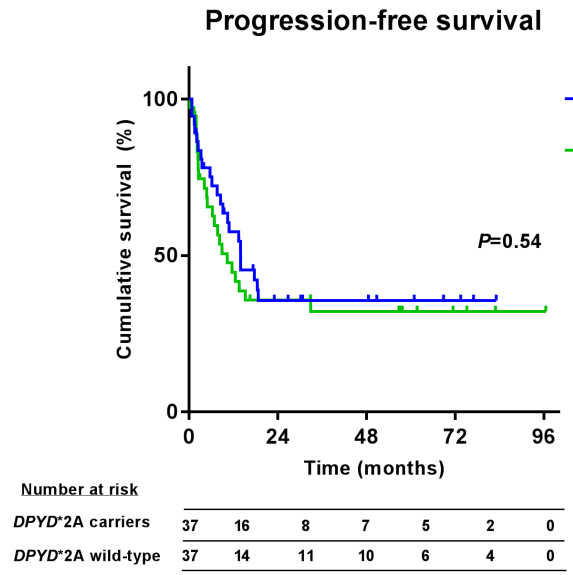
Patient number	Patient characteristic of group 1	Patient characteristic of group 2
2	WHO: 1	WHO: 0
4	WHO: ND	WHO: 1
9	WHO: ND	WHO: 0
11	WHO: ND	WHO: 0
13	WHO: 0	WHO: ND
14	WHO: 1	WHO: ND
16	WHO: 1 Line of treatment: 2	WHO: 0 Line of treatment: 3
17	WHO: 0 Regimen: CAP + RT	WHO: ND Regimen: CAP + RT + MMC
18	Age: 80.8 years	Age: 72.1 years
19	WHO: 2	WHO: ND
21	WHO: 1	WHO: ND
22	WHO: 1	WHO: 1-2
26	WHO: 1 Line of treatment: 1	WHO: ND Line of treatment: 2
27	Regimen: CAP + TRA + VIN	Regimen: CAP + TRA
29	WHO: 2	WHO: ND
31	Regimen: FOLFIRI switched to FOLFOX	Regimen: FOLFOX + bevacizumab
33	Regimen: CAPOX	Regimen: CAPIRI
34	WHO: 0 Regimen: FOLFIRI	WHO: 1 Regimen: 5-FU + cisplatin
37	Age 61.1 years	Age: 55.3 years
39	Line of treatment: 3	Line of treatment: 2

Abbreviations: CAP: capecitabine; CAPIRI: capecitabine combined with irinotecan; CAPOX: capecitabine combined with oxaliplatin; FOLFIRI: 5-fluorouracil combined with irinotecan; FOLFOX: 5-fluorouracil combined with oxaliplatin; MMC: mitomycin; ND: not determined; RT: radiotherapy; TRA: trastuzumab; VIN: vincristine; WHO: performance status according to World Health Organization (WHO) classification.

Supplementary Table 2. Results of comparison of disease control rate.

Response according to RECIST criteria	Group 1 (*DPYD**2A carriers treated with reduced dose) (N=37)	Group 2 (wild-type patients treated with standard dose (N=37)	P-value [a]
Disease controlled	12 (60%)	10 (48%)	>.99
Complete response	0 (0%)	1 (5%)	
Partial response	4 (20%)	6 (29%)	
Stable disease	8 (40%)	3 (14%)	
Progressive disease	8 (40%)	11 (30%)	
Not evaluable	17	16	

[a] *P*-value was calculated using the McNemar's test comparing the disease control rate and progressive disease rate, but excluding the non-evaluable patients. This resulted in only 13 matched pairs that could be included in this statistical analysis.



Supplementary Figure 1. Progression-free survival analysis of *DPYD2A carriers with reduced dose versus wild-type patients with standard dose.**
Shown is the Kaplan-Meier survival curve for progression-free survival. The *P*-value was calculated using the log-rank test for equality of survival curves. Patients alive at last follow-up were censored.

Supplementary Table 3. Individual patient data of historical literature cohort.

	Study	5-FU or CAP	RT	Sex	Tumor type	Grade ≥3 overall toxicity	Grade ≥3 GI toxicity	Grade ≥3 hematological toxicity	Treatment-related death	Total *N* evaluable patients	*N* of pts with *DPYD**2A	Study design	Treatment regimen
1	Schwab 2008[1]	5-FU	No	M	Unknown	No	No	No	No	683	13	prospective	high-dose infusion (24h)
2		5-FU	No	M	Unknown	Yes	Yes	Yes	No				high-dose infusion (24h)
3		5-FU	No	M	Unknown	Yes	Yes	No	No				high-dose infusion (24h)
4		5-FU	No	M	Unknown	Yes	Yes	Yes	No				mayo (bolus)
5		5-FU	No	M	Unknown	Yes	Yes	Yes	No				mayo (bolus)
6		5-FU	No	M	Unknown	Yes	Yes	No	No				mayo (bolus)
7		5-FU	No	F	Unknown	No	No	No	No				Unknown
8		5-FU	No	F	Unknown	No	No	No	No				Unknown
9		5-FU	No	F	Unknown	No	No	No	No				mayo (bolus)
10		5-FU	No	F	Unknown	No	No	No	No				Unknown
11		5-FU	No	F	Unknown	No	No	No	No				mayo (bolus)
12		5-FU	No	F	Unknown	No	No	No	No				mayo (bolus)
13		5-FU	No	F	Unknown	Yes	Yes	Yes	No				mayo (bolus)
14	Largillier 2006[2]	CAP	No	F	adv BC	Yes	Yes	Yes	Yes	105	1	prospective	CAP mono
15[a,b]	Boisdron-Celle 2007[3]	5-FU	No	M	adv CRC	Yes	Yes	Yes	Yes	252	3[c]	prospective	modified de Gramont or weekly regimen
16		5-FU	No	Unknown	adv CRC	Yes	Unknown	Unknown	No				modified de Gramont or weekly regimen
17[d]	Morel 2006[4]	5-FU	No	F	BC	Yes	Yes	Yes	No	487	10[e]	prospective	FEC
18		5-FU	No	Unknown	CRC	Yes	Yes	Yes	Yes[f]				de Gramont
19		5-FU	No	Unknown	adv CRC	Yes	Yes	Yes	No[f]				FOLFIRI
20		5-FU	No	Unknown	adv CRC	Yes	Yes	Yes	No[f]				FOLFIRI
21		5-FU	No	Unknown	adv CRC	Yes	Yes	Yes	No[f]				FOLFOX
22		5-FU	No	Unknown	head and neck cancer	Yes	Yes	Yes	No[f]				4 day infusional 5-FU + CIS
23		5-FU	No	Unknown	Unknown	No	No	No	No				Unknown
24		5-FU	No	Unknown	Unknown	No	No	No	No				Unknown

	Study	5-FU or CAP	RT	Sex	Tumor type	Grade ≥3 overall toxicity	Grade ≥3 GI toxicity	Grade ≥3 hematological toxicity	Treatment-related death	Total *N* evaluable patients	*N* of pts with *DPYD**2A	Study design	Treatment regimen
25	Deenen 2011[5]	CAP	No	F	adv CRC	Yes	Yes	Yes	Yes[f]	568	7	retrospective	CAPOX + BEV + CETUX
26		CAP	No	M	adv CRC	Yes	Yes	Yes	No[f]				CAPOX + BEV + CETUX
27		CAP	No	F	adv CRC	Yes	No	Yes	No[f]				CAPOX + BEV + CETUX
28		CAP	No	M	adv CRC	Yes	Yes	Yes	No[f]				CAPOX + BEV + CETUX
29		CAP	No	F	adv CRC	Yes	Yes	Yes	No[f]				CAPOX + BEV
30		CAP	No	M	adv CRC	Yes	Yes	Yes	No[f]				CAPOX + BEV
31		CAP	No	M	adv CRC	Yes	Yes	Yes	No[f]				CAPOX + BEV + CETUX
32	Capitain 2008[6]	5-FU	No	Unknown	adv CRC	Yes	Unknown	Unknown	No	76	1	retrospective	5-FU mono
33	Braun 2009[7]	5-FU	No	Unknown	adv CRC	Yes	Unknown	Unknown	No	629	5	retrospective	modified de Gramont
34		5-FU	No	Unknown	adv CRC	Yes	Unknown	Unknown	No				modified de Gramont
35		5-FU	No	Unknown	adv CRC	No	Unknown	Unknown	No				modified de Gramont
36		5-FU	No	Unknown	adv CRC	No	Unknown	Unknown	No				modified de Gramont
37		5-FU	No	Unknown	adv CRC	Yes	Unknown	Unknown	No				FOLFIRI
38	McLeod 2010[8]	5-FU	No	Unknown	adv CRC	Yes	No	Yes	No	114	2	retrospective	FOLFIRI
39		5-FU	No	Unknown	adv CRC	No	No	No	No				FOLFIRI
40	Boige 2010[9]	5-FU	No	Unknown	adv CRC	Yes	No	Yes	No	346	2	prospective	Unknown
41		5-FU	No	Unknown	adv CRC	Yes	No	Yes	No				Unknown
42	Cerić 2010[10]	CAP	No	F	adv BC	Yes	Yes	Yes	Yes	50	1	prospective	CAP mono
43	Cellier 2011[11]	UFT	Yes	Unknown	adv CRC	Yes	Yes	Yes	No	85	1	prospective	UFT + leucovorin
44	Sulzyc-Bielecka 2008[12]	5-FU	Unknown	M	adv CRC	Yes	No	Yes	No	252	1	retrospective	Unknown
45	Salgueiro 2004[13]	5-FU	No	M	adv CRC	Yes	No	Yes	No	73	1	retrospective	5-FU bolus
46	Jennings 2013[14]	5-FU	No	F	adv CRC	Yes	No	Yes	No	254	3	prospective	5-FU mono
47		5-FU	No	F	adv CRC	Yes	Yes	No	No				5-FU combination
48		5-FU	No	Unknown	adv CRC	No	No	No	No				Unknown
49	Joerger 2015[15]	CAP	No	Unknown	adv GEC	Yes	Yes	No	No	140	1	prospective	ECC

	Study	5-FU or CAP	RT	Sex	Tumor type	Grade ≥3 overall toxicity	Grade ≥3 GI toxicity	Grade ≥3 hematological toxicity	Treatment-related death	Total *N* evaluable patients	*N* of pts with *DPYD**2A	Study design	Treatment regimen
50[a]	Lee 2014[16]	5-FU	No	Unknown	adv CRC	Yes	Yes[g]	Yes[g]	Yes	2594	25	prospective	FOLFIRI/FOLFOX ± CETUX
51		5-FU	No	Unknown	adv CRC	Yes	Yes[g]	Yes[g]	No				FOLFIRI/FOLFOX ± CETUX
52		5-FU	No	Unknown	adv CRC	Yes	Yes[g]	Yes[g]	No				FOLFIRI/FOLFOX ± CETUX
53		5-FU	No	Unknown	adv CRC	Yes	Yes[g]	Yes[g]	No				FOLFIRI/FOLFOX ± CETUX
54		5-FU	No	Unknown	adv CRC	Yes	Yes[g]	Yes[g]	No				FOLFIRI/FOLFOX ± CETUX
55		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Yes[g]	No				FOLFIRI/FOLFOX ± CETUX
56		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Yes[g]	No				FOLFIRI/FOLFOX ± CETUX
57		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Yes[g]	No				FOLFIRI/FOLFOX ± CETUX
58		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Yes[g]	No				FOLFIRI/FOLFOX ± CETUX
59		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Yes[g]	No				FOLFIRI/FOLFOX ± CETUX
60		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Yes[g]	No				FOLFIRI/FOLFOX ± CETUX
61		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Yes[g]	No				FOLFIRI/FOLFOX ± CETUX
62		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Yes[g]	No				FOLFIRI/FOLFOX ± CETUX
63		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Yes[g]	No				FOLFIRI/FOLFOX ± CETUX
64		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Yes[g]	No				FOLFIRI/FOLFOX ± CETUX
65		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Yes[g]	No				FOLFIRI/FOLFOX ± CETUX
66		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Unknown[g]	No				FOLFIRI/FOLFOX ± CETUX
67		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Unknown[g]	No				FOLFIRI/FOLFOX ± CETUX
68		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Unknown[g]	No				FOLFIRI/FOLFOX ± CETUX
69		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Unknown[g]	No				FOLFIRI/FOLFOX ± CETUX
70		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Unknown[g]	No				FOLFIRI/FOLFOX ± CETUX
71		5-FU	No	Unknown	adv CRC	Yes	Unknown[g]	Unknown[g]	No				FOLFIRI/FOLFOX ± CETUX
72		5-FU	No	Unknown	adv CRC	No	No	No	No				FOLFIRI/FOLFOX ± CETUX
73		5-FU	No	Unknown	adv CRC	No	No	No	No				FOLFIRI/FOLFOX ± CETUX
74		5-FU	No	Unknown	adv CRC	No	No	No	No				FOLFIRI/FOLFOX ± CETUX

	Study	5-FU or CAP	RT	Sex	Tumor type	Grade ≥3 overall toxicity	Grade ≥3 GI toxicity	Grade ≥3 hematological toxicity	Treatment-related death	Total *N* evaluable patients	*N* of pts with *DPYD**2A	Study design	Treatment regimen
75	Toffoli 2015[17]	CAP	No	Unknown	CRC	Yes	Yes	Yes	No	603	12	prospective	CAP mono
76		5-FU	No	Unknown	nasopharynx	Yes	Yes	No	No				TPF
77		CAP	No	Unknown	CRC	Yes	No	Yes	No				CAP mono
78		CAP	No	Unknown	GEC	Yes	No	Yes	No				DOC
79		CAP	No	Unknown	CRC	Yes	Yes	No	No				CAPOX
80		5-FU	No	Unknown	CRC	Yes	No	Yes	No				FOLFOX
81		5-FU	No	Unknown	CRC	Yes	No	Yes	No				FOLFOX
82		5-FU	No	Unknown	mCRC	No	No	No	No				FOLFIRI
83		5-FU	No	Unknown	mCRC	No	No	No	No				FOLFIRI
84		5-FU	No	Unknown	mCRC	No	No	No	No				FOLFIRI
85		5-FU	No	Unknown	mCRC	No	No	No	No				FOLFIRI
86[h]		5-FU	No	Unknown	GEC	Yes	Yes	No	Yes				5-FU

[a] Also carrier of the c.2846A>T *DPYD* variant.
[b] Also carrier of c.85T>C *DPYD* variant.
[c] 1 out of 3 patients ineligible due to initial dose reduction.
[d] Homozygous carrier of *DPYD**2A.
[e] 2 out of 10 patients ineligible due to initial dose reduction.
[f] One patient reported to have fatal toxicity, not certain which individual patient.
[g] At least 16 patients experienced hematological toxicity and at least 5 patients GI toxicity, from the publication it is not certain which individual patients and also the exact number could not be interpreted from the publication.
[h] Also carrier of c.1679T>G *DPYD* variant.

Abbreviations: 5-FU: 5-fluorouracil; adv: advanced; BC: breast cancer; BEV: bevacizumab; CAP: capecitabine; CAPOX: capecitabine + oxaliplatin; CETUX: cetuximab; CIS: cisplatin; CRC: colorectal cancer; DOC: docetaxel + oxaliplatin + capecitabine; ECC: capecitabine + cisplatin + epirubicin; F: female; FOLFIRI: 5-fluorouracil + irinotecan; FEC: 5-fluorouracil + epirubicin + cyclophosphamide; FOLFOX: 5-fluorouracil + oxaliplatin; GI: gastrointestinal; GEC: gastric or gastroesophegeal cancer, M: male; mCRC: metastatic colorectal cancer; mono: monotherapy; RT: radiotherapy; TPF: docetaxel + cisplatin + 5-FU; UFT: tegafur + uracil.

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11

DPYD genotype-guided dose individualization of fluoropyrimidine therapy: a prospective safety and cost-analysis on four relevant *DPYD* variants

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SUMMARY

Background

Fluoropyrimidines are generally well tolerated drugs, but can result in severe toxicity in up to 30% of patients. A major cause of toxicity is reduced activity of the key metabolic enzyme dihydropyrimidine dehydrogenase (DPD), often caused by genetic variants in *DPYD*; the gene encoding DPD. In a prospective clinical trial we determined whether toxicity of fluoropyrimidines can be reduced by upfront screening for four *DPYD* variants and *DPYD*-guided dose individualization.

Methods

Prospective genotyping for *DPYD**2A, c.2846A>T, c.1679T>G, and c.1236G>A was performed in patients prior to start of fluoropyrimidine-based therapy. Heterozygous *DPYD* variant allele carriers received an initial dose reduction of 25% (c.2846A>T, c.1236G>A) or 50% (*DPYD**2A, c.1679T>G). Incidence of severe (grade≥3) fluoropyrimidine-related toxicity in *DPYD* variant allele carriers was compared to wild-type patients from the study and to a historical cohort of *DPYD* variant allele carriers treated with full dose.

Findings

1103 evaluable patients were enrolled, of whom 85 *DPYD* variant allele carriers (7.7%). Compared to the historical cohort, *DPYD* genotype-guided dosing markedly reduced the incidence of grade≥3 toxicity for *DPYD**2A and c.1679T>G carriers, moderately reduced incidence for c.2846A>T carriers, and resulted in a similar incidence for c.1236G>A carriers. Toxicity-related hospitalization incidence in *DPYD* variant allele carriers was similar to wild-type patients. A cost-analysis showed that reduced toxicity incidence resulted in lower average total treatment costs per patient for screening compared to non-screening.

Interpretation

Upfront *DPYD* genotyping was feasible in routine practice, and improved patient safety of fluoropyrimidine treatment, without increasing costs. For *DPYD**2A and c.1679T>G carriers, a 50% initial dose reduction seems adequate. For c.1236G>A and c.2846A>T carriers, the applied dose reductions of 25% did not reduce toxicity sufficiently, and therefore larger dose reductions of 50% or closer monitoring (applying dose reductions when treatment is not tolerated well) need to be investigated.

INTRODUCTION

Fluoropyrimidine anticancer drugs, including 5-fluorouracil (5-FU) and its oral prodrug capecitabine, have been widely used for over sixty years in the treatment of different solid tumor types, such as colorectal, breast, and gastric cancer. Although these drugs are relatively well tolerated, up to 30% of patients experiences severe treatment-related toxicity, including diarrhea, mucositis, myelosuppression and hand-foot syndrome.[1–3] In addition, severe fluoropyrimidine-related toxicity can lead to treatment-related death in up to 1% of patients.[4,5] The occurrence of these severe side-effects can lead to treatment discontinuation and toxicity-related hospitalization, which puts a heavy burden on health-care costs in addition.

Fluoropyrimidine-related toxicity is often caused by reduced activity of the enzyme dihydropyrimidine dehydrogenase (DPD), the main metabolic enzyme for fluoropyrimidine inactivation.[6,7] A partial DPD deficiency (i.e. a ~50% reduced DPD activity compared to normal) is present in 3-5% of the Western population, and these DPD deficient patients have a highly increased risk of developing severe treatment-related toxicity when treated with a standard dose of fluoropyrimidines.[8–10] Complete DPD deficiency is much rarer, with an estimated prevalence of 0.01-0.1%.[8,11,12] DPD deficiency is most often caused by genetic variants in *DPYD*, the gene encoding DPD. The four *DPYD* variants currently considered most clinically relevant and with convincingly demonstrated association with severe toxicity are *DPYD**2A (rs3918290, c.1905+1G>A), c.2846A>T (rs67376798), c.1679T>G (rs55886062, *DPYD**13), and c.1236G>A (rs56038477, in haplotype B3).[10,13,14] For these variants, available evidence suggests that heterozygous carriers of these variants have an average reduction in DPD enzyme activity of approximately 25% (c.2846A>T, c.1236G>A) to 50% (*DPYD**2A, c.1679T>G).[14]

Prospective *DPYD* genotyping and dose reduction in heterozygous *DPYD* variant allele carriers is a promising strategy for preventing severe and potentially fatal fluoropyrimidine-related toxicity in clinical practice without affecting efficacy of treatment. In a previous study by Deenen *et al.,* prospective genotyping and dose-individualization for one *DPYD* variant, *DPYD**2A, in a cohort of 1631 patients showed that severe fluoropyrimidine-related toxicity could be decreased from 73% in *DPYD**2A carriers receiving a standard fluoropyrimidine dose (*N*=48) to 28% by genotype-guided dosing, i.e. *DPYD**2A carriers receiving a 50% dose reduction (*N*=18, *P*<0.001).[15] This study showed that by reducing the fluoropyrimidine dose by 50% in *DPYD**2A variant allele carriers, severe toxicity was reduced to a frequency (28% severe toxicity) comparable to that in *DPYD**2A wild-type patients treated with a standard dose of fluoropyrimidines (23%). It is expected that patient safety can be further improved by expanding the number of prospectively tested *DPYD* variants beyond *DPYD**2A alone. The objective of the current study was to assess the impact on patient safety and costs of prospective screening for the four most relevant *DPYD* variants and subsequent *DPYD* genotype-guided dose individualization in daily clinical care.

PATIENTS AND METHODS

Study design and patient population

This study was a prospective multicenter clinical trial in which 17 hospitals in the Netherlands participated (NCT02324452). The study was approved by the institutional review board of The Netherlands Cancer Institute, Amsterdam, the Netherlands, and approval from the board of directors of each individual hospital was obtained for all participating centers. All patients provided written informed consent before enrollment in the study. Additional informed consent was

obtained for *DPYD* variant allele carriers who participated in pharmacokinetic and DPD enzyme activity measurements.

The study population consisted of patients intended to start with a fluoropyrimidine-based anticancer therapy, either as single agent or in combination with other chemotherapeutic agents and/or radiotherapy. Prior chemotherapy was allowed, except for prior use of fluoropyrimidines. Patients were genotyped before start of fluoropyrimidine therapy for the previously mentioned four *DPYD* variants. Heterozygous *DPYD* variant allele carriers received an initial dose reduction of either 25% (for c.2846A>T and c.1236G>A) or 50% (for *DPYD**2A and c.1679T>G), in line with current recommendations from Dutch and international pharmacogenomic guidelines.[13,16] To achieve maximal safe exposure, dose escalation was allowed after the first two cycles provided that treatment was well tolerated, and the decision to escalate was left to the discretion of the treating physician. The dose of other chemotherapeutic agents or radiotherapy were left unchanged at start of treatment. Homozygous or compound heterozygous *DPYD* variant allele carriers (patients carrying multiple *DPYD* variants simultaneously) were excluded from the study, and were treated with personalized regimens outside this protocol.[17] Non-carriers of the above mentioned *DPYD* variants are considered wild-type patients in this study, and were treated according to existing standard of care. Full inclusion and exclusion criteria can be found in the Supplement.

Toxicity was graded by participating centers according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTC-AE; version 4.03),[18] and severe toxicity was defined as grade 3 or higher. Patients were followed for toxicity during the entire treatment period. Toxicity defined as possibly, probably or definitely related to fluoropyrimidine-treatment was considered treatment-related toxicity. Other relevant toxicity-related parameters that were investigated included toxicity-related hospitalization and treatment discontinuation due to adverse events. Patients were considered evaluable if they received at least one fluoropyrimidine administration.

Procedures

DPYD genotyping: Genotyping for the four *DPYD* variants *DPYD**2A, c.2846A>T, c.1679T>G and c.1236G>A was performed before start of treatment. Genotyping was performed in a clinical laboratory of the local hospital or in one of the other participating centers of this trial. Validated assays were used and all laboratories participated in and met the criteria of a Dutch national proficiency testing program for all four *DPYD* variants.[19]

Pharmacokinetics and DPD enzyme activity: In *DPYD* variant allele carriers who provided written informed consent for additional tests, plasma levels of capecitabine, 5-FU and their metabolites were determined at the first day of a capecitabine/5-FU cycle (preferably the first cycle) to assess the pharmacokinetic profile in these patients. A validated ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS) method was used (details in Supplement). Results of pharmacokinetic parameters, including the area under the plasma concentration-time curve (AUC) and half-life ($t_{1/2}$) were calculated using non-compartmental analysis and compared to control values derived from a previous study.[20] DPD enzyme activity in peripheral blood mononuclear cells (PBMCs) was determined in a pretreatment sample in the *DPYD* variant allele carriers and compared to DPD enzyme activity measured in wild-type patients in this study, using a validated assay developed by Van Kuilenburg *et al.*[21]

Cost-analysis: A cost-analysis was conducted using a decision analytic model from a health care payer perspective, taking only direct medical costs into account. The model, as previously published by Deenen *et al.,*[15] was used and updated with data from the current study and current cost prices. The model compared prospective screening for four *DPYD* variants (screening strategy) with no *DPYD* screening (non-screening strategy, details in Supplement).

Outcomes

The primary end point of the study was the frequency of severe overall fluoropyrimidine-related toxicity across the entire treatment duration. A comparison was made between *DPYD* variant allele carriers treated with reduced dose, and 1) wild-type patients treated with standard dose in this study, or 2) *DPYD* variant allele carriers treated with full dose in a historical cohort derived from a previously published meta-analysis.[10] Secondary endpoints included a cost-analysis of individualized dosing based on upfront genotypic assessment, and pharmacokinetics of capecitabine and 5-FU in *DPYD* variant allele carriers.

Sample size calculation and statistical analysis

The sample size was calculated under the assumption that overall fluoropyrimidine-related severe toxicity could be reduced from 60% (in *DPYD* variant allele carriers receiving standard dose)[10,15] to 20% by individualized dosing in *DPYD* variant allele carriers (details in Supplement). This resulted in a required sample size of 1100 evaluable patients, with an one-sided type I error probability α of 2.83% and power of 83.9%.

Associations between dichotomous outcomes, e.g. occurrence of severe toxicity or hospitalization, and genotype status were tested using χ^2 or Fisher's exact test. DPD enzyme activity was compared between carriers of individual *DPYD* variants and wild-type patients using Student's *t*-tests. *P* values <0.05 were considered statistically significant. Statistical analyses were performed using SPSS (version 22.0) and R (version 3.1.2).

RESULTS

Patient and treatment characteristics

Between April 2015 and January 2018, a total of 1181 patients intended to start fluoropyrimidine-based treatment were enrolled in this study. In total, 78 patients were considered non-evaluable (Figure 1), as they retrospectively were identified as not meeting the inclusion criteria (*N*=48), did not start fluoropyrimidine-based treatment (*N*=26), or were homozygous or compound heterozygous *DPYD* variant allele carriers (*N*=4). This resulted in a total of 1103 evaluable patients, of whom 85 heterozygous *DPYD* variant allele carriers (7.7%). Baseline characteristics of *DPYD* variant allele carriers and *DPYD* wild-type patients are described in Table 1 and Supplementary Table 1. The most common tumor type was colorectal cancer (64%), and 83% of patients were treated with a capecitabine-based regimen.

Mean relative dose intensities for each patient group are presented in Table 2. In general, dose recommendations as described in the study protocol were followed by the treating physicians, which resulted in mean dose intensities in the first cycle of 74%, 73%, 51% and 50% for c.1236G>A, c.2846A>T, *DPYD**2A and c.1679T>G, respectively. The performed dose reductions were therefore in line with the pre-specified dose reductions of 25% (for c.1236G>A and c.2846A>T) or 50% (for *DPYD**2A and c.1679T>G) However, for four patients carrying *DPYD* variants, dose reductions were not applied at start of treatment (details in Supplement). One of these patients, a c.2846A>T

carrier was treated by mistake with a full capecitabine dose for the first two cycles, and this resulted in fatal fluoropyrimidine-related toxicity. Although dosing recommendations were not followed in four patients, these patients were included in the analysis (intention-to-treat analysis). Doses were escalated during treatment in 11 out of 85 *DPYD* variant allele carriers (13%). In five of these patients the higher dose was not well tolerated and the dose was reduced again. Also, one patient discontinued treatment after the dose escalation due to toxicity. Five patients were able to continue treatment with the escalated dose.

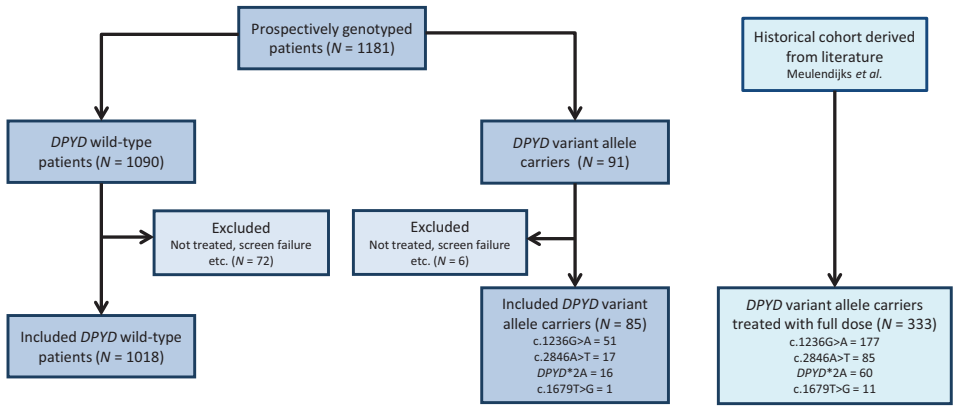

Figure 1. Consort diagram of included patients.

Toxicity in DPYD variant allele carriers versus wild-type patients

Frequencies of severe toxicity for *DPYD* variant allele carriers who received genotype-guided dosing and wild-type patients who received standard dosing are depicted in Table 2. A total of 33 out of 85 (39%) *DPYD* variant allele carriers experienced severe (grade≥3) fluoropyrimidine-related toxicity, which was significantly higher than the frequency in wild-type patients (23%), *P*=0.001. The percentage of toxicity in *DPYD* variant allele carriers was mainly driven by the two most common variants, who also had higher toxicity frequencies. A total of 20 out of 51 c.1236G>A carriers experienced severe toxicity (39%) and 8 out of 17 c.2846A>T carriers (47%). For *DPYD**2A carriers, 5 out of 16 patients (31%) experienced severe toxicity. The single c.1679T>G carrier, who did receive reduced-dose treatment, did not experience severe treatment-related toxicity (0%).

For 16 out of 85 *DPYD* variant allele carriers (19%) fluoropyrimidine-related toxicity resulted in hospitalization, compared to 140 out of 1018 wild-type patients (14%), *P*=0.20. Median duration of hospitalization was five days for both *DPYD* variant allele carriers and wild-type patients (range 1-19 days, and 1-42 days, respectively).

As described above, one c.2846A>T carrier experienced fatal fluoropyrimidine-related toxicity, but the intended dose reductions were not applied for this patient. When disregarding this patient for the critical protocol violation, no treatment-related death occurred in *DPYD* variant allele carriers. In the wild-type cohort, three patients died due to fluoropyrimidine-related toxicity (0.3%), which is comparable to literature data.[4,5]

Table 1. Demographic and clinical characteristics of patients.

Characteristic	*DPYD* variant allele carriers *N* = 85	Wild-type patients *N* = 1018	Total *N* = 1103
Sex			
Male	48 (56%)	545 (54%)	593 (54%)
Female	37 (44%)	473 (46%)	510 (46%)
Age			
Median [range]	63 [29-82]	64 [19-89]	64 [19-89]
Ethnic origin			
Caucasian	84 (99%)	964 (95%)	1048 (95%)
African	0	19 (2%)	19 (2%)
Asian	1 (1%)	23 (2%)	24 (2%)
Other [a]	0	12 (1%)	12 (1%)
Tumor type			
Non-metastatic CRC	32 (38%)	440 (43%)	472 (43%)
Metastatic CRC	24 (28%)	208 (20%)	232 (21%)
BC	10 (12%)	131 (13%)	141 (13%)
GC	6 (7%)	57 (6%)	63 (6%)
Other [b]	13 (15%)	182 (18%)	195 (18%)
Type of treatment regimen			
CAP mono	14 (16%)	191 (19%)	205 (19%)
CAP + RT	18 (21%)	246 (24%)	264 (24%)
CAPOX	31 (36%)	343 (34%)	374 (34%)
CAP other	5 (6%)	67 (7%)	72 (7%)
5-FU mono	1 (1%)	1 (0%)	2 (0%)
5-FU + RT	6 (7%)	57 (6%)	63 (6%)
FOLFOX	5 (6%)	38 (4%)	43 (4%)
5-FU other	5 (6%)	75 (7%)	80 (7%)
BSA			
Median [range]	1.9 [1.5-2.6]	1.9 [1.3-2.7]	1.9 [1.3-2.7]
WHO performance status			
0	39 (46%)	515 (51%)	554 (50%)
1	36 (42%)	412 (40%)	448 (41%)
2	4 (5%	38 (4%)	42 (4%)
NS [c]	6 (7%)	53 (5%)	59 (5%)
Number of treatment cycles			
Median [range]	4 [1-24]	3 [1-37]	3 [1-37]
DPYD status			
Wild-type	0	1018 (100%)	1018 (92%)
c.1236G>A heterozygous	51 (60%)	0	51 (5%)
c.2846A>T heterozygous	17 (20%)	0	17 (2%)
*DPYD*2A heterozygous*	16 (19%)	0	16 (1%)
c.1679T>G heterozygous	1 (1%)	0	1

[a] Other ethnic origins included Hispanic descent, mixed-racial parentage and unknown ethnic origin.
[b] Other tumor types included anal cancer, esophageal cancer, head and neck cancer, pancreas cancer, bladder cancer, unknown primary tumor, vulva carcinoma, and several rare tumor types.
[c] WHO performance status was not specified for these patients, but was either 0,1, or 2, as this was required by the inclusion criteria of the study.

Abbreviations: 5-FU mono: 5-fluorouracil monotherapy; 5-FU other: 5-fluorouracil combined with other anticancer drugs (excluding the FOLFOX regimen); 5-FU + RT: 5-fluorouracil combined with radiotherapy (with or without mitomycin); BC: breast cancer; BSA: body surface area; CAP mono: capecitabine monotherapy (with or without bevacizumab); CAPOX: capecitabine combined with oxaliplatin (with or without bevacizumab); CAP other: capecitabine combined with other anticancer drugs; CAP + RT: capecitabine combined with radiotherapy (with or without mitomycin); CRC: colorectal cancer; *DPYD*: gene encoding dihydropyrimidine dehydrogenase; FOLFOX: 5-fluorouracil combined with oxaliplatin and leucovorin (with or without bevacizumab); GC: gastric cancer; NS: not specified.

Table 2. Treatment outcome of patients included in this study.

Type of event	*DPYD* variant allele carriers	Wild-type patients	*P* value [a]			
	N = 85	*N* = 1018				
				c.1236G>A	c.2846A>T	*DPYD**2A	c.1679T>G
				N = 51	*N* = 17	*N* = 16	*N* = 1
Relative dose intensity whole treatment mean [range] [b]	69.1% [36.7% - 96.6%]	94.1% [48.8% - 127.6%]	NA	73.6% [50.9% - 96.6%]	71.6% [48.8% - 96.2%]	52.9% [36.7% - 74.1%]	54.2%
Relative dose intensity first cycle mean [range] [b]	69.3% [24.8% - 96.2%]	96.3% [37.2% - 127.6%]	NA	74.0% [50.9% - 87.5%]	73.4% [55.3% - 96.2%]	51.1% [24.8% - 81.5%]	50.0%
Overall grade≥3 toxicity [c]	33 (39%)	231 (23%)	0.001	20 (39%)	8 (47%)	5 (31%)	0
Grade≥3 gastrointestinal toxicity	17 (20%)	86 (8%)	0.001	11 (22%)	4 (24%)	2 (13%)	0
Grade≥3 hematological toxicity	13 (15%)	65 (6%)	0.006	7 (14%)	4 (24%)	2 (13%)	0
Grade3 hand-foot syndrome [d]	1 (1%)	36 (4%)	0.35	0	1 (6%)	0	0
Grade≥3 cardiac toxicity	1 (1%)	9 (1%)	0.55	1 (2%)	0	0	0
Grade≥3 other treatment-related toxicity	9 (11%)	78 (8%)	0.30	7 (14%)	1 (6%)	1 (6%)	0
Fluoropyrimidine-related hospitalization	16 (19%)	140 (14%)	0.20	10 (20%)	4 (24%)	2 (13%)	0
Stop of fluoropyrimidines due to adverse events	15 (18%)	175 (17%)	0.88	8 (16%)	3 (18%)	4 (25%)	0
Fluoropyrimidine-related death	1 (1%) [e]	3 (0%)	0.27	0	1 (6%) [e]	0	0

[a] *P* value determined with Fisher's exact test. Values in bold are statistically significant ($P < 0.05$).
[b] The relative dose intensity is calculated as the given dose in mg/m^2 divided by the standard dose in mg/m^2 given for the indication and treatment schedule which was applicable for the patient. The relative dose intensity was calculated for the first cycle and for the entire treatment duration.
[c] Overall toxicity includes all toxicities evaluated as possibly, probably or definitely related to fluoropyrimidine-treatment.
[d] Defined as palmar-plantar erythrodysesthesia syndrome by the Common Terminology Criteria for Adverse Events (CTC-AE) version 4.03.[18]
[e] This patient (c.2846A>T carrier) was wrongly treated with a full capecitabine dose for two cycles, which resulted in fatal fluoropyrimidine-related toxicity.
Abbreviations: DPYD: gene encoding dihydropyrimidine dehydrogenase; NA: not applicable.

Toxicity of genotype-guided dosing versus standard dosing in DPYD variant allele carriers

As another primary comparison, the toxicity risk of *DPYD* variant allele carriers with genotype-guided dosing was compared to *DPYD* variant allele carriers from a historical cohort of a previously performed meta-analysis.[10] *DPYD* variant allele carriers described in the meta-analysis were not identified prior to start of treatment and were therefore treated with a full dose. Relative risks of severe toxicity for each *DPYD* variant obtained in the meta-analysis are described in Table 3 and were compared to calculated relative risks in the current study. This analysis showed that genotype-guided dosing reduced the relative risk of severe toxicity in *DPYD**2A carriers from 2.87 (95% confidence interval [95%CI]: 2.14-3.86) when treated with full dose to 1.30 (95%CI: 0.63-2.72) when treated with individualized dose, thus showing a clinically relevant reduction of toxicity risk. Interestingly, for c.1236G>A and c.2846A>T, a reduction in toxicity risk comparable to that of *DPYD* wild-type patients could not be demonstrated, despite treatment with a reduced starting dose. The risk for c.1236G>A in the historical cohort was 1.72 (95%CI: 1.22-2.42), and in our study it was 1.69 (95%CI: 1.18-2.42), showing that toxicity risk was still increased even when applying a 25% dose reduction. For c.2846A>T, the risk of severe toxicity determined in the meta-analysis was 3.11 (95%CI: 2.25-4.28), which was decreased to 2.00 (95%CI: 1.19-3.34) after 25% dose reduction. However, this risk was still higher compared to non-carriers of this variant.
For the c.1679T>G variant no relative risk could be calculated, as only one patient with this variant was included. This patient tolerated the reduced dose of the fluoropyrimidine drug well and did not experience severe toxicity over the course of treatment (three cycles).

Pharmacokinetics of DPYD-guided dosing

A total of 26 *DPYD* variant allele carriers (of which 16 c.1236G>A carriers, five c.2846A>T carriers, four *DPYD**2A carriers and one c.1679T>G carrier) treated with a reduced fluoropyrimidine dose gave informed consent to draw blood for pharmacokinetic analysis. Mean AUC values of the *DPYD* variant allele carriers and control values are depicted in Figure 2. Mean exposure to capecitabine and all metabolites, including 5-FU, was comparable between patients dosed based on *DPYD* genotype and control values,[20] showing that mean drug exposure of all combined *DPYD* variant allele carriers treated with a reduced dose is adequate. However, in line with toxicity data, AUC values for 5-FU were markedly higher for c.1236G>A carriers and especially for c.2846A>T carriers, compared to *DPYD**2A and c.1679T>G carriers as shown in Supplementary Table 2.

DPD enzyme activity

In 56 *DPYD* variant allele carriers and 82 wild-type patients (participating in a subgroup of the study where DPD phenotyping tests were investigated), pretreatment DPD enzyme activity was determined (Figure 3). Mean DPD activity in *DPYD* wild-type patients was 9.4±3.6 nmol/(mg*h), similar as previously published.[22] For the c.1236G>A variant (*N*=35), the mean DPD activity was 7.5±2.8 nmol/(mg*h) (i.e. a 20% reduction compared to wild-type). The mean DPD activity for c.2846A>T (*N*=12) was 6.2±1.9 nmol/(mg*h) (34% reduction), and for *DPYD**2A (*N*=8) 5.2±0.6 nmol/(mg*h) (45% reduction). The single patient carrying c.1679T>G had a DPD enzyme activity of 3.8 nmol/(mg*h) (60% reduction). For c.1236G>A, c.2846A>T and *DPYD**2A, the mean DPD enzyme activity was significantly lower than the mean for wild-type patients. Statistical analysis was not possible for c.1679T>G.

Cost-analysis

In the cost-analysis the expected total costs for a patient in the screening strategy were €2599, compared to €2650 in the non-screening strategy, thereby resulting in a net cost saving of €51 per patient treated. Results of the probabilistic sensitivity analysis and one-way sensitivity analysis are depicted in Supplementary Figure 2 and 3. Results of these analyses demonstrated that, even when varying parameters in the model, the screening strategy is very likely to be cost saving.

Table 3. Relative risk for severe toxicity of *DPYD* variant carriers compared to historical controls.

***DPYD* variant**	***DPYD* variant carriers treated with reduced dose (this study)**	***DPYD* variant carriers treated with full dose (historical controls)**
	Relative risk overall grade≥3 toxicity (95%CI) [a]	Relative risk overall grade≥3 toxicity (95%CI) [b]
c.1236G>A	1.69 (1.18 – 2.42)	1.72 (1.22 - 2.42)
c.2846A>T	2.00 (1.19 – 3.34)	3.11 (2.25 – 4.28)
*DPYD**2A	1.30 (0.63 – 2.72)	2.87 (2.14 – 3.86)
c.1679T>G	NA [c]	4.30 (2.10 - 8.80)

[a] Relative risk for overall grade≥3 fluoropyrimidine-related toxicity compared to non-carriers of this variant as described in Table 2.

[b] Relative risk for overall grade≥3 fluoropyrimidine-related toxicity compared to non-carriers of this variant, as determined in an random-effects meta-analysis by Meulendijks *et al.*[10] Unadjusted relative risks for the meta-analysis are depicted, as the relative risk in the current study was also calculated as an unadjusted value (as patient numbers were low).

[c] Relative risk cannot be calculated as only one patient who carried c.1679T>G was present. This patient did not experience severe toxicity.

Abbreviations: 95%CI: 95% confidence interval; NA: not applicable.

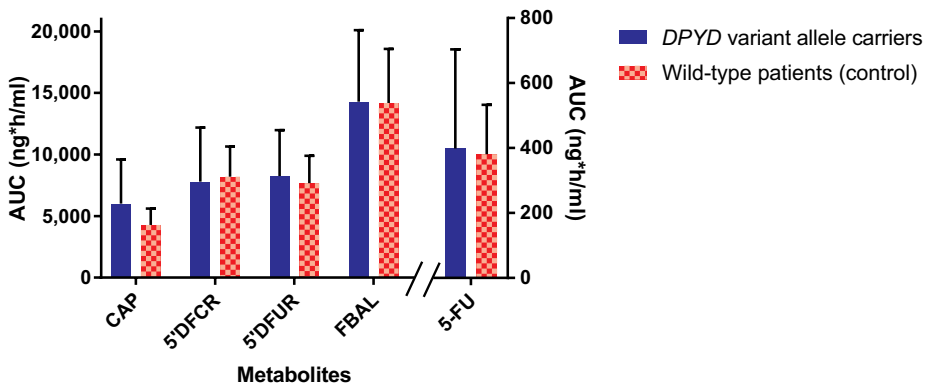

Figure 2. Pharmacokinetics of *DPYD*-guided capecitabine dosing.
Depicted are the mean AUCs of capecitabine, and the metabolites 5'DFCR, 5'DFUR, 5-FU and FBAL of the *DPYD* variant allele carriers treated with *DPYD*-genotype guided dose (blue) and control values from wild-type patients from a published study (red).[20] Error bars represent the standard deviation.
Abbreviations: 5'DFCR: 5-deoxy-5-fluorocytidine; 5'DFUR: 5-deoxy-5-fluorouridine; 5-FU: 5-fluorouracil; AUC: area under the plasma concentration-time curve; CAP: capecitabine; FBAL: fluoro-β-alanine.

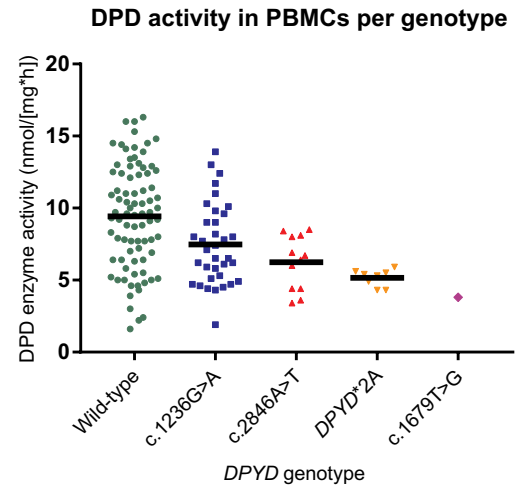

Figure 3. DPD enzyme activity in *DPYD* variant allele carriers and wild-type patients.
Wild-type patients were wild-type for the four *DPYD* variants that were prospectively tested. Mean DPD enzyme activity was statistically significantly lower than wild-type (mean 9.4±3.6 nmol/[mg*h]) for the *DPYD* variants as determined by a *t*-test: c.1236G>A (7.5±2.8 nmol/[mg*h], *P*=0.005), c.2846A>T (6.2±1.9 nmol/[mg*h], *P*=0.003), and *DPYD**2A (5.5±0.6 nmol/[mg*h], *P*=0.001). As only one patient carried c.1679T>G, no statistical test could be performed for this variant. However, the single measurement in this patient was in the range of DPD deficiency (3.8 nmol/[mg*h]).
Abbreviations: DPD: dihydropyrimidine dehydrogenase; PBMCs: peripheral blood mononuclear cells.

DISCUSSION

This is the first prospective study to investigate the effect on fluoropyrimidine-related toxicity by dose individualization based on four *DPYD* variants. Our results demonstrate that genotype-guided dosing is feasible in clinical practice. Dose individualization markedly decreased the risk of severe toxicity for *DPYD**2A and c.1679T>G carriers, and moderately decreased the risk for c.2846A>T carriers. For c.1236G>A carriers, a 25% dose reduction was not enough to decrease severe treatment-related toxicity. This shows that *DPYD* genotype-guided dose-individualization improves patient safety for three of the four variants. Also, implementation of *DPYD* genotype-guided dosing resulted in similar frequencies of toxicity-related hospitalization for wild-type patients and *DPYD* variant allele carriers.

Interestingly, for *DPYD**2A carriers, the frequency of severe toxicity found in this study was 31%; dramatically lower than the frequency in the historical cohort (72%). DPD enzyme activity measurements in this study showed that activity for *DPYD**2A carriers was approximately 50% reduced compared to wild-type patients, which endorses the dose recommendation of 50% for this variant.

As only one carrier of the rare c.1679T>G variant was identified in our current study, this made statistical comparisons impossible. However, we showed that this patient did not experience severe toxicity in a completed treatment with 50% reduced dose, showing that this patient could be treated safely. The DPD enzyme activity was around 50% decreased as well for this patient, in line with expectations based on previous studies.[23]

For carriers of the c.1236G>A and c.2846A>T variant, risk of severe toxicity remained relatively high despite dose individualization. In this study, 39% of the c.1236G>A carriers experienced severe toxicity and 47% of the c.2846A>T carriers. For these two variants, an initial dose reduction of 25% was applied in this study, because these variants have a less deleterious effect on DPD activity than the non-functional variants *DPYD**2A and c.1679T>G.[14,16] However, the Clinical Pharmacogenetics Implementation Consortium (CPIC), an international organization providing evidence based pharmacogenetics guidelines, is more cautious in their recommendations for c.1236G>A and c.2846A>T and advices a 25%-50% dose reduction in heterozygous carriers.[13] This guideline mentions that evidence is limited regarding the optimal degree of dose reduction for decreased function variants, and the 25% dosing recommendation is mainly based on one small retrospective study. Our current results suggest that applying 25% dose reduction might be insufficient for some patients, as toxicity risk was increased for carriers of c.1236G>A and c.2846A>T, compared to wild-type patients. In line with these findings, our pharmacokinetic analyses showed that exposure to 5-FU was markedly higher in c.2846A>T carriers than in wild-type controls.

The mean DPD enzyme activity for c.1236G>A was approximately 20% reduced, but a large variation in DPD activity was found (Figure 3), which suggests that a proportion of patients need a larger dose reduction, while other patients might even tolerate a full dose. This is also in line with the large variation in pharmacokinetic exposure seen in c.1236G>A carriers. Individual dose titration based on tolerance is important to ensure an adequate and safe dose for all patients. Therefore, we would recommend an initial 25% dose reduction for the c.1236G>A variant, but a closer monitoring of patient tolerability and applying dose escalations or reductions, so dosing is adequate in all patients.

The mean value for c.2846A>T DPD enzyme activity was approximately 35% reduced compared to normal. The DPD activity measurements show that 25% dose reduction might not be sufficient for most of the patients, and could be an explanation for the higher toxicity risk in this patient group. A more cautious initial dose reduction of 50% should be considered in these patients, followed by close monitoring in addition.

The cost-analysis showed that prospective *DPYD* screening and dose individualization is very likely to not increase healthcare costs and even be cost-saving. This is in line with two previous studies investigating costs of *DPYD* genotyping and toxicity[15,24]

Our study was performed in a daily clinical care setting in general regional hospitals and a few academic centers, demonstrating the feasibility of implementation of upfront *DPYD* screening. In a few cases the *DPYD* genotyping result was not awaited prior to start of fluoropyrimidine-based chemotherapy, although this was not in line with the study protocol. When *DPYD* variant allele carriers are treated with a full dose or doses are escalated too quickly, fatal toxicity can occur, as was shown in the one *DPYD* variant allele carrier inadvertently treated with a full dose. In order to make *DPYD*-guided dosing feasible in all hospitals, it is important that the turn-around time for *DPYD* genotyping is short to prevent a delay in the start of treatment. Participating laboratories in our study had a turn-around time of a few days to a maximum of a week.

A historical cohort of *DPYD* variant allele carriers treated with full dose was used as control cohort in this study. This study design was chosen as a randomized clinical trial is considered unethical, since it is known that *DPYD* variant allele carriers are at increased risk of severe toxicity when treated with a full dose.[25] A previously performed randomized clinical trial was stopped

prematurely as a patient in the arm without dose individualization died due to treatment-related toxicity.[26]

The four *DPYD* variants investigated in this study are especially relevant to a Caucasian population. For ethnicities other than Caucasians, more research on the frequency and clinical relevance of these and other *DPYD* variants is recommended.

In conclusion, we showed that patient safety was improved by dose individualization based on *DPYD* genotype. Dose reductions of 50% in heterozygous *DPYD**2A and c.1679T>G variant allele carriers reduced toxicity risk significantly. The applied dose reductions of 25% in heterozygous c.1236G>A and c.2846A>T carriers appear to be insufficient for all patients to lower the risk of fluoropyrimidine-related toxicity to the background risk in wild-type patients. A larger initial dose reduction of 50% for c.2846A>T carriers should therefore be considered. Especially for 1236G>A carriers, with a large variation in DPD activity, close monitoring of tolerability and individual dose titration remain important to achieve the maximal safe exposure in these patients.

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FINANCIAL DISCLOSURE / CONFLICTS OF INTEREST

The authors have declared no conflicts of interest. LM Henricks, CATC Lunenburg and the NCT02324452 study were sponsored by the Dutch Cancer Society (Alpe-d'HuZes/KWF-fund, project number: NKI 2013-6249). Carin Lunenburg was previously supported by an unrestricted grant from Roche Pharmaceuticals. There was no involvement in the study design, data collection, analysis or interpretation.

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SUPPLEMENT

SUPPLEMENTARY METHODS

Inclusion and exclusion criteria

Patients with a pathologically confirmed malignancy for which treatment with a fluoropyrimidine drug was considered to be in the patient's best interest could be included in this study. Eligible patients were 18 years or older, and were willing to undergo blood sampling for the purpose of this study (pharmacogenetic and phenotyping analysis). Patients had to have a WHO performance status of 0, 1 or 2, a life expectancy of at least 12 weeks, and acceptable safety laboratory values (neutrophil count of ≥1.5 x 10^9/L, platelet count of ≥100 x 10^9/L, hepatic function as defined by serum bilirubin ≤1.5 x upper limit of normal (ULN), alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) ≤2.5 x ULN or in case of liver metastases ALAT and ASAT≤5 x ULN, renal function as defined by serum creatinine ≤1.5 x ULN or creatinine clearance ≥60 ml/min (by Cockcroft-Gault formula).

Exclusion criteria were prior treatment with fluoropyrimidines, patients with known substance abuse, psychotic disorders, and/or other diseases expected to interfere with study or the patient's safety, women who were pregnant or breast feeding, man and women who refused to use reliable contraceptive methods throughout the study, and patients with a homozygous *DPYD* polymorphic genotype or compound heterozygous *DPYD* genotype.

Sample size calculation

A sample size calculation was made based on the primary aim of the study, which was to determine whether fluoropyrimidine-related severe toxicity can be reduced by individualized dosing in *DPYD* variant allele carriers compared to standard dosing in these patients. Using a one stage Phase II design and a null hypothesis of a probability of toxicity of 60% (the estimated severe treatment-related toxicity probability if *DPYD* variant allele carriers received standard dose)[1,2] and an alternative hypothesis of 20% (estimated toxicity probability of *DPYD* variant allele carriers receiving individualized dose), a sample size of 11 *DPYD* variant allele carriers would give a one-sided type I error probability α of 2.83% and power of 83.9%. It was decided that the frequency of c.2846A>T carriers (approximately 1.0%)[3] would determine the total number of patients required in the study. These patients would then arise from an expected minimum population of 1100 treated patients. To account for a proportion of patients not evaluable for the study, the target accrual was set at 1250 patients. Given the very low allele frequency of the c.1679T>G variant, it was considered not feasible to power this study for this particular variant. The estimated frequency of c.1236G>A is 3% and of *DPYD**2A 1%, which means that the calculated sample size would be adequate for those individual variants, or when analyzing all four variants together (estimated frequency of 5%).

Pharmacokinetic analyses

For pharmacokinetic analyses, peripheral blood was collected on the first day of treatment. Blood was collected in heparin tubes at nine different time points up to eight hours after capecitabine intake (pre-dose, 0.25 hours, 0.5 hours, 1 hour, 2 hours, 3 hours, 4 hours, 6 hours and 8 hours after capecitabine intake). Samples were centrifuged immediately after the blood was drawn and plasma was stored at -80°C until analysis.

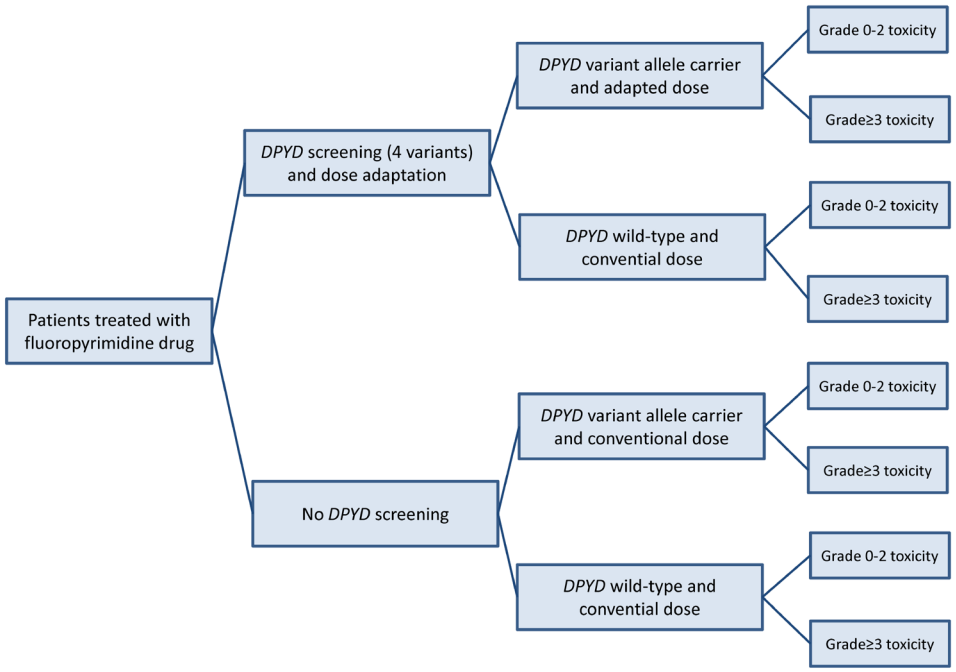
Capecitabine and the metabolites 5'-deoxy-5-fluorocytidine (5'DFCR), 5'-deoxy-5-fluorourdine (5'DFUR), 5-fluorouracil (5-FU) and fluoro-β-alanine (FBAL) were quantified in plasma samples using a validated ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry (MS/MS) method. Lower limit of quantifications were 25 ng/ml for capecitabine, 10 ng/ml for 5'DFCR, 5'DFUR and 5-FU and 50 ng/ml for FBAL. Stable isotopes were used as internal standard for all analytes. Protein precipitation was performed using 900 µl of methanol-acetonitrile (50:50 v/v). Samples were vortex-mixed for 10 s, shaken for 10 min at 1250 rpm and centrifuged at 14,000 rpm for 10 minutes. The clear supernatants were dried under a stream of nitrogen at 40°C and reconstituted in 100 µl of 0.1% formic acid in water. An Acquity UPLC® HSS T3 column was used for chromatographic separation, at a flow rate of 300 µl/min and a gradient of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The following gradient was used: 100% A from 0-2.5 min, an increase from 0% to 90% B from 2.5-7.5 min, and 100% A from 7.5-9 min. For detection an API5500 triple quadruple mass spectrometer (AB Sciex) equipped with a turbo ionspray interphase was used, using the mass transitions 360.0 → 243.9 for capecitabine, 244.9 → 128.8 for 5'DFUR, 128.9 → 42.1 for 5-FU, and 105.9 → 85.9 for FBAL.

Pharmacokinetic parameters were calculated using non-compartmental analysis and the calculated area under the plasma concentration-time curve (AUC) and half-life ($t_{1/2}$) were compared with pharmacokinetic data described in literature,[4] measured at the same laboratory as the current study.

Cost-analysis

A cost-minimization analysis was conducted using a decision analytical model from a health care payer perspective, similar as described previously.[1] Parameter estimations incorporated in the model were derived from data of the present trial and relevant data from literature.[5,6] Interventions for treatment-related toxicity were prospectively collected for all patients during the trial. An overview of the decision tree is depicted in Supplementary Figure 1. In the model, a comparison between the screening strategy (prospective screening for four *DPYD* variants and dose adjustments in heterozygous *DPYD* variant allele carriers) and the non-screening strategy was made. Expected differences in costs of both strategies were calculated.

Costs included were restricted to direct medical costs only. Cost parameters included costs for genotyping, fluoropyrimidine drug therapy including visits to the medical doctor and day care, costs for treatment of adverse events (e.g. extra medication, extra doctor visits, extra assessments) and costs for hospitalization due to adverse events. Costs for other anticancer drugs than the fluoropyrimidine drugs were not included in the model, as they were expected to be equal in both arms (as can be seen in Table 1 of the main manuscript). Cost-saving was calculated as the difference between the net direct costs of the *DPYD* screening strategy versus the non-screening strategy. One-way and probabilistic sensitivity analyses were performed to examine the effect on variations in parameter values. In the one-way sensitivity analysis, each parameter was varied individually at ±20% of the baseline value. In the probabilistic sensitivity analysis, all parameters were varied simultaneously by running 1000 Monte Carlo simulations. Since the parameter values of the wild-type patients for both the screening and the non-screening arm are identical, these parameters remained fixed in the probabilistic sensitivity analysis.



Supplementary Figure 1. Decision tree for cost-analysis.

SUPPLEMENTARY RESULTS

Detailed information of DPYD variant allele carriers not treated according to dosing recommendations

For four patients dosing recommendations were not followed according to protocol. One patient carrying *DPYD**2A started with a full dose as genotyping results were not awaited before start of treatment. After one week of treatment the *DPYD* genotyping result became available and the dose was reduced to 50%. The patient did not experience severe treatment-related toxicity in this course. However, from the third cycle onwards the dose was quickly titrated upwards (75% in the third cycle and 90% in the fourth cycle), hereafter treatment-related toxicity (anorexia grade 2, fatigue grade 3) occurred and the dose was reduced again. A second patient (*DPYD**2A carrier) also started with a full dose as genotyping results were not awaited before starting treatment. As results were known the following day, the patient had only taken a full dose for one day, which did not result in severe toxicity. The patient was treated with a 50% dose from the second day onwards. A third patient carrying c.2846A>T, used a full dose for four days, but continued with a 50% dose after an interruption of 5 days. The overall dose intensity of this cycle was approximately 55% and no toxicity occurred. The fourth patient (c.2846A>T carrier) was wrongly treated with a full dose for two cycles due to miscommunication with the patient. The patient experienced severe diarrhea, pancytopenia and sepsis, and passed away.

Pharmacokinetic analyses

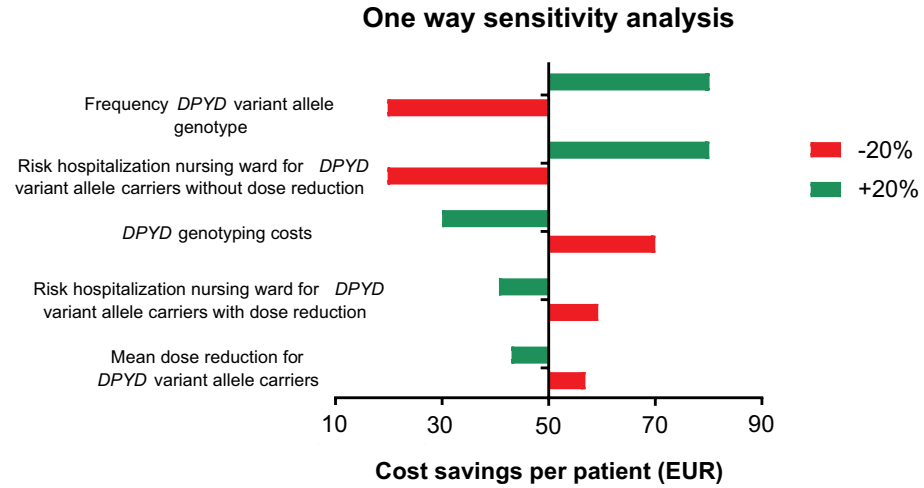
A total of 26 *DPYD* variant allele carriers treated with reduced dose of capecitabine was included in the analysis. Pharmacokinetic results are shown in Supplementary Table 1. In 24 out 26 patients pharmacokinetic sampling was performed at day 1 of cycle 1, in two patients this was done later during treatment, but also at start of a cycle, after a resting period of one week without capecitabine intake.

Of five patients who were treated with 5-FU, pharmacokinetic blood samplings was performed as well, but results were considered unreliable, most likely as drawing of blood was not done correctly. Results of the 5-FU treated patients are therefore not included in the analysis.

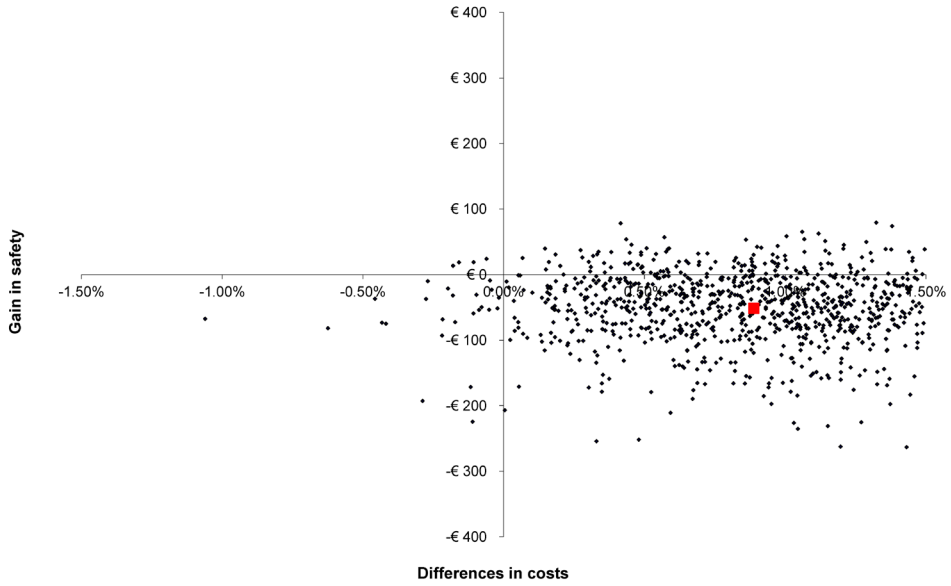
Cost-analysis

All parameter estimates used in the model are provided in Supplementary Table 3. Results of the one-way sensitivity analysis are depicted in Supplementary Figure 2. This showed that the parameter with the largest influence on outcome of the cost-analysis was the frequency of the *DPYD* variant allele genotype followed by the risk of hospitalization at the nursing ward for *DPYD* variant allele carrier receiving standard dose, and *DPYD* genotyping costs. However, in all cases, the cost saving remained positive. Results of the simulations for the probabilistic sensitivity analysis are depicted in Supplementary Figure 3.

Average cost savings from the simulation in the probabilistic sensitivity analysis were €52 per patient (range -€70 to €263). Average gain in safety was 0.89% (range -1.06% to 2.48%). This gain in safety represents the difference between the proportion of patients treated without severe toxicity (both wild-type patients and *DPYD* variant allele carriers taken together) in the screening strategy and the non-screening strategy.



Supplementary Figure 2. One-way sensitivity analysis of upfront *DPYD* genotyping versus non-screening. All parameters were individually varied by ±20%, effects of which cost savings are indicated by horizontal bars. The vertical line indicates the baseline costs savings of €50.



Supplementary Figure 3. Probabilistic sensitivity analysis of the cost-analysis.
For this sensitivity analysis, all parameters were varied simultaneously by running 1000 Monte Carlo simulations.

Supplementary Table 1. Demographic and clinical characteristics of *DPYD* variant allele carriers.

Characteristic	*DPYD* variant allele carriers	c.1236G>A	c.2846A>T	*DPYD**2A	c.1679T>G
	***N* = 85**	***N* = 51**	***N* = 17**	***N* = 16**	***N* = 1**
Sex					
Male	48 (56%)	26 (51%)	11 (65%)	10 (63%)	1 (100%)
Female	37 (44%)	25 (49%)	6 (35%)	6 (38%)	0
Age					
Median [range]	63 [29-82]	62 [29-82]	62 [46-82]	64 [47-80]	70
Ethnic origin					
Caucasian	84 (99%)	51 (100%)	17 (100%)	15 (94%)	1 (100%)
African	0	0	0	0	0
Asian	1 (1%)	0	0	1 (6%)	0
Other [a]	0	0	0	0	0
Tumor type					
Non-metastatic CRC	32 (38%)	15 (29%)	7 (40%)	9 (56%)	1 (100%)
Metastatic CRC	24 (28%)	17 (33%)	4 (24%)	3 (19%)	0
BC	10 (12%)	5 (10%)	3 (18%)	2 (13%)	0
GC	6 (7%)	4 (8%)	1 (6%)	1 (6%)	0
Other [b]	13 (15%)	10 (20%)	2 (12%)	1 (6%)	0
Type of treatment regimen					
CAP mono	14 (16%)	8 (16%)	4 (24%)	2 (13%)	0
CAP + RT	18 (21%)	8 (16%)	5 (29%)	5 (31%)	0
CAPOX	31 (36%)	19 (37%)	5 (29%)	6 (38%)	1 (100%)
CAP other	5 (6%)	3 (6%)	1 (6%)	1 (6%)	0
5-FU mono	1 (1%)	0	0	1 (6%)	0
5-FU + RT	6 (7%)	6 (12%)	0	0	0
FOLFOX	5 (6%)	2 (4%)	2 (12%)	1 (6%)	0
5-FU other	5 (6%)	5 (10%)	0	0	0
BSA					
Median [range]	1.9 [1.5-2.6]	1.9 [1.5-2.5]	2.0 [1.7-2.6]	2.0 [1.5-2.5]	2.1
WHO performance status					
0	39 (46%)	26 (51%)	8 (47%)	4 (25%)	1 (100%)
1	36 (42%)	18 (35%)	9 (53%)	9 (56%)	0
2	4 (5%	3 (6%)	0	1 (6%)	0
NS [c]	6 (7%)	4 (8%)	0	2 (13%)	0
Number of treatment cycles					
Median [range]	4 [1-24]	4 [1-16]	3 [1-24]	3 [1-8]	3

[a] Other ethnic origins included Hispanic descent, mixed-racial parentage and unknown ethnic origin.
[b] Other tumor types included anal cancer, esophageal cancer, head and neck cancer, pancreas cancer, bladder cancer, unknown primary tumor, vulva carcinoma, and several rare tumor types.
[c] WHO performance status was not specified for these patients, but was either 0,1, or 2, as this was required by the inclusion criteria of the study.

Abbreviations: 5-FU mono: 5-fluorouracil monotherapy; 5-FU other: 5-fluorouracil combined with other anticancer drugs (excluding the FOLFOX regimen); 5-FU + RT: 5-fluorouracil combined with radiotherapy (with or without mitomycin); BC: breast cancer; BSA: body surface area; CAP mono: capecitabine monotherapy (with or without bevacizumab); CAPOX: capecitabine combined with oxaliplatin (with or without bevacizumab); CAP other: capecitabine combined with other anticancer drugs; CAP + RT: capecitabine combined with radiotherapy (with or without mitomycin); CRC: colorectal cancer; *DPYD*: gene encoding dihydropyrimidine dehydrogenase; FOLFOX: 5-fluorouracil combined with oxaliplatin and leucovorin (with or without bevacizumab); GC: gastric cancer; NS: not specified.

Supplementary Table 2. Pharmacokinetic parameters of capecitabine and metabolites in *DPYD* variant allele carriers and controls.

Metabolite	Mean $AUC_{0-\infty}$ (ng*h/ml) [CV%] [a]		Mean $T_{1/2}$ (h) [CV%]	
	***DPYD* variant allele carriers (N=26)**	**Wild-type control patients (N=23) [b]**	***DPYD* variant allele carriers (N=26)**	**Wild-type control patients (N=23) [b]**
Capecitabine	6007 [60%]	4281 [31%]	0.73 [49%]	0.76 [55%]
5'DFCR	7792 [56%]	8192 [30%]	0.83 [43%]	1.0 [45%]
5'DFUR	8243 [45%]	7673 [29%]	0.85 [38%]	0.9 [34%]
5-FU	398 [77%]	381 [40%]	0.92 [112%]	1.0 [57%]
FBAL	14295 [41%]	14177 [31%]	2.2 [133%]	2.6 [33%]

Metabolite	Mean $AUC_{0-\infty}$ (ng*h/ml) [CV%] [a]				Mean $T_{1/2}$ (h) [CV%]			
	c.1236G>A (*N*=16)	**c.2846A>T (*N*=5)**	***DPYD**2A (*N*=4)**	**c.1679T>G (*N*=1)**	**c.1236G>A (*N*=16)**	**c.2846A>T (*N*=5)**	***DPYD**2A (*N*=4)**	**c.1679T>G (*N*=1)**
Capecitabine	6579 [65%]	5944 [26%]	4460 [51%]	3350	0.77 [48%]	0.68 [50%]	0.66 [61%]	0.53
5'DFCR	9162 [45%]	8320 [50%]	2552 [25%]	4185	0.84 [51%]	0.88 [28%]	0.83 [21%]	0.51
5'DFUR	9319 [41%]	8150 [46%]	4824 [17%]	5161	0.84 [38%]	0.73 [41%]	0.84 [19%]	1.6
5-FU	346 [49%]	765 [64%]	197 [54%]	219	1.1 [120%]	0.75 [47%]	0.54 [20%]	0.82
FBAL	16217 [30%]	15627 [36%]	6244 [18%]	9082	2.9 [78%]	2.5 [14%]	2.2 [15%]	2.9

[a] Note that for all metabolites the AUC is calculated until infinity ($AUC_{0-\infty}$, extrapolated from the last time point). Only for FBAL, the metabolite with the longest half-life, this resulted in a difference between the AUC until last time point (AUC_{0-t}) and $AUC_{0-\infty}$, with the $AUC_{0-\infty}$ being on average 26% higher than AUC_{0-t}. For the metabolites capecitabine, 5'DFCR, 5'DFUR and 5-FU the difference between AUC_{0-t} and $AUC_{0-\infty}$ was respectively 0.5%, 0.8%, 1%, and 4%.

[b] Control values are derived from Deenen *et al.*[4] for patients with advanced cancer of the stomach or gastroesophageal junction after administration of capecitabine 850 mg/m2 (dose level 2 of the study).

Abbreviations: 5'DFCR: 5'-deoxy-5-fluorocytidine; 5'DFUR: 5'-deoxy-5-fluorouridine; 5-FU: 5-fluorouracil; AUC: area under the plasma concentration-time curve; CV%: coefficient of variation; FBAL: fluoro-β-alanine; $T_{1/2}$: half-life.

Supplementary Table 3. Cost and probability parameters used in the cost-analysis.

Variable	Probabilities and other parameters				
	Baseline value	**Standard error[a]**	**Sensitivity range [b]**	**Reference**
Frequency *DPYD* genotype				
DPYD wild-type	0.9229	0.0080	fixed	This study
DPYD variant allele carrier	0.0771	0.0080	0.0617 – 0.0925	This study
Risk severe toxicity				
DPYD wild-type	0.2269	fixed	fixed	This study
DPYD variant allele carrier, reduced dose	0.3882	0.0526	0.3106 – 0.4658	This study
DPYD variant allele carrier, standard dose	0.5015	0.0274	0.4012 – 0.6018	Meta-analysis[2]
DPYD wild-type				
Hospitalization nursing ward	0.1356	fixed	fixed	This study
Mean duration (days)	7.9855	fixed	fixed	This study
Hospitalization ICU	0.0088	fixed	fixed	This study
Mean duration (days)	3.1111	fixed	fixed	This study
DPYD variant allele carrier, reduced dose				
Hospitalization nursing ward	0.1647	0.0400	0.1318 – 0.1976	This study
Mean duration (days)	5.7857	1.3350	4.6286 – 6.9428	This study
Hospitalization ICU	0.0235	0.0163	0.0188 – 0.0282	This study
Mean duration (days)	1.0000	0.1000	0.8000 – 1.2000	This study
DPYD variant allele carrier, standard dose				
Hospitalization nursing ward	0.2350	0.0422	0.1880 – 0.2820	Analysis on previous study[1,7]
Mean duration (days)	13.1000	3.0000	10.4800 – 15.7200	Analysis on previous study[1,7]
Hospitalization ICU	0.0310	0.0172	0.0248 – 0.0372	Analysis on previous study[1,7]
Mean duration (days)	7.0000	3.0000	5.6000 – 8.4000	Analysis on previous study[1,7]
Mean number of cycles				
Capecitabine	5.0208	0.1567	4.0166 – 6.0250	This study
5-FU	5.0426	0.3639	4.0341 – 6.0511	This study
Type of fluoropyrimidine drug				
Capecitabine	0.83	fixed	fixed	This study
5-FU	0.17	fixed	fixed	This study
Mean dose intensity for *DPYD* variant allele carriers	0.6910	0.0124	0.5528 – 0.8292	This study

Variable	Cost parameters (expressed in €)			
	Baseline value	**Standard error[a]**	**Sensitivity range [a]**	**Reference**
DPYD genotyping costs	100	Fixed	80-120	This study
Hospitalization nursing ward (per day)	636	Fixed	Fixed	Guideline[5]
Hospitalization ICU (per day)	2015	Fixed	Fixed	Guideline[5]
Additional costs for interventions related to toxicity (expect hospitalization)				
Grade 0-2	86.00	fixed	fixed	This study
Grade ≥3	234.00	fixed	fixed	This study
Treatment costs capecitabine (per cycle)				
Capecitabine medication	144.06	30	fixed	This study/Price info drugs[6]
Medical doctor visit	132	Fixed	fixed	Guideline[5]
Treatment costs 5-FU per cycle				
5-FU medication + pharmacy preparation	59.29	20	fixed	This study/Price info drugs[6]
Administration at day care	276	fixed	fixed	Guideline[5]
Medical doctor visit	132	fixed	fixed	Guideline[5]

[a] The standard error is calculated on data of this study, or otherwise estimated for parameters not derived from this study. The standard error is used for the probabilistic sensitivity analysis.

[b] The sensitivity range is calculated by varying the baseline value ±20%. The sensitivity range is used for the one way sensitivity analysis.

Abbreviations: 5-FU: 5-fluorouracil; *DPYD*: gene encoding dihydropyrimidine dehydrogenase; ICU: intensive care unit.

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Phenotyping of dihydropyrimidine dehydrogenase

12

Pretreatment serum uracil concentration as a predictor of severe and fatal fluoropyrimidine-associated toxicity

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SUMMARY

Background

We investigated the predictive value of dihydropyrimidine dehydrogenase (DPD) phenotype, measured as pretreatment serum uracil and dihydrouracil concentrations, for severe as well as fatal fluoropyrimidine-associated toxicity in 550 patients treated previously with fluoropyrimidines during a prospective multicenter study.

Methods

Pretreatment serum concentrations of uracil and dihydrouracil were measured using a validated LC-MS/MS method. The primary endpoint of this analysis was global (any) severe fluoropyrimidine-associated toxicity, that is, grade ≥3 toxicity according to the NCI CTC-AE v3.0, occurring during the first cycle of treatment. The predictive value of uracil and the uracil/dihydrouracil ratio for early severe fluoropyrimidine-associated toxicity were compared. Pharmacogenetic variants in *DPYD* (c.2846A>T, c.1679T>G, c.1129-5923C>G, and c.1601G>A) and *TYMS* (*TYMS* 5′-UTR VNTR and *TYMS* 3′-UTR 6-bp ins/del) were measured and tested for associations with severe fluoropyrimidine-associated toxicity to compare predictive value with DPD phenotype. The Benjamini-Hochberg false discovery rate method was used to control for type I errors at level $q<0.050$ (corresponding to $P<0.010$).

Results

Uracil was superior to the dihydrouracil/uracil ratio as a predictor of severe toxicity. High pretreatment uracil concentrations (>16 ng/ml) were strongly associated with global severe toxicity (OR 5.3, $P=0.009$), severe gastrointestinal toxicity (OR 33.7, $P<0.0001$), toxicity-related hospitalization (OR 16.9, $P<0.0001$), as well as fatal treatment-related toxicity (OR 44.8, $P=0.001$). None of the *DPYD* variants alone, or *TYMS* variants alone, were associated with severe toxicity.

Conclusions

High pretreatment uracil concentration was strongly predictive of severe, including fatal, fluoropyrimidine-associated toxicity, and is a highly promising phenotypic marker to identify patients at risk of severe fluoropyrimidine-associated toxicity.

INTRODUCTION

Chemotherapy with fluoropyrimidines is used by over two million cancer patients each year.[1] Of the patients treated, 10–30% experience severe treatment-related toxicity, which is lethal in 0.5–1% of the patients.[2] Identifying biomarkers that are predictive of patients' risk of fluoropyrimidine-associated toxicity has the potential to greatly improve the safety of large numbers of patients.

The most well-known biochemical cause of intolerance to fluoropyrimidines is deficiency of the key 5-fluorouracil (5-FU) metabolic enzyme, dihydropyrimidine dehydrogenase (DPD).[3] The fact that decreased DPD activity in peripheral blood mononuclear cells (PBMCs) is found in 39–61% of the patients who experience severe toxicity, demonstrates the critical relationship between DPD activity and fluoropyrimidine-associated toxicity.[4–6] Polymorphisms in *DPYD*, the gene encoding DPD, have received wide-spread attention as predictors of fluoropyrimidine-associated toxicity, and dose adaptation based on several of these *DPYD* variants is now recommended by the Clinical Pharmacogenetics Implementation Consortium (CPIC).[7] Upfront screening for the most well-known variant, IVS14+1G>A (*DPYD**2A), and dose individualization in *DPYD**2A allele carriers has recently been shown to improve the safety of these patients.[7,8]

Importantly, however, *DPYD* genotyping inherently has suboptimal sensitivity and positive predictive value (PPV), as a result of the fact that activity of DPD is regulated not only at the level of *DPYD*, but also to a relevant extent at the transcriptional level (e.g., by transcription factors SP1 and SP3) and the post-transcriptional level (e.g., by microRNA 27-a and 27-b).[3,9–12] Genetic variants in *TYMS*, the gene encoding 5-FU's target thymidylate synthase (TS), have also been associated with risk of fluoropyrimidine-associated toxicity,[13] but in contrast to *DPYD* variants there is currently insufficient evidence for clinical validity of *TYMS* variants.

Measuring the DPD phenotype has the potential to greatly improve the performance of an upfront test to identify patients at risk of severe and potentially fatal fluoropyrimidine-associated toxicity. However, measuring DPD activity upfront on a routine basis is technically and logistically challenging, laborious, and expensive.[14]

DPD converts its endogenous substrate uracil (U) into dihydrouracil (DHU), and the pretreatment ratio of serum concentrations of DHU to U – the DHU/U ratio – has been investigated as a phenotypic measure of systemic DPD activity. Several studies have shown that the DHU/U ratio correlates with clearance of 5-FU and with patients' risk of toxicity.[15–23] However, the clinical applicability of the DHU/U ratio has thus far been limited, mainly due to lack of robust evidence on clinical validity. Importantly, bioanalytical issues in previous studies that mainly used HPLC-UV techniques for quantification of U and DHU might have contributed to inconsistent results, as recently emphasized.[24] Importantly, it is unclear whether the DHU/U ratio or U concentrations alone best correlates with systemic DPD activity and risk of fluoropyrimidine-associated toxicity.[15,24]

While most available studies have correlated the DHU/U ratio to fluoropyrimidine-associated toxicity, a large study by Boisdron-Celle *et al.* in 252 patients showed that while U concentration correlated with 5-FU plasma clearance ($r=-0.221$, $P=0.006$ for 5-FU *de Gramont* regimen and $r=-0.219$, $P=0.05$ for weekly 4h 5-FU), the DHU/U ratio did not correlate with 5-FU clearance.[15]

We assessed, in 550 patients treated with fluoropyrimidine-based chemotherapy during a previous prospective multicenter study, the association between pretreatment U and DHU concentrations and early severe and fatal fluoropyrimidine-associated toxicity.[8] In addition, we investigated correlations of the DHU/U ratio and U concentrations with PBMC DPD activity. Genotyping of

DPYD and *TYMS* were also performed, to compare predictive value for severe toxicity. In this largest study to date investigating pretreatment serum U and DHU concentrations in relation to fluoropyrimidine-associated toxicity, we show that pretreatment U concentration may be of great value as a clinical predictor of severe and fatal fluoropyrimidine-associated toxicity which may complement current genotyping strategies.

MATERIALS AND METHODS

Patients and study design

The primary objective of this study was to assess the performance of pretreatment DPD phenotype, measured as pretreatment U concentrations or the DHU/U ratio, to identify patients at risk of severe fluoropyrimidine-associated toxicity. Genotyping of pharmacogenetic variants in *DPYD* and *TYMS* was performed to compare predictive value for severe toxicity.

A prospective multicenter study of *DPYD**2A genotype-guided dosing of fluoropyrimidines (NCT00838370) in which 1631 patients were enrolled and treated with standard fluoropyrimidine-based regimens (as monotherapy or in combination with other chemotherapy or radiotherapy) was the basis for this analysis.[8] The primary endpoint of NCT00838370 was severe toxicity (grade ≥3) according to the NCI CTC-AE v3.0 (Bethesda, MD, USA).

For the current analysis, a subset of patients from NCT00838370 was selected to investigate the performance of pretreatment DPD phenotype to identify patients at risk of severe and fatal toxicity (Figure 1). Pretreatment serum was only collected at the main center at which NCT00838370 was performed (The Netherlands Cancer Institute) and these samples were used for determination of U and DHU concentrations. All patients of whom a serum sample was available were included in this analysis (see consent procedures below). Patients of whom no serum sample was available were excluded. Patients treated with fluoropyrimidine-based chemoradiotherapy regimens were excluded, to avoid interference by radiotherapy-related toxicities which are not related to DPD deficiency. Further inclusion and exclusion criteria for enrolment in NCT00838370 have been published previously.[8] Eighteen patients carrying the *DPYD**2A allele were treated in NCT00838370 with an a priori reduced dose of fluoropyrimidines, and these patients were therefore also excluded from the analysis (Figure 1).

This analysis was performed on patient material collected previously for diagnostic purposes (secondary use). The patient material was used in accordance with the Dutch 'Code of conduct for responsible use of human tissue for medical research', drawn up by the Federation of Dutch Medical Scientific Societies (FEDERA, www.federa.org). In accordance with the code, Institutional Review Board (IRB) approval was obtained for the study, and anonymized patient material was used. All patients were informed in writing about the secondary use of their tissue, and in line with the code an opt-out procedure was in place (patients could object against the use of their material at any time). Only patients who did not object to the use of their tissue were included in this study. No additional informed consent was obtained from individual patients.

Determination of pretreatment DPD phenotype and associations with fluoropyrimidine-associated toxicity

Pretreatment serum U and DHU concentrations were measured using a validated LC-MS/MS method (Jacobs *et al.*[25] and Supplement). We first determined whether U or the DHU/U ratio best correlated with DPD activity as measured in PBMCs (which is considered the gold standard

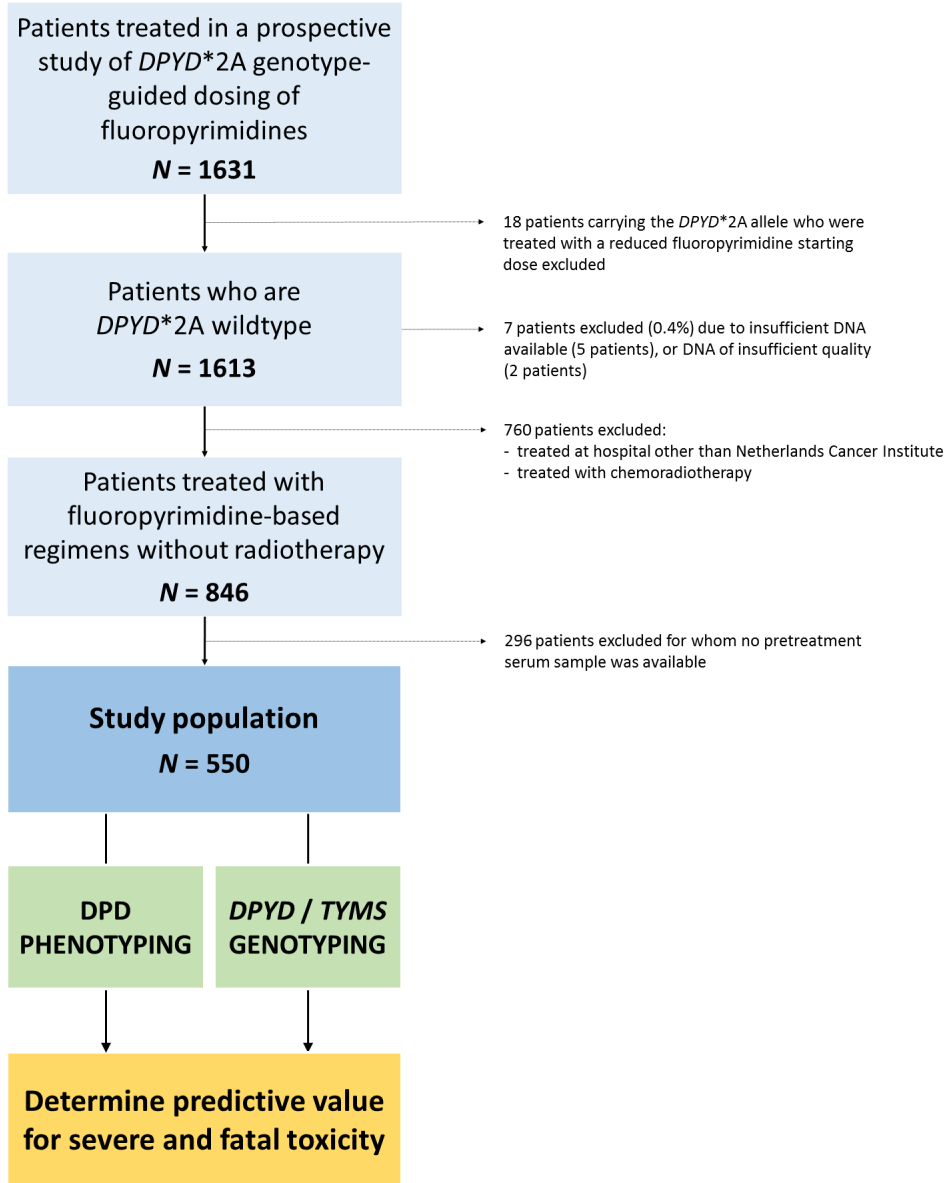


Figure 1. Selection of study population for analysis.
Abbreviations: DPD: dihydropyrimidine dehydrogenase; *DPYD*: dihydropyrimidine dehydrogenase (gene); *TYMS*: thymidylate synthase (gene).

for measurement of DPD phenotype) of healthy volunteers. This was done in an independent dataset of measurements in healthy volunteers (*N*=20).[26] Akaike information criterion (AIC) was used to determine model performance, and *r* was calculated to assess correlations. Subsequently, it was tested in 100 bootstrap samples whether U or the DHU/U ratio resulted in the best model (lowest AIC).

It was then determined whether U or the DHU/U ratio provided the best model describing risk of global severe toxicity in the main dataset of 550 patients treated with fluoropyrimidine-based chemotherapy. This was done by comparing the AIC of a logistic regression model to predict global severe toxicity which included either U or the DHU/U ratio (as continuous variables), with adjustment for age, gender, and treatment regimen (i.e., concomitant chemotherapy; details below). This was done in the original dataset and, subsequently, in 1000 bootstrap samples to assess internal validity.

No cutoff for pretreatment DPD phenotype has yet been defined to classify patients at increased risk of fluoropyrimidine-associated toxicity. Based on the previously estimated frequency of DPD deficiency of at least 3%, we hypothesized that the patients in the lowest 3 percentiles of DPD phenotypes would be at clinically relevant increased risk, corresponding to the highest 3 percentiles of pretreatment U concentrations, percentiles 98–100.[6,27–29] We investigated the risk of toxicity for this group of patients. Because several studies estimated a much higher frequency of DPD deficiency (up to 61% of the patients who experience severe toxicity),[3] we defined two additional groups of patients in which we investigated risk of toxicity, corresponding to percentiles 95–97 and percentiles 92–94 of pretreatment U concentrations. The remaining patients (in percentiles 1–91) were considered the reference group for tests of associations with severe toxicity.

Sensitivity analyses to determine associations between pretreatment DPD phenotype and fluoropyrimidine-associated toxicity

Sensitivity analyses were performed to investigate the robustness of observed associations between pretreatment DPD phenotype and risk of global severe toxicity. First, at each possible cutoff within the observed range of serum concentrations of U, an OR for severe toxicity and its 95% confidence interval (CI) was determined using a logistic regression model that included a factor with two levels (U above cutoff vs U below cutoff), with adjustment for age, gender, and treatment regimen. This was done first in the original dataset, and ORs and their 95% CIs were plotted against the range of cutoffs. In addition, this analysis was repeated in a bootstrap analysis in which at each cutoff 1000 bootstrap samples were drawn to estimate the risk of severe toxicity for patients with pretreatment U above the cutoff vs patients below the cutoff. For this bootstrap analysis, median ORs and their bias-corrected 95% CIs were plotted against the range of cutoffs.

Pharmacogenetic variants in DPYD and TYMS and associations with fluoropyrimidine-associated toxicity

Candidate pharmacogenetic variants in *DPYD* and *TYMS* were identified and selected based on a systematic literature search, to determine their clinical validity (Further details are available in the Supplement). The selected variants were: *DPYD* c.2846A>T (rs67376798), *DPYD* c.1679T>G (rs55886062), *DPYD* c.1129-5923C>G (rs75017182), *DPYD* c.1601G>A (rs1801158), *TYMS* 5′-UTR

VNTR (variable number of 28-bp tandem repeats, rs34743033/rs45445694), and *TYMS* 3′-UTR 6-bp ins/del (rs11280056). *DPYD**2A was not included, as patients carrying this allele were already excluded from this analysis in view of the dose-adapted treatment they received in NCT00838370. The PCR methods used to determine genotypes are detailed in the Supplement.

All variants were tested for deviation from Hardy–Weinberg equilibrium using the exact test.[30] Associations between pharmacogenetic variants in *DPYD* and severe toxicity were analyzed under dominant models. For the *TYMS* variants, log-additive, recessive, and dominant models were investigated. In addition, the *TYMS* risk score (= the total number of risk alleles present for the *TYMS* 5′-UTR VNTR and *TYMS* 3′-UTR 6-bp ins/del polymorphisms, as proposed by Rosmarin *et al.*[13]), was investigated. The pharmacogenetic analysis was performed in the same patients as selected for analysis of DPD phenotype (Figure 1). Because DNA samples were available for all 1613 patients who were found to be *DPYD**2A wild type in study NCT00838370, a secondary pharmacogenetic analysis was performed in this entire cohort, in order to further characterize the predictive value of these genotypes for severe toxicity.

Endpoints and data analysis

The primary endpoint of this analysis was global (any) severe fluoropyrimidine-associated toxicity, that is, grade ≥3 toxicity according to the NCI CTC-AE v3.0, occurring during the first cycle of treatment. Global (any) severe toxicity and individual types of severe toxicity, gastrointestinal toxicity, hematological toxicity, hand-foot syndrome, and cardiological toxicity were dichotomized as absent to moderate (grade 0–2) vs severe (grade 3–5). Considering only cycle one for severe toxicity assessment was considered most adequate, because in an analysis of the entire treatment duration using logistic regression there are large differences in treatment duration and consequent fluoropyrimidine-exposure between patients which are not corrected for. This potentially biases the results, for example, as a result of attrition bias or treatment modifications during the course of therapy, such as dose reductions. Also, with increasing treatment duration the cumulative incidence of severe toxicity increases, thereby reducing sensitivity to estimate differences in risk for severe toxicity between groups, due to the fact that the OR will gradually trends towards 1.0 with increasing cumulative incidence (further detailed in Supplement of Meulendijks *et al*.[11]).

Associations of the DPD phenotype and of the pharmacogenetic variants with severe toxicity were tested in logistic regression models, with adjustment for age (continuous), gender, and treatment regimen (categorized as capecitabine monotherapy, capecitabine plus platinum, capecitabine plus taxane, capecitabine-based triplet combination, capecitabine plus other drug, or 5-FU-based chemotherapy). The starting dose of capecitabine was highly collinear with type of regimen and was not predictive of toxicity after adjustment for treatment regimen; it was therefore not included in the models. Associations with toxicity-related hospitalization during the first cycle, and with fatal fluoropyrimidine-associated toxicity were assessed in separate analyses, using the same covariables. For testing associations with fatal fluoropyrimidine-associated toxicity, the whole treatment duration was taken into account, in view of the low number of events of fatal toxicity.

In order to control for type I errors as a result of testing associations with toxicity for four variants in *DPYD*, two variants in *TYMS*, and three groups based on low DPD phenotype, the Benjamini-Hochberg false discovery rate (FDR) method was used.[31] A FDR rate of $q<0.050$ was used, which corresponded to $P<0.010$ for testing associations with global severe toxicity. The same threshold

was applied for the individual types of toxicity and toxicity-related outcomes, because they were assumed to be dependent on global toxicity. Statistical tests resulting in $P<0.010$ can therefore be considered formally significant and those that achieved $P<0.050$ as nominally significant. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and F1 score (the harmonic mean of sensitivity and PPV) for predicting severe toxicity were calculated for *DPYD* variants and the DPD phenotype.

The effects of *DPYD* variants on DPD phenotype were assessed. Kruskal–Wallis and Mann–Whitney U-tests were used to test for differences in DPD phenotype between patients according to *DPYD* variants. The threshold for significance for the latter analysis was $P<0.050$. All statistical analyses were performed using R v3.1.0 (Vienna, Austria).

RESULTS

Patients

The process of patient selection for analysis is shown in Figure 1. Patient and treatment characteristics, and frequencies of adverse events are summarized in Table 1.

Pretreatment U concentration as a predictor of severe and fatal fluoropyrimidine-associated toxicity

It was first analyzed whether U or the DHU/U ratio best correlated with PBMC DPD activity. This analysis showed that pretreatment U was superior to the DHU/U ratio (Supplement and Figure 2A). Subsequently, the association between pretreatment U concentration and severe toxicity was analyzed. As expected based on correlations with PBMC DPD activity, also in relation to predicting severe toxicity U was superior to the DHU/U ratio based on AIC (AIC 363.5 for basic clinical model with age, gender, and treatment regimen as covariables, AIC 357.7 for basic clinical model plus U concentration, and AIC 362.0 for basic clinical model plus the DHU/U ratio). Also in bootstrap analysis U concentration was the superior predictor (Supplement).

Uracil as a continuous variable was strongly predictive of global severe toxicity (OR 2.75 per 10 ng/ml, 95% CI 1.39–5.44, $P=0.004$), gastrointestinal toxicity (OR 5.58, 95% CI 2.08–14.9, $P=0.0006$), toxicity-related hospitalization (OR 2.53, 95% CI 1.23–5.19, $P=0.011$), and fatal treatment-related toxicity (OR 5.11, 95% CI 1.56–16.7, $P=0.007$), but not significantly associated with hematological toxicity (OR 1.53, 95% CI 0.59–3.96, $P=0.383$). Pretreatment U concentrations were between 3.2 and 38.2 ng/ml (Figure 2B). Based on the observed distribution for pretreatment U, the upper three percentiles of pretreatment U concentrations corresponded to U >16 ng/ml ($N=17$). The next two groups were 13.9–16 ng/ml ($N=17$) and 13–13.8 ng/ml ($N=16$), respectively (Figure 2B).

As shown in Figure 3, patients with pretreatment U >16 ng/ml, as well as patients with U ≥13.9–16 ng/ml, were at significantly increased risk of global severe toxicity compared to patients with low pretreatment U (<13 ng/ml), with OR 8.2 ($P=0.0004$) and OR 5.3 ($P=0.0087$) for groups U ≥13.9–16 ng/ml and U >16 ng/ml, respectively. Pretreatment U concentration was also strongly associated with risk of gastrointestinal toxicity and toxicity-related hospitalization in patients with pretreatment U ≥13.9 ng/ml. For patients with pretreatment U concentrations 13–13.8 ng/ml, risks of toxicity outcomes were not significantly increased.

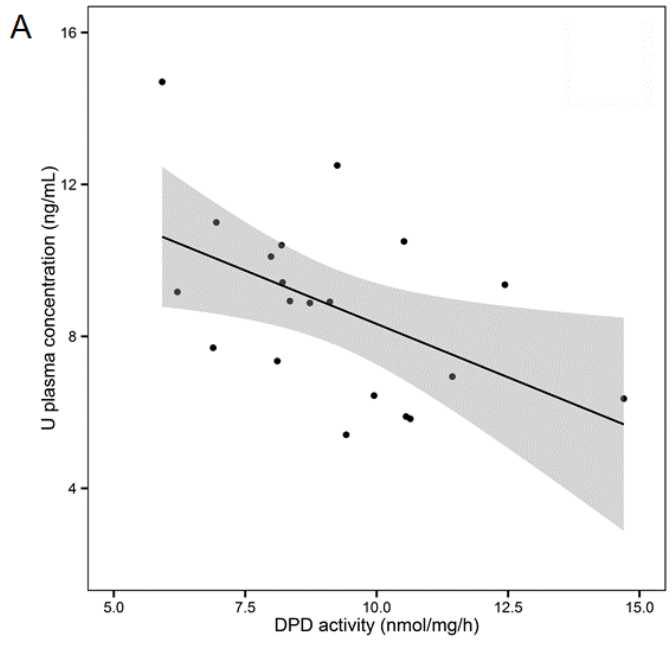
While fatal treatment-related toxicity was rare in the group of patients with normal pretreatment U (<13 ng/ml), with 2/500 patients (0.4%) suffering fatal treatment-related toxicity, in the group of patients with pretreatment U concentrations >16 ng/ml, two out of 18 patients (11%) suffered fatal toxicity ($P=0.0011$, Figure 3).

Table 1. Patient characteristics and frequencies of early severe toxicity (*N*=550).

Patient characteristics	
Age	
Median (range)	58 (21–89)
Sex	
Male	232 (42%)
Female	318 (58%)
Tumor type	
Colorectal cancer	190 (35%)
Gastric cancer	126 (23%)
Breast cancer	175 (32%)
Other	59 (11%)
Treatment	
Capecitabine monotherapy	187 (34%)
Capecitabine plus taxane	46 (8%)
Capecitabine plus platinum	148 (27%)
Capecitabine triplet combination	83 (15%)
Capecitabine plus other	16 (3%)
5-FU-based chemotherapy	70 (13%)
Origin	
Caucasian	521 (95%)
Other	29 (5%)
Previous chemotherapy	
No	407 (74%)
Yes	143 (26%)
Frequencies of severe toxicity, toxicity-related hospitalization, and fatal toxicity	
Global (overall) toxicity	
Grade 0-2	485 (88%)
Grade ≥3	65 (12%)
Gastrointestinal toxicity	
Grade 0-2	532 (97%)
Grade ≥3	18 (3%)
Hematological toxicity	
Grade 0-2	511 (93%)
Grade ≥3	39 (7%)
Hand-foot syndrome	
Grade 0-2	536 (97%)
Grade ≥3	14 (3%)
Cardiological toxicity	
Grade 0-2	539 (98%)
Grade ≥3	11 (2%)
Toxicity-related hospitalization	
No	516 (94%)
Yes	34 (6%)
Fatal treatment-related toxicity [a]	
No	546 (99.3%)
Yes	4 (0.7%)

[a] In the overall population ($N=550$), four patients (0.7%) suffered fatal fluoropyrimidine-associated toxicity. These cases were associated with the following toxicities: grade 3 diarrhea with dehydration, kidney failure, and circulatory decompensation; grade 4 cardiological toxicity; grade 2 diarrhea with sepsis; and grade 3 diarrhea with dehydration and circulatory decompensation.

Abbreviations: 5-FU: 5-fluorouracil.



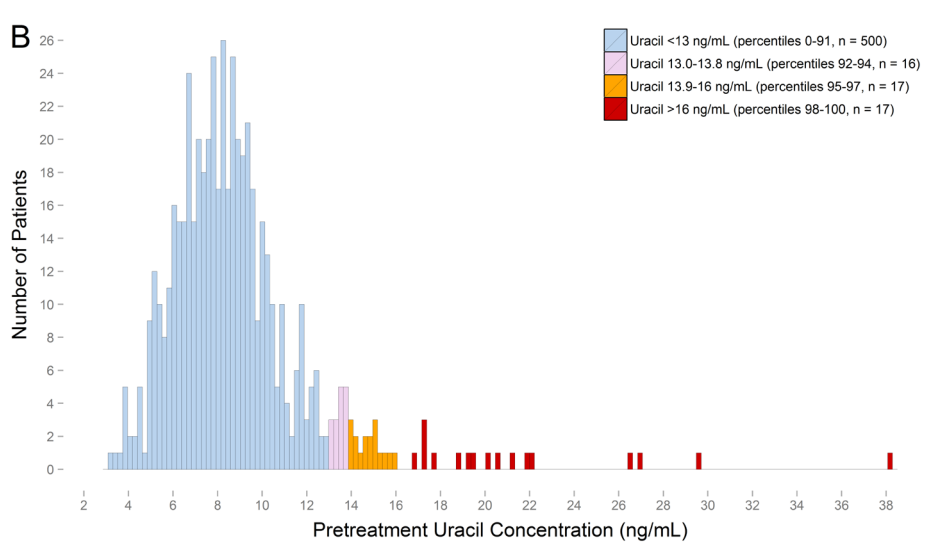


Figure 2. Correlation between pretreatment serum uracil concentrations and DPD activity in healthy volunteers and distribution of uracil concentrations in patients.
Correlation between dihydropyrimidine dehydrogenase activity in peripheral blood mononuclear cells and uracil plasma levels (r=-0.51, P=0.023) in 20 healthy volunteers (A, Figure adapted from Jacobs *et al.*[26] with permission). Distribution of pretreatment serum uracil concentrations in the entire cohort of 550 patients treated with fluoropyrimidine-based chemotherapy (B).
Abbreviations: U: uracil; DPD: dihydropyrimidine dehydrogenase.

Figure 4 depicts the results of the sensitivity analysis in which at each possible cutoff between serum U 6–16 ng/ml an OR for severe toxicity with its corresponding 95% CI were determined using logistic regression. The OR reflects risk of severe toxicity for patients with serum U above the cutoff depicted on the x-axis vs patients who are below the cutoff with adjustment for age, gender, and treatment regimen. Risk of global severe toxicity (Figure 4A) and severe gastrointestinal toxicity (Figure 4B) were found to increase proportionally with increasing pretreatment U concentration. Bootstrap analysis confirmed these results (Supplement), and when pretreatment U was plotted against the log odds for severe toxicity using spline regression, a linear increase in the risk of severe toxicity was observed over the range of serum U concentration of 8–20 ng/ml (Supplement).

Associations between pharmacogenetic variants in DPYD and TYMS and fluoropyrimidine-associated toxicity

All pharmacogenetic variants were in Hardy–Weinberg equilibrium (P<0.05). None of the individual *DPYD* variants were found to be significantly associated with global severe toxicity (Figure 5A). With regard to individual subtypes of toxicity outcomes, associations were found between *DPYD* c.1129-5923C>G and toxicity-related hospitalization (OR 3.2, P=0.047), *DPYD* c.1601G>A and gastrointestinal toxicity (OR 5.0, P=0.026), and *DPYD* c.1601G>A and toxicity-related hospitalization (OR 3.1, P=0.018). When the *DPYD* variants were combined, they were found to be associated with gastrointestinal toxicity (OR 4.1, P=0.017) and associated with toxicity-related hospitalization (OR 3.3, P=0.002). None of the *TYMS* variants were associated with toxicity outcomes (Figure 5B).

Pretreatment Uracil	*N*	Outcome	OR (95% CI)	*p* value
<13 ng/mL	500	-	1.00 (reference)	-
13-13.8 ng/mL	16	Global severe toxicity	1.2 (0.24-6.14)	0.8089
		Severe gastrointestinal toxicity	2.9 (0.32-25.5)	0.3428
		Severe hematological toxicity	0.8 (0.10-7.78)	0.8413
		Toxicity-related hospitalization	2.7 (0.54-13.5)	0.2298
13.9-16 ng/mL	17	Global severe toxicity	8.2 (2.55-26.1)	0.0004
		Severe gastrointestinal toxicity	6.3 (1.13-34.7)	0.0359
		Severe hematological toxicity	4.6 (1.19-18.1)	0.0269
		Toxicity-related hospitalization	4.1 (1.02-16.6)	0.0472
>16 ng/mL	17	Global severe toxicity	5.3 (1.53-18.7)	0.0087
		Severe gastrointestinal toxicity	33.7 (6.42-176)	<0.0001
		Severe hematological toxicity	1.0 (0.11-8.26)	0.9685
		Toxicity-related hospitalization	16.9 (4.41-64.7)	<0.0001
		Fatal treatment-related toxicity*	44.8 (4.55-441)	0.0011

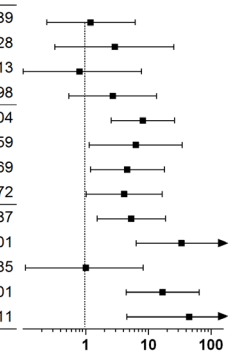


Figure 3. Associations of pretreatment serum uracil concentrations with toxicity outcomes.
Associations of pretreatment serum uracil concentrations with toxicity outcomes in the entire population of 550 patients.
* 2/17 patients (12%) in the uracil >16 ng/ml group had fatal treatment-related toxicity, compared to 2/500 patients (0.4%) among patients with uracil concentrations <13ng/ml. Fatal treatment-related toxicity did not occur among patients with pretreatment U concentrations of 13–13.8 or 13.9–16 ng/ml. Associations with fatal toxicity were determined with adjustment for age and gender but not treatment regimen (due to the low number of events).
Abbreviations: OR: odds ratio; CI: confidence interval.

When the entire cohort of 1613 patients was genotyped, none of the individual *DPYD* variants were found to be associated with global severe toxicity, nor were the *TYMS* variants (Figure 5C). For c.2846A>T and c.1679T>G combined, there was evidence for an association with global severe toxicity (OR 3.0, 95% CI 1.05–8.77, *P*=0.040). In addition, *DPYD* c.1679T>G alone was associated with hematological toxicity (OR 24.9, 95% CI 1.74–354, *P*=0.018). The four *DPYD* variants, combined, were associated with toxicity-related hospitalization (OR 2.1, 95% CI 1.20–3.63, *P*=0.009). There were no significant associations between *DPYD* variants and fatal treatment-related toxicity (OR 3.0, *P*=0.202, for all *DPYD* variants combined, not shown in Figure 5C).

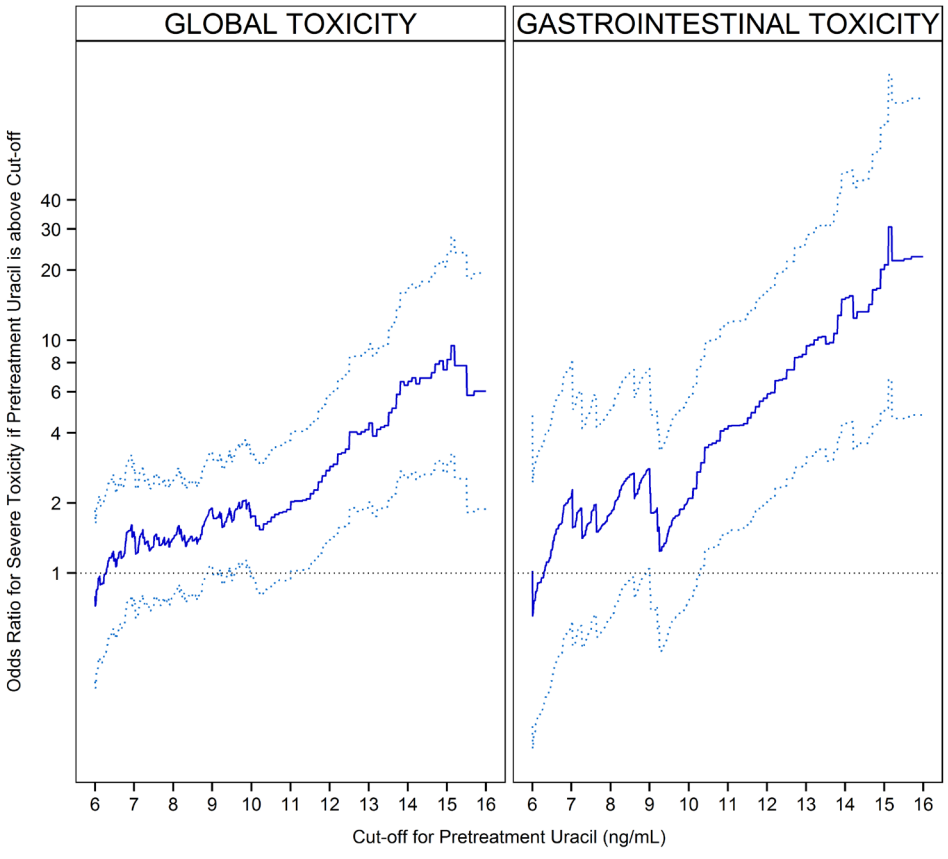


Figure 4. Risk of global severe toxicity and severe gastrointestinal toxicity at varying cutoff levels for pretreatment serum uracil.
Results from the analysis to estimate the risk of global severe (grade ≥3) toxicity and severe gastrointestinal toxicity at varying cutoffs for pretreatment uracil concentration in the original dataset, adjusted for age, gender, and treatment regimen. The solid line depicts the estimated odds ratio for risk of severe toxicity for patients with pretreatment uracil concentrations above the cutoff, vs patients with uracil concentrations below the cutoff. The dashed lines represent 95% confidence intervals. Odds ratios and 95% confidence intervals are shown on a log scale.

Associations between DPYD variants and increased pretreatment U concentration
Figure 6 shows the relationship between *DPYD* variants and pretreatment U concentration. Overall, the *DPYD* variants (combined) were significantly associated with U concentrations (*P*=0.009). Both c.2846A>T and c.1679T>G were individually associated with increased U concentrations (*P*<0.001 and *P*=0.024, respectively). In contrast, c.1129-5923C>G and c.1601G>A were not associated with pretreatment U (*P*=0.105 and *P*=0.431, respectively).

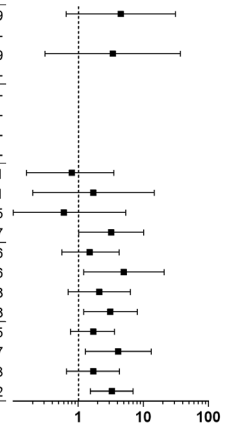
Performance of DPYD variants and pretreatment U to predict early severe toxicity
Sensitivity, specificity, PPV, NPV, and F1 score for first cycle global toxicity were calculated for *DPYD* genotyping and pretreatment phenotyping, as well as for combination strategies. To assess diagnostic accuracy of genotyping, we combined the *DPYD* variants which were clinically validated in a recent meta-analysis which was published while preparing the current manuscript, that is, c.2846A>T, c.1679T>G, and c.1129-5923C>G.[32] The variant *DPYD* c.1601G>A was not included is these calculations as this variant could not be clinically validated in the meta-analysis.[32]
For combined genotyping of c.2846A>T, c.1679T>G, and c.1129-5923C>G, sensitivity was 6%, PPV 13%, specificity 95%, and NPV 88%. For pretreatment U (at a cutoff of ≥13.9 ng/ml), sensitivity was 18%, PPV 35%, specificity 95%, and NPV 90%. Since sensitivity and PPV are considered the most relevant parameters in this context, we also calculated the F1 score, the harmonic mean of sensitivity and PPV. F1 score was 8% for genotyping of variants c.2846A>T, c.1679T>G, and c.1129-5923C>G, while it was 24% for phenotyping alone (cutoff for U concentration ≥13.9 ng/ml). For genotyping and phenotyping combined, sensitivity was 22%, PPV 24%, specificity 91%, NPV 90%, and F1 score 23%. Further data on diagnostic accuracy are detailed in the Supplement.

DISCUSSION

In this study we showed that high pretreatment serum U concentration (>16 ng/ml) was strongly associated with severe fluoropyrimidine-associated toxicity (OR 5.3, *P*=0.0087), as well as fatal fluoropyrimidine-associated toxicity (OR 44.8, *P*=0.0011), and risk of severe toxicity increased proportionally with increasing serum U concentration. Sensitivity to identify patients at risk of early severe toxicity was three times higher for phenotyping (18%), than for genotyping of the established *DPYD* variants c.2846A>T, c.1679T>G, and c.1129-5923C>G (6%).[32] Similarly, PPV of phenotyping was found to be 35 vs 13% for genotyping of the established *DPYD* variants. Combined genotyping and phenotyping did not further improve diagnostic accuracy compared to phenotyping alone.
In contrast to the strong predictive value of U concentration, known *DPYD* genotypes appeared only moderately predictive of severe toxicity in the same patient population. The *TYMS* variants, which were previously found to have a modest association with severe fluoropyrimidine-associated toxicity in a meta-analysis (OR 1.36 for *TYMS* 5'-VNTR, *P*<0.001; and OR 1.25 for *TYMS* 3'-UTR, *P*=0.02),[13] were not associated with toxicity in our study. In view of the modest effect size observed for these variants, both in this study and the previous meta-analysis, the value of these pharmacogenetics variants in *TYMS* in clinical practice should be questioned.
Our results suggest that pretreatment serum U concentration can potentially strongly improve an upfront test to identify patients with DPD deficiency who are at high risk of severe and potentially fatal toxicity. Concentration of U was found to correlate better with PBMC DPD activity (in healthy volunteers) and better predicted toxicity than the DHU/U ratio in patients.

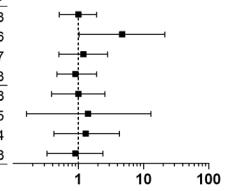
A

variant (risk allele)	N	Outcome	OR (95% CI)	p value
DPYD c.2846A>T *(T)*	6/547	Global toxicity	4.5 (0.65-31.0)	0.129
		Gastrointestinal toxicity	Not estimable	-
		Hematological toxicity	3.4 (0.31-36.9)	0.319
		Hospitalization	Not estimable	-
DPYD c.1679T>G (G)	2/546	Global toxicity	Not estimable	-
		Gastrointestinal toxicity	Not estimable	-
		Hematological toxicity	Not estimable	-
		Hospitalization	Not estimable	-
DPYD c.1129-5923C>G (G)	22/547	Global toxicity	0.8 (0.16-3.53)	0.721
		Gastrointestinal toxicity	1.7 (0.20-14.7)	0.621
		Hematological toxicity	0.6 (0.08-5.35)	0.685
		Hospitalization	3.2 (1.02-10.1)	0.047
DPYD c.1601G>A (A)	34/546	Global toxicity	1.5 (0.56-4.25)	0.396
		Gastrointestinal toxicity	5.0 (1.22-21.0)	0.026
		Hematological toxicity	2.1 (0.70-6.30)	0.188
		Hospitalization	3.1 (1.22-8.08)	0.018
DPYD all variants	64/546	Global toxicity	1.7 (0.76-3.59)	0.205
		Gastrointestinal toxicity	4.1 (1.29-13.3)	0.017
		Hematological toxicity	1.7 (0.66-4.30)	0.273
		Hospitalization	3.3 (1.54-6.91)	0.002



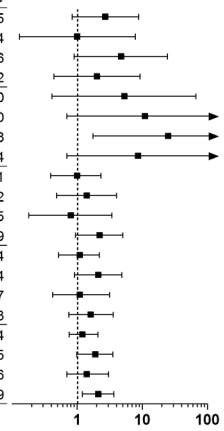
B

Variant	N			Outcome	OR (95% CI)	p value
TYMS 5'-UTR	150	262	150	Global toxicity	1.0 (0.51-1.90)	0.963
(VNTR)	(2R/2R)	(2R/3R)	(3R/3R)	Gastrointestinal toxicity	4.7 (1.03-21.35)	0.046
				Hematological toxicity	1.2 (0.50-2.80)	0.697
		n = 545		Hospitalization	0.9 (0.47-1.90)	0.873
TYMS 3'-UTR	239	239	67	Global toxicity	1.0 (0.39-2.56)	0.988
(6-bp ins/del)	(ins/ins)	(ins/del)	(del/del)	Gastrointestinal toxicity	1.4 (0.16-13.0)	0.745
				Hematological toxicity	1.3 (0.42-4.28)	0.624
		n = 545		Hospitalization	0.9 (0.33-2.39)	0.818

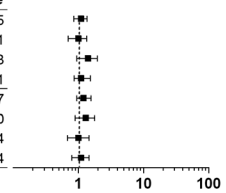


C

Variant (risk allele)	N	Outcome	OR (95% CI)	p value
DPYD c.2846A>T (T)	19/1604	Global toxicity	2.7 (0.84-8.79)	0.095
		Gastrointestinal toxicity	1.0 (0.13-7.78)	0.994
		Hematological toxicity	4.7 (0.90-24.3)	0.066
		Hospitalization	2.0 (0.44-9.22)	0.362
DPYD c.1679T>G (G)	3/1605	Global toxicity	5.3 (0.41-66.5)	0.200
		Gastrointestinal toxicity	10.9 (0.69-173)	0.090
		Hematological toxicity	24.9 (1.74-354)	0.018
		Hospitalization	8.6 (0.69-105)	0.094
DPYD c.1129-5923C>G (G)	58/1606	Global toxicity	1.0 (0.39-2.31)	0.911
		Gastrointestinal toxicity	1.4 (0.48-4.03)	0.542
		Hematological toxicity	0.8 (0.18-3.42)	0.735
		Hospitalization	2.2 (0.94-5.02)	0.069
DPYD c.1601G>A (A)	84/1601	Global toxicity	1.1 (0.52-2.19)	0.864
		Gastrointestinal toxicity	2.1 (0.91-4.84)	0.084
		Hematological toxicity	1.1 (0.42-3.13)	0.797
		Hospitalization	1.6 (0.74-3.56)	0.223
DPYD all variants	164/1600	Global toxicity	1.2 (0.75-2.08)	0.404
		Gastrointestinal toxicity	1.9 (0.99-3.51)	0.055
		Hematological toxicity	1.4 (0.69-3.03)	0.336
		Hospitalization	2.1 (1.20-3.63)	0.009



Variant	N			Outcome	OR (95% CI)	p value
TYMS 5'-UTR	380	801	421	Global toxicity	1.1 (0.85-1.36)	0.545
(VNTR)	(2R/2R)	(2R/3R)	(3R/3R)	Gastrointestinal toxicity	1.0 (0.70-1.35)	0.871
				Hematological toxicity	1.4 (0.94-1.96)	0.103
		n = 1602		Hospitalization	1.1 (0.86-1.53)	0.361
TYMS 3'-UTR	732	676	194	Global toxicity	1.2 (0.95-1.56)	0.127
(6-bp ins/del)	(ins/ins)	(ins/del)	(del/del)	Gastrointestinal toxicity	1.3 (0.89-1.78)	0.200
				Hematological toxicity	1.0 (0.68-1.45)	0.984
		n = 1602		Hospitalization	1.1 (0.79-1.45)	0.664



◄ Figure 5. Results of the pharmacogenetic analysis.
Results of the pharmacogenetic analysis investigating associations between *DPYD* variants (A) and *TYMS* variants (B) in the primary cohort of 550 patients. The frequencies of early hand-foot syndrome and cardiological toxicity were too low in the population to investigate associations with these individual types of toxicity. The associations mentioned as 'not estimable' could not be estimated due to too few events of severe toxicity. For the *TYMS* variants, the results are shown for the log-additive pharmacogenetic model. The other models (dominant, recessive, or risk score) resulted in similar, non-significant, associations between *TYMS* genotypes and toxicity outcomes (details not shown). Results of the pharmacogenetic analysis in the larger cohort of 1613 patients are shown in C.
Abbreviations: OR: odds ratio; CI: confidence interval, *DPYD*: dihydropyrimidine dehydrogenase (gene); *TYMS*: thymidylate synthase (gene).

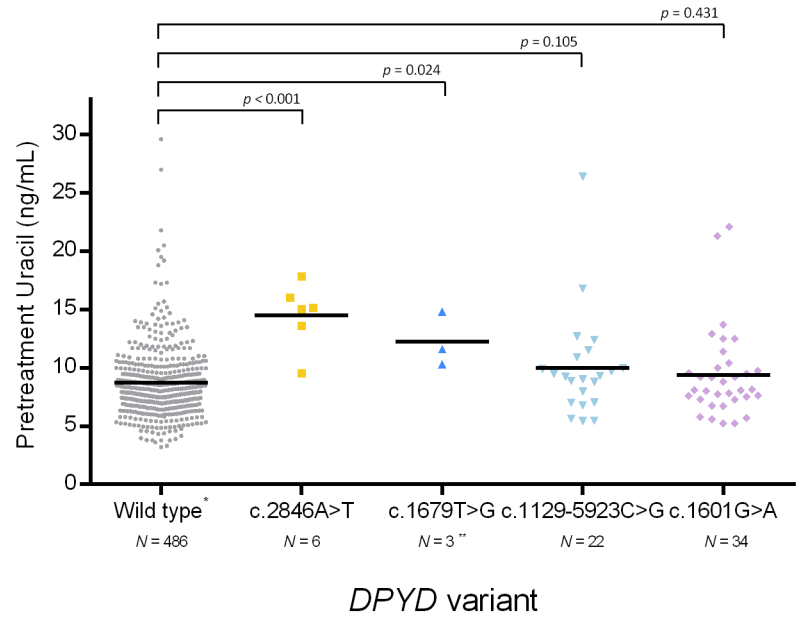


Figure 6. Relationships between *DPYD* variants and pretreatment uracil concentrations.
The figure shows pretreatment serum uracil concentrations by *DPYD* variant. The horizontal lines represent median concentrations. Overall, *DPYD* variants were associated with an increase of pretreatment uracil concentrations of 12% (*P*=0.003). The c.2846A>T and c.1679T>G variants were associated with significantly higher uracil concentrations (+82%, *P*<0.001 and +41%, *P*=0.024, respectively). In contrast, c.1129-5923C>G and c.1601G>A were not significantly associated with pretreatment uracil concentrations (+12%, *P*=0.105 and -1%, *P*=0.431, respectively).
* Wild type for *DPYD**2A, c.2846A>T, c.1679T>G, c.1129-5923C>G, and c.1601G>A.
** The cohort of 550 patients contained 2 patients with the c.1679T>G variant. In view of the low frequency of this variant, the third patient who carried c.1679T>G from the entire cohort of 1613 patients was phenotyped solely for the purpose of this analysis investigating the association between *DPYD* variants and pretreatment serum uracil concentration (this patient received chemoradiotherapy and was therefore excluded from the main analysis).

These findings may be explained by the metabolism of U and DHU. Uracil is metabolized via three sequential reactions: U is converted into DHU by DPD, which is converted into beta-ureidopropionate by dihydropyrimidinase, which is converted into beta-alanine, ammonia, and CO_2 by beta-ureidopropionase. The activity of the first enzyme in this scheme, DPD, is the main determinant of toxicity upon treatment with 5-FU because 5-FU is converted by DPD into the non-cytotoxic compound 5,6-dihydrofluorouracil. Ito *et al.* modelled the catabolism of U, and showed that in the overall cascade of enzymes, dihydropyrimidinase (which converts DHU into beta-ureidopropionate) is rate-limiting.[33] This is reflected by the approximately 10 times higher concentrations of DHU compared to U in plasma, and indicates that concentrations of DHU are mainly determined by dihydropyrimidinase, and not by DPD. This may explain our finding that taking into account concentrations of DHU in addition to U – by incorporating it in the DHU/U ratio – did not lead to better prediction of toxicity than by using U concentration alone. Further supportive data come from a study in which 500 mg/m^2 U was administered to a group of individuals with DPD deficiency and a group with normal DPD activity.[34] It was found that while exposure to U (measured as AUC) was 230% in DPD deficient subjects (130% increased) compared to subjects with normal DPD activity, the difference in DHU exposure was much smaller (25% reduction in exposure in DPD deficient subjects compared to DPD proficient subjects).

In our study U and DHU concentrations in relation to fluoropyrimidine-associated toxicity were measured using a validated LC-MS/MS method.[25] This is of relevance, since previous studies mainly used methods based on UV detection and, as shown recently, there has been large variability in the reported ranges for the DHU/U ratio in these studies, which could indicate that bioanalytical issues may have negatively affected results in previous studies.[24]

Determination of an optimal cutoff for pretreatment U concentration to identify patients at risk of severe toxicity was not a formal aim in our study. Based on our results, however, it appears that it can be safely assumed that there is a clinically relevant increase in risk of severe toxicity above 16 ng/ml, and possibly for patients with pretreatment U ≥13.9–16 ng/ml.

Dose adaptation in patients with elevated pretreatment U concentration has the potential to increase the safety of DPD deficient patients at high risk of severe and fatal toxicity, and prescreening could be an effective approach to improve patient safety. Determination of the threshold for dose adaptation should therefore be an important objective of future studies.

A limitation of our study is that we did not externally validate the clinical validity of U concentration in an independent cohort of patients treated with fluoropyrimidines. However, we did validate the association between U concentration and PBMC DPD activity in a small independent dataset of healthy volunteers. We also performed different sensitivity analyses, which showed that risk of severe toxicity increased proportionally with increasing pretreatment U concentration, supporting the validity of our findings, which are further backed up by previous smaller studies suggesting the clinical validity of pretreatment DPD phenotype.[15–18] We are currently undertaking a prospective validation study to replicate our current findings in 1250 patients (NCT02324452).

Our findings should be interpreted in the context of the fact that patients with the *DPYD**2A variant were excluded from the analysis (*N*=18, 1% of a typical Caucasian population). Calculations of diagnostic accuracy could therefore be affected as a result, but it is expected that this will only affect sensitivity and PPV to a minor extent in view of the low frequency of *DPYD**2A. Furthermore, we found that *DPYD**2A genotype strongly correlates with high pretreatment U concentration

(unpublished observations), indicating that pretreatment U concentration is also able to identify these patients.

In conclusion, this study indicates that pretreatment U is a highly promising phenotypic marker with high sensitivity and PPV to identify patients at high risk of fluoropyrimidine-associated toxicity, that could be used alone or in combination with *DPYD* genotype-based dose-individualization to improve patient safety. The safety of 3–6% of the patients treated with fluoropyrimidines could thereby be improved. Prospective investigations to confirm the clinical validity and, importantly, the clinical utility of pretreatment U concentration are now warranted.

DISCLOSURE

The study was funded by The Netherlands Cancer Institute. The authors declare no conflict of interest.

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SUPPLEMENT

SUPPLEMENTARY METHODS

PUBMED search for identification of pharmacogenetic variants

For selection of candidate pharmacogenetic variants in *DPYD* and *TYMS* a computerized literature search was conducted in PUBMED for published articles in English up to April 1st, 2014. Appropriate trials were identified from the following search definition: (*DPYD* OR DPD OR dihydropyrimidine dehydrogenase OR thymidylate synthase OR *TYMS*) AND (polymorphism OR Polymorphism, Single Nucleotide[MeSH] OR Polymorphism, Genetic[MeSH] OR pharmacogenet*[tiab] OR Pharmacogenetics[MeSH] OR mutation/genetics OR genotype[MeSH] OR polymorphisms OR variant OR variants OR SNP) AND (toxicity OR fluorouracil/adverse effects OR capecitabine/adverse effects OR adverse drug reaction OR side effects OR Antineoplastic Combined Chemotherapy Protocols/adverse effects[MeSH).

All titles and abstracts were screened to identify studies of relevance to the subject. All relevant publications were retrieved in full-text. References listed in the retrieved articles were searched manually for additional relevant publications.

Based on the published data on the functional and clinical relevance of *DPYD* and *TYMS* variants, we selected four variants in *DPYD*: c.2846A>T (rs67376798), c.1679T>G (rs55886062), c.1129-2953C>G (rs75017182) and c.1601G>A (rs1801158); and two variants in *TYMS*: *TYMS* 5'-UTR VNTR (variable number of 28-bp tandem repeats, rs34743033/rs45445694) and *TYMS* 3'-UTR 6-bp ins/del (rs11280056) as candidate pharmacogenetic variants of which we aimed to validate clinical validity (See Supplementary Table 1).

Genotyping of pharmacogenetic variants in DPYD and TYMS

Genomic DNA was isolated from peripheral blood cells using the MagNA Pure Total Nucleic Acid Isolation Kit I on MagNA Pure LC (Roche Diagnostics).

DPYD genotyping: The *DPYD* variants c.2846A>T, c.1679T>G, and c.1601G>A were determined using real-time PCR assays with allele-specific TaqMan probes (Applied Biosystems, Bleijswijk, The Netherlands). Each well contained ± 40 ng genomic DNA, 2.5 µL 2X Universal PCR Master Mix (Applied Biosystems), and 0.25 µL of pre-designed allele-specific primers with FAM and VIC dye-labeled TaqMan probes (20X) (Applied Biosystems) in a total reaction volume of 5 µL. In each run sequenced wild type, heterozygous, and homozygous controls, as well as two negative controls, were included. For c.1679T>G only wild type and heterozygous controls were included as no homozygous control was available. PCR reactions were performed using an ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems) under the following conditions: 50 °C for 2 min followed by 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 sec and 60 °C for 1 min. Data was analyzed using 7500 Fast System SDS Software (Applied Biosystems).

To determine c.1129-2953C>G in intron 10 we measured the c.1236G>A mutation, which is in the same haplotype (Haplotype B3) and is in complete linkage disequilibrium with c.1129-2953C>G.[13,14] A custom made Taqman assay was designed by Applied Biosystems for determination of c.1236G>A. To confirm that carriers of the c.1236G>A SNP were carriers of c.1129-2953C>G, the intron 10 mutation was subsequently determined using PCR followed by sequencing according to previously published methods.[14] The region of interest in intron 10 was amplified using the following primers: 5'- TCAGACCAAATCATCGCATT-3' (forward) and 5'- TTCTCCTCATGGCACCCATA-3'

Supplementary Table 1. Pharmacogenetic variants selected for analysis.

Pharmacogenetic variant	RefSNP number	Star allele designation	Amino acid change	Biochemical consequences	Clinical findings
***DPYD* c.2846A>T**	rs67376798	-	D949V	Interferes with cofactor binding and/or electron transport.[1] Homozygous *in vitro* expression of this variant in HEK293 cells results in significantly decreased enzyme activity (rest activity 59% compared to wild-type *DPYD*, *P*=0.0031).[2]	Different high-quality studies and a meta-analysis have demonstrated an association between this variant and increased risk of fluoropyrimidine-associated toxicity.[3–10]
***DPYD* c.1679T>G**	rs55886062	*DPYD**13	I560S	Change of hydrophobic into hydrophilic residue; thought to result in destabilization of the protein. Homozygous *in vitro* expression of this variant in HEK293 cells results in significantly decreased enzyme activity (rest activity 25% compared to wild-type *DPYD*, *P*=5.2 x 10^{-9}).[11]	An association between this variant and increased risk of severe toxicity was found in different studies.[3,7] No significant effect was found in some other studies, possibly due to a low frequency.[8,12,13]
***DPYD* c.1129-2953C>G**	rs75017182	-	-	Deep intronic mutation that leads to aberrant pre-mRNA splicing. In complete linkage with the synonymous mutation c.1236G>A, and three intronic polymorphisms, c.483+18G>A, c.959–51T>G, and c.680+139G>A. Together these polymorphisms constitute a haplotype that has been termed haplotype B3.[12,14]	Several studies have demonstrated an association between this variant and the risk of fluoropyrimidine-associated toxicity.[6,12–14] No significant effect on risk of severe toxicity was found in other studies.[5,7,10,15–17]
***DPYD* c.1601G>A**	rs1801158	*DPYD**4	S534N	Has been associated with a significant decrease in DPD activity, as measured in PBMCs.[18] *In vitro* expression of this variant in HEK293 cells resulted in significantly increased enzyme function (36% more active than wild-type *DPYD*, *P*=3.4 x 10^{-7}).[11]	One study found an association between this variant and increased risk of severe toxicity.[7] Multiple other studies did not find a relationship.[5,6,10,12,15,17]
***TYMS* 5'-UTR VNTR**	rs34743033 or rs45445694	-	-	A variable number of 28 bp tandem repeats in the 5'UTR promoter enhancer region of *TYMS*. A higher number of tandem repeats is associated with increased *TYMS* gene transcription and translational activity.[19–21] The most common alleles in Caucasians are the double repeat (2R) and the triple repeat (3R).	Several independent studies demonstrated an association with fluoropyrimidine-associated toxicity,[5,22–24] as well as a meta-analysis.[10] A decreased risk of developing fluoropyrimidine-associated toxicity is found with increasing number of tandem repeats (*e.g.* 2R/3R or 3R/3R vs. 2R/2R).[5,10,22–24] Other studies did not confirm these associations.[7,25–28]
***TYMS* 3'-UTR 6-bp ins/del (c.1494del6b)**	rs11280056	-	-	A 6 bp insertion/deletion in the 3'UTR of *TYMS,* which affects mRNA stability.[29]	An association with increased risk of treatment-related toxicity was found in several studies.[7,10] while others did not find an association.[5,22–28]

(reverse). Purified PCR reaction products were sequenced using BigDye® Terminator v3.1 (Applied Biosystems), and sequence fragments analyzed using an automated sequencer (ABI3730, Applied Biosystems). All carriers of c.1236G>A that we identified were carriers of c.1129-2953C>G.

TYMS genotyping: The variable number of 28 bp tandem repeats (VNTR) in the 5'-UTR of *TYMS* (rs34743033), the G>C SNP in the second repeat of the 3R allele of *TYMS* (rs2853542), and the 6-bp ins/del polymorphism in the 3'-UTR of *TYMS* (rs11280056) were determined using PCR followed by sequencing. For amplification of the 5'-UTR the following primers were used: forward 5'-AAAAGGCGCGCGGAAGGGGTCCT-3' and reverse 5'-TCCGAGCCGGCCACAGGCAT-3'. For the 3'-UTR 6-bp ins/del polymorphism the following primers were used: 5'-CAAATCTGAGGGAGCTGAGT-3' (forward) and 5'- CAGATAAGTGGCAGTACAGA-3' (reverse). Each well contained PCR Buffer II (Applied Biosystems), DNTPs 0.2 mM (each) (Qiagen, Valencia, CA, USA), 5% DMSO (Sigma-Aldrich, St. Louis, MO, USA), 0.4 µM forward and reverse primer (Invitrogen), 0.5 units of Amplitaq Gold® Polymerase (Applied Biosystems), and ± 40 ng of genomic DNA in a total reaction volume of 30 µL for the amplification of 5'-UTR, whereas the amplification of the 3'-UTR was performed in a total reaction volume of 15 µL.

PCR conditions for amplification of the 5'-UTR were as follows: 5 min at 94 °C, followed by 45 cycles of 1 min at 94 °C, 1 min at 61 °C, and 1 min at 72 °C. PCR conditions for amplification of the 3'-UTR region was as follows: 5 min at 94 °C, followed by 39 cycles of 1 min at 94 °C, 1 min at 58 °C, and 1.5 min at 72 °C. PCR reactions were performed using a T100 thermal cycler (Bio-Rad Laboratories).

After PCR, the VNTR was visualized on 3% agarose with TAE buffer and ethidium bromide. To determine the G>C SNP a separate sample of the same PCR product was incubated overnight with HaeIII (Thermo Scientific), and subsequently visualized on 3% agarose with TAE buffer and ethidium bromide which was run at 4°C. To determine the presence of the 6-bp ins/del polymorphism in the 3'-UTR of *TYMS* the PCR product was incubated overnight with DraI (New England Biolabs), and subsequently visualized on a 3% agarose gel run at 4°C.

Sequencing: PCR products were sequenced using the BigDye® Terminator Cycle sequencing v3.1 kit (Applied Biosystems, Foster City, CA). 5 µL of PCR product was first purified using 2 µL Illustra ExoProStar 1-step (GE Healthcare) incubated for 15 min at 37 °C followed by 15 min at 80 °C. Subsequently, 7 µL purified PCR product was sequenced using 1 µL BigDye® Terminator v3.1 and 0.4 µM forward or reverse primer in a total reaction volume of 20 µL, under the following conditions: 96 °C for 10 sec followed by 50 °C for 5 sec and 60 °C for 4 min, for 24 cycles. Sequence data were analyzed using Unipro UGENE software (Novosibirsk, Russia).

Determination of pretreatment uracil and dihydrouracil concentrations

U and DHU concentrations were measured in a pretreatment serum sample using LC-MS/MS.[30] Briefly, a volume of 20 µL of internal standard working solution containing 1,3-U-$^{15}N_2$ and 5,6-UH_2-$^{13}C_4$,$^{15}N_2$ was added to 300 µL serum. Protein precipitation was performed using 900 µL of methanol and acetonitrile (50:50 v/v). The samples were vortex-mixed for 10 s, shaken for 10 min at 1250 rpm (Labinco, Breda, The Netherlands) and centrifuged at 14,000 rpm for 10 minutes. The clear supernatants were dried under a stream of nitrogen at 40°C and reconstituted in 100 µL 0.1% formic acid in water. Chromatographic separation was performed on an Acquity UPLC® HSS T3 column (150 x 2.1 mm ID, particle size 1.8 µm), and a gradient of 0.1% formic acid in water (Eluent A) and 0.1% formic acid in acetonitrile (eluent B) at a flow of 0.3 mL/min. The following

Supplementary Table 2. Performance of genotyping and phenotyping strategies to predict first cycle global severe toxicity.

Summary of diagnostic performance of genotyping, phenotyping, or combinations of genotyping and phenotyping. The F1 score is a measure of test accuracy in which both sensitivity and positive predictive value are considered.

Screening strategy	Pharmacogenetic variants/ phenotype cut-off	Sensitivity	Specificity	Positive predictive value	Negative predictive value	F1 score*
Genotyping	*DPYD* c.2846A>T	3%	99%	33%	88%	6%
Genotyping	*DPYD* c.1679T>G	0%	1%	0%	88%	0%
Genotyping	*DPYD* c.1129-2953C>G	3%	96%	9%	88%	5%
Genotyping	*DPYD* c.2846A>T *DPYD* c.1679T>G *DPYD* c.1129-2953C>G	6%	95%	13%	88%	8%
Phenotyping	Cut-off uracil: ≥13.9 ng/ml	18%	95%	35%	90%	24%
Genotyping and phenotyping	*DPYD* c.2846A>T *DPYD* c.1679T>G *DPYD* c.1129-2953C>G Cut-off uracil: ≥13.9 ng/ml	22%	91%	24%	90%	23%

*F1 score: harmonic mean of sensitivity (SEN) and positive predictive value (PPV): 2 * (SEN * PPV)/(SEN + PPV).

gradient was used: 100% A from 0-3.2 min, 90% B from 3.2-3.7 min, 100% A from 3.7-5 min. A triple quadrupole mass spectrometer (API5500, AB Sciex, USA) was operated in the negative mode for quantification of U and in the positive mode for quantification of DHU, using the mass transitions m/z 110.9 → 42.0 for U and m/z 114.9 → 55 for DHU. The method was validated over a concentration range of 1 to 100 ng/mL for U and 10 to 1000 ng/mL for DHU.

SUPPLEMENTARY RESULTS

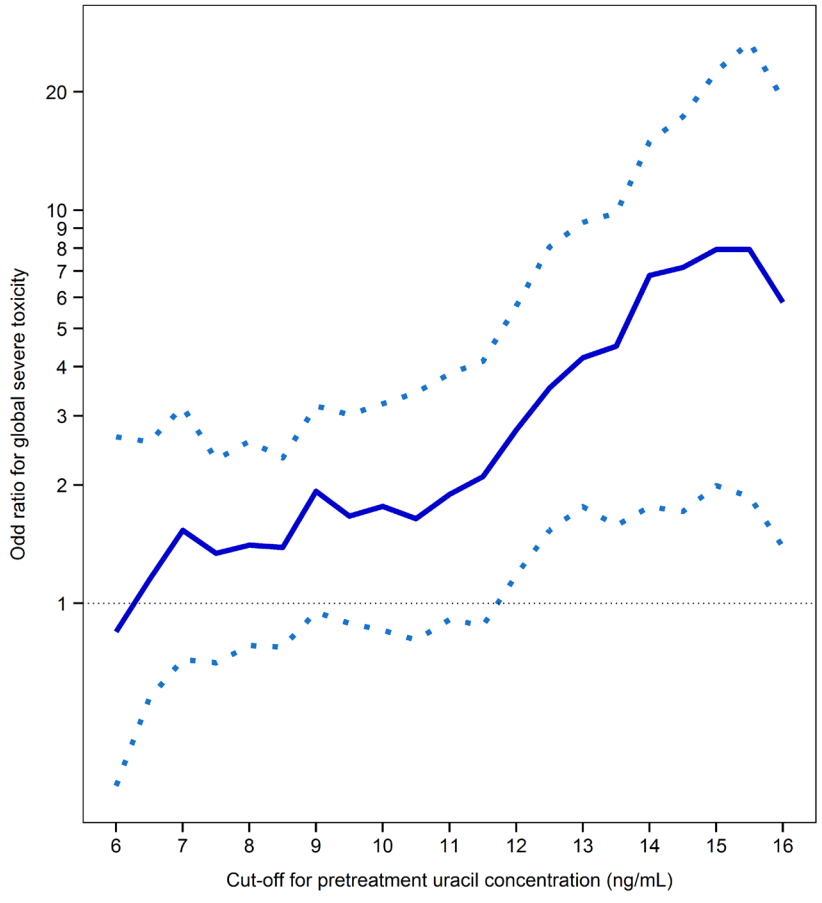
Associations of pretreatment uracil and dihydrouracil concentrations with peripheral blood mononuclear cell DPD activity in healthy individuals

To determine whether U or the DHU/U ratio best correlated with peripheral blood mononuclear cell (PBMC) DPD activity, we tested correlations between U or the DHU/U ratio and PBMC DPD activity in an independent cohort of healthy volunteers from a previous study (*N*=20).[31] Uracil concentrations resulted in a better model than the DHU/U ratio with AIC 86.4 for U and AIC 88.6 for the DHU/U ratio, with *r*=-0.51, *P*=0.023 for U and *r*=0.41, *P*=0.072 for DHU/U ratio. The bootstrap analysis showed that U was superior to the DHU/U ratio in 72% of bootstrap samples. Based on these findings, pretreatment U was selected as the measure of DPD phenotype for further analysis of associations with severe toxicity.

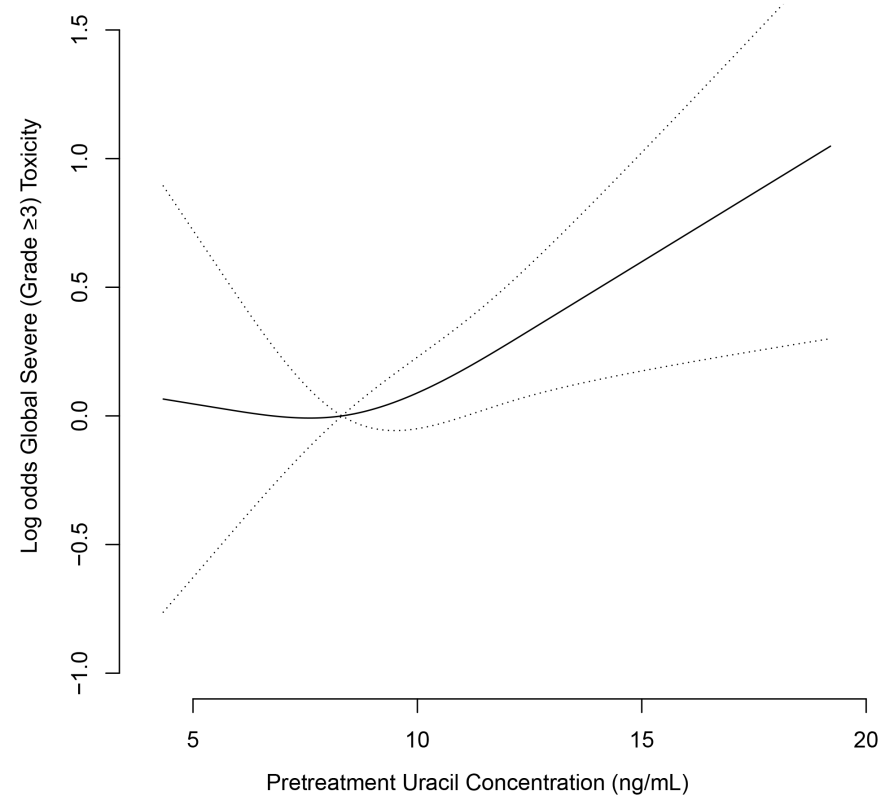
Associations of pretreatment uracil and dihydrouracil concentrations with risk of severe fluoropyrimidine-associated toxicity in patients

We then determined which phenotypic parameter best correlated with risk of severe toxicity in

patients. The AIC value corresponding to the basic clinical model for global severe toxicity (including age, gender, and treatment regimen as covariables) was 363.5. Addition of pretreatment U concentrations resulted in an improvement of the model (AIC 357.7). The DHU/U ratio resulted in less improvement (AIC 362.0). Similar results were obtained for gastrointestinal toxicity: 156.0 for the basic model, 146.5 for U, and 152.0 for the DHU/U ratio. In 1000 bootstrap samples in which both models were compared, U was superior to the DHU/U ratio in 83% of bootstrap samples.



Supplementary Figure 1. Risk of global severe toxicity at varying pretreatment uracil cut-off in bootstrap analysis.
Results of the bootstrap analysis. The figure depicts median ORs (solid line) and their corresponding bias-corrected 95% confidence intervals (dashed lines). The range in which the cut-off for pretreatment U was varied was restricted to 6-16 ng/mL, because estimates of the OR and 95%CI were unstable outside of this range due to a too small number of patients outside of this range.



Supplementary Figure 2. Spline curve for the estimated association between pretreatment uracil and global severe toxicity.
Spline curve showing the association between pretreatment U concentration and global severe toxicity. To allow for possible non-linearity of the association between pretreatment U and overall toxicity, U was entered into a logistic regression model as a continuous variable using a restricted cubic spline with 3 knots placed at 5.63 ng/mL, 8.32 ng/mL, and 12.50 ng/mL. The reference was chosen at 8.32 ng/mL (the median pretreatment U concentration).

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13

Food-effect study on uracil and dihydrouracil plasma levels as marker for dihydropyrimidine dehydrogenase activity in human volunteers

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SUMMARY

Aims

This study aimed to determine the effect of food intake on uracil and dihydrouracil plasma levels. These levels are a promising marker for dihydropyrimidine dehydrogenase activity and for individualizing fluoropyrimidine anticancer therapy.

Methods

A randomized, cross-over study in sixteen healthy volunteers was performed, in which subjects were examined in fasted and fed state on two separate days. In fed condition, a high-fat, high-caloric breakfast was consumed between 8:00h-8:30h. Whole blood for determination of uracil, dihydrouracil and uridine plasma levels was drawn on both test days on predefined time points between 8:00h and 13:00h.

Results

Uracil levels showed to be statistically significantly different between fasting and fed state. At 13:00h, the mean uracil level in fasting state was 12.6±3.7 ng/ml and after a test meal 9.4±2.6 ng/ml (P<0.001). Dihydrouracil levels were influenced by food intake as well (mean dihydrouracil level at 13:00h in fasting state 147.0±36.4 ng/ml and in fed state 85.7±22.1 ng/ml, P<0.001). Uridine plasma levels showed curves with similar patterns as for uracil.

Conclusions

It was shown that both uracil and dihydrouracil levels were higher in fasting state than in fed state. This is hypothesized to be an direct effect of uridine plasma levels, which were previously shown to be elevated in fasting state and reduced after intake of food. These findings show that, when assessing plasma uracil and dihydrouracil levels for adaptive fluoropyrimidine dosing in clinical practice, sampling should be done between 8:00h-9:00h in the fasting state to avoid bias caused by circadian rhythm and food effects.

INTRODUCTION

The fluoropyrimidine anticancer drugs 5-fluorouracil (5-FU) and its oral prodrug capecitabine are commonly used in the treatment of solid tumors, including early and advanced breast, colorectal, gastric and head-and-neck cancer. The enzyme dihydropyrimidine dehydrogenase (DPD), encoded by the gene *DPYD*, plays an important role in the metabolism of fluoropyrimidines. Over 80% of the administered dose of 5-FU is inactivated in the liver by DPD, which makes DPD the key metabolizing enzyme of fluoropyrimidines.[1,2] DPD enzyme activity is known to have a high interindividual variability and reduced DPD activity is present in up to 5% of the population. DPD deficiency is an important risk factor for developing severe, potentially fatal, fluoropyrimidine-related toxicity when patients are treated with a standard fluoropyrimidine dose.[3–6]

DPD deficiency is often caused by single nucleotide polymorphisms (SNPs) in the *DPYD* gene. Pre-treatment *DPYD* screening and dose individualization based on *DPYD* polymorphisms have shown to significantly improve patient safety.[7] However, as not all DPD deficiency can be attributed to genetic *DPYD* variants, other methods to identify DPD deficient patients at risk of fluoropyrimidine-related toxicity are being investigated, including DPD phenotyping approaches. A frequently used phenotyping method is measuring DPD activity in peripheral blood mononuclear cells (PBMCs), as liver DPD activity correlates relatively well with DPD activity in PBMCs.[8] However, this method seems less suitable for routine clinical use, as this method is expensive, laborious, logistically difficult, and requires specific equipment which is not available in most hospitals.[9]

Another promising phenotyping approach to identify DPD deficient patients is determining the levels of uracil (U), the endogenous substrate for DPD, and its product dihydrouracil (DHU). Multiple studies have shown an association between high endogenous U levels or a low DHU/U ratio and severe fluoropyrimidine-associated toxicity.[10–15] These results support the idea that U and DHU levels could be used to individualize fluoropyrimidine therapy in order to increase patient safety. However, an important uncertainty is that there is limited information on potential factors influencing the U and DHU levels and DHU/U ratio, such as circadian rhythm[16] or intake of food containing high levels of U. Therefore, the aim of this study was to determine the effect of oral food intake on plasma U and DHU levels, in order to investigate if a fasting state is necessary when U and DHU levels will be used as diagnostic marker for DPD activity in routine clinical practice.

Information on food containing high levels of U or its precursor uridine is limited. Uridine can be converted *in vivo* to U by a phosphorolysis reaction. This reaction is catalyzed by the enzyme uridine phosphorylase,[17] (Figure 1). U is also one of the four bases in RNA, so intake is also influenced by RNA contents in food. Daily RNA and DNA intake is typically in the range of 0.1 to 1 g/person/day.[18] In the gastrointestinal tract, RNA is broken down to nucleic bases including U. Relatively high concentrations of RNA and DNA can be found in edible offal, animal muscle tissues and mushrooms, whereas plant-derived foods contain lower concentrations.[18,19]

In this study, a breakfast containing food expected to have a high U content was consumed by healthy volunteers in a randomized, cross-over study. It was hypothesized that U levels, and potentially also DHU levels, would increase after consumption of the test breakfast, compared to the fasting state. This hypothesis was based on the assumption that the U present in food would increase the U plasma concentrations after absorption.

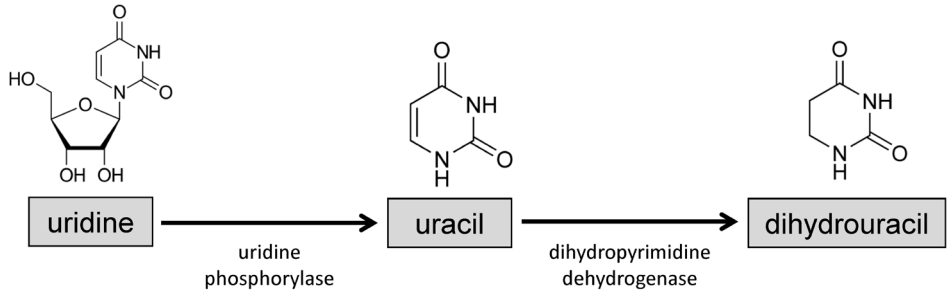

Figure 1. Metabolism of uridine, uracil and dihydrouracil.

METHODS

Study design and sample collection

Sixteen healthy volunteers participated in the study. Enrolled subjects were eighteen years or older, not pregnant and able and willing to consume the prescribed breakfast and undergo blood sampling. The study (clinical trials.gov identifier: NCT02718664) was approved by the Medical Ethics Committee of The Netherlands Cancer Institute, Amsterdam, The Netherlands and was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent prior to study assessments. The study had a randomized, cross-over design, consisting of two test days: day A (fasting state, no food allowed from 22:00h the previous night until 13:00h on the test day) and day B (a test meal was consumed between 8:00h and 8:30h, no other food allowed from 22:00h the previous night until 13:00h). On both test days, consumption of tap water was allowed during the study period. The test days were planned on two consecutive days, participants were 1:1 randomized for the order of the test days (AB or BA).

Blood for determination of U, DHU and uridine plasma levels was collected on both days on eleven predefined time points, between 8:00h and 13:00h (8:00h, 8:45h, 9:00h, 9:15h, 9:30h, 10:00h, 10:30h, 11:00h, 11:30h, 12:00h, 13:00h). On one of the test days, an additional blood sample was taken at 8:00h for determination of DPD enzyme activity in PBMCs. Also, a blood sample was collected for *DPYD* genotyping.

Determination of U, DHU and uridine plasma levels

Peripheral blood for assessment of U and DHU was drawn in a heparin tube (4 ml) and centrifuged directly (1500g, 10 min, 4°C). Plasma was stored at -80°C until analysis. A validated ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) assay was used for quantification of U and DHU levels as described by Jacobs *et al.*[20]

As an exploratory analysis, uridine levels were determined in the plasma samples that were drawn for determination of U and DHU levels. The same UPLC-MS/MS assay developed by Jacobs *et al.*[20] was used for quantification of uridine levels, using the same sample pre-treatment methods and analytical system settings. The concentration range for uridine was 50 to 5000 ng/ml and uridine-2-^{13}C-1,3-$^{15}N_2$ was used as internal standard.

Determination of DPD enzyme activity in PBMCs

10 ml peripheral blood, drawn in a heparin tube, was collected for assessment of DPD activity in PBMCs. PBMCs were isolated directly, using Ficoll-Paque density gradient centrifugation as described previously.[21] Isolated PBMCs were stored at -80°C until further analysis. A validated radio-assay was used, where DPD activity was expressed as the amount of ^{3}H-dihydrothymine formed per mg of protein of PBMC after 1 hour of *ex vivo* incubation with ^{3}H-thymine.[21]

DPYD genotyping

Genotyping for four *DPYD* variants was performed. *DPYD* variants tested were *DPYD**2A (c.1905+1G>A, IVS14+1G>A, rs3918290), c.1679T>G (rs55886062), c.2846A>T (rs67376798) and c.1236G>A (rs56038477, in haplotype B3). DNA was isolated from 4 ml EDTA peripheral blood, and *DPYD* genotyping was performed with real time PCR, using the Roche LightCycler® 480II platform and commercially available primers and probes.

Test meal

On day B of the study (fed condition), a standardized breakfast had to be consumed. This test meal was in accordance with a high-fat (approximately 50 percent of total caloric content of the meal) and high-caloric (approximately 800 to 1000 kcal) meal as described in the guidance on food-effect bioavailability and fed bioequivalence studies of the U.S. Food and Drug Administration (FDA).[22] The breakfast consisted of two slices of whole-wheat bread, two boiled eggs, two tomatoes, one portion (30 g) of liverwurst (liver sausage), one portion (30 g) of steak tartare, one portion (30 g) unsalted butter and 200 ml of whole milk. The total breakfast contained approximately 820 kcal, of which 490 kcal provided by fat. Ingredients were included which were expected to have a potentially large effect on U levels, e.g. liverwurst containing pig liver. The test meal had to be consumed between 8:00h and 8:30h and it was monitored if the whole meal was finished within this time period. We estimated that the test meal would contain at least 15 mg of U, and based on the published value of 474L for the volume of distribution divided by the bioavailability (Vd/F),[23] we calculated that intake of this amount of U could potentially result in plasma levels of 32 ng/ml (15/474=0.032 mg/l), and therefore might significantly increase U levels.

Sample size calculation and statistical analyses

The primary objective of the study was to determine the effect of oral food intake on plasma U and DHU levels. A required sample size of sixteen was calculated, which is also in compliance with the FDA guidance, where it is stated that a minimum of twelve subjects should be included in food-effect studies.[22] For sample size calculation, two null hypotheses were taken into account: one that the difference in mean for U levels in both conditions is below -4 ng/ml and one that it is above 4 ng/ml. A 90% power was chosen to reject both null hypotheses, in favor of the alternative hypothesis that the means of the two conditions (A and B) are equivalent. This assumed that the expected difference in means is zero, the Crossover ANOVA root mean squared error is 3.16 (so the standard deviation of differences is 4.47), and that each test is made at the 5.0% alpha level. The standard deviation of differences was calculated assuming that the standard deviation is 2 under the first condition (A), 4 under the second condition (B) and that the correlation between the two is zero.

Descriptive statistics were used to describe DPD activity in PBMCs, U, DHU and uridine levels and DHU/U ratio. Paired t-tests were used for comparison of U levels, DHU levels and DHU/U ratio between condition A and B at different time points (8:00h, 10:30h, 13:00h). Pearson correlation coefficients were estimated to examine the association between DPD activity in PBMCs and U levels, DHU levels, DHU/U ratio and uridine. The threshold for statistical significance was set at $P<0.05$.

RESULTS

A total of sixteen participants (eight females, eight males) were included, with a median age of 27 years (range 25-46 years). All participants were Caucasian. Participants were equally randomized for the order of the test days (eight subjects randomized as AB, eight as BA). Baseline characteristics are summarized in Table 1.

DPD activity in PBMCs was shown to have a relatively high interindividual variability, with a mean value of 14.2 nmol/(mg*h) and standard deviation (SD) of 5.5 nmol/(mg*h); individual values for PBMC DPD activity are depicted in Supplementary Table 1. The participant with the lowest PBMC DPD activity (subject six; 3.4 nmol/(mg*h)) was identified as a heterozygous carrier of the *DPYD**2A variant. Another participant (subject eight) carried the *DPYD* c.1236G>A variant heterozygously. This variant, however, did not result in decreased DPD activity in PBMCs for this subject (value of 13.4 nmol/(mg*h)). All other participants were tested as wild-type for the four *DPYD* variants analyzed in this study.

Table 1. Baseline characteristics of participants.

Characteristic	Participants (N=16)
Age	
median (range)	27 (25 – 46)
Gender	
male (%)	8 (50%)
female (%)	8 (50%)
Height (m)	
mean (range)	1.77 (1.64 – 1.93)
Weight (kg)	
mean (range)	73 (58 – 96)
Body mass index (kg/m^2)	
mean (range)	23.2 (20.4 – 27.5)
Randomization	
AB (%)	8 (50%)
BA (%)	8 (50%)
DPYD genotype	
Wild-type [a]	14 (87.5%)
*DPYD**2A heterozygous	1 (6.25%)
c.1236G>A heterozygous	1 (6.25%)

[a] Wild-type for four *DPYD* variants: *DPYD**2A, c.1236G>A, c.2846A>T and c.1679T>G.

In Figure 2 the individual and mean day curves for U levels, DHU levels, DHU/U ratio and uridine levels are depicted. Results are shown separately for condition A (fasting state) and condition B (consumption of test meal between 8:00h and 8:30h). As shown in Figure 2A, mean U levels at 8:00h were higher for fasting state compared to fed state (14.5 ± 4.3 ng/ml for fasting state and 13.4 ± 3.5 ng/ml for fed state, $P=0.03$), but with a difference of 1.1 ng/ml between the two conditions, this was considered not clinically relevant. On this time point, participants were in fasting state on both days. In both conditions, U levels declined during the day. However, after consumption of the test meal (condition B), U levels declined significantly more than in fasting state (condition A). A drop between 8:45h and 10:30h was observed after consumption of the test meal, after which the mean value remained relatively constant. At 10:30h mean U levels in fasting state were 13.5 ± 4.7 ng/ml and in fed state 9.2 ± 2.4 ng/ml ($P<0.001$). At 13:00h the U level had a mean value of 12.6 ± 3.7 ng/ml for fasting state and 9.4 ± 2.6 ng/ml for fed state ($P<0.001$).

For DHU levels, results are summarized in Figure 2B. Mean DHU levels at 8:00h, when participants were in a fasting state on both days, were not significantly different between both test days (102.2 ± 25.2 ng/ml for fasting state and 111.0 ± 23.6 ng/ml for fed state, $P=0.23$). However, in the fasting state, mean levels were found to increase over the day, with a maximum of 147.0 ± 36.4 ng/ml at 13:00h, and in the fed state mean levels declined over the day, to a mean level of 85.7 ± 22.1 ng/ml at 13:00h. This difference at 13:00h was found to be statistically significant ($P<0.001$). At 10:30h mean DHU levels in fasting state were 121.0 ± 32.1 ng/ml and in fed state 87.3 ± 26.9 ng/ml ($P=0.003$). When combining U and DHU levels into the DHU/U ratio, as depicted in Figure 2C, both at 8:00h and 13:00h the mean DHU/U ratio values were significantly different (8:00h: fasting state=7.5±2.2, fed state=8.6±2.1, $P=0.012$; 13:00h: fasting state=12.6±4.7; fed state=9.6±3.1, $P=0.012$), but not at 10:30h (fasting state=9.7±3.0, fed state=10.1±4.0, $P=0.65$). Individual results per subject for U levels, DHU levels and DHU/U ratio are included in the Supplement (Supplementary Table 1 and Supplementary Figure 1).

In an exploratory analysis, uridine levels were measured in the same plasma samples in which U and DHU levels were quantified. For nine plasma samples, insufficient plasma was available to determine uridine levels. Uridine measurements showed that curves for uridine showed similar patterns as for U, with higher (stable) levels in the fasting state, and a drop in levels after intake of the breakfast. The uridine results are depicted in Figure 2D and individual results in Supplementary Figure 1.

Associations between DPD activity in PBMCs and U plasma levels, DHU plasma levels, DHU/U ratio and uridine plasma levels were investigated (Supplementary Figure 2). There was a significant negative correlation coefficient between PBMC DPD activity and U plasma levels ($r^2=0.4220$; $P=0.0065$) and a significant positive correlation between PBMC DPD activity and DHU/U ratio ($r^2=0.6162$; $P=0.0003$). DHU levels were not significantly correlated with PBMC DPD activity ($r^2=0.1402$; $P=0.153$), neither were uridine levels ($r^2=0.2018$; $P=0.093$).

DISCUSSION

As far as we know, this is the first study investigating the effect of oral food intake on U and DHU plasma levels. U levels and DHU/U ratio are promising biomarkers for DPD enzyme activity, 5-FU clearance and as a predictor of fluoropyrimidine-related toxicity.[10–15,24–27] Our recent retrospective study in 550 patients showed that a high pre-treatment U level (>16 ng/ml) was strongly associated with severe fluoropyrimidine related-toxicity (OR 5.3, $P=0.009$).[14] Additionally, several prospective

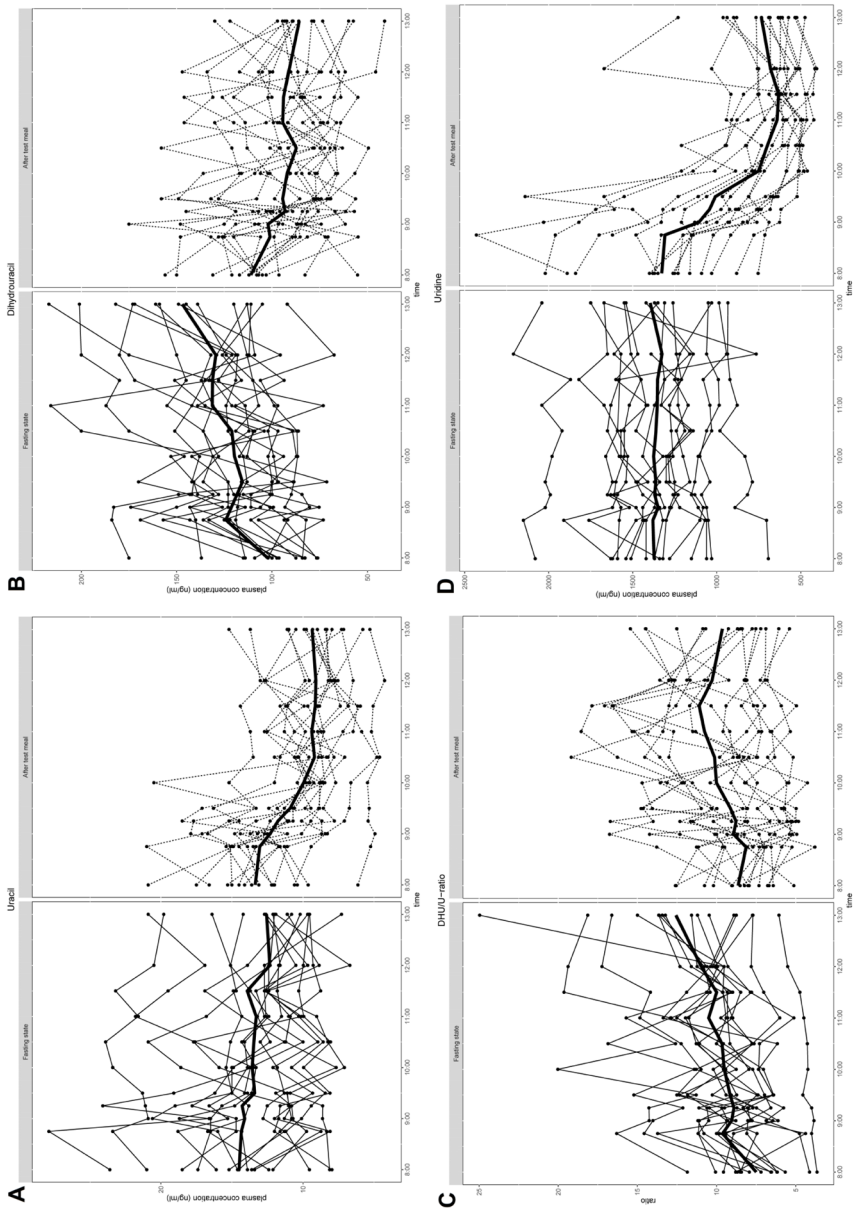

Figure 2. Uracil levels (A), dihydrouracil levels (B), dihydrouracil/uracil ratio (C) and uridine levels (D) in plasma.
Levels were determined on predetermined time points between 8:00h and 13:00h in 16 participants in a fasting state (arm A) and after consuming a standardized breakfast between 8:00h and 8:30h (arm B). Individual levels are depicted and the mean values (bold line). *Abbreviations:* DHU: dihydrouracil; U: uracil.

studies have been performed, in which dose reduction of fluoropyrimidine-based chemotherapy was performed based on DHU/U ratio[25,28] or a combination of *DPYD* genotype and DHU/U ratio,[29] resulting in lower incidence of severe fluoropyrimidine-related toxicity. However, a concern is that U and DHU levels might not only be influenced by systemic DPD activity, but also by other factors, e.g. circadian rhythm[16] or food containing high levels of U or uridine. In several studies investigating DHU and U levels, blood is therefore drawn in the morning and after a fasting period.[12,24,26,30] However, direct evidence showing the effect of oral food intake on U and DHU levels was not yet available.

In this study, we showed that U and DHU levels were not only influenced by interindividual variation and the time of the day (indicating a circadian rhythmicity), but that indeed intake of food was a statistically significant contributing factor as well. However, the influence had the opposite direction as initially hypothesized. Instead of an increase in U levels after food intake, we found that U levels both declined from 8:00h to 13:00h in a fasting state and after food intake, but that the decline was more pronounced after food intake compared to the fasting state. For DHU levels the effect of oral food intake seemed even more pronounced, as the levels from 8:00h to 13:00h increased in the fasting state and decreased after the intake of the test breakfast. When combining U and DHU levels into the DHU/U ratio, the effect of food status is most significant after 12.00h, influenced by the big difference between DHU levels in fasting and fed state at this time period.

These effects of food intake on U and DHU levels have not been shown previously and the exact mechanism behind these findings is uncertain. The results of our study suggest that certain metabolic processes in the body which are influenced by a prolonged fasting state or, on the contrary, the intake of a high-caloric meal, influence plasma U and DHU levels. In the fasting condition, participants had to abstain from food from 22:00h to 13:00h the following day, meaning a period of 15 hours. For uridine, the precursor of U, it has recently been shown by Deng *et al.* that plasma uridine levels are elevated during fasting state and show a rapid drop in a post-prandial state;[31] thus showing a similar pattern as U levels in our study. Adipose tissue dominates uridine biosynthetic activity in the fasted state, resulting in elevated plasma uridine levels, but after food intake, a rapid reduction of plasma uridine is seen, both caused by reduction of uridine synthesis in adipocytes and enhancement of its clearance through the bile.[31] Assuming that endogenous U plasma levels are largely dependent on uridine homeostasis and not on the intake of U by food, this phenomenon could be an explanation for the findings in our study. It has been shown that uridine homeostasis is tightly regulated by the enzyme uridine phosphorylase, the enzyme which converts uridine to U.[17,32,33] This supports the hypothesis that U levels are mainly dependent on uridine homeostasis. When radioactively labelled ^{3}H-uridine was administered intraperitoneally to mice, ^{3}H-uridine was metabolized rapidly with a half-life of less than 2 minutes, and radioactive ^{3}H-uracil levels were detected in plasma, already 5 minutes after administration.[32] For uridine present in food, it was shown that gut-derived uridine is not released in the systemic circulation, but subsequently circulates with bile within the enterohepatic circulation.[31] This is also in line with our findings that food intake did not result in an increase of U levels.

The exploratory analyses in our study confirmed that uridine levels showed a similar pattern as U levels, suggesting that the differences between fed and fasted state of U and DHU are indeed likely to be the direct result of the homeostatic control of uridine. However, when comparing the uridine and U curves over the day, uridine levels show a larger drop in plasma levels after intake

of food than the U levels, where the drop is more modest. This could be caused by the high uracil-containing test meal, from which potentially some uracil is directly taken up in the systemic circulation, resulting in higher U plasma levels. Another possible explanation for this difference between uridine and U is that the formation of U from uridine is rather slow, thereby limiting the direct effect on plasma levels of U by uridine homeostasis. This is however in contrast to the rapid conversion from uridine to U earlier described.[32]

Our study shows that mean levels of uridine, U and DHU clearly have a distinct pattern for fasting state and fed state. The mean difference for U levels between fed and fasted state was between 2 to 5 ng/ml (depending on the time point), showing the relevance of taking food status into account when assessing U plasma levels. The results of our study also implicate that findings of previous studies investigating U levels or the DHU/U ratio in association with fluoropyrimidine-related toxicity might have been influenced by unknown food status. Results of our study also indicate that both inter- and intra-individual variation of uridine, U and DHU levels are high, even in a homogenous healthy volunteer group. When setting a threshold for dose-individualization of fluoropyrimidines based on U levels or the DHU/U ratio, this variation is important to keep in mind, as this can influence the chance of incorrectly classifying someone as DPD deficient based on a measured plasma levels.

In conclusion, with this study we showed that both U levels and DHU levels are generally lower after the intake of a high-caloric breakfast, compared to a fasting state. This means that oral food intake of patients should be taken into account, when blood is drawn for determination of U and DHU levels. We recommend choosing fixed circumstances for blood collection for measuring U and DHU levels, such as a fasting state and a collection time between 8:00h and 9:00h, as this will minimize the effects of potential confounders as much as possible.

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SUPPLEMENT

Supplementary Figure 1. Uracil levels (A), dihydrouracil levels (B), DHU/U ratio (C) and uridine levels (D) in plasma per individual participant.
Levels were determined on predetermined time points between 8:00h and 13:00h in 16 participants in a fasting state (arm A) and after consuming a standardized breakfast between 8:00h and 8:30h (arm B).
Abbreviations: DHU: dihydrouracil; U: uracil.

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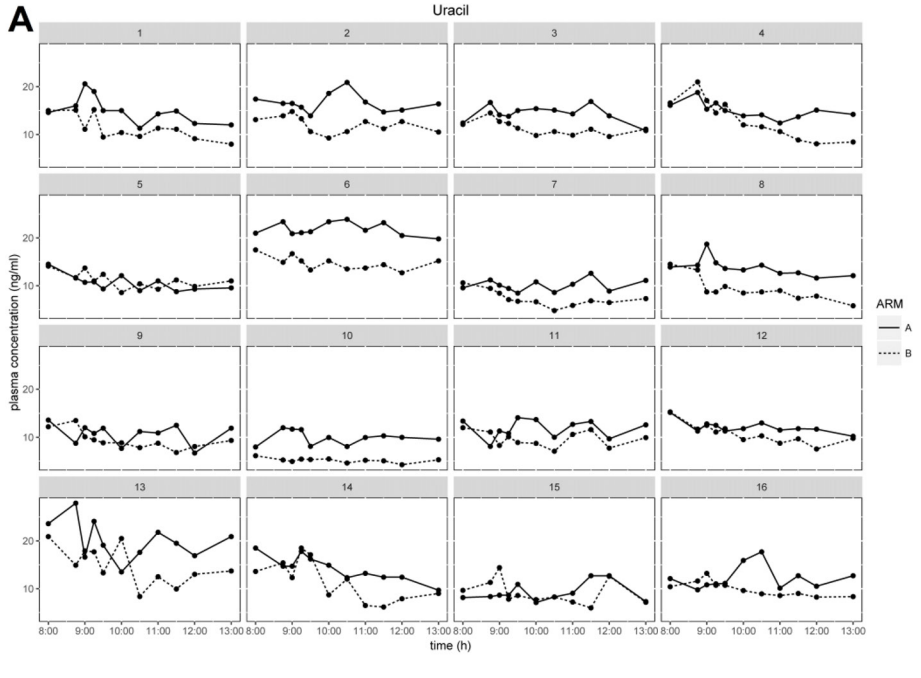
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plasma concentration (ng/ml)
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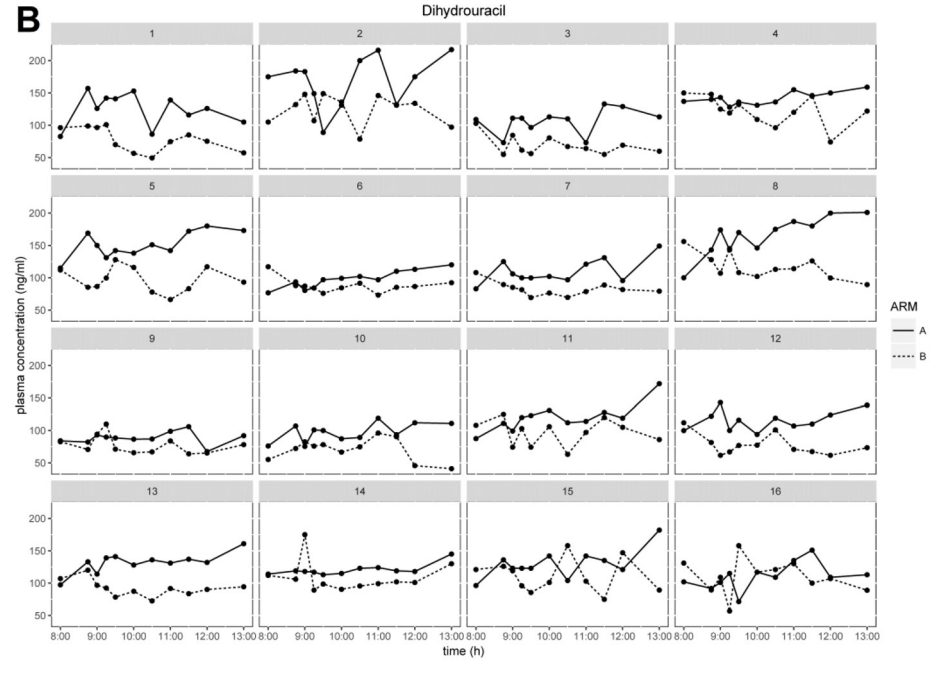
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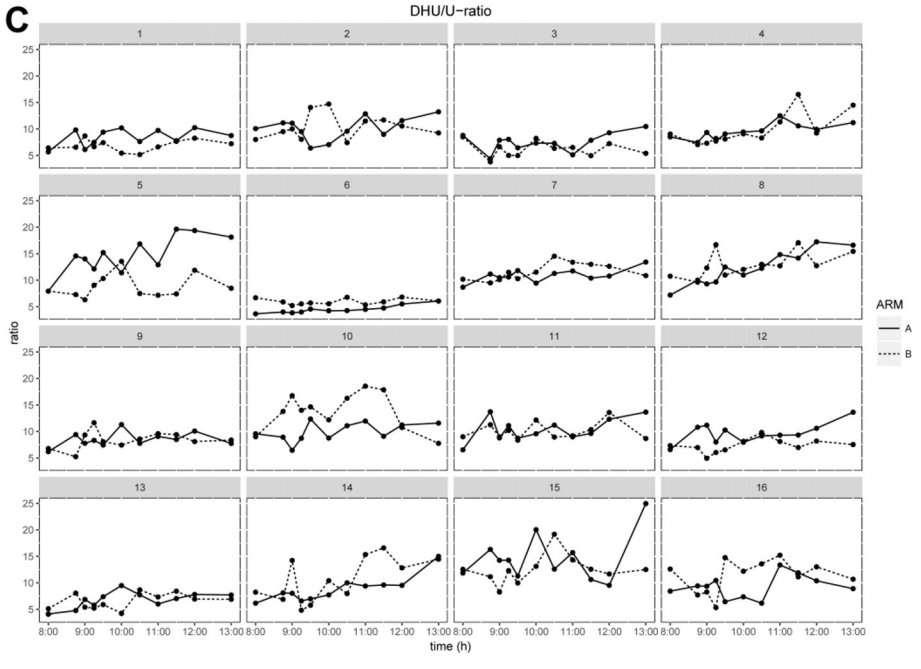
Dihydrouracil
plasma concentration (ng/ml)
time (h)
ARM
A
B


C
DHU/U−ratio
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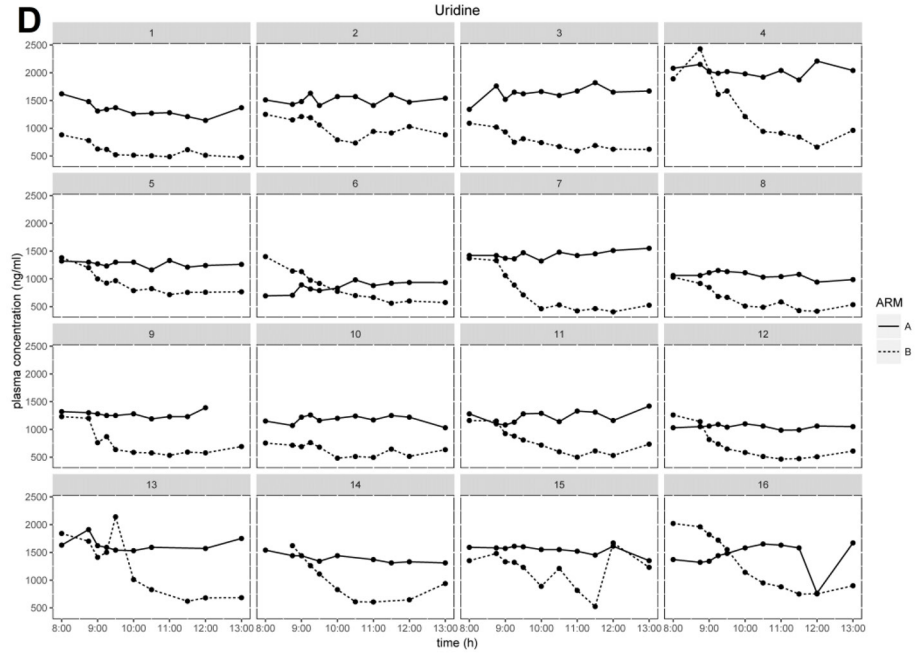
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Uridine
plasma concentration (ng/ml)
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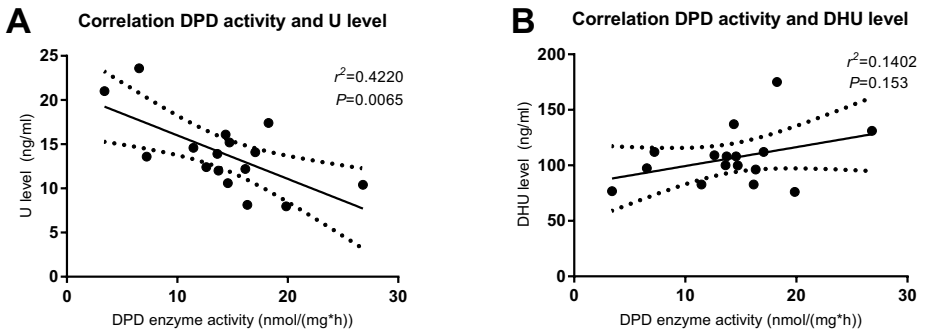

Supplementary Figure 2. Correlation between DPD activity in PBMCs and uracil plasma level (A), dihydrouracil plasma level (B), DHU/U ratio (C) and uridine plasma level (D).
The values for uracil, dihydrouracil and uridine were determined in the sample that was drawn at the same time and day as the sample for determination of DPD activity in PBMCs.
Abbreviations: DHU: dihydrouracil; DPD: dihydropyrimidine dehydrogenase; PBMCs: peripheral blood mononuclear cells; U: uracil.

Supplementary Table 1. Results for DPD activity, uracil and dihydrouracil levels per individual participant.

Participant	DPD activity in PBMCs (nmol/(mg*h))	Average U level arm A (ng/ml)	Average U level arm B (ng/ml)	*P* value [a]	Average DHU level arm A (ng/ml)	Average DHU level arm B (ng/ml)	*P* value [a]	Average DHU/U ratio arm A	Average DHU/U ratio arm B	*P* value [a]
1	11.5	15.0	11.4	<0.001	124.9	78.3	<0.001	8.4	6.9	0.033
2	18.3	16.6	12.1	<0.001	168.3	124.0	0.020	10.1	10.4	0.81
3	12.6	14.4	11.4	<0.001	106.5	68.7	<0.001	7.5	6.1	0.031
4	14.4	15.0	13.2	0.079	141.8	121.9	0.029	9.6	9.9	0.68
5	17.1	10.6	11.2	0.35	151.2	96.8	<0.001	14.7	8.8	<0.001
6	3.4	21.8	14.8	<0.001	97.5	87.7	0.14	4.5	6.0	<0.001
7	14.6	10.1	7.3	<0.001	109.9	82.5	0.0029	10.9	11.6	0.26
8	13.6	13.8	9.3	<0.001	165.4	117.1	0.0072	12.2	13.0	0.44
9	16.2	10.7	9.4	0.16	88.7	77.5	0.041	8.5	8.4	0.90
10	19.9	9.9	5.2	<0.001	97.4	70.8	0.0036	10.0	13.8	0.013
11	13.7	11.8	9.6	0.009	119.7	96.5	0.028	10.4	10.1	0.64
12	14.7	12.1	10.7	0.014	115.8	77.4	<0.001	9.7	7.3	0.0089
13	6.5	20.1	14.8	0.010	131.8	92.2	<0.001	6.8	6.6	0.75
14	7.2	14.3	11.6	0.016	120.5	109.0	0.14	8.8	10.7	0.10
15	16.3	9.2	9.1	0.91	129.8	111.0	0.17	14.7	12.5	0.20
16	26.8	12.2	9.9	0.047	110.5	109.8	0.96	9.3	11.3	0.13

[a] Paired t-test comparing plasma levels on individual time points between 8:00h and 13:00h in arm A and B. *P* values in bold are statistically significant (*P*<0.05).
Abbreviations: DHU: dihydrouracil; DPD: dihydropyrimidine dehydrogenase; PBMCs: peripheral blood mononuclear cells; U: uracil.

14

Clinical value of four dihydropyrimidine dehydrogenase phenotyping assays in predicting fluoropyrimidine-induced toxicity

Manuscript in preparation

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SUMMARY

Background

Fluoropyrimidines are widely used anticancer drugs which cause severe toxicity in up to 30% of patients. Dihydropyrimidine dehydrogenase (DPD), encoded by the gene *DPYD*, is the key metabolic enzyme of fluoropyrimidines. Several *DPYD* variants were identified to result in reduced DPD activity and to be associated with the onset of severe toxicity. However, these *DPYD* variants can only predict ~50% of the severe fluoropyrimidine-induced toxicity resulting from DPD deficiency. In this study, four phenotyping assays developed to determine DPD activity were investigated, aiming to investigate which phenotyping assay is best in identifying DPD deficiency and patients at risk for fluoropyrimidine-induced toxicity.

Methods

Four DPD phenotyping assays were executed in the same patients before starting fluoropyrimidine-based therapy; the endogenous dihydrouracil/uracil (DHU/U) ratio, endogenous uracil levels, the uracil loading dose, and the 2-^{13}C-uracil breath assay. Results of the phenotyping assays were compared to direct measurement of DPD enzyme activity in peripheral blood mononuclear cells (PBMCs), i.e. the presumed gold standard to measure DPD activity. Sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) were calculated per phenotyping assay as predictive measures for DPD deficiency and severe (grade ≥3) fluoropyrimidine-induced toxicity.

Results

In total, 92 patients participated in this study and completed the DPD phenotyping assays prior to fluoropyrimidine-based therapy. None of the phenotyping assays correlated with DPD activity in PBMCs (r^2 for all assays <0.064). Maximum clinical validity parameters to predict severe toxicity were 45% sensitivity (2-^{13}C-uracil breath assay), 97% specificity (endogenous DHU/U ratio and uracil loading dose), 88% NPV (2-^{13}C-uracil breath assay), and 33% PPV (uracil loading dose).

Conclusions

The lack of correlation between the phenotyping assays and DPD activity in PBMCs was surprising, and could possibly be caused by flaws in execution of the tests. Besides this, neither of the phenotyping assays was able to predict the onset of severe toxicity very well, possibly due to the fact that only ~50% of fluoropyrimidine-induced toxicity can be explained by DPD deficiency.

INTRODUCTION

Fluoropyrimidines, including 5-fluorouracil (5-FU) and its oral pro-drug capecitabine, play a key role in the treatment of multiple types of cancer.[1] Although these drugs have been used for over 60 years, toxicity remains a major clinical problem, as severe fluoropyrimidine-induced side effects occur in up to 30% of patients, resulting in lethal outcome in up to 1% of treated patients.[1,2] With over two million patients treated with fluoropyrimidines each year, many patients are at risk of developing severe toxicity.[3] Common side effects are diarrhea, mucositis, hand-foot syndrome, and myelosuppression. Abundant research has been performed on dihydropyrimidine dehydrogenase (DPD), the key metabolic enzyme of fluoropyrimidines, and the gene encoding DPD (*DPYD*). Low DPD activity and *DPYD* variants have been associated with severe fluoropyrimidine induced toxicity.[4–6] Prospective phenotyping or genotyping, followed by dose adjustments in DPD deficient patients or *DPYD* variant allele carriers, could reduce the risk for severe toxicity. As was previously shown by Deenen *et al.* for *DPYD**2A-genotype guided dosing.[7] In recent years, it has become clear that additional *DPYD* variants besides *DPYD**2A are clinically relevant predictors of severe fluoropyrimidine-induced toxicity.[8] In a clinical trial (NCT02324452), prospective *DPYD* genotyping was expanded to four *DPYD* variants (c.1236G>A, c.2846A>T, *DPYD**2A and c.1679T>G), and fluoropyrimidine dose reductions in heterozygous carriers of any of these four variants were applied. Wild-type patients for these four *DPYD* variants experienced 23% severe toxicity.

Sensitivity of genotyping for prediction of severe fluoropyrimidine-induced toxicity is inherently limited, as other genetic and also non-genetic factors are known to play a role in causing variation in DPD activity. Phenotyping of the DPD enzyme, an indirect way to predict DPD activity, can potentially better predict severe fluoropyrimidine-induced toxicity as it takes both pharmacogenetic and other factors influencing DPD activity into account. Therefore, this study focuses on DPD phenotyping.

DPD enzyme activity in peripheral blood mononuclear cells (PBMCs) is considered the gold standard to determine DPD activity. The activity in PBMCs is well-correlated to DPD enzyme activity in the liver, and reference values have been well-established.[6,9] However, the method is not very feasible in clinical practice and implementation in routine clinical practice remains limited due to the high costs, logistical difficulties, laborious aspects and specific equipment required for this test. There are a number of DPD phenotyping assays that have previously been investigated. One phenotyping assay is the measurement of endogenous plasma levels of uracil (U) and dihydrouracil (DHU). Several studies have shown an association between pre-treatment endogenous DHU/U ratio in plasma and 5-FU pharmacokinetics (PK),[10–13] and also with severe fluoropyrimidine-induced toxicity.[13–16] Recently, Meulendijks *et al.* have shown that high pre-treatment serum uracil concentrations were also strongly related to severe and fatal fluoropyrimidine-induced toxicity.[17] Another method for determining DPD activity is the uracil loading dose assay.[18,19] In this assay, a high dose of uracil is administered orally and uracil and DHU levels are measured using a limited sampling strategy.[18] Finally, the DPD activity can also be determined using the 2-^{13}C-uracil breath test assay.[20] This method uses a personalized dose of 2-^{13}C uracil (6 mg/kg), a stable isotope of uracil, and is based on the conversion of 2-^{13}C uracil into $^{13}CO_2$ which can be measured in exhaled breath.[20]

Each of these assays has been reviewed independently;[21,22] however never head to head and in clinical practice. Therefore, in this prospective study, we tested four DPD phenotyping assays in patients prior to treatment with fluoropyrimidines, investigated correlations between phenotyping

assays and DPD activity in PBMCs, determined the predictive value of each of the assays in detecting DPD deficiency (defined as DPD enzyme activity in PBMCs below the cut-off value) and predicting severe fluoropyrimidine-induced toxicity.

MATERIALS AND METHODS

Study design

This study was a preplanned part of a large prospective multicenter clinical trial (clinicaltrials.gov identifier NCT02324452). Four out of 17 Dutch hospitals participating in the clinical trial collaborated in the current DPD phenotyping study. Patient recruitment for this study was open from February 2016 until January 2018. Approval for the study was granted by the medical ethical committee of The Netherlands Cancer Institute, Amsterdam, the Netherlands, and each participating hospital obtained approval from their board of directors. All patients provided written informed consent before enrolment in the current study. Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE; version 4.03)[23] and severe toxicity was defined as CTCAE grade ≥3. Only toxicity defined as definitely, probably and possibly related to fluoropyrimidines was taken into account. Patients were followed for toxicity during the entire treatment period and were evaluable for toxicity if they received at least one fluoropyrimidine drug administration. The primary aims of this study were to determine the correlation of each of the four DPD phenotyping assays with the gold standard to determine DPD activity in patients (i.e. direct DPD enzyme activity measurements in PBMCs) and to determine their clinical value (i.e. the predictive value for the onset of grade ≥3 toxicity). All assays were executed before start of fluoropyrimidine therapy, and patients were asked to participate in all phenotyping assays, which made intra-patient comparisons possible. Results of the phenotyping assays were determined after start of treatment, and not used for dose individualization. However, dose adjustments of the fluoropyrimidine drug were done based on *DPYD* genotype as per protocol of study NCT02324452. Pre-therapeutic screening for four *DPYD* variants was performed; heterozygous *DPYD**2A and c.1679T>G variant allele carriers received a 50% initial dose reduction, and c.2846A>T and c.1236G>a carriers a 25% dose reduction.[24]

Patients

Patients were eligible for this study if they were eligible to start with fluoropyrimidine-based therapy, were 18 years or older, had an adequate performance status, had adequate renal and liver function and hematological values, and had not been treated previously with fluoropyrimidines. The endogenous DHU/U ratio and endogenous uracil levels assays were measured in patients from the NCT02324452 trial as well, these patients were therefore included in the analyses in this study.

Study scheme

During two random days prior to start of fluoropyrimidine-based treatment, all four phenotyping assays were performed in each patient. On the first day, blood draws for the DPD enzyme activity in PBMCs and two phenotyping assays (endogenous DHU/U ratio and endogenous uracil levels assay) were taken. Immediately thereafter, the third phenotyping assay (uracil loading dose) was performed. At least one day later, but prior to start of fluoropyrimidines, the fourth phenotyping assay (2-^{13}C-uracil breath assay) was performed including blood draws for ^{13}C-uracil plasma

measurements. In one of four participating hospitals the 2-^{13}C-uracil breath assay was not incorporated in the study design, due to logistic difficulties.

The uracil loading dose and 2-^{13}C-uracil breath assay were performed on two separate days to exclude any interference, as uracil was administered orally in both assays. Also, a minimum time interval of 24 hours between the phenotyping assays and start of fluoropyrimidine treatment was taken into account as a safety precaution, although it was expected that the administered uracil would not affect the efficacy and safety of patients when starting their fluoropyrimidine-based treatment, since uracil has a very short half-life of around 40 minutes.[25] Each assay is described in more detail in the Supplement.

Sample size calculation and statistical analyses

The required sample size was based on one of the primary endpoints (the association between the result of a DPD phenotyping assay and severe fluoropyrimidine-associated toxicity) and calculated to be 260 (see detailed description in the Supplement). Pearson's correlation coefficients were estimated to examine the association between DPD enzyme activity in PBMCs, and the results of the endogenous DHU/U ratio, the endogenous uracil levels, the uracil loading dose, and the 2-^{13}C-uracil breath assay, respectively. Also, the association between plasma samples (measured ^{13}C-DHU/U-ratio and ^{13}C-uracil levels at 50 minutes) and breath samples (calculated as DOB_{50}) of the 2-^{13}C-uracil breath assay was evaluated by estimating Pearson's correlations coefficients.

For assessing clinical validity, measures to determine diagnostic performance (i.e. sensitivity, specificity, positive predictive value [PPV], negative predictive value [NPV] and F1-score [harmonic mean of sensitivity and PPV]) of the assays with regard to DPD deficiency were determined. In addition, the same measures were determined with regard to the onset of severe toxicity. *DPYD* variant allele carriers received an initial dose reduction based on their genotype and they were excluded in the latter calculations. The assessment of clinical validity with regard to prediction of DPD deficiency and severe fluoropyrimidine-induced toxicity were both considered primary endpoints of the study. Receiver Operating Characteristic (ROC)-curves were plotted per phenotyping assay for prediction of severe toxicity. DPD deficiency was defined as low DPD activity in PBMCs (<6.9 nmol/[mg*h]).[6] Patient characteristics or toxicity differences between patient groups were tested using Chi-Square test or Mann-Whitney U test. The level of significance was set at $P<0.05$. Analyses were performed using SPSS, version 23 (IBM SPSS Inc., Chicago, IL, USA).

RESULTS

Patients

In total, 92 evaluable patients were included in this study (baseline characteristics in Table 1). Patient and treatment characteristics were compared with those of the main study cohort of NCT02324452 (N=1103) and found similar (Supplementary Table 1), with the exception that patients participating in the current study were slightly younger (median age of 60 versus 64 years, P=0.003). Endogenous DHU/U ratio and endogenous uracil levels from all patients of the trial could thus be taken in to account in the analysis of this study. Details on fluoropyrimidine-induced toxicity for this study and the main study cohort are depicted in Supplementary Table 2. In this study 19 out of 92 patients (21%) experienced severe fluoropyrimidine-induced toxicity, which is comparable to the main study in which 264 out of 1103 patients (24%) experienced severe

toxicity (*P*=0.441). Details on fluoropyrimidine-induced toxicity for *DPYD* wild-type and variant allele carriers were presented in Supplementary Table 3, showing an increased percentage of hematological toxicity in *DPYD* variant allele carriers compared to wild-type patients (*P*=0.002).

Phenotyping assays

DPD enzyme activity assay: The mean DPD enzyme activity found was 9.27±3.60 nmol/(mg*h), and ranged from 1.6-16.3 nmol/(mg*h) (*N*=92). Wild-type patients had a mean DPD enzyme activity of 9.43±3.61 nmol/(mg*h) (*N*=82), compared to the *DPYD* variant allele carriers who had a mean DPD enzyme activity of 7.96±3.41 nmol/(mg*h) (*N*=10).

Endogenous DHU/U ratio and endogenous uracil levels: The endogenous DHU/U ratio was determined in the main study cohort (*N*=1037). The average endogenous DHU/U ratio was 9.40±3.94 (range 0.64-29.86) and average endogenous uracil levels were 12.13±9.54 ng/ml (range 3-188 ng/ml). Wild-type patients had a mean endogenous DHU/U ratio of 9.52±3.92 (*N*=955) and mean uracil levels of 11.87±9.59 ng/ml (*N*=955), compared to *DPYD* variant allele carriers who had a mean endogenous DHU/U ratio of 7.99±3.96 (*N*=82) and mean uracil levels of 15.19±8.46 ng/ml (*N*=82).

Uracil loading dose: The uracil loading assay is determined by the U/DHU ratio at 120 minutes after administration of uracil, and showed an average U/DHU ratio at 120 minutes of 0.93±0.86, with a range from 0.07-4.82 (*N*=92). Wild-type patients had a mean U/DHU ratio at 120 minutes of 0.82±0.74 (*N*=82), compared to *DPYD* variant allele carriers who had a mean U/DHU ratio at 120 minutes of 1.82±1.23 (*N*=10).

2-^{13}C-uracil breath assay: A personalized dose of 6 mg/kg 2-^{13}C-uracil was given to the patients. On average, 488 mg was administered, ranging from 312 to 840 mg. For the 2-^{13}C-uracil breath assay, an average delta-over-baseline ratio at t=50 minutes (DOB_{50}) value of 160.2±33.9 was found, ranging from 71.8-227.4 (*N*=82). Wild-type patients had a mean DOB_{50} value of 162.2±34 (*N*=74), compared to *DPYD* variant allele carriers who had a mean DOB_{50} value of 141.2±28.3 (*N*=8). Blood samples were taken to correlate the DOB_{50} value from breath samples to ^{13}C-uracil plasma levels and ^{13}C-DHU/U ratio. No significant correlation could be shown with the DOB_{50} determined in breath samples, for both the ^{13}C-U plasma levels (r^2<0.001, *P*=0.81) and the ^{13}C-DHU/U ratio (r^2=0.014, *P*=0.29). Results are shown in Supplementary Figure 1.

Correlations between phenotyping assays

Pearson's correlation coefficients were estimated to correlate results of the phenotyping assays to DPD enzyme activity in PBMCs. Patients were included in the analyses if results for both DPD enzyme activity and the other phenotyping assay were known. For endogenous uracil levels and the endogenous DHU/U ratio, no significant correlation was found (r^2=0.004, *P*=0.54 and r^2=0.010, *P*=0.35, respectively), as depicted in Figure 1. The same counted for the 2-^{13}C-uracil breath assay, determined as the DOB_{50} value (r^2=0.036, *P*=0.09). However, a small but significant correlation between the uracil loading dose (defined as the U/DHU ratio at 120 minutes) and DPD enzyme activity in PBMCs was found (r^2=0.064, *P*=0.02).

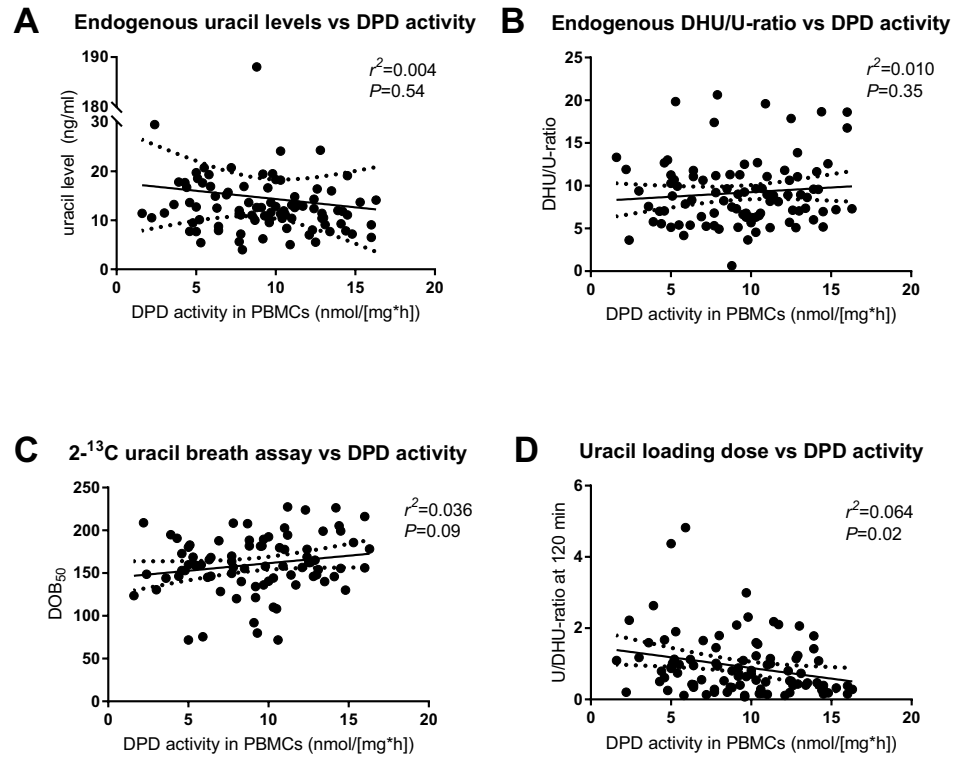

Figure 1. Correlations of DPD phenotyping assays with *DPD* enzyme activity in PBMCs.
Abbreviations: DHU: dihydrouracil; DOB_{50}: delta-over-baseline ratio at 50 minutes; DPD: dihydropyrimidine dehydrogenase; PMBCs: peripheral blood mononuclear cells; U: uracil; vs: versus.

Clinical validity for DPD deficiency

Clinical validity parameters, i.e. sensitivity, specificity, NPV, PPV and F1-score, were determined comparing the phenotyping assay results to DPD deficiency (determined as low DPD activity <6.9 nmol/[mg*h] in PBMCs). Results are shown in Table 2. None of the tests showed a combination of both high sensitivity and specificity parameters.

Clinical validity for severe toxicity

Per phenotyping assay, boxplots are shown separated on occurrence of severe toxicity (Figure 2). ROC-curves of each phenotyping assay for prediction of severe toxicity are shown in Figure 3. Clinical validity parameters were determined comparing the phenotyping assay results to the onset of severe grade ≥3 fluoropyrimidine-induced toxicity (Table 3). The above-mentioned table and figures show wild-type patients alone, as *DPYD* variant carriers in this study received initial dose reductions which influenced their risk of toxicity. Limited differences between patients who experienced severe toxicity or did not, were observed between phenotyping assays. ROC-curves show limited added value of the phenotyping assays to predict severe toxicity.

Table 1. Baseline characteristics of patients in this study.

Characteristic	This study (*N*=92)
Sex	
Male	56 (61%)
Female	36 (39%)
Age	
Median [range]	60 [19-78]
Ethnic origin	
Caucasian	87 (95%)
African descent	1 (1%)
Asian	2 (2%)
Other	2 (2%)
Tumor type	
Non-metastatic CRC	38 (41%)
Metastatic CRC	23 (25%)
BC	7 (8%)
GC	7 (8%)
Other	17 (18%)
Type of treatment regimen	
CAP mono	12 (13%)
CAP + RT	23 (25%)
CAPOX	37 (40%)
CAP other	5 (5%)
5-FU mono	0
5-FU + RT	6 (7%)
FOLFOX	4 (4%)
5-FU other	5 (5%)
BSA	
Median [range]	2.0 [1.46-2.73]
WHO performance status	
0	49 (53%)
1	41 (45%)
2	1 (1%)
NS	1 (1%)
Number of treatment cycles	
Median [range]	3 [1-16]
DPYD status	
Wild-type	82 (89%)
DPYD variant allele carrier	10 (10.9%)
c.1236G>A heterozygous	6 (6.5%)
c.2846A>T heterozygous	3 (3.3%)
*DPYD*2A heterozygous*	1 (1.1%)
c.1679T>G heterozygous	0

Abbreviations: 5-FU mono: 5-fluorouracil monotherapy; 5-FU other: 5-fluorouracil combined with other anticancer drugs; 5-FU + RT: 5-fluorouracil combined with radiotherapy (with or without mitomycin); BC: breast cancer; BSA: body surface area; CAP mono: capecitabine monotherapy (with or without bevacizumab); CAP other: capecitabine combined with other anticancer drugs; CAP + RT: capecitabine combined with radiotherapy (with or without mitomycin); CAPOX: capecitabine combined with oxaliplatin (with or without bevacizumab); CRC: colorectal cancer; *DPYD*; gene encoding dihydropyrimidine dehydrogenase; FOLFOX: 5-fluorouracil combined with oxaliplatin and leucovorin (with or without bevacizumab); GC: gastric cancer; NS: not specified, either WHO 0, 1 or 2.

Table 2. Comparison of phenotyping assays in performance for prediction of DPD deficiency.
DPD deficiency is defined by gold standard DPD enzyme activity in PBMCs; <6.9 nmol/(mg*h) is considered DPD deficient.

Assay	*N* patients	Mean (range)	Cut-off for DPD deficiency	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)	F1-score [a] (%)
Endogenous DHU/U ratio	92	9.17 (0.64-20.63)	<3.60 [b]	0	98	71	0	NA
			<4.31 [b]	8	97	73	50	13
Endogenous uracil levels	92	14.57 (3.98-188) ng/ml	>16.0 ng/ml [17]	38	82	77	45	42
			≥13.9 ng/ml [17]	42	73	76	38	40
2-^{13}C-uracil breath assay	82	DOB_{50}: 160.2 (71.8-227.4)	DOB_{50} <128.9 [20,26]	13	86	70	27	17
			DOB_{50} ≤161.4 [28]	63	50	76	34	44
Uracil loading dose	92	U/DHU-ratio at 120 min: 0.93 (0.07-4.82)	U/DHU-ratio at 120 min>2.4 [18]	12	98	74	75	20

[a] The F1-score represents the harmonic mean of sensitivity and PPV.
[b] These cut-off values are determined by calculating the 3% and 6% lower limits of the data, as was described by Meulendijks *et al*.[17]
Abbreviations: DHU: dihydrouracil; DOB_{50}: delta-over-baseline ratio at 50 minutes; DPD: dihydropyrimidine dehydrogenase; NA: not applicable; NPV: negative predictive value; PBMCs: peripheral blood mononuclear cells; PPV: positive predictive value; U: uracil.

Phenotyping assays and DPYD genotype

Supplementary Figure 2 displays the phenotyping assay results separated per *DPYD* genotype. Wild-type patients show a large variation in results for all phenotyping assays. As this is an interventional study, and patients received initial dose reductions based on genotype, it was not possible to determine the clinical validity parameters of genotyping and phenotyping combined. However, we did calculate the chance of carrying a *DPYD* variant when having reduced DPD activity in PBMCs, and next to this, the chance of having reduced DPD activity when experiencing severe toxicity.

In the main study cohort and this study combined, DPD activity was measured in 138 patients. A total of 54 patients had a DPD activity below the cut-off value (<6.9 nmol/[mg*h]), of whom 33 patients a *DPYD* variant (61%). Thus, (only) 61% of DPD deficient patients could be identified by genotyping four *DPYD* variants.

DPD activity was measured in 82 wild-type patients in this study. 15 out of 82 wild-types experienced severe toxicity, of whom four had a DPD activity below 6.9 nmol/(mg*h) (27%). Thus, 27% of severe toxicity in this study could be predicted by presence of DPD deficiency.

DISCUSSION

Four phenotyping assays were evaluated in this study which was part of a large clinical trial (NCT02324452), and compared to DPD enzyme activity measurements in PBMCs. Results of wild-types of the phenotyping assays showed no deviations from previously described values.[6,17,18,26]

Table 3. Comparison of phenotyping assays in performance for prediction of severe fluoropyrimidine-induced toxicity.
In this table, sensitivity, specificity, NPV, PPV and F1-score were calculated, excluding *DPYD* variant allele carriers. Since *DPYD* variant allele carriers received an initial dose reduction based on their genotype, bias could develop in the onset of severe fluoropyrimidine-induced toxicity.

Assay	*N* patients	Mean (range)	Cut-off for DPD deficiency	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)	F1-score [a] (%)
DPD enzyme activity PBMCs	82	9.43 (1.6-16.3)	<6.9 nmol/(mg*h) [6]	27	75	82	19	22
Endogenous DHU/U ratio	955	9.52 (0.64-29.86)	<3.60 [b]	3	97	77	26	6
			<4.31 [b]	6	95	77	26	10
Endogenous uracil levels	955	11.87 (2.96-188)	>16.0 ng/ml [17]	14	85	77	22	17
			≥13.9 ng/ml [17]	24	78	78	25	24
2-^{13}C-uracil breath assay	74	DOB_{50}: 162.2 (71.8-227.4)	DOB_{50} <128.9 [20,26]	27	89	88	30	29
			DOB_{50} ≤161.4 [28]	45	49	84	14	21
Uracil loading dose	82	U/DHU ratio at 120 min: 0.82 (0.07-4.37)	U/DHU-ratio at 120 min>2.4 [18]	7	97	82	33	11

[a] The F1-score represents the harmonic mean of sensitivity and PPV.
[b] These cut-off values are determined by calculating the 3% and 6% lower limits of the data, as was described by Meulendijks *et al.*[17]
Abbreviations: DHU: dihydrouracil; DOB_{50}: delta-over-baseline ratio at 50 minutes; DPD: dihydropyrimidine dehydrogenase; NPV: negative predictive value; PBMCs: peripheral blood mononuclear cells; PPV: positive predictive value; U: uracil.

Unfortunately, for none of the DPD phenotyping assays, a good correlation was found with DPD enzyme activity measurements in PBMCs. This lack of correlation could either be caused by outliers in the assays, differences within the execution of the assays between hospitals, or the fact that all DPD phenotyping assays measure DPD enzyme activity indirectly. For example, the 2-^{13}C-uracil breath assay measures $^{13}CO_2$, which is not only formed by the DPD enzyme, but also by two other enzymes (dihydropyrimidinase and β-ureidopropionase).[26]
This study is the first to investigate several DPD phenotyping assays in the same patients, prior to administration of fluoropyrimidine-based therapy. Our patient cohort was not selected based on –or enriched for– (severe) toxicity, but represents a patient cohort representative of daily clinical care. The main goal of the study was to determine clinical validity of DPD phenotyping assays in terms of predictive value for DPD activity and severe fluoropyrimidine-induced toxicity, based on cut-off values that were previously established. By using the current *DPYD* genotyping panel, only 61% of DPD deficient patients could be identified. Therefore, there is a need for reliable DPD phenotyping assays to better identify DPD deficient patients, as these patients are at high risk of severe, and potentially fatal fluoropyrimidine-induced toxicity.

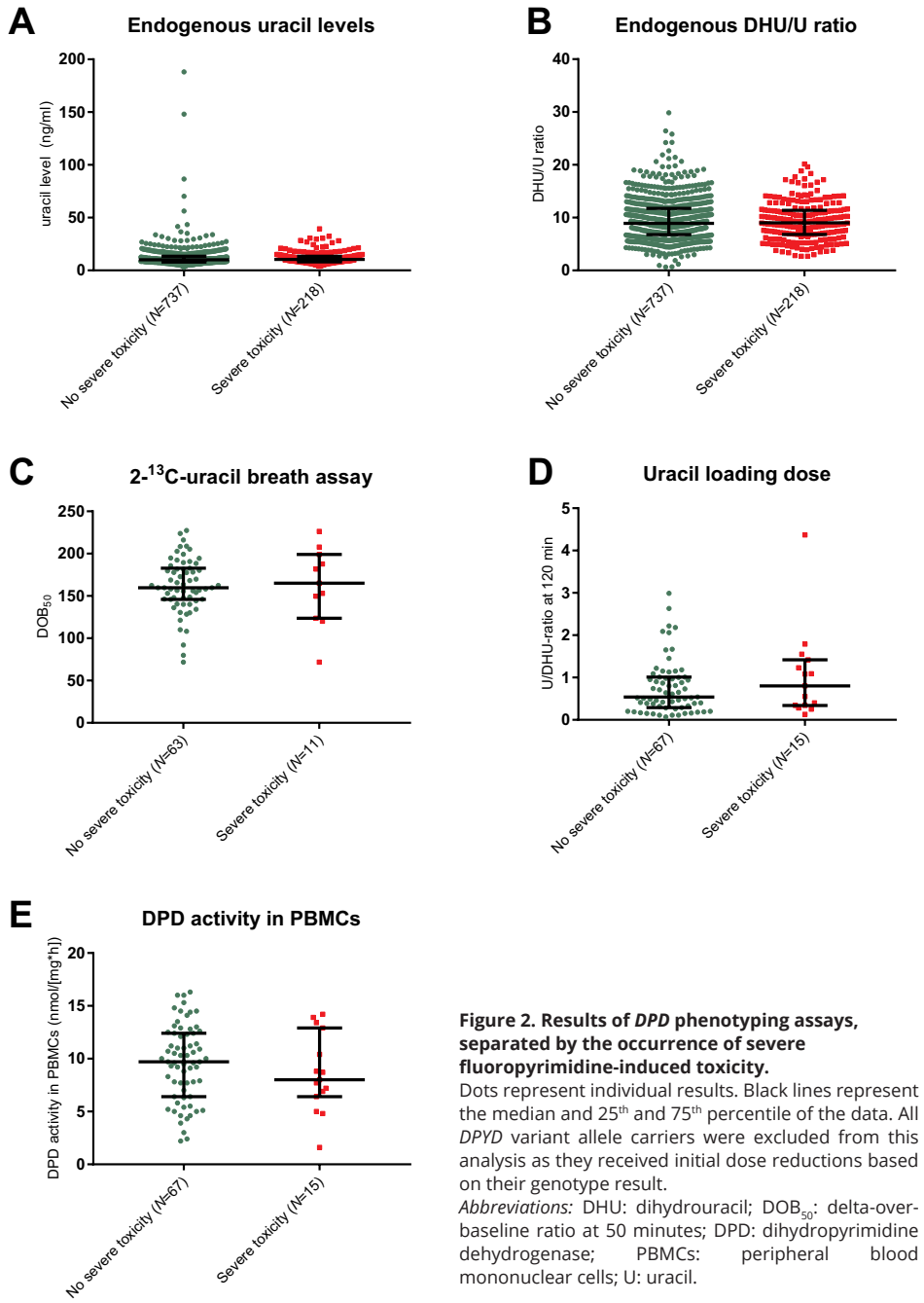

Figure 2. Results of *DPD* phenotyping assays, separated by the occurrence of severe fluoropyrimidine-induced toxicity.
Dots represent individual results. Black lines represent the median and 25th and 75th percentile of the data. All *DPYD* variant allele carriers were excluded from this analysis as they received initial dose reductions based on their genotype result.
Abbreviations: DHU: dihydrouracil; DOB_{50}: delta-over-baseline ratio at 50 minutes; DPD: dihydropyrimidine dehydrogenase; PBMCs: peripheral blood mononuclear cells; U: uracil.

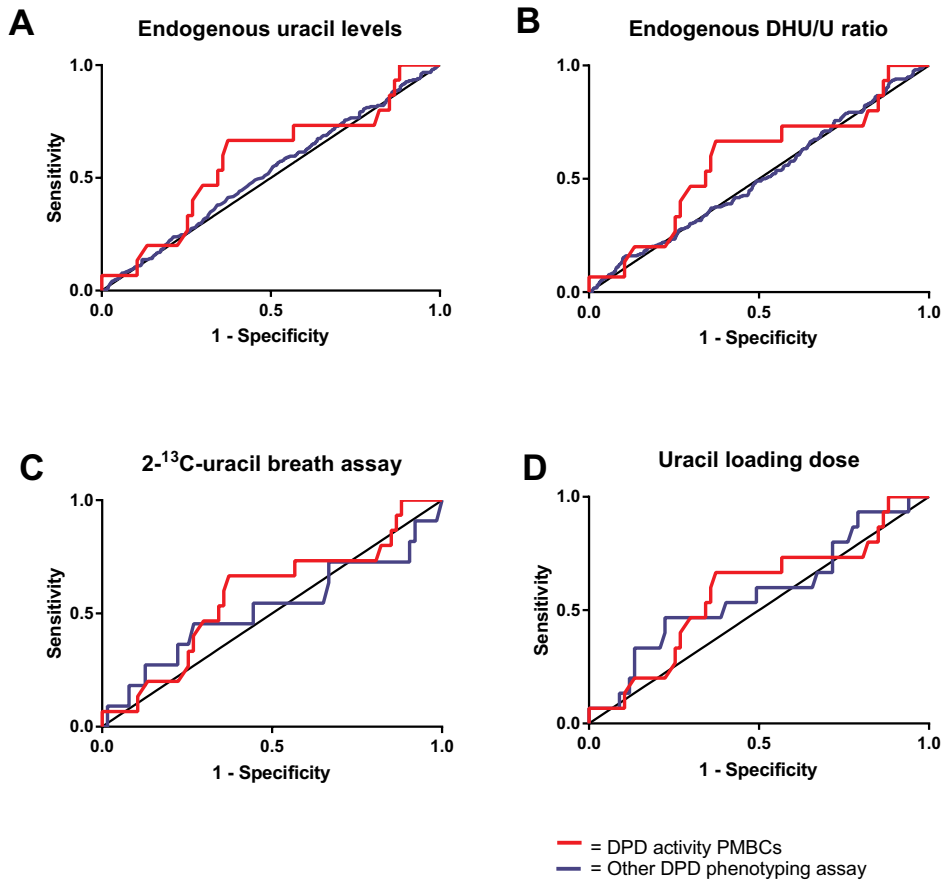

Figure 3. ROC-curves of *DPD* phenotyping assays for prediction of severe toxicity.
All *DPYD* variant allele carriers were excluded from this analysis as they received initial dose reductions based on their genotype result.
Abbreviations: DHU: dihydrouracil; DPD: dihydropyrimidine dehydrogenase; PBMCs: peripheral blood mononuclear cells; ROC: Receiver Operating Characteristic; U: uracil.

In terms of clinical validity, the parameters sensitivity, specificity, NPV, PPV and F1-score were calculated per phenotyping assay to predict DPD deficiency and severe toxicity. Severe toxicity must be prevented at all cause; therefore a phenotyping assay with a high PPV (individual parameter) should be selected for further research. However, underdosing of patients is also not desirable, and therefore NPV values should not be too low either. Tonk *et al.* have previously described that clinical validity parameters not only depend on the odds ratio (relationship between a certain genotype or phenotyping result and occurrence of toxicity), but also on the frequency of the adverse events and the frequency of patients with a genetic variant or the frequency of patients who are below a certain cut-off value in a phenotyping assay.[27] Sensitivity and PPV of an assay will remain limited even though there is a high odds ratio, if adverse events are frequent

and deficient patients are rare. This is also the case in our study. The phenotyping assays in this study show PPV values up to 33%. The assays with the highest sensitivity are the 2-^{13}C-uracil breath assay, DPD enzyme activity assay and endogenous uracil levels.
Previously, reported clinical validity parameters of a DPD phenotyping test calculated to predict DPD deficiency were higher (sensitivity 80%, specificity 98%, NPV 99%, PPV 67%),[18] possibly biased by the selection of patients who experienced severe toxicity. Interestingly, previously calculated clinical validity parameters for the endogenous uracil levels to predict severe fluoropyrimidine-induced toxicity were comparable to our results (sensitivity 18%, specificity 95%, NPV 90%, PPV 35%).[17] The reason for the low sensitivity and PPV is that fluoropyrimidine-induced toxicity is also caused by factors other than solely DPD deficiency. Indeed, in our cohort of patients, only 27% of severe toxicity could be predicted by presence of DPD deficiency (defined as <6.9 nmol/[mg*h] DPD activity in PBMCs).
A limitation of our study is the sample size of 92 evaluable patients (of whom 82 wild-types). The study was powered to 240 wild-types plus 10 to 20 *DPYD* variant allele carriers, but even after prolongation of the inclusion period of the study this number could not be reached and resulted in an insufficient power to predict severe toxicity. A substantial number of *DPYD* variant allele carriers was included in this study, however mostly carriers of the c.1236G>A and c.2846A>T variants. These variants have a smaller effect on DPD activity compared to the *DPYD**2A and c.1679T>G variants, and could explain the limited observed effect on some phenotyping assays between wild-type patients and *DPYD* variant allele carriers.
In terms of clinical utility, the health outcome of the use of the assays could not be determined, as there was no intervention based on the results of the phenotyping assays. Another clinical utility parameter is the risk of the assay for the patient, which is limited to a blood draw, as the intake of uracil or ^{13}C-uracil was considered without any risk.
Feasibility of the assays was not included as a formal endpoint in this study, but can be discussed in a qualitative way. The reduced sample size immediately points towards a limited feasibility of two of the phenotyping assays (2-^{13}C-uracil breath assay and uracil loading dose), as many patients were reluctant to visit the hospital on a separate day and did therefore not want to participate in this study. Four centers recruited patients for this study. In one center, the 2-^{13}C-uracil breath assay could not be performed due to logistical difficulties. Therefore, the feasibility in routine clinical practice of this assay could be questioned. The burden for the patient is much lower for the DPD enzyme activity, endogenous uracil levels and endogenous DHU/U ratio phenotyping assays, as only one blood draw is required, compared to the additional burden of the uracil loading dose and 2-^{13}C-uracil breath assay. For the latter two assays, patients are required to visit the hospital on a separate day after overnight fasting, after which they had to drink a ^{13}C-uracil or uracil solution and wait for a second assessment (50 or 120 minutes after start of the test, respectively). The feasibility in terms of the analyses in the laboratory is high for the 2-^{13}C-uracil breath assay, however due to the individualized dose a pharmacy with compounding facilities must be available. The DPD enzyme activity assay is laborious, logistically difficult and requires specific equipment not available in every hospital, making the feasibility lower. For the endogenous uracil and DHU levels, laboratory feasibility is moderate. All of the above mentioned factors influence the results of the phenotyping assays. As the execution of the phenotyping assays were not validated per center, it is possible that differences in results could arise due to differences between participating centers.

The lack of correlation between the phenotyping assays and DPD activity in PBMCs was surprising, and could possibly be caused by flaws in execution of the tests. Besides this, neither of the phenotyping assays was able to predict the onset of severe toxicity very well, possibly due to the fact that not all toxicity can be explained by DPD deficiency. Previously it was described that clinical validity and utility was not yet determined for all phenotyping assays,[22] yet with this study we were unable to complement this lack of evidence. Taking all of the above mentioned factors into account, we question if DPD phenotyping tests, as currently executed, have added value to *DPYD* genotyping in predicting the occurrence of severe fluoropyrimidine-induced toxicity.

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SUPPLEMENT

SUPPLEMENTARY METHODS

Calculation of sample size for this study

Within *DPYD* wild-type patients a variability in DPD enzyme activity exists. We assumed that 95% of the *DPYD* wild-type patients would be classified as having normal enzyme activity and 5% of the *DPYD* wild-type patients would be classified with a low DPD enzyme activity (DPD deficient), with an increased risk of toxicity. This results in an unequal sample size, therefore a total sample size of 240 evaluable patients was required to achieve at least 80% power at significance level α=0.05 to detect an increase in the probability of toxicity from an estimated 20% in non-DPD deficient patients to 60% in DPD deficient patients. Furthermore 10 to 20 extra *DPYD* variant allele carriers would be included, to be able to better investigate the correlation between *DPYD* genotype and DPD phenotype. This made the total required sample size 260.

Methods phenotyping assays

DPD enzyme activity assay:[1,2] In this study, the DPD enzyme activity in PBMCs was determined using a validated radio-assay, which is based on conversion of the radiolabeled probe 4-^{14}C thymine to 4-^{14}C dihydrothymine.[2] As this phenotyping assay is considered the gold standard in DPD phenotyping, other assays were correlated to this assay. This DPD phenotyping assay was also determined in *DPYD* variant allele carriers in the main study cohort (NCT02324452 trial). Between 8 and 9 am, after overnight fasting, 20 ml blood (EDTA tube) was drawn, combined with a blood draw for determining the endogenous DHU/U ratio. Depending on the hospital of inclusion, whole blood was either shipped overnight to the Academic Medical Center, Amsterdam for further processing, or was processed at the hospital of blood draw as described before, to isolate PBMCs.[2] After processing, isolated PMBCs were kept at -80°C before measurement of DPD activity at the Academic Medical Center, Amsterdam.

Endogenous DHU/U ratio and endogenous uracil levels:[3,4] In this study, the uracil and DHU levels were determined in plasma using a validated ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS) method.[4] All samples were measured at the Netherlands Cancer Institute, Amsterdam. 4 ml blood (heparin tube) was drawn between 8 and 9 am, after overnight fasting, and centrifuged at 4°C at 1500g for 10 min. Plasma was kept at -80°C until measurement. This DPD phenotyping assay was also determined in the main study cohort (NCT02324452 trial). However, in these patients blood could be drawn throughout the day and in non-fasting state, but information was collected on how long before the blood draw the patient had eaten a meal, as food status could influence the uracil levels in patients (Henricks *et al*., submitted for publication).

Uracil loading dose:[5,6] Previously, a loading dose uracil of 500 mg/m^2 was used in this assay. To increase feasibility, a standardized dose of 1000 mg uracil was administered in this study. Patients had to fast overnight for a minimum of 8 hours. Food and drinks had to be abstained for the duration of the assay as well. Uracil was dissolved in warm water and administered between 8 and 9 am, to minimize effects of circadian rhythm. 4 ml blood (EDTA tube) was taken at 60 and 120 min after oral intake of uracil. Sample processing consisted of adding 0.15 ml of the DPD inhibitor gimeracil to a 4 ml sample and centrifuging at 4°C at 1500g for 10 min. Plasma was kept at -80°C until measurement. Uracil and its metabolite dihydrouracil were determined in plasma

using a high-performance liquid chromatography ultra-violet (HPLC-UV) method in the laboratory of the Department of Pharmacy at the Scheper Hospital in Emmen.

2-^{13}C-uracil breath assay:[7–9] A personalized dose of 6 mg/kg 2-^{13}C uracil was administered to patients after overnight fasting (minimum 8 hours) and alcohol abstaining (minimum 24 hours). Food and drinks had to be abstained for the duration of the assay as well. The 2-^{13}C uracil was dissolved in hot water and administered between 8 and 9 am, to minimize effects of circadian rhythm. Just prior to the administration of the 2-^{13}C uracil solution the patients had to ingest two tablets of Alka-Seltzer Gold® (containing anhydrous citric acid, potassium bicarbonate and sodium bicarbonate) with water, to stimulate uniform and fast absorption of the 2-^{13}C uracil solution. Breath samples (300 ml in a Otsuka Pharmaceuticals breath bag, Japan®) and blood samples (4 ml in a heparin tube) were taken pre-dose and 50 min after administration of uracil. Blood samples were centrifuged immediately at 4°C at 1500g for 10 min. Plasma was kept at -80°C until analysis. Quantification of ^{13}C-uracil and ^{13}C-dihydrouracil levels was done using the same UPLC-MS/MS method as for the endogenous DHU/U ratio at the Netherlands Cancer Institute, Amsterdam, but with uracil-$^{13}C_4$,$^{15}N_2$ and dihydrouracil-$^{13}C_4$,$^{15}N_2$ as internal standards. $^{13}CO_2$ and $^{12}CO_2$ concentrations were determined in the exhaled breath samples by infrared spectrometry using the FDA approved POCone IR spectrometer (Photal Electronics, Japan®) at the laboratory of the department of Clinical Pharmacy and Toxicology at the Leiden University Medical Center or at the Division of Pharmacology at the Netherlands Cancer Institute, Amsterdam. A delta-over-baseline (DOB) ratio at 50 minutes was calculated that represents a change in the $^{13}CO_2/^{12}CO_2$ ratio of two breath samples.

TS activity:[10] In patients at three centers where DPD enzyme activity samples were taken, an extra blood sample pre-dose was taken (20 ml in heparin tube) to determine the activity of thymidylate synthase (TS) in PBMCS, as TS activity has previously be shown to be an important factor for fluoropyrimidine-induced toxicity as well.[11,12] Samples were processed as previously described[10] and kept at -80°C until measurement at the Netherlands Cancer Institute, Amsterdam.

SUPPLEMENTARY RESULTS

Results food intake

The endogenous uracil levels and endogenous DHU/U ratio were correlated to time of last meal that was eaten, to study the influence of food on the uracil levels. No correlation was found (Supplementary Figure 3), therefore time of food intake was not taken into account as covariate in any further analyses.

Results TS activity

The mean TS activity level was 0.187±0.148, with a range of 0.027-0.985 (*N*=75). Previously mean reported TS activity levels in human volunteers were 0.072 nmol/(mg*h) with a range of 0.024-0.596 nmol/(mg*h).[13] Supplementary Figure 4 shows the TS activity plotted against the development of severe fluoropyrimidine-induced toxicity. This did not show a difference in TS-activity between patients with and without severe fluoropyrimidine-induced toxicity.

Supplementary Table 1. Baseline characteristics of patients in this study and the main study cohort.

Characteristic	This study (*N*=92)	Main study cohort (*N*=1103)	*P* value[a]
Sex			
Male	56 (61%)	593 (54%)	0.153
Female	36 (39%)	510 (46%)	
Age			
Median [range]	60 [19-78]	64 [19-89]	**0.003**
Ethnic origin			
Caucasian	87 (95%)	1048 (95%)	0.723
African descent	1 (1%)	19 (2%)	
Asian	2 (2%)	24 (2%)	
Other	2 (2%)	12 (1%)	
Tumor type			
Non-metastatic CRC	38 (41%)	472 (43%)	ND
Metastatic CRC	23 (25%)	232 (21%)	
BC	7 (8%)	141 (13%)	
GC	7 (8%)	63 (6%)	
Other	17 (18%)	195 (18%)	
Type of treatment regimen			
CAP mono	12 (13%)	205 (19%)	0.832
CAP + RT	23 (25%)	264 (24%)	
CAPOX	37 (40%)	374 (34%)	
CAP other	5 (5%)	72 (7%)	
5-FU mono	-	2 (0%)	
5-FU + RT	6 (7%)	63 (6%)	
FOLFOX	4 (4%)	43 (4%)	
5-FU other	5 (5%)	80 (7%)	
BSA			
Median [range]	2.0 [1.46-2.73]	1.91 [1.31-2.73]	0.071
WHO performance status			
0	49 (53%)	554 (50%)	0.115
1	41 (45%)	448 (40%)	
2	1 (1%)	42 (4%)	
NS	1 (1%)	59 (5%)	
Number of treatment cycles			
Median [range]	3 [1-16]	3 [1-37]	ND
DPYD status			
Wild-type	82 (89%)	1018 (92%)	0.235
DPYD variant allele carrier	10 (10.9%)	85 (7.7%)	
c.1236G>A heterozygous	6 (6.5%)	51 (4.6%)	
c.2846A>T heterozygous	3 (3.3%)	17 (1.5%)	
*DPYD*2A heterozygous*	1 (1.1%)	16 (1.5%)	
c.1679T>G heterozygous	-	1 (0.1%)	

[a] All *P*-values represent patients in this study compared to patients from the main study cohort, not included in this study.

Abbreviations: 5-FU mono: 5-fluorouracil monotherapy; 5-FU other: 5-fluorouracil combined with other anticancer drugs; 5-FU + RT: 5-fluorouracil combined with radiotherapy (with or without mitomycin); BC: breast cancer; BSA: body surface area; CAP mono: capecitabine monotherapy (with or without bevacizumab); CAP other: capecitabine combined with other anticancer drugs; CAP + RT: capecitabine combined with radiotherapy (with or without mitomycin); CAPOX: capecitabine combined with oxaliplatin (with or without bevacizumab); CRC: colorectal cancer; *DPYD*; gene encoding dihydropyrimidine dehydrogenase; FOLFOX: 5-fluorouracil combined with oxaliplatin and leucovorin (with or without bevacizumab); GC: gastric cancer; ND: not determined; NS: not specified, either WHO 0, 1 or 2.

Supplementary Table 2. Data on toxicity of patients in this study and the main study cohort.

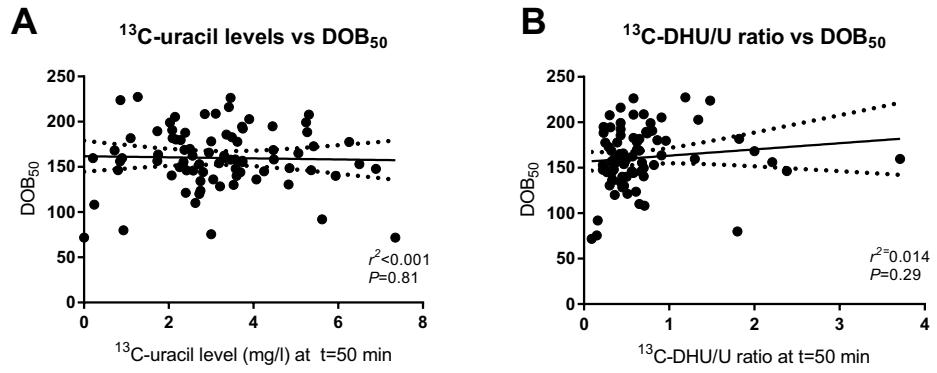
Type of event	This study (*N*=92)	Main study cohort (*N*=1103)	*P* value[a]
Overall grade ≥3 toxicity	19 (21%)	264 (24%)	0.441
Grade ≥3 gastrointestinal toxicity	6 (7%)	103 (9%)	0.332
Grade ≥3 hematological toxicity	10 (11%)	78 (7%)	0.138
Grade 3 hand-foot syndrome	4 (4%)	37 (3%)	0.580
Grade ≥3 cardiological toxicity	0	10 (1%)	0.338
Grade ≥3 other treatment-related toxicity	3 (3%)	87 (8%)	0.085
Fluoropyrimidine-related hospitalization	7 (8%)	156 (14%)	0.066
Stop of fluoropyrimidines due to adverse events	20 (22%)	190 (17%)	0.231
Fluoropyrimidine-related death	0	2 (0%)	ND

[a] All *P*-values represent patients in this study compared to the patients from the main study cohort not included in this study.
Abbreviations: ND: not determined.

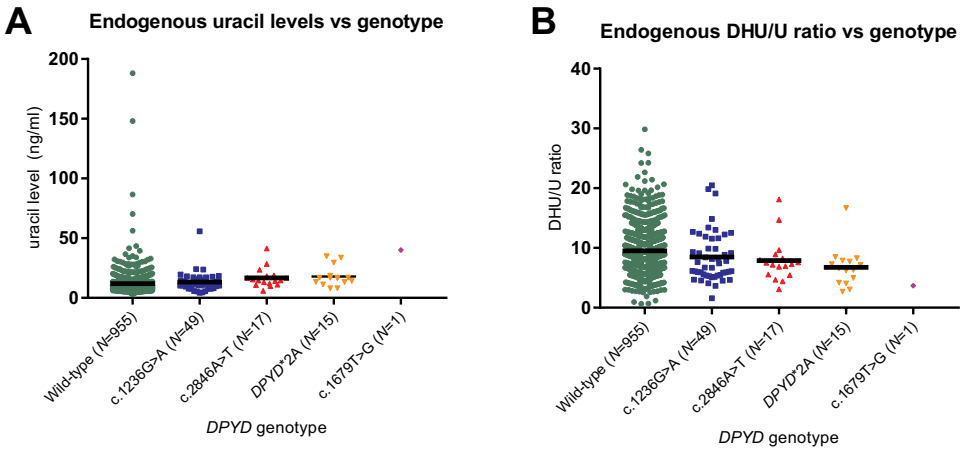
Supplementary Table 3. Toxicity for wild-type patients and *DPYD* variant allele carriers included in this study.

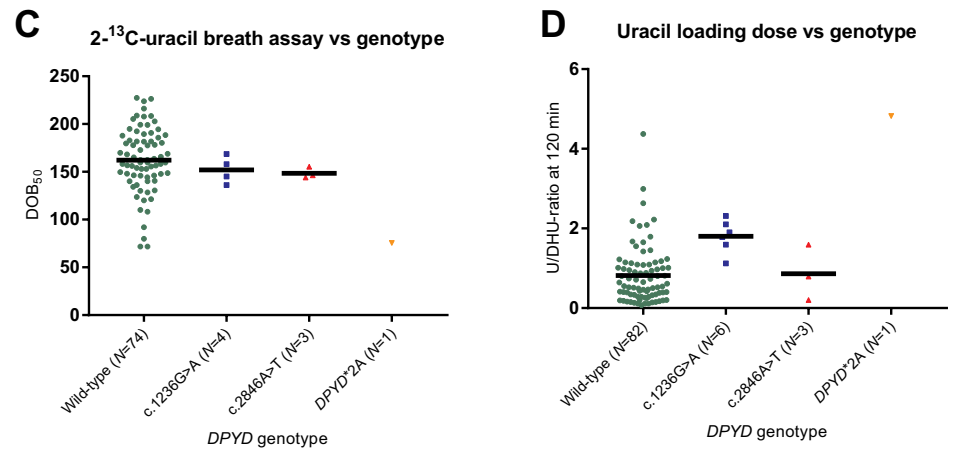
Type of event	*DPYD* variant allele carriers (*N*=10)	Wild-type patients (*N*=82)	*P* value
Overall grade ≥3 toxicity	4 (40%)	15 (18%)	0.109
Grade ≥3 gastrointestinal toxicity	1 (10%)	5 (6%)	0.637
Grade ≥3 hematological toxicity	4 (40%)	6 (7%)	0.002
Grade 3 hand-foot syndrome	0	4 (5%)	0.475
Grade ≥3 cardiological toxicity	0	0	ND
Grade ≥3 other treatment-related toxicity	0	3 (4%)	0.539
Fluoropyrimidine-related hospitalization	1 (10%)	6 (7%)	0.763
Stop of fluoropyrimidines due to adverse events	1 (10%)	19 (23%)	0.340
Fluoropyrimidine-related death	0	0	ND

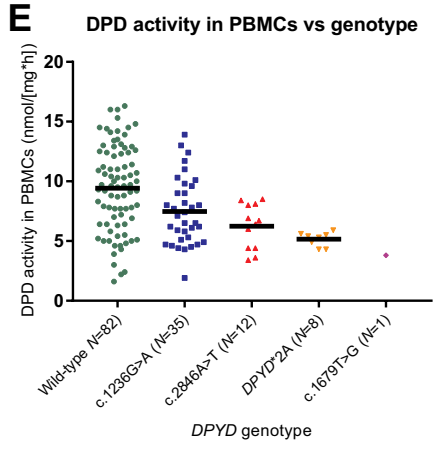
Abbreviations: ND: not determined.



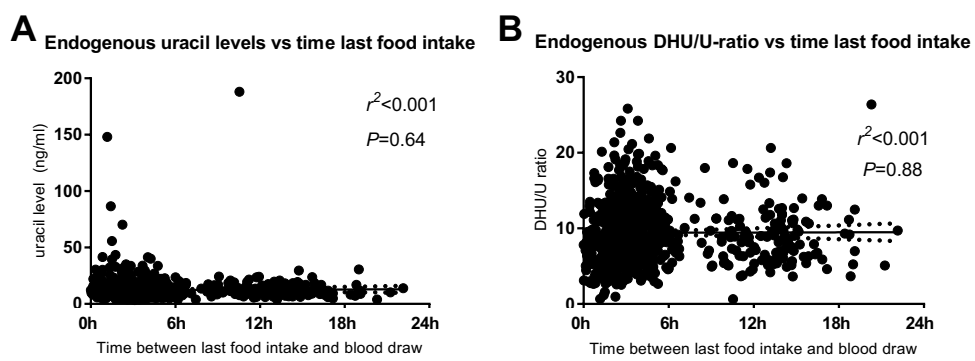
Supplementary Figure 1. Correlation between breath samples and plasma samples of the 2-^{13}C-uracil breath assay.
Abbreviations: DHU: dihydrouracil; DOB_{50}: delta-over-baseline ratio at 50 minutes; U: uracil; vs: versus.



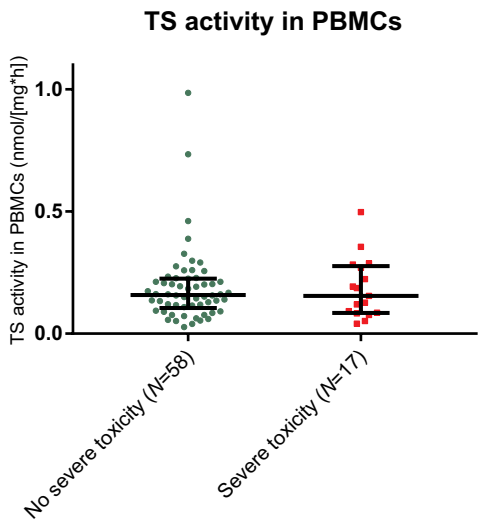




Supplementary Figure 2.
Phenotyping assay results plotted against *DPYD* genotype.
Dots represent individual results. Black lines represent the median of the data. Figure 2E shows the DPD enzyme activity measured in PBMCs of 138 patients. This includes both patients from this study and 46 additional measurements in *DPYD* variant allele carrying patients in the main study cohort of NCT02324452.
Abbreviations: DHU: dihydrouracil; DOB_{50}: delta-over-baseline ratio at 50 minutes; DPD: dihydropyrimidine dehydrogenase; *DPYD*: gene encoding dihydropyrimidine dehydrogenase; PBMCs: peripheral blood mononuclear cells; U: uracil; vs: versus.



Supplementary Figure 3. Endogenous uracil levels and endogenous DHU/U ratio plotted against the time between the blood draw and last food intake.
Abbreviations: DHU: dihydrouracil; U: uracil; vs: versus.



Supplementary Figure 4.
Results of thymidylate synthase activity in PBMCs, separated by the occurrence of severe fluoropyrimidine-induced toxicity.
Dots represent individual results. Black lines represent the median and 25th and 75th percentile of the data.
Abbreviations: PBMCs: peripheral blood mononuclear cells; TS: thymidylate synthase.

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Conclusions and perspectives

Conclusions and perspectives

CONCLUSIONS AND PERSPECTIVES

Implementation of DPYD genotype-guided dosing

Fluoropyrimidine drugs including 5-fluorouracil (5-FU), capecitabine and tegafur are valuable chemotherapeutic agents in the treatment of colorectal, gastric and breast cancer and other solid tumor types, and are used by approximately two million patients each year.[1] However, severe fluoropyrimidine-related toxicity remains a major clinical limitation that affects up to 30% of patients treated with fluoropyrimidines.[2]

As shown in this thesis, a substantial proportion of fluoropyrimidine-related toxicity is the result of deficiency in the enzyme dihydropyrimidine dehydrogenase (DPD). This deficiency is most often caused by genetic single nucleotide polymorphisms in *DPYD*, the gene encoding DPD.[3] There is convincing clinical evidence that carriers of genetic *DPYD* variants are at strongly increased risk of developing severe fluoropyrimidine-related toxicity, and that this risk can be significantly reduced by prospective *DPYD* genotyping and dose reductions in *DPYD* variant allele carriers. However, despite the wealth of available evidence, *DPYD* genotype-guided dosing has not been implemented universally as routine clinical care. As described in Chapter 3, we advocate that prospective *DPYD* genotyping should become standard of care.

Currently, in the drug labels of capecitabine and 5-FU no recommendations on individualized dosing based on *DPYD* genotype are included. In Chapter 4 we summarize available evidence on *DPYD* genotype-guided dosing and how this strategy can improve patient safety. Based on the currently available data, we recommend adjusting the labels of capecitabine and 5-FU by including recommendations on screening for *DPYD* variants before start of fluoropyrimidine-treatment and *DPYD* genotype-guided dose adjustments. In this way, DPD deficient patients can be safely treated with a reduced fluoropyrimidine dose, and potentially effective anticancer therapy does not have to be withheld from these patients. We have sent our proposal for a fluoropyrimidine drug label update to the European Medicines Agency (EMA), and after reviewing our proposal, the EMA has now asked the involved pharmaceutical companies to update the drug labels and include recommendations on *DPYD* genotype-guided dosing. When these label changes will be implemented, hopefully in the near future, we expect that *DPYD* genotype-guided dosing will become standard of care in more and more hospitals worldwide, which is expected to result in an important improvement in patient safety of fluoropyrimidine treatment.

For clinical implementation of *DPYD* genotype-guided dosing, it is important that clinical guidelines are available on how to adjust the fluoropyrimidine dose when a *DPYD* polymorphism is identified in a patient about to start treatment. The Clinical Pharmacogenetics Implementation Consortium (CPIC) aims to fulfill this need by creating evidence-based guidelines to help clinicians translating pharmacogenetic laboratory results into actionable prescribing decisions for affected drugs.[4] For *DPYD*, the first CPIC guideline was published in 2013.[5] As multiple new studies on *DPYD* and risk of fluoropyrimidine-associated toxicity became available in recent years, an update of this guideline was needed. This updated version of the CPIC guideline is described in Chapter 2. In this guideline a differentiation between different *DPYD* variants and corresponding dose recommendations is made, as not all variants have the same deleterious effect on DPD activity. This phenomenon is discussed in Chapter 1 as well.

Implementation of pharmacogenetic-based dosing in clinical practice could benefit from integration of pharmacogenetic information in the electronic patient file and the prescribing system.[6] In this way, clinicians will be able to make dose decisions based on available genetic information and automatic warnings can be given when a drug-gene interaction occurs.

Genotyping of dihydropyrimidine dehydrogenase

As mentioned, DPD deficiency is most often the result of genetic *DPYD* polymorphisms. *DPYD* is a highly polymorphic gene, as over 100 *DPYD* variants have been described,[7] and currently, clinical validity has been established for four *DPYD* variants: *DPYD**2A, c.2846A>T, c.1679T>G and c.1236G>A, as is described in a meta-analysis in Chapter 5.

A previous study by Deenen *et al.* demonstrated the safety, feasibility and cost-effectiveness of *DPYD**2A genotype-guided dosing. In our new prospective study (NCT02324452), described in Chapter 11, prospective *DPYD* screening was expanded to the four *DPYD* variants considered clinically relevant, to investigate whether patient safety will be further improved by screening for four variants instead of *DPYD**2A alone. This confirmed that a 50% initial dose reduction in *DPYD**2A and c.1679T>G carriers markedly reduced the frequency of severe toxicity in these patients. For c.1236G>A and c.2846A>T carriers however, a 25% dose reduction resulted in a toxicity risk that was still significantly increased compared to *DPYD* wild-type patients. More research on these genetic variants and the corresponding effect on DPD activity is warranted. In future studies stronger dose reductions of 50% could be considered, and it should be investigated if this strategy will result in a reduced toxicity risk and adequate drug exposure.

By dose reductions in *DPYD* variant allele carriers according to dosing recommendations, levels of capecitabine, 5-FU and metabolites are shown to be in the range of wild-type patients treated with standard dose. It is therefore expected that effectiveness of fluoropyrimidine therapy will not be negatively affected by *DPYD* genotype-guided dosing. However, as potential loss of effectiveness remains an important concern of those who are critical to *DPYD* genotyping, this was investigated in the study described in Chapter 10. This study showed that effectiveness appeared to be equal between *DPYD**2A carriers treated with reduced dose and matched wild-type patients treated with standard dose. Although an underpowered study, this is the strongest evidence so far endorsing the assumption that effectiveness is not negatively affected by *DPYD* genotype-guided dosing. Ideally, a randomized clinical trial on *DPYD*-guided dosing would be performed to determine safety and effectiveness, but as this is considered unethical and not feasible due to a very large sample size, it is unlikely that this evidence will ever be available.

It is known that DPD activity is not only influenced by *DPYD* variants itself, but can be regulated at a post-transcriptional level as well, for example by microRNA 27a (miR-27a).[8] Polymorphisms in *MIR27A*, the gene encoding miR-27a, were previously shown to decrease DPD activity and increase the risk of fluoropyrimidine-associated toxicity when present in combination with *DPYD* variants.[9] These findings were replicated in the study described in Chapter 9. These data suggest that it is likely that by combining *DPYD* genotyping with *MIR27A* genotyping, positive predictive value for a patients' risk of toxicity can be improved. Before clinical implementation of upfront *MIR27A* screening and dose adjustments based on *MIR27A* genotype, additional evidence on the relationship between *MIR27A* genotype and DPD activity is desired.

Most research is focused on heterozygous *DPYD* variant allele carriers who are partially DPD deficient, as these heterozygous carriers are quite common in the Western population, with an estimated frequency of 5% for the four variants taken together. Completely DPD deficient patients, as described in Chapter 7 and 8, are much rarer. For those patients, with no residual DPD activity,

fluoropyrimidine-based chemotherapy is generally discouraged.[5] We showed however in two completely DPD deficient patients, that treatment with drastically reduced capecitabine doses is safe and results in adequate exposure as determined by pharmacokinetic measurements.
A homozygous *DPYD* genotype or compound heterozygous *DPYD* genotype (carrying multiple *DPYD* variants simultaneously) does not always result in complete DPD deficiency, as described for the patients in Chapter 6 and 7. Those patients can be safely treated with reduced fluoropyrimidine doses. As evidence on the effect of *DPYD* genotype on DPD activity is limited, additional measurements, such as determining DPD activity in peripheral blood mononuclear cells (PBMCs) is recommended, to determine a safe and adequate dosing recommendation for each individual patient. The studies described in Chapter 6, 7 and 8 show that clinical decision making for patients with rare *DPYD* genotypes can be guided by *DPYD* genotype, measurements of DPD activity and pharmacokinetic measurements of capecitabine and 5-FU. This strategy can also be useful when patients with other rare *DPYD* variants will be identified, for which clinical validity has not been established due to the very low allele frequency. Since genetic testing is developing fast and single *DPYD* variant testing might be replaced by whole exome sequencing or even whole genome sequencing in the near future, it is likely that more and more carriers of *DPYD* variants with unknown clinical validity will be identified. Additional investigations including DPD phenotyping and pharmacokinetics are then useful tools for determining individual dosing recommendations.
The four *DPYD* variants highlighted in this thesis are especially relevant to Caucasians, as most studies focus on patients of this ethnic origin. For ethnicities other than Caucasians, more research on the frequency and clinical relevance of these and other *DPYD* variants is recommended. For example, Offer *et al.* showed that the *DPYD* variant Y186C was unique to patients of African ancestry and DPD activity was 46% lower in carriers compared to non-carriers.

Phenotyping of dihydropyrimidine dehydrogenase
DPD deficiency can also not always be traced back to a genetic alteration in *DPYD*. Therefore, a combined genotyping and phenotyping approach is likely to improve sensitivity of identifying patients at risk of fluoropyrimidine-related toxicity. One of the most promising DPD phenotyping methods is measurement of endogenous uracil levels, as described in Chapter 12. In this retrospective study it was shown that a much larger proportion of patients at risk of toxicity could be identified by this DPD phenotyping test than by *DPYD* genotyping. It was shown in Chapter 13 that food status should be taken into account when measuring these endogenous uracil levels. Uracil levels were markedly increased in a prolonged fasting state, compared to fed state. Preferably, sampling should therefore be done in the morning after overnight fasting.
Next to measurement of endogenous uracil levels, other DPD phenotyping methods, including a uracil breath test, uracil loading dose and measurement of DPD activity in PBMCs, are of interest as well. In the study described in Chapter 14, all these four DPD phenotyping tests were compared, to identify the test that is best performing in identifying patients at risk of severe fluoropyrimidine-related toxicity. Results of this analysis will be used for a follow-up study where upfront *DPYD* genotyping and DPD phenotyping will be combined. By dose individualization based on a combination of *DPYD* genotype and DPD phenotype it is expected that patient safety of fluoropyrimidine treatment can be improved even further.

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SUMMARY

5-fluorouracil (5-FU) and its oral prodrug capecitabine belong to the group of fluoropyrimidines and are among the most commonly used anticancer drugs for various types of solid tumors. The studies described in this thesis are focused on improving patient safety of fluoropyrimidine therapy by individualized dosing based on dihydropyrimidine dehydrogenase (DPD) activity. DPD activity can be determined either by genotyping of the *DPYD* gene or phenotyping of the DPD enzyme.

Implementation of DPYD genotype-guided dosing

Chapter 1 describes the gene activity score, a tool which can be used for translating *DPYD* genotype into DPD phenotype. This method can be used to standardize fluoropyrimidine dose adjustments. Several single nucleotide polymorphisms (SNPs) in the *DPYD* gene have been described that result in decreased DPD activity. However, these SNPs differ in their effect on DPD enzyme activity, therefore a differentiated dose adaptation for each *DPYD* variant is recommended. With the gene activity score the amount of dose reduction for a certain *DPYD* variant can be calculated.

In **Chapter 2** the international guideline on fluoropyrimidine dosing based on *DPYD* genotype is presented. This guideline is made by the Clinical Pharmacogenetics Implementation Consortium (CPIC), a consortium aimed to facilitate implementation of pharmacogenetic testing by creating evidence-based guidelines for clinical practice. In the *DPYD* guideline dosing recommendations are made which are in line with the gene activity score that was described in Chapter 1. For heterozygous carriers of *DPYD* variants, a 25% or 50% dose reduction of the fluoropyrimidine drug is recommended, depending on the *DPYD* variant identified.

Although *DPYD* genotype-guided dosing of fluoropyrimidines is known to improve patient safety, as risk of severe and life-threatening toxicity is significantly reduced by applying dose reductions in *DPYD* variant carriers, this strategy is most often not yet implemented in daily clinical care. In **Chapter 3** available evidence in favor of prospective *DPYD* genotyping is summarized, aiming to convince physicians to implement this strategy as standard of care.

Chapter 4 focuses on implementing *DPYD* genotype-guided dosing as well, but now by recommending a label update of fluoropyrimidine drugs. By including prospective screening for *DPYD* variants and *DPYD* genotype-guided dose adjustments in the drug label, patient safety can be even further improved. We have sent our call for a drug label adjustment to the European Medicines Agency (EMA) and after reviewing our proposal, the EMA has now asked the involved pharmaceutical companies to update the drug labels.

Genotyping of dihydropyrimidine dehydrogenase

In **Chapter 5** a systematic review and meta-analysis is presented that was performed to determine the clinical validity of the *DPYD* variants c.1679T>G, c.1236G>A and c.1601G>A. Individual patient data from included studies were retrieved and analyzed in a multivariable analysis. The association between each *DPYD* variant and severe fluoropyrimidine-associated toxicity was determined by calculating a relative risk (RR). A total of 7365 patients from eight studies were included in the analysis. *DPYD* c.1679T>G was found to be significantly associated with fluoropyrimidine-associated toxicity (RR: 4.40, 95% confidence interval (CI): 2.08–9.30, *P*<0.0001), as was c.1236G>A (RR: 1.59, 95%CI: 1.29–1.97, *P*<0.0001). There was no significant association between c.1601G>A and fluoropyrimidine-associated toxicity (RR: 1.52, 95%CI 0.86–2.70, *P*=0.15). This meta-analysis showed that the *DPYD* variants c.1679T>G and c.1236G>A variants are clinically relevant. Upfront screening for these two variants is recommended, in addition to the established variants *DPYD**2A and c.2846A>T.

Chapter 6 describes three patients who were retrospectively identified as homozygous c.1129-5923C>G carriers (this variant is in complete linkage with c.1236G>A). An approximately 50% reduction of DPD enzyme activity was found in two of the patients, which was shown to be associated with aberrant mRNA processing, thereby confirming the functional relevance of c.1129-5923C>G. The presented clinical data show that the patients were able to tolerate only low doses of fluoropyrimidines. The presented data support an upfront dose reduction of approximately 50% in homozygous carriers of c.1129-5923C>G, which is in line with the 25% dose reduction which is recommended for heterozygous carriers of this variant.

In **Chapter 7** data of six patients with a homozygous or compound heterozygous *DPYD* genotype (carrying two different *DPYD* variants simultaneously) are presented. All patients were identified with these rare genotypes before start of fluoropyrimidine treatment and individualized dosing recommendations were made for all patients based on *DPYD* genotype and DPD enzyme activity. One patient was identified as a homozygous *DPYD**2A carrier with complete DPD deficiency. It was shown that treatment with an extremely low dose of capecitabine (0.65% of standard dose) was safe and feasible in this patient. If the patients' genotype had not been identified upfront and he had received a standard dose, this most likely would have been fatal. The other patients, carrying homozygous c.2846A>T genotypes, homozygous c.1236G>A genotypes or compound heterozygous c.2846A>T/c.1236>A genotypes, all had partial remaining DPD activity and could therefore be safely treated with more moderate dose reductions.

Chapter 8 describes another patient with complete DPD deficiency. Genetic analyses showed that this complete absence of DPD activity was likely to be caused by a novel *DPYD* genotype, consisting of a combination of amplification of exon 17 and 18 of *DPYD* and heterozygosity for *DPYD**2A. The patient was treated with a strongly reduced capecitabine dose of 150 mg every five days (0.8% of standard dose), which was based on our experience with the previously identified completely DPD deficient patient described in Chapter 7. Pharmacokinetic analyses confirmed adequate exposure and treatment was completed without occurrence of capecitabine-related toxicity. The case report demonstrated that a more comprehensive genotyping and phenotyping approach, combined with pharmacokinetically-guided dose administrations enables save fluoropyrimidine treatment in completely DPD deficient patients.

The objective of the study described in **Chapter 9** was to determine whether genotyping of *MIR27A* polymorphisms rs895819A>G and rs11671784 can be used to improve the predictive value of *DPYD* variants to identify patients at risk of severe fluoropyrimidine-related 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. In *DPYD* wild-type patients, *MIR27A* variants did not affect risk of fluoropyrimidine-associated toxicity (odds ratio (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 fluoropyrimidine-associated toxicity (OR: 4.9, 95%CI: 1.24–19.7, *P*=0.023). Rs11671784 was not associated with fluoropyrimidine-associated toxicity (OR: 2.9,

95%CI: 0.47–18.0, *P*=0.253). This study demonstrated the clinical validity of combined *MIR27A/DPYD* screening to identify patients at risk of severe fluoropyrimidine-associated toxicity.

In **Chapter 10** effectiveness of reduced-dose fluoropyrimidine therapy in patients carrying the *DPYD**2A variant was investigated. A cohort of 40 prospectively identified *DPYD**2A carriers treated with an approximately 50% reduced fluoropyrimidine dose was described. For effectiveness analyses, a matched pair analysis was performed where for each *DPYD**2A carrier treated with reduced dose a matched wild-type patient treated with full dose was identified. It was shown that there was no statistically significant difference between *DPYD**2A carriers and wild-type patients in overall survival (*P*=0.47) and progression-free survival (*P*=0.54). Risk of severe toxicity in *DPYD**2A carriers treated with reduced dose was 18%, comparable to wild-type patients treated with full dose, where the risk was 23% (*P*=0.57). Compared to a historical literature cohort of *DPYD**2A carriers treated with full dose, severe risk dropped significantly from 77% to 18% by dose reductions based on *DPYD**2A genotype (*P*<0.001). This study endorsed that *DPYD**2A genotype-guided dosing does not have a negative effect on effectiveness of fluoropyrimidine-based chemotherapy, while resulting in significantly improved patient safety.

Chapter 11 describes a prospective clinical trial, in which it was investigated whether the risk of fluoropyrimidine-related toxicity can be reduced by *DPYD* genotype-guided dosing, by testing for four *DPYD* variants (*DPYD**2A, c.2846A>T, c.1679T>G and c.1236G>A). Heterozygous *DPYD* variant allele carriers received an initial dose reduction of 25% or 50%, dependent on the genetic variant. Toxicity was compared to a historical cohort of *DPYD* variant allele carriers treated with full dose, and to *DPYD* wild-type patients from the current study. Next to this, pharmacokinetic parameters in *DPYD* variant allele carriers were investigated and a cost-analysis was performed. A total of 1103 evaluable patients was enrolled and prospectively genotyped, of whom 85 *DPYD* variant allele carriers (7.7%). When comparing to a historical cohort, *DPYD* genotype-guided dosing markedly reduced toxicity risk for *DPYD**2A and c.1679T>G carriers, moderately reduced risk for c.2846A>T carriers and did not result in a risk reduction for c.1236G>A carriers. Pharmacokinetic analyses in *DPYD* variant allele carriers treated with reduced dose showed that fluoropyrimidine exposure was comparable to wild-type patients treated with a full dose. The cost-analysis showed that a *DPYD* screening strategy is likely to be cost saving, as the average total treatments costs per patient were lower for screening than for non-screening. This study showed that upfront *DPYD* genotyping improves patient safety of fluoropyrimidine therapy and is feasible in routine practice, without increasing costs. A 50% initial dose reduction is recommended for heterozygous *DPYD**2A and c.1679T>G carriers. The applied dose reductions of 25% for c.1236G>A and c.2846A>T carriers could not lower toxicity risk to background risk, so stronger dose reductions of 50% or closer monitoring are recommended.

Phenotyping of dihydropyrimidine dehydrogenase

In **Chapter 12** it was studied whether endogenous uracil concentrations are a useful predictor of severe fluoropyrimidine-related toxicity. Pre-treatment serum concentrations of uracil and dihydrouracil were measured in 550 patients, derived from a previous prospective study of patients treated with fluoropyrimidine-based chemotherapy. High pre-treatment uracil concentrations (>16 ng/ml) were strongly associated with global severe toxicity (OR: 5.3, 95%CI: 1.53–18.7, *P*=0.009), toxicity-related hospitalization (OR: 16.9, 95%CI: 4.41–64.7, *P*<0.0001) and fatal treatment-related toxicity (OR: 44.8, 95%CI: 4.55–441, *P*=0.001). It was shown that pre-

treatment uracil concentration is a highly promising phenotypic marker to identify patients at risk of severe fluoropyrimidine-associated toxicity.

Chapter 13 focused on uracil concentrations as well, but investigated if food intake had a relevant influence on uracil and dihydrouracil plasma levels. In a randomized, cross-over study in sixteen healthy volunteers, subjects were examined in fasted and fed state (after consuming a test mail with a high uracil content). The study showed that uracil and dihydrouracil levels were significantly higher in fasting state than in fed state. This is thought to be a direct effect of uridine homeostasis as measured uridine curves showed similar patterns as for uracil. These findings show that, when assessing plasma uracil and dihydrouracil levels for adaptive fluoropyrimidine dosing in clinical practice, sampling should be done between 8:00h-9:00h in the fasting state to avoid bias caused by food effects.

In **Chapter 14** four phenotyping assays were investigated, with the aim to determine which phenotyping assay is best in identifying DPD deficient patients at risk for fluoropyrimidine-related toxicity. Four assays developed to measure DPD activity (DPD enzyme activity in peripheral blood mononuclear cells [PBMCs], the endogenous dihydrouracil//uracil [DHU/U] ratio or endogenous uracil levels alone, uracil loading dose and 2-^{13}C-uracil breath assay) were compared in a clinical trial of patients starting fluoropyrimidine-based chemotherapy. In total, 92 patients participated in this study. None of the phenotyping assays did correlate well to the gold standard of DPD phenotyping (DPD enzyme activity in PBMCs). Clinical validity parameters to predict DPD deficiency and severe toxicity were calculated per phenotyping assay. None of the phenotyping assays was able to predict the onset of severe fluoropyrimidine-related toxicity well.

NEDERLANDSE SAMENVATTING

5-fluorouracil (5-FU) en de orale prodrug capecitabine behoren tot de groep van fluoropyrimidines en zijn veelgebruikte antikankergeneesmiddelen voor diverse solide tumoren. De studies die worden beschreven in dit proefschrift zijn gericht op het verbeteren van de patiëntveiligheid van fluoropyrimidines door geïndividualiseerd doseren op basis van dihydropyrimidine dehydrogenase (DPD)-activiteit. DPD-activiteit kan zowel worden bepaald door genotypering van het *DPYD*-gen of door fenotypering van het DPD-enzym.

Implementatie van doseren gebaseerd op DPYD-genotype

Hoofdstuk 1 beschrijft de genactiviteitsscore, een methode om *DPYD*-genotype te vertalen naar DPD-fenotype. Deze methode kan worden gebruikt om dosisaanpassingen van fluoropyrimidines te standaardiseren. Meerdere *single nucleotide polymorphisms* (SNP's) in het *DPYD*-gen die leiden tot een verlaagde DPD-activiteit zijn bekend. Deze SNP's verschillen echter in de mate waarop DPD-enzymactiviteit verminderd wordt en daarom wordt een gedifferentieerde dosisaanpassing aangeraden, die onderscheid maakt tussen de verschillende *DPYD*-varianten. Met de genactiviteitsscore kan de mate van dosisaanpassing voor een bepaalde *DPYD*-variant worden berekend.

In **Hoofdstuk 2** wordt de internationale richtlijn over het doseren van fluoropyrimidines op basis van *DPYD*-genotype gepresenteerd. Deze richtlijn is gemaakt door het *Clinical Pharmacogenetics Implementation Consortium* (CPIC). Dit is een consortium met als doel het faciliteren van implementatie van farmacogenetische testen door *evidence-based* richtlijnen te maken voor de praktijk. In de *DPYD*-richtlijn worden dosisaanpassingen beschreven die overeenkomen met de genactiviteitsscore uit Hoofdstuk 1. Voor heterozygote dragers van een *DPYD*-variant wordt een 25% of 50% dosisreductie van de fluoropyrimidine aangeraden, afhankelijk van de gevonden *DPYD*-variant.

Het is bekend dat doseren van fluoropyrimidines op basis van *DPYD*-genotype de patiëntveiligheid verbetert, aangezien het risico op ernstige en levensbedreigende toxiciteit significant verlaagd wordt door het toepassen van dosisreducties in dragers van een *DPYD*-variant. Helaas wordt deze strategie meestal nog niet toegepast in de dagelijkse praktijk. In **Hoofdstuk 3** wordt het beschikbare bewijs dat pleit voor prospectief *DPYD*-genotyperen samengevat, met als doel artsen te overtuigen om deze strategie te implementeren als standaardzorg.

Hoofdstuk 4 is ook gericht op de implementatie van doseren op basis van *DPYD*-genotype, maar ditmaal door het aanbevelen van een aanpassing van de bijsluiter van fluoropyrimidines. Als aanbevelingen voor prospectief screenen voor *DPYD*-varianten en dosisaanpassingen op basis van *DPYD*-genotype in de bijsluiter worden opgenomen, kan patiëntveiligheid nog verder worden verbeterd. Wij hebben onze aanbevelingen voor een aanpassing van de bijsluiter naar de *European Medicines Agency* (EMA) gestuurd, en na beoordeling van ons voorstel heeft de EMA aan de betrokken farmaceutische bedrijven gevraagd om de bijsluiter aan te passen.

Genotypering van dihydropyrimidine dehydrogenase

In **Hoofdstuk 5** wordt een systematische review en meta-analyse gepresenteerd die is uitgevoerd om de klinische validiteit van de *DPYD*-varianten c.1679T>G, c.1236G>A en c.1601G>A te bepalen. Individuele patiëntdata van de geïncludeerde studies werden opgevraagd en zijn geanalyseerd

in een multivariabele analyse. De associatie tussen elke *DPYD*-variant en ernstige fluoropyrimidine-geassocieerde toxiciteit werd bepaald door het berekenen van een relatief risico (RR). In totaal werden 7365 patiënten uit acht studies geïncludeerd in de analyse. *DPYD* c.1679T>G bleek significant geassocieerd met fluoropyrimidine-gerelateerde toxiciteit (RR: 4,40; 95% betrouwbaarheidsinterval (CI): 2,08–9,30; *P*<0,0001). Hetzelfde gold voor c.1236G>A (RR: 1,59, 95%CI: 1,29–1,97, *P*<0,0001). Er werd geen significante associatie tussen c.1601G>A en fluoropyrimidine-gerelateerde toxiciteit gevonden (RR: 1,52; 95%CI 0,86–2,70; *P*=0,15). Deze meta-analyse toont aan dat de *DPYD*-varianten c.1679T>G en c.1236G>A klinisch relevant zijn. Prospectieve screening voor deze twee varianten wordt aangeraden, naast screening voor de reeds bekende varianten *DPYD**2A en c.2846A>T.

Hoofdstuk 6 beschrijft drie patiënten die retrospectief werden geïdentificeerd als homozygote c.1129-5923C>G dragers (deze variant komt altijd gelijktijdig voor met c.1236G>A). In twee van de patiënten werd een reductie van DPD-enzymactiviteit gevonden van ongeveer 50% en afwijkingen in mRNA-productie, wat de functionele relevantie van c.1129-5923C>G bevestigt. De klinische gegevens van deze patiënten lieten zien dat de patiënten slechts lage doses van fluoropyrimidines konden verdragen. Deze gepresenteerde data onderbouwen een dosisreductie van ongeveer 50% in homozygote dragers van c.1129-5923C>G, wat overeenkomt met de 25% dosisreductie die wordt aangeraden voor heterozygote dragers van deze variant.

In **Hoofdstuk 7** worden zes patiënten gepresenteerd met een homozygoot of dubbel heterozygoot *DPYD* genotype (het gelijktijdig dragen van twee verschillende *DPYD* varianten). Alle patiënten werden geïdentificeerd met deze zeldzame genotypes voor start van fluoropyrimidine-bevattende chemotherapie en geïndividualiseerde dosisadviezen werden gemaakt voor alle patiënten, gebaseerd op *DPYD*-genotype en DPD-enzymactiviteit. Eén patiënt was een homozygote drager van de *DPYD**2A variant en had complete DPD-deficiëntie. Er werd aangetoond dat behandeling met een extreem lage dosering capecitabine (0,65% van de standaarddosering) mogelijk was en ook veilig was voor deze patiënt. Als het genotype van deze patiënt niet bepaald was voor start van de behandeling en een volledige dosering was toegediend, dan zou dit waarschijnlijk een dodelijk gevolg hebben gehad. De andere patiënten, met een homozygoot c.2846A>T genotype, een homozygoot c.1236G>A genotype of een compound heterozygoot c.2846A>T/c.1236G>A genotype, hadden allen deels resterende DPD-enzymactiviteit en konden daarom behandeld worden met kleinere dosisreducties.

Hoofdstuk 8 beschrijft een andere patiënt met complete DPD-deficiëntie. Genetische analyses toonden aan dat deze complete DPD-deficiëntie waarschijnlijk werd veroorzaakt door een nieuw *DPYD*-genotype, bestaande uit een combinatie van amplificatie van exon 17 en 18 van *DPYD* en heterozygotie voor *DPYD**2A. De patiënt werd behandeld met een sterk gereduceerde capecitabinedosering van 150 mg elke vijf dagen (0,8% van standaarddosering). Dit was gebaseerd op onze ervaring met de compleet DPD-deficiënte patiënt die is beschreven in Hoofdstuk 7. Farmacokinetiekanalyses bevestigden dat de blootstelling adequaat was en de behandeling werd afgerond zonder het optreden van capecitabine-gerelateerde toxiciteit. Dit *case report* toont aan dat een gecombineerde genotyperings- en fenotyperingsaanpak, gecombineerd met farmacokinetiekbepalingen, een veilige fluoropyrimidine-behandeling in compleet DPD-deficiënte patiënten mogelijk maakt.

Het doel van de studie die wordt beschreven in **Hoofdstuk 9** was om te bepalen of genotypering van de *MIR27A*-polymorfismen rs895819A>G en rs11671784 gebruikt kan worden om de

voorspellende waarde van *DPYD*-varianten te verbeteren voor het identificeren van patiënten met een verhoogd risico op ernstige fluoropyrimidine-gerelateerde toxiciteit. Patiënten die behandeld waren met fluoropyrimidine-bevattende chemotherapie in een eerdere prospectieve studie werden gegenotypeerd voor rs895819 en rs11671784 en voor *DPYD* c.2846A>T, c.1679T>G, c.1129-5923C>G en c.1601G>A. In patiënten die *DPYD*-wildtype waren, beïnvloedden *MIR27A*-varianten het risico op fluoropyrimidine-geassocieerde toxiciteit niet (odds ratio (OR) 1,3 voor ≥1 variant *MIR27A* allel vs. geen, 95%CI: 0,87–1,82, *P*=0,228). In tegenstelling tot patiënten met *DPYD*-wildtype, was de aanwezigheid van ≥1 rs895819 variant allel geassocieerd met een verhoogd risico op fluoropyrimidine-gerelateerde toxiciteit (OR: 4,9; 95%CI: 1,24–19,7; *P*=0,023) in patiënten die drager waren van een *DPYD*-variant. Rs11671784 was niet geassocieerd met fluoropyrimidine-gerelateerde toxiciteit (OR: 2,9; 95%CI: 0,47–18,0; *P*=0,253). Deze studie toont de klinische validiteit aan van een gecombineerde *MIR27A/DPYD* screening om patiënten te identificeren met een verhoogd risico op ernstige fluoropyrimidine-geassocieerde toxiciteit.

In **Hoofdstuk 10** werd de effectiviteit van verlaagde doseringen van fluoropyrimidinebehandeling bij patiënten die drager zijn van de *DPYD**2A variant onderzocht. Een cohort van 40 prospectief geïdentificeerde *DPYD**2A dragers die behandeld werden met een ongeveer 50% gereduceerde dosis van de fluoropyrimidine werd beschreven. Voor effectiviteitsanalyses werd een *matched pair*-analyse uitgevoerd, waarbij voor iedere *DPYD**2A drager die behandeld werd met gereduceerde dosis, een passende wildtype patiënt die behandeld werd met een volledige dosis, werd gezocht. Er werd geen statistisch significant verschil gevonden voor *DPYD**2A dragers en wildtype patiënten voor algemene overleving (*P*=0,47) en progressievrije overleving (*P*=0,54). Het risico van ernstige toxiciteit in *DPYD**2A dragers behandeld met gereduceerde dosis was 18%, wat vergelijkbaar is met het risico voor wildtype patiënten behandeld met volledige dosis, namelijk 23% (*P*=0,57). Vergeleken met een historisch literatuurcohort van *DPYD**2A dragers met volledige dosis, daalde het risico van ernstige toxiciteit significant van 77% naar 18% door het toepassen van dosisreductie gebaseerd op *DPYD**2A genotype (*P*<0,001). Deze studie laat zien dat doseren op basis van *DPYD**2A genotype geen negatief effect op effectiviteit van fluoropyrimidine-bevattende chemotherapie heeft, terwijl het wel de patiëntveiligheid significant verbetert.

Hoofdstuk 11 beschrijft een prospectieve klinische studie, waarin werd onderzocht of het risico op fluoropyrimidine-gerelateerd toxiciteit verlaagd kan worden door doseren op basis van *DPYD*-genotype. Hierbij werd getest op vier *DPYD*-varianten (*DPYD**2A, c.2846A>T, c.1679T>G en c.1236G>A). Heterozygote *DPYD*-variantdragers ontvingen een 25% of 50% dosisreductie, afhankelijk van de genetische variant. Toxiciteit werd vergeleken met een historisch cohort van *DPYD*-variantdragers die werden behandeld met een volledige dosis, en met de *DPYD*-wildtype-patiënten uit de huidige studie. Daarnaast werden farmacokinetiekparameters bij de *DPYD*-variantdragers onderzocht en werd een kostenanalyse uitgevoerd. Een totaal van 1103 evalueerbare patiënten werd geïncludeerd in de studie en prospectief gegenotypeerd. Hierdoor werden 85 *DPYD*-variantdragers (7,7%) geïdentificeerd. Bij het vergelijken met het historisch cohort, bleek dat door dosisindividualisatie het toxiciteitsrisico sterk gedaald was voor de *DPYD**2A- en c.1679T>G-dragers, enigszins verlaagd voor de c.2846A>T-dragers en niet verlaagd voor de c.1236G>A-dragers. De farmacokinetiekanalyses in de *DPYD*-variantdragers die behandeld werden met een gereduceerde dosis lieten zien dat de fluoropyrimidineblootstelling vergelijkbaar was met *DPYD*-wildtype-patiënten die behandeld werden met een volledige dosis. De kostenanalyse toonde aan dat een *DPYD*-screeningsstrategie waarschijnlijk kostenbesparend is, aangezien de

gemiddelde totale behandelkosten per patiënt lager waren voor screenen dan voor niet screenen. Deze studie liet zien dat prospectieve *DPYD*-genotypering de patiëntveiligheid van fluoropyrimidinetherapie kan verbeteren en haalbaar is in de dagelijkse praktijk, zonder voor extra kosten te zorgen. Voor heterozygote *DPYD**2A- en c.1679T>G-dragers wordt een initiële dosisreductie van 50% aangeraden. Voor c.1236G>A- en c.2846A>T-dragers kon de toegepaste dosisreductie van 25% het toxiciteitsrisico niet verlagen tot het achtergrondrisico. Daarom worden voor deze *DPYD*-varianten sterkere dosisreducties van 50% of meer nauwlettende controles aangeraden.

Fenotypering van dihydropyrimidine dehydrogenase

In **Hoofstuk 12** werd onderzocht of endogene uracilconcentraties een goede voorspeller zijn van ernstige fluoropyrimidine-gerelateerde toxiciteit. Serumconcentraties van uracil en dihydrouracil werden bepaald in 550 patiënten, gemeten in bloed voor start van de behandeling. Deze patiënten waren geïncludeerd in een eerdere prospectieve studie en werden behandeld met fluoropyrimidine-bevattende chemotherapie. Een hoge uracilspiegel (>16 ng/ml) bleek sterk geassocieerd met ernstige toxiciteit (OR: 5,3; 95%CI: 1,53–18,7; *P*=0,009), toxiciteits-gerelateerde ziekenhuisopnames (OR: 16,9; 95%CI: 4,41–64,7, *P*<0,0001) en dodelijke fluoropyrimidine-gerelateerde toxiciteit (OR: 44,8, 95%CI: 4,55–441; *P*=0,001). Er werd aangetoond dat uracilspiegels een veelbelovende fenotype-marker zijn om patiënten te identificeren met een verhoogd risico op ernstige fluoropyrimidine-gerelateerde toxiciteit.

Ook in **Hoofdstuk 13** lag de focus op uracilconcentraties, maar in deze studie werd onderzocht of voedselinname een relevante invloed had op uracil- en dihydrouracilplasmaspiegels. In een gerandomiseerde *cross-over*-studie in zestien gezonde vrijwilligers werden de uracil- en dihydrouracilspiegels van deelnemers zowel in gevaste toestand als na het eten van een testmaaltijd die veel uracil bevatte, onderzocht. De studie liet zien dat uracil- en dihydrouracilspiegels significant hoger waren in gevaste toestand dan in gevoede toestand. Er wordt gedacht dat dit een direct effect is van de uridinehomeostase, aangezien de gemeten uridinecurves in de studie een vergelijkbaar patroon lieten zien als de uracilcurves. Deze resultaten tonen aan dat, wanneer uracil- en dihydrouracilspiegels gemeten worden voor het aanpassen van de fluoropyrimidinedosering, de bloedafname gedaan zou moeten worden tussen 8.00h en 9.00h en in nuchtere toestand, om bias door voedseleffecten te voorkomen.

In **Hoofdstuk 14** werden vier fenotyperingstesten onderzocht met het doel om te bepalen welke fenotyperingstest het beste DPD-deficiënte patiënten kan identificeren die een risico hebben op fluoropyrimidine-gerelateerde toxiciteit. De vier testen die ontwikkeld zijn voor het meten van DPD-activiteit (DPD-enzymactiviteit in perifeer bloed-mononucleaire cellen [PBMC's], de endogene dihydrouracil/uracil [DHU/U]-ratio of endogene uracilspiegels alleen, de uracilbelastingtest en de 2-^{13}C-uracil-ademtest) werden vergeleken in een klinische studie van patiënten die gingen starten met fluoropyrimidine-therapie. In totaal deden 92 patiënten mee aan deze studie. Geen van de fenotyperingstesten had een goede correlatie met de gouden standaard in DPD-fenotypering (DPD-enzymactiviteit in PBMC's). Parameters voor het beschrijven van klinische validiteit voor zowel het voorspellen van DPD-deficiëntie als ernstige toxiciteit werden berekend voor elke test. Geen van de fenotyperingstesten kon het optreden van fluoropyrimidine-gerelateerde toxiciteit goed voorspellen.

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CURRICULUM VITAE

Linda Henricks was born on February 6th, 1989 in Utrecht. After graduating from high school at the St. Bonifatiuscollege in Utrecht *summa cum laude*, she started studying Pharmacy at Utrecht University in 2007. She completed both the bachelor and master program *cum laude*.
As part of her master program, she did a six month research project at the University of California, San Francisco in the research group of Jeroen Roose, investigating the Ras pathway in colorectal cancer. She was awarded the KNMP student award in 2014 and the Vliegenthart thesis award for her research project in San Francisco in 2015.
During her study, Linda was active in both the local and national student organization for pharmacy students, including a fulltime year as executive board of the royal Dutch pharmaceutical student association (K.N.P.S.V.).
After graduating as a pharmacist, Linda Henricks started as a PhD candidate at the Netherlands Cancer Institute (NKI) in Amsterdam in September 2014, under supervision of prof. dr. Jan H.M. Schellens and dr. Annemieke Cats of the NKI, and prof dr. Henk-Jan Guchelaar of the Leiden University Medical Center (LUMC). Her main project was setting up, coordinating and finishing a large prospective clinical trial in seventeen hospitals throughout the Netherlands. This trial aimed to investigate and implement *DPYD* genotype-guided individualized dosing of fluoropyrimidines, in order to improve patient safety of patients treated with fluoropyrimidine-based chemotherapy.
In addition to her PhD project, Linda Henricks also followed the clinical pharmacology training of the Dutch Society of Clinical Pharmacology and Biopharmacy (NVKFB), and became a clinical pharmacologist in August 2017.

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