Personalised medicine of fluoropyrimidines using DPYD pharmacogenetics

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PERSONALISED MEDICINE OF FLUOROPYRIMIDINES USING *DPYD* PHARMACOGENETICS

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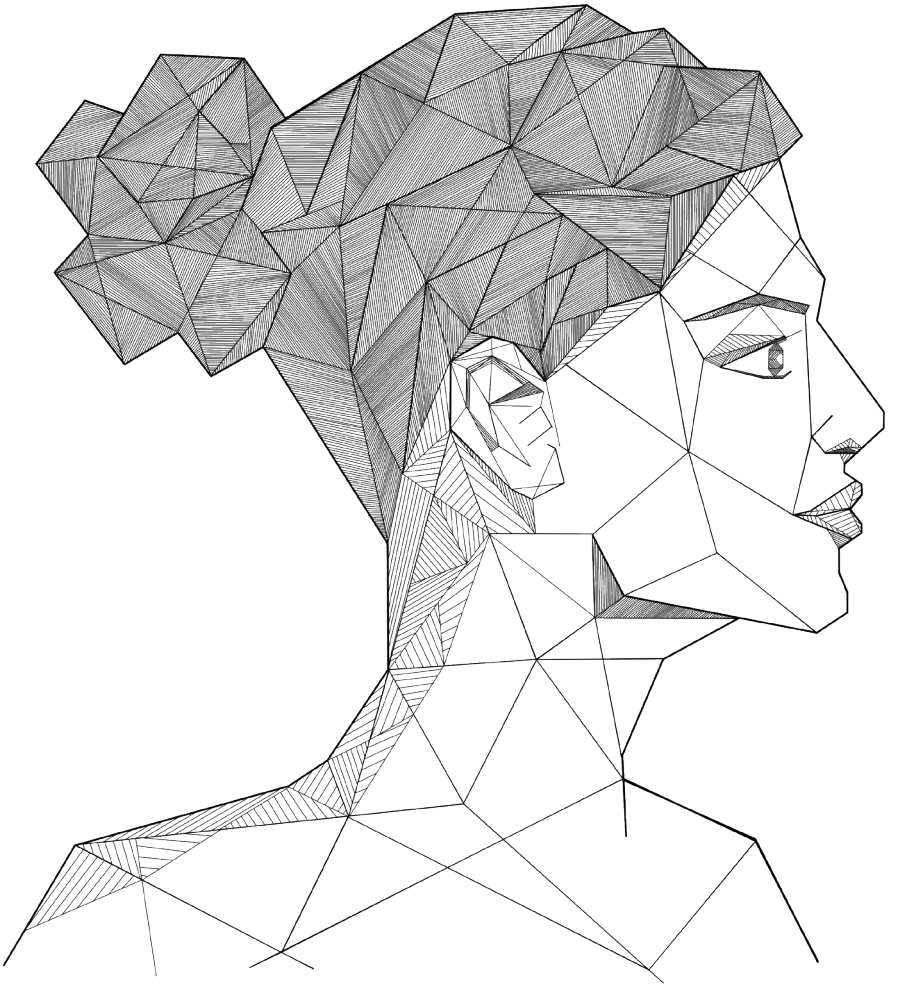

Carin Lunenburg

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You can never cross the ocean
unless you have the courage to lose sight of the shore
-
Christopher Columbus
1451-1506

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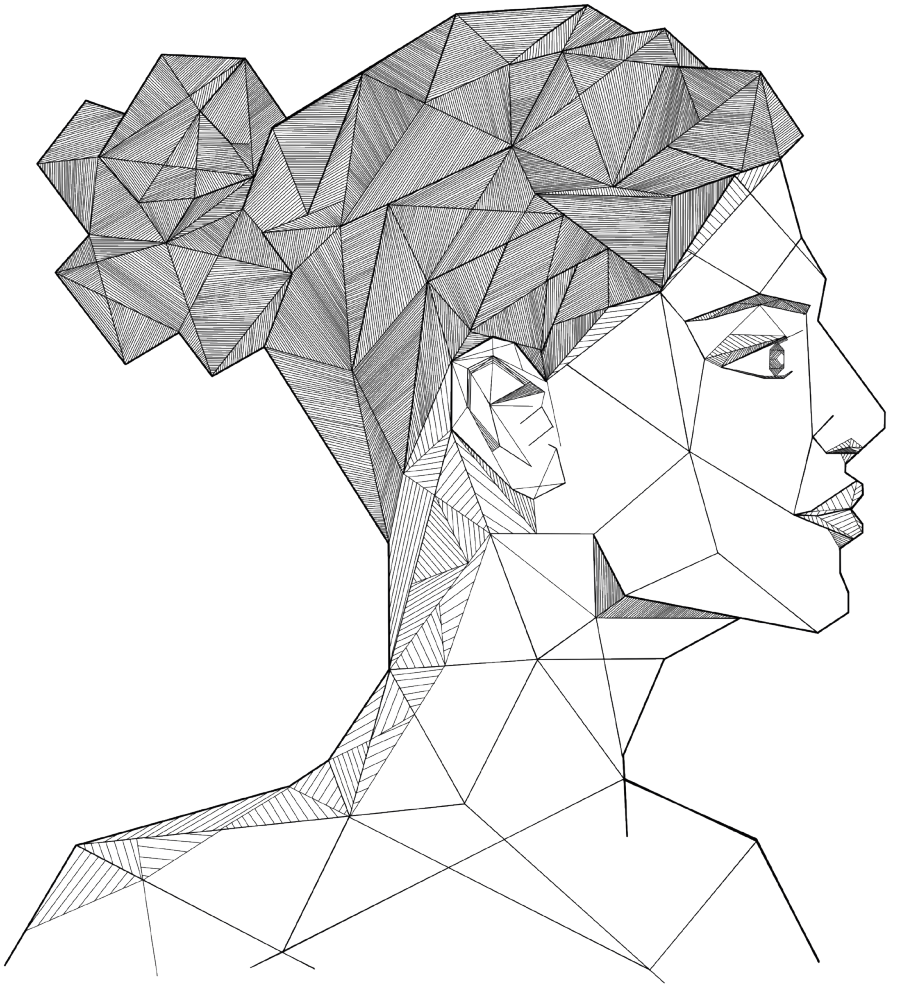

CHAPTER 1

General introduction

Fluoropyrimidines

5-Fluorouracil (5-FU) and capecitabine belong to the group of fluoropyrimidines, which represent the backbone of anti-cancer treatment for various types of cancer, such as colorectal, breast and gastric cancer. Fluoropyrimidines are used by millions of patients worldwide each year[1-3] and are often combined with other chemotherapeutic drugs (e.g. irinotecan or oxaliplatin), immunotherapeutic drugs or act as a radio-sensitizer in chemo-radiotherapy.[4,5]

5-FU was developed by Heidelberger *et al.* in the 1950's.[6] The anti-cancer effect of 5-FU is caused by three active metabolites, as shown in Figure 1. The first is 5-fluoro-2'-deoxyuridine-5'-monophosphate (5-FdUMP), which inhibits the enzyme thymidylate synthase (TS). The inhibition of TS leads to a reduced production of deoxythymidine monophosphate (dTMP), resulting in the inhibition of DNA synthesis and repair. Two other metabolites, fluorouridine triphosphate (FUTP) and fluorodeoxyuridine triphosphate (FdUTP), are incorporated into RNA and DNA, respectively. This results in RNA and DNA damage and ultimately cell death.[7]

In February 2001, European approval and market authorization for Xeloda® (capecitabine) was given, the first oral pro-drug of 5-FU used in the treatment of metastatic colorectal cancer. Besides the advantage of oral administration, capecitabine is also a tumour-specific therapy for colorectal and breast cancer. Thymidine phosphorylase (TP), the third enzyme converting capecitabine into 5-FU, was found to be more expressed in breast and colorectal tumour cells compared to normal tissue. This leads to higher 5-FU levels in tumour cells compared to plasma, and thus a higher anti-cancer effect of capecitabine with less toxicity.[8-10]

5-FU has a relatively narrow therapeutic index and, depending on the type of treatment regimen, up to 30% of patients suffer from severe toxicity such as diarrhoea, nausea, (oral) mucositis, myelosuppression and hand-foot syndrome (HFS). These side-effects can lead to mortality in approximately 1% of patients.[11,12] Toxicity is classified using the common terminology criteria for adverse events (CTC-AE) and grades 3 and higher are considered severe toxicity (range 0–5).

Dihydropyrimidine dehydrogenase

The enzyme dihydropyrimidine dehydrogenase (DPD) plays a key role in the metabolism of 5-FU. It is the rate limiting enzyme degrading over 80% of the drug into the inactive metabolite 5-fluoro-5,6-dihydrouracil (DHFU). Because of this, DPD plays an important role in the development of toxicity.[13-16] DPD is mainly expressed in the liver, but also in other tissues.[17,18] DPD shows great interpatient and intrapatient variability, is influenced by circadian rhythm[19,20] and possibly gender.[21-24] Some patients are partially DPD deficient (incidence 3–8%) or completely DPD deficient (incidence 0.2%).[23-25] DPD deficient patients have higher levels of active 5-FU metabolites and therefore an increased risk to develop severe or even fatal fluoropyrimidine-induced toxicity.[26-28] In addition, the onset of toxicity occurs faster in DPD deficient patients compared to patients with a normal DPD enzyme activity.[28] Up to 60% of the patients who experienced severe fluoropyrimidine-induced toxicity were DPD deficient.[21,22,27,28]

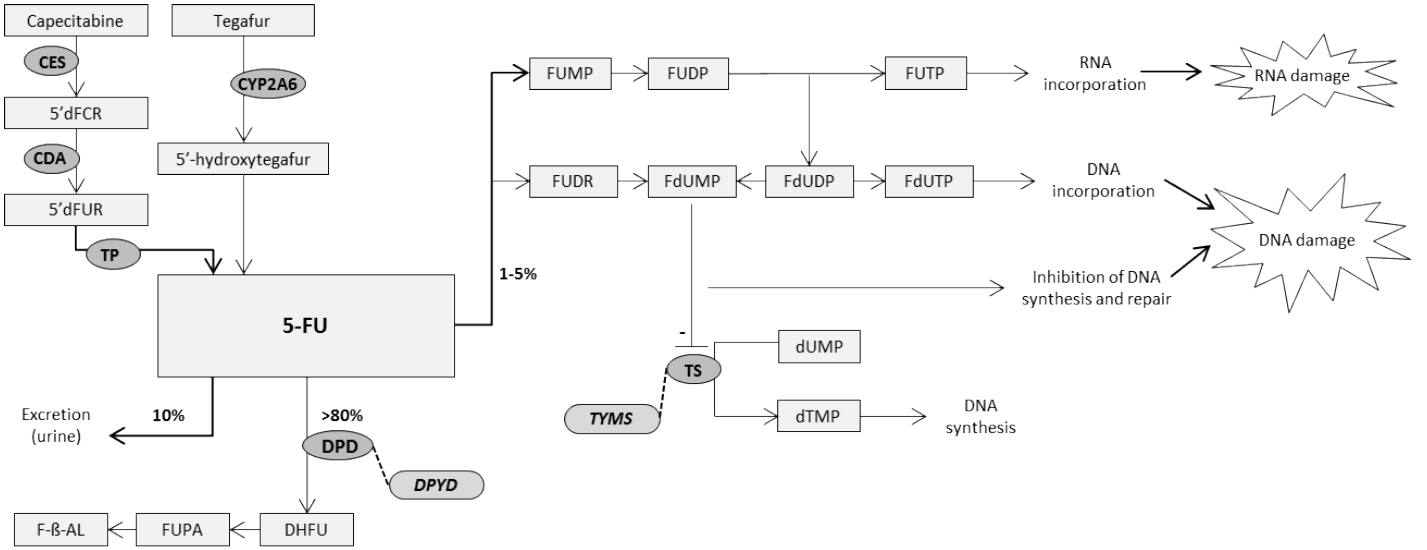

Figure 1. Metabolic pathway of fluoropyrimidines

Abbreviations: CES: carboxylesterase; 5'dFCR: 5'-deoxy-5-fluorocytidine; CDA: cytidine deaminase; 5'dFUR: 5'-deoxy-5-fluorouridine; TP: thymidine phosphorylase; 5-FU: 5-fluorouracil; FUMP: fluorouridine monophosphate; FUDP: fluorouridine diphosphate; FUTP: fluorouridine triphosphate; RNA: ribonucleic acid; FUDR: fluorodeoxyuridine; FdUMP: fluorodeoxyuridine monophosphate; FdUDP: fluorodeoxyuridine diphosphate; FdUTP: fluorodeoxyuridine triphosphate; DNA: deoxyribonucleic acid; TS: thymidylate synthase; *TYMS*: gene encoding TS; dUMP: deoxyuridine monophosphate; dTMP: deoxythymidine monophosphate; DPD: dihydropyrimidine dehydrogenase; *DPYD*: gene encoding DPD; DHFU: 5,6-dihydrofluorouracil; FUPA: fluoro-ß-ureidopropionate; F-ß-AL: fluoro-ß-alanine.

Personalised medicine

In order to prevent severe fluoropyrimidine-induced toxicity, interpatient differences must be overcome and treatments must be individualized (personalised medicine). As DPD is an important factor for the onset of severe fluoropyrimidine-induced toxicity, DPD deficient patients are an interesting target for personalised medicine. Yet, DPD deficient patients generally do not show specific phenotypic features and must be identified otherwise. One way to use personalised medicine, is through pharmacogenetics or pharmacogenomics (PGx). In PGx, the influence of human genetic variation in drug metabolic pathways or molecular drug targets on drug therapy response (both efficacy as toxicity) is studied.

DPD is encoded by the *DPYD* gene, which consists of 26 exons and is located on chromosome 1p21.3.[29,30] Over 1,000 variants or single nucleotide polymorphisms (SNPs) are known in *DPYD*, some leading to altered DPD enzyme activity.[31-33] A well-known example is the variant *DPYD**2A, which is located at the intron downstream of exon 14. This point mutation at a splice donor site leads to skipping of exon 14 and results in a catalytically inactive enzyme.[34]

Heterozygous carriers of *DPYD**2A are partially DPD deficient. Of four variants (*DPYD**2A, rs3918290, c.1905+1G>A, IVS14+1G>A; *DPYD**13, rs55886062, c.1679T>G, I560S; c.2846A>T, rs67376798, D949V; c.1236G>A/HapB3, rs56038477, E412E) sufficient evidence has been provided showing the association with severe fluoropyrimidine-induced toxicity.[13,35-41] Other *DPYD* variants have been described, however evidence on the association with toxicity is limited or missing.

Previously, Deenen *et al.* have shown that prospective genotyping of *DPYD**2A, followed by initial dose reductions in heterozygous carriers, resulted in a reduction of severe fluoropyrimidine-induced toxicity in these patients.[42] In this study, 28% of the *DPYD**2A variant allele carriers treated with reduced dosages experienced severe fluoropyrimidine-induced toxicity compared to 73% of *DPYD**2A variant allele carriers treated with regular dosages in a historic cohort. The risk of toxicity for *DPYD**2A variant allele carriers was reduced to the wild-type level of 23%. Efficacy of the treatment was not expected to be reduced, as exposure to active metabolites of 5-FU were similar in *DPYD**2A variant allele carriers treated with a reduced dose and wild-types. In addition, the study showed that prospective screening was feasible and did not increase costs.

Over time, genotyping in general has become very attractive for routine diagnostics, with decreasing costs of the assays and better interpretation of the data. Yet, implementation of prospective *DPYD* genotyping remained limited for a substantial period, as evidence of its effectivity from a randomized clinical trial (RCT) was lacking.

Aim and outline of this thesis

The general aim of this thesis is to study how to further reduce severe fluoropyrimidine-induced toxicity, in addition to genotyping of *DPYD**2A, while keeping aspects of implementation of any method in clinical practice in mind.

The first part of the thesis is entitled "*DPYD* genotyping: proof of principle and implementation in clinical practice". In chapter 2 we present a review, in which we summarize the evidence on the association with severe fluoropyrimidine-induced toxicity for four *DPYD*

variants. In addition, we discuss the advantages and disadvantages of *DPYD* genotyping.[43] In chapter 3, literature is extensively checked to discuss the effect of four *DPYD* variants on DPD enzyme activity. This is converted into a gene activity score for each *DPYD* variant, which will be used in PGx guidelines to translate the *DPYD* genotype into a DPD phenotype.[44] Chapter 4 contains the Dutch Pharmacogenetics Working Group (DPWG) PGx guideline for *DPYD* and fluoropyrimidines. The guideline provides a dose reduction advice for heterozygous *DPYD* variant allele carriers of *DPYD**2A, *DPYD**13, c.2846A>T and c.1236G>A. In addition, a statement is made that *DPYD* genotyping should be performed for all patients prior to treatment with fluoropyrimidines, as the clinical implication score for *DPYD* is essential. Then, in chapter 5, *DPYD* genotyping is applied prospectively in a nationwide clinical trial.[45] Patients with an intention to treatment with fluoropyrimidines are genotyped for *DPYD**2A, *DPYD**13, c.2846A>T and c.1236G>A. Heterozygous carriers are treated with an initially reduced dose of fluoropyrimidines according to the DPWG PGx guidelines at the start of the study. The goal of the study is to show that *DPYD* genotyping improves patient safety. In chapter 6 we show a cost analysis of prospective *DPYD* genotyping of four *DPYD* variants.[46] In chapter 7, we look into severe toxicity in patients who receive fluoropyrimidines as part of chemoradiation therapy.[47] Fluoropyrimidine dosages in chemoradiation therapy are substantially lower compared to fluoropyrimidine dosages in other treatment regimens. Current PGx guidelines do not distinguish fluoropyrimidine dosing recommendations between treatment regimens. Therefore, in this chapter we compare severe toxicity between wild-type patients and *DPYD* variant allele carriers, either treated with standard or reduced fluoropyrimidine dosages, who receive chemoradiation therapy. In chapter 8, the first 21 months of implementation of *DPYD* genotyping at Leiden University Medical Center is evaluated, to study the feasibility of *DPYD* genotyping in daily clinical care.[48] Clinical acceptance of *DPYD* genotyping as well as adherence to the genotyping results are the main objectives of this study. In chapter 9 we look into the aspect of quality control of genotyping in the laboratory, in specific confirmation practice.[49] We use *DPYD* genotyping as an example. We discuss if it should be required to have two independent genotyping assays to correctly determine a genotype. Implementation of *DPYD* genotyping in clinical practice can improve if there is consensus on laboratory requirements.

In the first part of this thesis we describe how to reduce severe fluoropyrimidine-induced toxicity by *DPYD* genotyping of *DPYD**2A, *DPYD**13, c.2846A>T and c.1236G>A. Yet, is it known that not all severe fluoropyrimidine-induced toxicity can be predicted using *DPYD* genotyping of these four variants. Therefore, we investigate other options, beyond genotyping of the current four *DPYD* variants, to reduce severe fluoropyrimidine-induced toxicity. This is shown in the second part of this thesis, entitled “beyond current *DPYD* pharmacogenetics”.

In chapter 10 we investigate four DPD phenotyping assays. The goal of the study is to determine the clinical value of each DPD phenotyping assay, by assessing clinical validity parameters (e.g. sensitivity and specificity) for DPD deficiency and the onset of severe fluoropyrimidine-induced toxicity. In the following chapters, we focus on future application of genetics. In chapter 11 we investigate a special group of *DPYD* variant allele carriers, i.e.

the compound heterozygous patients.[50] These patients carry multiple *DPYD* variants and the effect of the *DPYD* variants on the DPD enzyme activity cannot be predicted using the gene activity score. We determine the prevalence of these patients using several publicly available databases. In addition, we describe a few patient cases and apply additional genotyping assays to determine the location of the *DPYD* variants on the alleles (phasing), in order to determine a gene activity score and predict the DPD phenotype. In chapter 12 we describe a genome-wide association study. It is expected that other enzymes besides DPD, and thus other genes besides *DPYD,* are involved in the onset of severe fluoropyrimidine-induced toxicity. With the genome-wide approach we aim to discover other variants, outside the *DPYD* gene, which are associated to the onset of severe fluoropyrimidine-induced toxicity.

This thesis ends with a general discussion, including future perspectives (chapter 13), followed by an English and Dutch summary (chapter 14).

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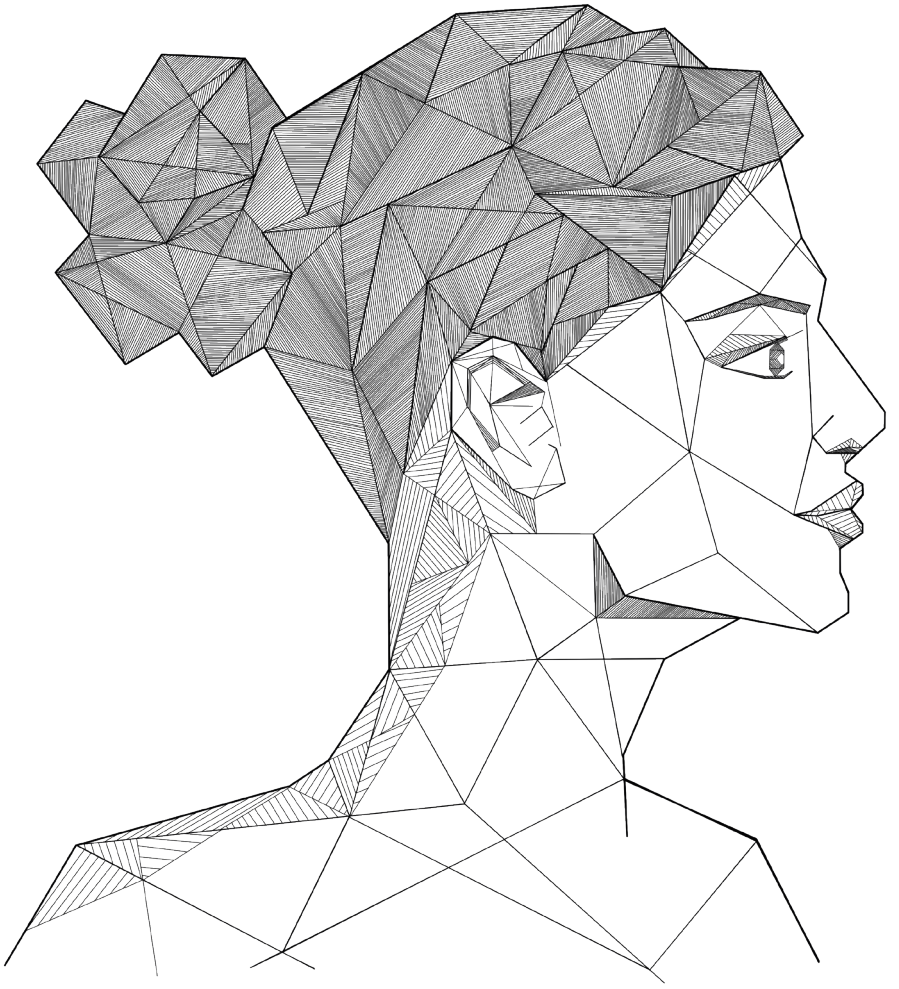
DPYD GENOTYPING: PROOF OF PRINCIPLE AND IMPLEMENTATION IN CLINICAL PRACTICE



CHAPTER 2

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

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Abstract
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 favour of prospective genotyping, including discussion of unjustified worries on cost-effectiveness, and potential underdosing. We conclude that there is convincing evidence to implement prospective *DPYD* genotyping with an upfront dose adjustment in DPD deficient patients. Immediate benefit in patient care can be expected through decreasing toxicity, while maintaining efficacy.

Case: fatal toxicity following treatment with capecitabine

A 52-year-old woman with human epidermal growth factor receptor 2 (HER2)-positive metastasised breast cancer was treated with capecitabine 1,250 mg/m^2 twice daily, for 14 days every three weeks, plus intravenous trastuzumab on day 1. The first cycle was fully completed; at day 18 of treatment mild diarrhoea and a herpes zoster infection located at her mouth were noticed during routine outpatient visit. Due to low haematological laboratory values (leucocytes, neutrophils CTC-AE grade 2, and thrombocytes CTC-AE grade 3), the second cycle was planned to be deferred by one week. However, three days later she returned to the hospital with now severe diarrhoea (CTC-AE 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 hospitalisation 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 diarrhoea, 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 healthcare, 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 summarised in three separate meta-analyses.[8,9,30] Terrazzino *et al.* evaluated 4,094 patients (15 studies) for *DPYD**2A (IVS14+1G>A; rs3918290) and 2,308 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 4,855 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 7,365 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). 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 pre-treatment 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.

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 on-going discussion on whether a randomised 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 favouring upfront genotyping, it may not be ethically feasible to randomise 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 randomised study was performed in France. Boisdron-Celle *et al.* presented a multicentre 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 1,130 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 randomised 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 EGFR therapy was influenced by updated registration texts for epidermal growth factor receptor (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 randomised study.

Table 1. Toxicity associations of *DPYD* variants

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)
Rosmarin *et al.* 2015[4]	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)

table continues

Group	*DPYD* variant	Association with 5-FU and/or capecitabine grade ≥3 toxicity (OR/*RR [95% CI], p-value)
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)

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 are explicitly marked. Rosmarin *et al.* have also tested 5-FU infusion and 5-FU bolus separately. Meulendijks *et al.* have described RR values, not OR values, as shown by *.

Abbreviations: 5-FU: 5-fluorouracil; in: infusion; bo: bolus; cap: capecitabine; CI: confidence interval; OR: odds ratio; RR: relative risk; AF: allele frequency.

Table 2. Test characteristics of genotyping for *DPYD2A and c.2846A>T**

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

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

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 centres in France, Germany, the Netherlands, Ireland and the United States of America (USA).[44,53,60,61] An established and well-recognised *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 centres in France use the 'Onco Drug Personalized Medicine' or ODPM Tox™ and 2,000 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 3–5 toxicity patients and 100% of mortalities.[63]

(Cost) Effectiveness of DPD deficiency testing

A prospective, multicentre study was conducted by Deenen *et al.,* in which 2,038 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 3–5 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 tumour-type specific while oncology guidelines are traditionally tumour-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:

'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.

'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.

'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]

'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 minimises 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.

'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.

'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 1,300 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]

Summary

Although pharmacogenomics in general has the potential to result in safer use of drugs by supporting individualised 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 pharmacogenomic 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.

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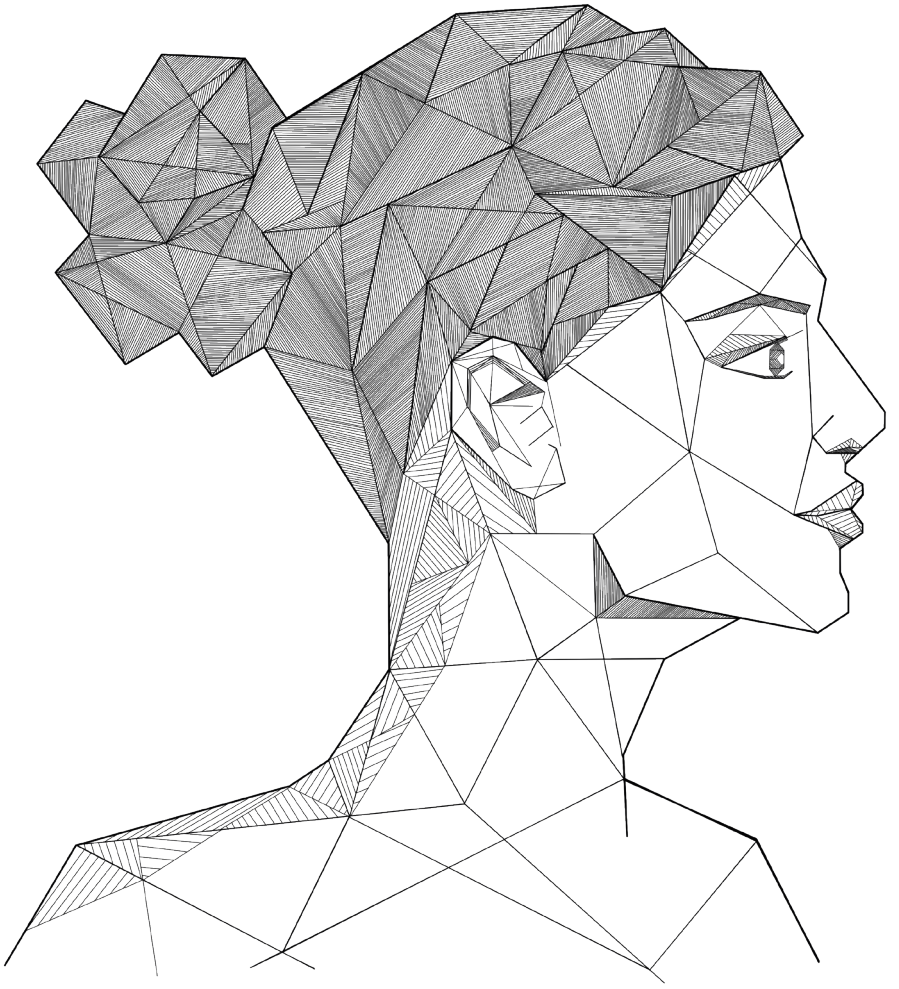

CHAPTER 3

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

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Abstract
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 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.

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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 nonfunctional 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 nonfunctional 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 and recommendations

According to the US FDA and 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, European Society for Medical Oncology and National Comprehensive Cancer Network 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 and 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 nonfunctionality 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 nonfunctional 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 as 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 nonfunctional 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—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.[18] 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 (author's unpublished data). 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 ($p=2x10^{-5}$) 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 nonfunctional 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.[57] 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,[58] 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 to 100 ng/ml for U and 10 to 1000 ng/ml for DHU. Genotyping of *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.

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.[59] 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).[60] 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.[60] 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 nonactive 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.

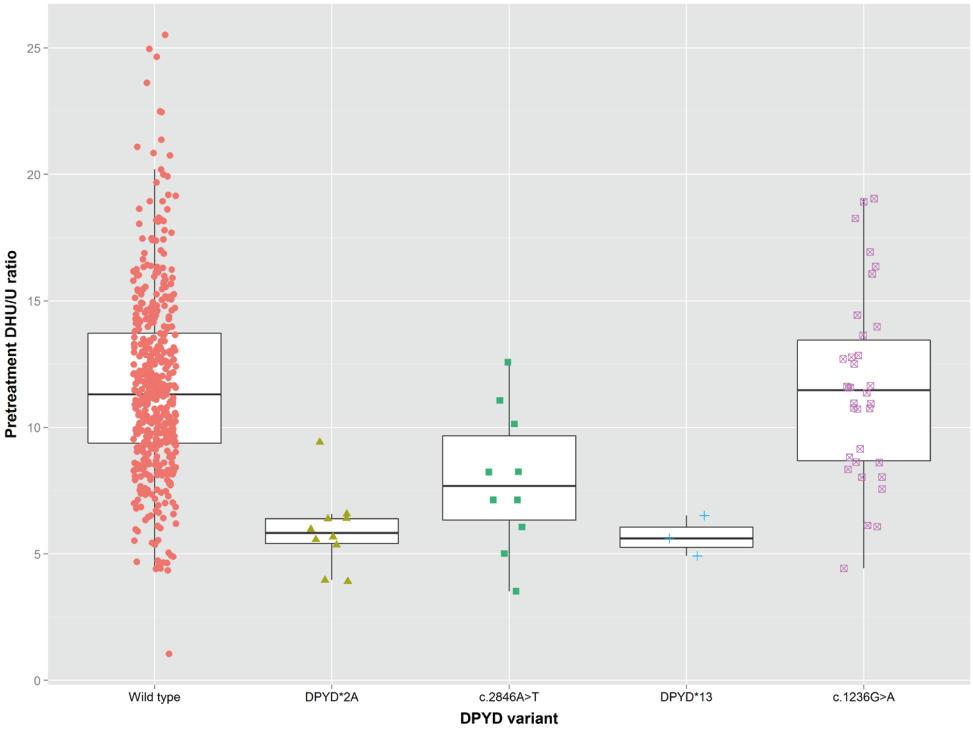

Figure 1. DHU/U ratio according to *DPYD* genotype

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

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. Patients heterozygous for *DPYD**2A or *DPYD**13 have one nonfunctional 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.

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

Activity Value	Alleles	Ref.
0	*DPYD**2A (rs3918290)	4,8,10,11,19,27,29-46
	*DPYD**13 (rs55886062)	4,19,30,32,33,46,49,50
0.5	c.2846A>T (rs67376798)	4,13,24,33,36,41,42,44,47
	c.1236G>A/HapB3 (rs556038477)	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%

Discussion and 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 an ~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 to 19 variants are expected to result in DPD deficiency[61,62]. 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.[63] 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 to 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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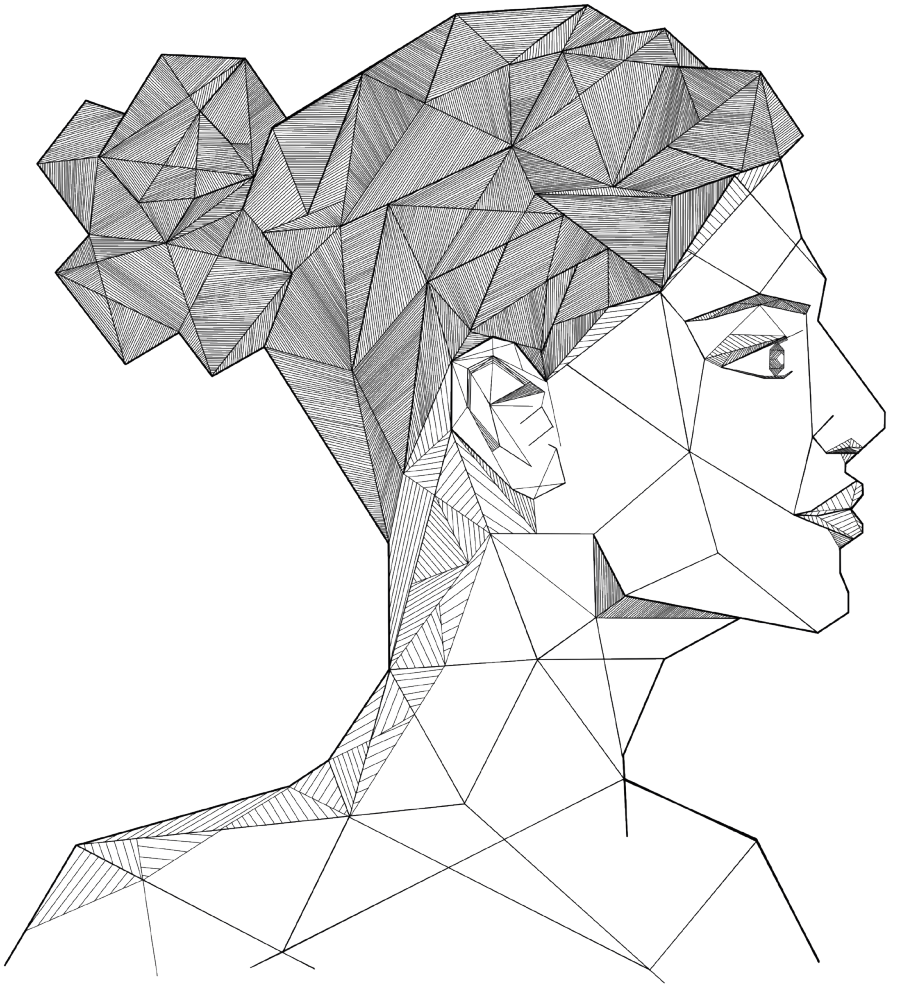

CHAPTER 4

Dutch Pharmacogenetics Working Group (DPWG) guideline for the gene-drug interaction of *DPYD* and fluoropyrimidines

Submitted (under review)

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Abstract
Despite advances in the field of pharmacogenetics (PGx), clinical acceptance has remained limited. The Dutch Pharmacogenetics Working Group (DPWG) aims to facilitate PGx implementation by developing evidence-based pharmacogenetics guidelines to optimize pharmacotherapy. This guideline describes the starting dose optimization of three anti-cancer drugs (fluoropyrimidines: 5-fluorouracil, capecitabine and tegafur) to decrease the risk of severe, potentially fatal, toxicity; such as diarrhoea, hand-foot syndrome, mucositis or myelosuppression. Dihydropyrimidine dehydrogenase enzyme (DPD) deficiency (encoded by the *DPYD* gene) increases risk of fluoropyrimidine-induced toxicity. The *DPYD*-gene activity score, determined by four *DPYD* variants, predicts DPD activity and can be used to optimize an individual's starting dose. The gene activity score ranges from 0 (no DPD activity) to 2 (normal DPD activity). Subjects with a gene activity score of 0 are recommended not to initiate fluoropyrimidines. Alternatively, DPD activity may be determined to adjust the dose accordingly. Subjects with a gene activity score of 0.5, 1 or 1.5 are recommended to initiate therapy with 25%, 50% or 75% of the normal dose of 5-fluorouracil or capecitabine, respectively. When initiating tegafur, an alternative chemotherapeutic agent, or a low dose is recommended. Dose may be increased in subsequent cycles in patients experiencing no or clinically tolerable toxicity. Subjects with a gene activity score of 2 (reference) should receive a normal dose. In case it is not possible to calculate the gene activity score based on *DPYD* genotype, we recommend to determine the DPD activity. Based on the DPWG clinical implication score, *DPYD* genotyping is considered "essential", therefore directing *DPYD* testing prior to initiating treatment with fluoropyrimidines.

Disclaimer
The Pharmacogenetics Working Group of the KNMP (DPWG) formulates the optimal recommendations for each phenotype group based on the available evidence. If this optimal recommendation cannot be followed due to practical restrictions, e.g. therapeutic drug monitoring or a lower dose is not available, then the health care professional should consider the next best option.

Introduction

The role of heritable genetic variation on drug response is referred to as pharmacogenetics (PGx). Germline mutations in pharmacogenetic loci can predict phenotypic differences in drug response and can be used to guide dose and drug selection to achieve safer and more (cost)effective pharmacotherapy. PGx guided pharmacotherapy is one of the first clinical applications of genomics in medicine. Despite scientific and clinical advances in the field of PGx, clinical adoption has remained limited. Barriers preventing implementation have been previously reported.[1] Some of these barriers have been overcome in the past years. One of these barriers was the lack of clear guidelines on how to interpret and apply PGx test results.

The Royal Dutch Pharmacists Association (KNMP) established the Dutch Pharmacogenetics Working Group (DPWG) in 2005 to overcome this barrier.[2] The main objectives of the DPWG are 1) to develop PGx informed therapeutic recommendations based on systematic literature review, and 2) to assist physicians and pharmacists by integrating the recommendations into computerized systems for drug prescription, dispensing, and automated medication surveillance. This manuscript thus provides both the content required for enabling local translation of assay results into the predicted phenotype (in this case the gene activity score) and for programming therapeutic recommendations into local clinical decision support systems. With the objective of implementing PGx into routine care, the DPWG has additionally developed the clinical implication score, which is given to every gene-drug interaction. The aim of this score is to direct clinicians on whether or not to order relevant PGx genotyping tests before initiating therapy. Recently, the DPWG guidelines were endorsed by the European Association of Clinical Pharmacology and Therapeutics (EACPT) and the European Association of Hospital Pharmacists (EAHP).[3,4]Other initiatives such as the Clinical Pharmacogenetics Implementation Consortium (CPIC) were also established to support clinical implementation.[5,6]

The DPWG is a multidisciplinary group in which (clinical) pharmacists, physicians, clinical pharmacologists, clinical chemists and epidemiologists are represented. From 2005 onwards, the DPWG has systematically executed 90 risk analyses for potential gene-drug interactions resulting in 49 guidelines providing therapeutic recommendations for one or more aberrant phenotypes.[7] Available DPWG guidelines and future updates will be published in an effort to provide transparency of their development and to fulfil the public demand for their publication.

This guideline describes the starting dose optimization of three anti-cancer drugs (fluoropyrimidines: 5-fluorouracil, capecitabine and tegafur) to decrease the risk of severe, potentially fatal, toxicity; such as diarrhoea, hand-foot syndrome, mucositis or myelosuppression. Dihydropyrimidine dehydrogenase enzyme (DPD) deficiency (which is encoded by the *DPYD* gene) increases the risk of fluoropyrimidine-induced toxicity. The gene activity score is currently based on the results of four *DPYD* variants, predicts DPD enzyme activity and is used to optimize an individual's starting dose. The gene activity score ranges from 0 (no DPD activity) to 2 (normal DPD activity). This manuscript provides an overview of the guideline development and summarizes the pharmacotherapeutic recommendations. Additionally, a comparison to alternative guidelines is presented. The *gene-drug interaction* section includes background on the pharmacological mechanism of the interaction. In

addition it also includes a list of the *DPYD* variants associated with toxicity and the method developed by DPWG for local translation of assay results into the gene activity score. This information may be useful for laboratories to select and design a *DPYD* genotyping assay and subsequently determine the patients' predicted phenotype based on the genotype results. Consequently, the literature review supporting the *DPYD*-fluoropyrimidine interaction is described and the DPWG guideline is presented. A summary of all references identified by the systematic review which were subsequently used to develop this guideline, can be found in Supplementary Tables 1 and 2. The recommendations provided in this manuscript can be used in combination with a patients' predicted phenotype to optimize starting dose of fluoropyrimidines, thereby decreasing the risk of severe and potentially fatal toxicity.

Drugs: fluoropyrimidines (5-fluorouracil, capecitabine and tegafur with DPD-inhibitors)

Fluoropyrimidines are antimetabolite drugs widely used in the treatment of colorectal, breast, stomach and skin cancer. Each year, over two million patients worldwide receive treatment with fluoropyrimidines. This includes 5-FU and its oral pro-drugs capecitabine and tegafur. Up to 30% of patients experience severe toxicity (common terminology criteria for adverse events, CTC-AE, grade ≥3), including diarrhoea, hand-foot syndrome, mucositis and myelosuppression. For ~1% of patients toxicity is fatal.[8,9] Toxicity may occur within the first treatment cycle (early onset), supporting the importance of optimizing the starting dose of fluoropyrimidine pharmacotherapy on a personalized basis, before initiating therapy.[10]

Capecitabine is metabolised into 5-FU in three consecutive steps. Capecitabine is firstly metabolised to 5'-deoxy-5-fluorocytidine (5'-DFCR) by carboxylesterase, subsequently, 5'-DFCR is converted into 5'-deoxy-5-fluorouridine (5'-DFUR) by cytidine deaminase, and to 5-FU by thymidine phosphorylase. 5-FU is metabolised in tissues to 5-fluoro-2'-deoxyuridine and then to 5-fluoro-2'-deoxyuridine-5'-monophosphate, the active metabolite of the drug. The active metabolite inhibits the enzyme thymidylate synthase, resulting in inhibition of DNA synthesis and repair, inducing cell apoptosis and thus, its effect. Additionally, toxic effects resulting from partial incorporation of 5-FU and its metabolites in DNA and RNA contribute to the drug's mechanism of action.[11]

Tegafur is metabolised into 5-FU and into the less cytotoxic metabolites 3-hydroxytegafur, 4-hydroxytegafur and dihydrotegafur by *CYP2A6*. The less toxic metabolites are renally cleared. Tegafur was combined with the DPD inhibitor uracil and is now combined with the DPD inhibitor gimeracil and the orotate phoshoribosyltransferase (OPRT) inhibitor oteracil. Oteracil diminishes the activity of 5-FU in normal gastrointestinal mucosa. The DPD inhibitors diminish the formation of functionally inactive metabolites of 5-FU that contribute to adverse events like stomatitis and mucositis. Both uracil and gimeracil inhibit DPD activity reversibly and have a shorter elimination half-life and thus shorter period of action than tegafur. For this reason, genetic variants influencing DPD enzyme activity are clinically relevant for tegafur in combination with DPD inhibitors.

Gene: dihydropyrimidine dehydrogenase (*DPYD*)

The *DPYD* gene encodes the enzyme DPD. *DPYD* is located on chromosome 1p21.3, and transcription variant 1 (NM_000110.3) has 26 exons, spanning approximately 900 kb.[12]

Over 160 different allele variants in *DPYD* have been identified and described in literature.[13] According to the gnomAD browser,[14] which contains whole exome data of almost 140,000 individuals, *DPYD* contains 2,190 known variants. The prevalence of individual variants is low. The effect of genetic variation on DPD enzyme activity is not fully established for the majority of variants and the size of the effect can differ between variants.

The frequency of the various *DPYD* variants and the associated phenotypes appears to vary significantly between nations and ethnic groups. For example, in the Caucasian population, approximately 3—5% has a partial DPD enzyme deficiency and 0.1—0.2% has a complete DPD enzyme deficiency. On the other hand, approximately 8% of the African American population has a partial DPD enzyme deficiency.[15,16]

Gene-drug interaction

Pharmacological mechanism

A schematic overview of fluoropyrimidine metabolism is shown in Figure 1. The DPD enzyme is mainly found in liver, but also intestinal mucosa, leucocytes, tumour cells and other tissues. Over 80% of 5-FU is inactivated to 5-fluoro-5,6-dihydrouracil (DHFU) by DPD. The decreased metabolic activity of DPD leads to increased intracellular concentrations of active metabolites of 5-FU.[17] The increased intracellular concentration of 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP) increases the risk of toxicity such as diarrhoea, hand-foot syndrome, mucositis and myelosuppression. Variants in the *DPYD* gene can result in reduced or even absent DPD enzyme activity, increasing the risk of severe toxicity. For example, 73% of the patients with *DPYD**2A experienced severe toxicity when treated with a full dose, compared to 23% of *1 allele carriers (wild-type patients) who experienced toxicity.[18] Many enzymes are involved in fluoropyrimidine metabolism, however, this guideline is limited to the role of the DPD enzyme in causing toxicity.

Since the genetic variation in *DPYD* only partially determines DPD enzyme activity, these guidelines for dose adjustment based on the predicted phenotype are no more than a tool that can be used to achieve the desired intracellular concentration of the active metabolite, to minimize risk of toxicity. The absence of tested variants does not eliminate the risk of toxicity. Pharmacokinetic dose adjustment (guided by steady-state plasma concentrations or AUC) may also be useful to optimize the dose of 5-FU. This is, however, currently not routinely used for capecitabine and tegafur, as they are mainly converted into 5-FU within tissue.

DPYD variants associated with toxicity

The variants known or suspected to have an effect on DPD enzyme activity, are listed in Table 1. These variants are mapped by the level of evidence for which association with toxicity has been established (columns) and the variant's effect on DPD enzyme activity (rows). Novel variants in *DPYD* will continue to be identified with the introduction of next generation sequencing techniques to clinical practice. However, in order for these variants to be included in Table 1, sufficient evidence regarding the effect on enzyme function or the onset of toxicity must be investigated, possibly by using the *DPYD*-Varifier[19] or by phenotyping patients who carry a novel variant. An update of this guideline will be published when a

renewed recommendation is given following newly published articles.

Translation of genotype to predicted phenotype

The DPWG has concluded that four variants have sufficient evidence to be implemented into clinical care: *DPYD**2A (IVS14+1G>A), *DPYD**13 (c.1679T>G), c.2846A>T and c.1236G>A (in linkage disequillibrium with c.1129-5923C>G). The current guideline only reports recommendations for these four variants; no recommendations are provided for other variants in *DPYD* or other genes. The results of this genotyping panel can be used to predict a patient's phenotype, i.e. the DPD enzyme activity. This predicted DPD activity can be expressed as the *DPYD*-gene activity score, which ranges from 0 (no or virtually no DPD enzyme activity) to 2 (normal DPD enzyme activity due to homozygosity for fully functional alleles, both assigned an activity score 1). The gene activity score is a sum of the two activities of protein isoforms expressed from both alleles. The development of the gene activity score is published elsewhere.[20]

The included variants are those for which substantial and sufficient evidence on the relation to severe toxicity has been established. It is a limitation to restrict to these four variants, as other variants may influence DPD activity as well. However, not all variants having a possible effect on DPD enzyme activity may have been identified yet or evidence for identified variants is insufficient. Therefore, this may result in the incorrect prediction of the DPD enzyme activity. Another limitation is that currently used genotyping methods are unable to determine the allelic location of the variants, but only the dichotomous presence or absence of the variant. This becomes a limitation when two or more different genetic variants are identified in a patient. In this case, either both genetic variants may be on the same allele, resulting in a genotype with one fully functional allele and one reduced functionality allele, or alternatively, both genetic variants may reside on different alleles, resulting in two alleles with inactive or reduced functionality. The latter is more likely to occur. The total gene activity score, however, differs between these cases. When the DPD enzyme activity cannot be predicted correctly, an additional phenotyping test is required to determine the DPD enzyme activity. The relationship between genotype result and predicted phenotype in patients carrying no variants or one or more variants leading to decreased DPD enzyme activity are shown in Supplementary Table 3. The frequency of individuals carrying two or more of four variants considered in the current guideline is rare, but can be assigned a gene activity score. A complete genotype to predicted phenotype translation table can be found in Supplementary Table 4, which can be used to program the translation of genotype results into predicted phenotypes in laboratory information systems.

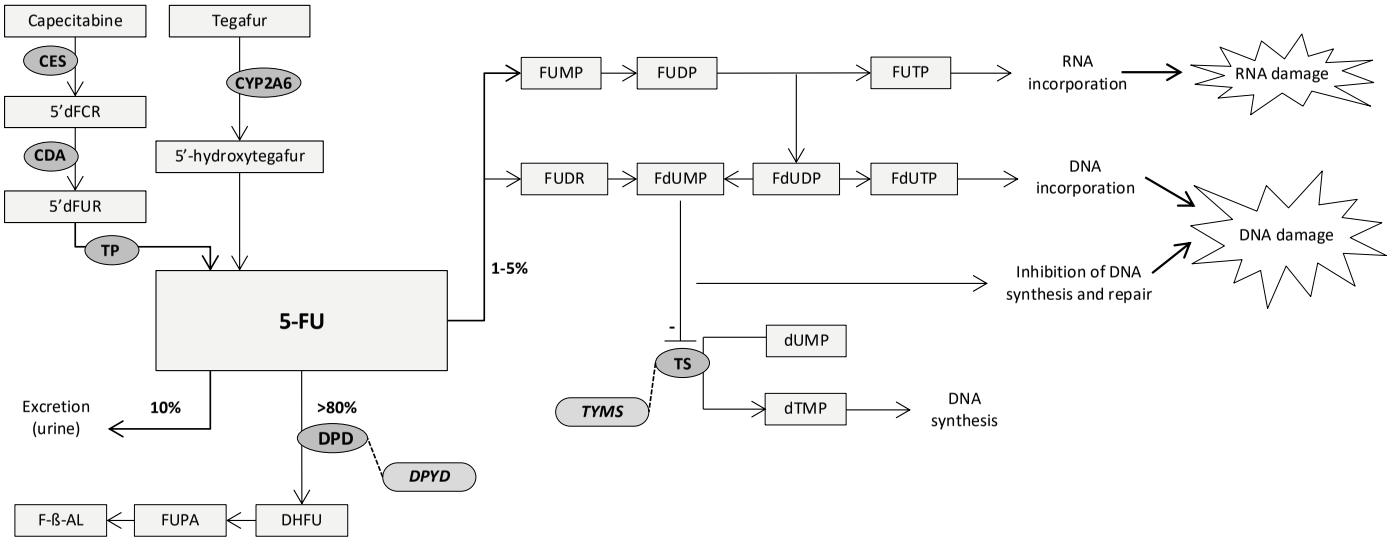

Figure 1. Schematic overview of fluoropyrimidine metabolism
In brief: tegafur, 5FU, capecitabine are metabolised into three major metabolites. FdUMP, which inhibits TS and prevents conversion of dUMP to dTMP, which is necessary for pyrimidine and DNA synthesis. FdUTP is incorporated in DNA, FdUTP is incorporated in RNA, both resulting in cell death.
Abbreviations: CES: carboxylesterase; 5'dFCR: 5'-deoxy-5-fluorocytidine; CDA: cytidine deaminase; 5'dFUR: 5'-deoxy-5-fluorouridine; TP: thymidine phosphorylase; 5-FU: 5-fluorouracil; FUMP: fluorouridine monophosphate; FUDP: fluorouridine diphosphate; FUTP: fluorouridine triphosphate; RNA: ribonucleic acid; FUDR: fluorodeoxyuridine; FdUMP: fluorodeoxyuridine monophosphate; FdUDP: fluorodeoxyuridine diphosphate; FdUTP: fluorodeoxyuridine triphosphate; DNA: deoxyribonucleic acid; TS: thymidylate synthase; *TYMS*: gene encoding TS; dUMP: deoxyuridine monophosphate; dTMP: deoxythymidine monophosphate; DPD: dihydropyrimidine dehydrogenase; *DPYD*: gene encoding DPD; DHFU: 5,6-dihydrofluorouracil; FUPA: fluoro-ß-ureidopropionate; F-ß-AL: fluoro-ß-alanine.

Table 1. Known *DPYD* variants stratified by level of evidence on the association with toxicity and predicted DPD enzyme activity

The variants in this table were selected based on literature in Supplementary Table 1 and 2. However, high allele frequency variants reported only in case reports with fluoropyrimidine toxicity were excluded. For these variants the association with DPD enzyme activity, and resulting severe fluoropyrimidine-induced toxicity, cannot be determined.

Level of evidence / DPD enzyme activity	Sufficient evidence[a]	Insufficient evidence[b]
Fully functional[c]	*DPYD* *4 = c.1601G>A *DPYD* *5 = c.1627A>G *DPYD* *9A = c.85T>C	
Reduced functionality[d]	c.2846A>T c.[1236G>A;1129-5923C>G] (hapB3)[e]	c.496A>G c.1129-15T>C (IVS10-15T>C) *DPYD*6 = c.2194G>A c.1896T>C *DPYD*3 = c.1897delC *DPYD*7 = c.299_302del *DPYD*8 = c.703C>T *DPYD* *9B = c.85T>C(;)c.2657G>A *DPYD* *10 = c.2983G>T *DPYD* *11 = c.1003G>T *DPYD* *12 = c.62G>A c.1156G>T c.1651G>A c.1845G>T
Fully dysfunctional[f]	*DPYD*2A = c.1905+1G>A (IVS14+1G>A) *DPYD*13 = c.1679T>G	c.300C>A[g] c.1024G>A[g] c.1025A>G[g] c.1475C>T[g] c.1774C>T[g] c.(2058_2059)_(2299_2300)dup

[a] DPWG has concluded an association between fully functional variants and no resulting toxicity, and an association between reduced functionality variants or fully dysfunctional variants and association with the onset of severe fluoropyrimidine-induced toxicity;

[b] DPWG has concluded there is insufficient evidence to associate a predicted DPD enzyme activity for these variants and the onset of severe fluoropyrimidine-induced toxicity;

[c] These variants are not included in the prospective *DPYD* genotyping panel, as there is no effect on predicted DPD enzyme activity, and therefore there is no association with the onset of severe fluoropyrimidine-induced toxicity;

[d] The effect of the variant on the protein sequence suggests that the protein may still be partially functional. Therefore residual metabolic DPD capacity may be present;

[e] Variant c.1236G>A, which does not lead to an alternative amino acid, is in complete linkage disequilibrium with variant c.1129-5923C>G, which leads to aberrant splicing in mRNA, which leads to a premature stop codon as a result. The resulting DPD enzyme activity is 50% of the normal activity.

Both variants are part of haplotype B3;
[f] The effect of the variant on the protein sequence suggests that the protein may be fully dysfunctional;
[g] These variants have decreased *in vitro* enzyme activity.
Variants from the table according to multiple nomenclatures (HGVS: NM_000110.3, NP_000101.2 and NC_000001.10):
(rs67376798, c.2846A>T, p.(Asp949Val), g.97547947T>A), (rs56038477, c.1236G>A, p.(Glu412=), g.98039419C>T, in haplotype B3), (rs75017182, c.1129-5923C>G, g.98045449G>C, in haplotype B3), (rs3918290, *2A, c.1905+1G>A, IVS14+1G>A, g.97915614C>G), (rs55886062, *13, c.1679T>G, p.(Ile560Ser), g.97981343A>C), (rs2297595, c.496A>G, p.(Met166Val), g.98165091T>C), (rs56293913, c.1129-15T>C, IVS10-15T>C, g:98039541A>G), (rs1801160, *6, c.2194G>A, p.(Val732Ile), g.97770920C>T), (rs17376848, c.1896T>C, p.(Phe632=), g.97915624A>G), (rs72549303, *3, c.1897delC/c.1898delC, p.(Pro633Glnfs), g.97915622delG), (rs72549309, *7, c.299_298delTCAT, p.(Phe100Serfs), g.98205971_98205974delATGA), (rs1801266, *8, c.703C>T, p.(Arg235Trp), g.98157332G>A), (rs1801265 + rs1801267, *9B, c.85T>C + c.2657G>A, p.(Cys29Arg) + p.(Arg886His), g.98348885G>A+ g.97564154C>T), (rs1801268, *10, c.2983G>T, p.(Val995Phe), g.97544627C>A), (rs72549306, *11, c.1003G>T, p.(Val335Leu), g.98058899C>A), (rs80081766, *12, c.62G>A, p.(Arg21Gln), g.98348908C>T), (rs78060119, c.1156G>T, p.(Glu386Ter), g.98039499C>A), (rs777425216, c.1651G>A, p.(Ala551Ser), g.97981371C>A), (c.1845G>T, p.(Glu615Asp)), (98205969, c.300C>A, (p.Phe100Leu)), (rs183385770, c.1024G>A, p.Asp342Asn, g.98058878C>T), (rs183385770, c.1025A>G, p.Asp342Asn, g.98058878C>T), (rs72549304, c.1475C>T, p.Ser492Leu, g.98015165G>A), (rs59086055, c.1774C>T, p.(Arg592Trp), g.97915746G>A), (g.(619762_619763)_(620801_620802) dup), (rs1801158, *4, c.1601G>A, p.(Ser534Asn), g.97981421C>T), (rs1801159, *5, c.1627A>G, p.(Ile543Val), g.97981395T>C), (rs1801265, *9A, c.85T>C, p.(Cys29Arg), g.98348885G>A).

Additional phenotyping test when genotype is unable to predict phenotype
In contrast to the *DPYD* genotyping test, which aims to predict DPD enzyme activity, a DPD phenotyping test can be performed to measure the actual DPD enzyme activity. Possible methods to perform phenotyping are to measure the DPD enzyme activity in peripheral blood mononuclear cells (PBMCs) or to measure the uracil concentrations in plasma or urine.[21] The average Caucasian DPD enzyme activity is 9.9±0.95 nmol/hour per mg protein.[22] Less commonly performed methods include: 1) the 2-^{13}C-uracil breath test,[23] where $^{13}C0_2$ is measured, which is a product of 2-C^{13}-uracil degradation by DPD and other enzymes involved in the katabolic route of pyrimidines; 2) the quantification of the uracil/dihydrouracil ratio in plasma, where endogenous substrates uracil and dihydrouracil are measured,[24,25] although recently it was shown that uracil levels were superior to the dihydrouracil/uracil ratio as a predictor of severe toxicity;[26] 3) measurement the metabolism of a single dose of uracil.[27] However, all DPD phenotyping tests have their limitations. Currently, the DPD enzyme activity measurements from PBMCs are considered the best developed DPD phenotyping test in The Netherlands.[27,28]

Supporting body of evidence
A detailed description of the methods used for literature collection, assessment and preparation of the gene-drug monograph has previously been published elsewhere.[2,7] In brief,

a systematic review of literature was performed and relevant articles were summarized by a scientist of the Royal Dutch Pharmacists Association (MN). The performed search strategy can be found in Supplementary Material 1. Each article was provided with two scores: 1) quality of evidence and 2) clinical impact. The quality of evidence was scored on a 5-point scale ranging from 0 (lowest; data on file) to 4 (highest; well performed controlled studies or meta-analysis) and the clinical impact of clinical effect was scored on a 7-point scale ranging from AA# (positive effect) to F (highest negative effect). The criteria used to develop these scores have been published in detail previously.[2,7] This clinical impact scale (AA#—F) runs parallel to the common terminology criteria for adverse events (CTC-AE); where CTC-AE grade 5 severity is equal to clinical relevance score F (death) and CTC-AE grade 1 severity is equal to clinical relevance score B. The clinical relevance score additionally includes the scores AA#, AA and A, since these do not exist in the CTC-AE. These regard "Positive clinical effect", "No clinical or kinetic effect", and "Significant kinetic effect or not clinically relevant effect", respectively. The summaries of articles, and their respective scores, reviewed to devise this guideline can be found in the Supplementary Table 1 and 2. The summaries of each article and their respective scores were checked by two independent DPWG members.

For 5-FU/capecitabine, the initial literature search was performed on March 24th 2009, followed by a second search on July 9th 2014. To update this guideline to the current date, an additional literature search was performed on October 19th 2017, resulting in eleven additional papers. Case reports concerning systemic 5-FU or capecitabine therapy were excluded in this literature review, due to a large number of case reports and other available publications of greater evidentiary quality. Kinetic studies from 2009 onwards were only included if the kinetic parameters were given per genotype. Clinical studies were only included if the patient numbers exceeded 500 (from 2009 onwards) or 1,000 (from May 2014 onwards) and the patient numbers with partially functional activity were at least ten or if the study investigated a variant for which no studies were as yet included or if the study investigated the effect of dose adjustment. From 2009, articles investigating the effect of a group containing both polymorphisms known to increase the risk of toxicity and polymorphisms not known to increase the risk of toxicity were not included. If more than one article described data of the same patient group and the same polymorphisms, only the article with data from the largest amount of patients was included.

For tegafur, the initial literature search was performed on August 20th 2009, followed by a second and third search on October 2nd 2012 and July 27th 2015. To update this guideline to current date, an additional literature search was performed on October 19th 2017, resulting in no additional papers.

General conclusion of evidence

In the systematic review performed for 5-FU/capecitabine, 16 of 18 studies and all three meta-analyses found an increased risk of grade ≥3 toxicity (either overall toxicity or at least one specified type of toxicity) for patients carrying variants resulting in reduced DPD enzyme activity (ranging from gene activity score 0 to 1.5). This increased risk was shown separately for patients assigned *DPYD*-gene activity scores 1 and 1.5, but gene activity scores 0 and 0.5 were only investigated when grouped with patients assigned other gene activity

scores. However, the increased risk of toxicity for patients assigned gene activity scores 0 and 0.5 can be concluded based on the confirmed association for gene activity scores 1 and 1.5, where deficiency is less, and is further supported by cases of patients assigned gene activity scores 0 and 0.5 who developed severe toxicity. Only one study investigating clinical outcome concluded there was no effect of variants on risk of toxicity. Based on the systematic review, the DPWG concludes that a gene-drug interaction is present and that DPD enzyme deficiency increases risk of severe toxicity in patients using capecitabine/5-FU. The highest quality of evidence concluding a gene-drug interaction was scored 4.

In the systematic review performed for tegafur with the DPD inhibitor uracil, one case report described four patients who used standard doses and developed severe toxicity. These patients were assigned *DPYD*-gene activity scores 1 and 1.5. Toxicity (CTC-AE grade 4) was similar to that reported in patients treated with 5-FU or capecitabine, both of which are given without a DPD inhibitor. There were no data available for patients assigned *DPYD*-gene activity score 0 or 0.5, however the increased risk of toxicity among these patients can be concluded based on the confirmed association with toxicity for gene activity scores 1 and 1.5, where deficiency is less. Based on the systematic review, the DPWG concludes that there is a clinically relevant gene-drug interaction present and that DPD enzyme deficiency increases risk of severe toxicity in patients using tegafur with DPD inhibitors. The highest quality of evidence concluding a gene-drug interaction was scored 2.

Pharmacotherapeutic recommendations

The DPWG therapeutic recommendation using a patient's pre-therapeutic PGx test result to optimize starting dose of 5-FU/capecitabine and tegafur with DPD inhibitors is summarized in Supplementary Table 5 and 6, respectively.

In brief, when initiating 5-FU, capecitabine or tegafur pharmacotherapy, a gene activity score of 0 recommends choosing an alternative chemotherapy or determining the residual DPD enzyme activity and adjusting the fluoropyrimidine starting dose accordingly. When initiating 5-FU or capecitabine, a gene activity score of 0.5, 1 or 1.5 recommends a starting dose of 25%, 50% or 75%, respectively. Further titration of the dose is possible, guided by toxicity. When initiating tegafur, a gene activity score of 0.5, 1 or 1.5 recommends choosing an alternative chemotherapy or starting with a lower dose and titrating dose based upon toxicity. A gene activity score of 2 (reference value) does not result in a recommendation for dose adaptation for 5-FU, capecitabine or tegafur. If genotype results cannot predict the gene activity score correctly, for example due to multiple identified variants, it is advised to determine the DPD enzyme activity to define an initial starting dose.

Where possible, dose adjustments have been calculated based on 5-FU clearance or AUC after administration of 5-FU or capecitabine. Data were also extrapolated to tegafur with DPD inhibitor, as this compound also follows the same catabolic and anabolic routes after conversion to 5-FU after clearance of the DPD inhibitor from the body. Data on 5-FU clearance are only available for patients carrying *DPYD**1/*DPYD**2A, *DPYD**1/c.2846A>T and *DPYD**2A/c.2846A>T. There are data from one patient with *DPYD**1/*DPYD**13 who developed severe toxicity after 5-FU use, from one patient with c.2846A>T/c.2846A>T and from one patient with c.1236G>A/c.2846A>T.

See Supplementary Table 7 and 8 for an overview of suggested pop-up texts for electronic prescribing systems for pharmacists and physicians. These can be used to program alerts into the clinical decision support system (CDSS). Spanish, Greek, Italian, German, Slovenian and Dutch translations of both the guidelines and background information are available on PharmGKB.org.

Implications for clinical practice

There is currently an ongoing debate regarding whether and which single-drug gene pairs should be implemented into routine care. Points of debate include the amount of evidence that is necessary supporting effectiveness of pre-emptive genotyping, the cost-effectiveness of the intervention and reimbursement of PGx testing.[29,30] This inconclusive debate seems to have hampered implementation of drug-gene pairs which seem ready for implementation.[1,31] In an effort to overcome this inconclusiveness and to direct clinicians on whether or not to order relevant PGx genotyping tests before initiating therapy, the DPWG has developed the clinical implication score. The pre-emptive PGx results for a certain drug-gene pair can be scored as: essential, beneficial, potentially beneficial or not required. The development of these categories and the systematic scoring criteria are discussed elsewhere.[32] In brief, the implications for clinical practice are based on a list of four criteria regarding the following: the clinical effect associated with the gene-drug interaction, the level of evidence supporting the clinical effect, the effectiveness of the intervention in preventing the clinical effect (which includes the number needed to genotype) and the PGx information included in the drug-label. The scores provided for each of these criteria by the DPWG can be found in Supplementary Table 9.

As a result, the DPWG has concluded the clinical implication score of *DPYD*-fluoropyrimidines to be "essential". This score dictates that *DPYD* genotyping prior to treatment must be performed for all patients initially being prescribed therapy with 5-FU, capecitabine or tegafur with DPD inhibitors, to optimize the initial dose and to prevent potentially fatal toxicity.

Differences between available guidelines

Other guidelines regarding the gene-drug interaction of *DPYD* and fluoropyrimidines have been developed. To the best of our knowledge, guidelines are available from CPIC,[11,33] French (French Network of Pharmacogenetics, RNPGx)[34] and Italian (Associazione Italiana di Oncologia Medica, AIOM-SIF) [unpublished guidelines, *edited by the AIOM-SIF Working Group*] initiatives. We have compared the DPWG guidelines to other available guidelines published in English. This regards only the CPIC guideline, since the French and Italian guidelines are unpublished or not in English.

CPIC

Differences between CPIC and DPWG methodology, genotype to phenotype conversion and recommendations have previously been described in detail.[6] However, both guidelines have been updated.[33,35] The current DPWG and CPIC guidelines[5] for *DPYD*/fluoropyrimidines differ regarding the therapeutic recommendations. In contrast to CPIC, DPWG distinguishes

between 5-FU/capecitabine and tegafur within the therapeutic recommendations for fluoropyrimidines, where the CPIC guideline does not provide any dosing recommendations for tegafur due to the limited available evidence. DPWG also further distinguishes between systemic and cutaneous routes of administration within the 5-FU/capecitabine recommendations. The therapeutic recommendations for 5-FU/capecitabine also differ regarding the following: 1) For patients with gene activity score 0: DPWG recommends phenotyping while CPIC does not when no alternative is available. 2) For patients with gene activity score 0.5: DPWG recommends initiating therapy with 25% of standard dose or an alternative whereas CPIC recommends an alternative or a strongly reduced dose with therapeutic drug monitoring, but does not provide an absolute percentage. 3) For patients with gene activity score 1.5 the DPWG recommends a 75% standard dose whereas CPIC recommends a 50% of standard dose.

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SUPPLEMENT CHAPTER 4

Dutch Pharmacogenetics Working Group (DPWG) guideline for the gene-drug interaction of *DPYD* and fluoropyrimidines

Submitted (under review)

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Supplementary Material
Search terms used to perform the literature review of *DPYD*-[5-FU/capecitabine/tegafur] interactions.

Search strategy
Pubmed was used to search English, Dutch, German articles were accepted. Keywords were the drugs of interest (fluorouracil, capecitabine and tegafur/S1), the gene and variations (*DPYD*, DPD, dihydropyrimidine dehydrogenase), and others (e.g. metabolizer, pharmacogenetics, polymorphism). The complete search string was;

Fluorouracil and capecitabine
Search performed in 2009: ("Fluorouracil"[Mesh] OR fluorouracil) AND ("Dihydropyrimidine Dehydrogenase Deficiency"[Mesh] OR metabolizer OR metaboliser OR polymorph* OR "Polymorphism, Genetic"[MeSH] OR "Pharmacogenetics"[MeSH]) AND (English[lang] OR German[lang] OR Dutch[lang])

("capecitabine "[Substance Name] OR capecitabine) AND ("Dihydropyrimidine Dehydrogenase Deficiency"[Mesh] OR metabolizer OR metaboliser OR polymorph* OR "Polymorphism, Genetic"[MeSH] OR "Pharmacogenetics"[MeSH]) AND (English[lang] OR German[lang] OR Dutch[lang])

("Fluorouracil"[Mesh] OR fluorouracil OR "capecitabine "[Substance Name] OR capecitabine) AND (("Dihydrouracil Dehydrogenase (NADP)"[Mesh] OR (dihydropyrimidine dehydrogenase)) AND mutation) AND (English[lang] OR German[lang] OR Dutch[lang])

Search performed in 2014: ("Fluorouracil"[Mesh] OR fluorouracil OR "capecitabine" [Supplementary Concept] OR capecitabine) AND ("Dihydropyrimidine Dehydrogenase Deficiency"[Mesh] OR "Dihydropyrimidine Dehydrogenase Deficiency" OR metabolizer OR metaboliser OR polymorph* OR "Polymorphism, Genetic"[MeSH] OR "Pharmacogenetics"[MeSH]) AND (English[lang] OR German[lang] OR Dutch[lang])

Search performed in 2017: ("Fluorouracil"[Mesh] OR fluorouracil OR "Capecitabine"[Mesh] OR capecitabine OR fluoropyrimidines) AND ("Dihydrouracil Dehydrogenase (NADP)"[Mesh] OR "Dihydropyrimidine Dehydrogenase Deficiency"[Mesh] OR "Dihydropyrimidine Dehydrogenase Deficiency" OR "Dihydropyrimidine Dehydrogenase" OR *DPYD* OR DPD) AND (English[lang] OR German[lang] OR Dutch[lang])

Tegafur
Search performed in 2009 and 2012: ("Tegafur"[Mesh] OR tegafur[Text Word]) AND ("Dihydropyrimidine Dehydrogenase Deficiency"[Mesh] OR metabolizer OR metaboliser OR polymorph* OR "Polymorphism, Genetic"[MeSH] OR "Pharmacogenetics"[MeSH]) AND (English[lang] OR German[lang] OR Dutch[lang])

Search performed in 2015: ("Tegafur"[Mesh] OR "S 1 (combination)" [Supplementary Concept] OR "tegafur-gimeracil-oteracil" [Supplementary Concept] OR tegafur[Text Word] OR S1 OR S-1 OR Teysuno) AND ("Dihydropyrimidine Dehydrogenase Deficiency"[Mesh] OR Dihydropyrimidine Dehydrogenase OR DPD OR *DPYD*) AND (English[lang] OR German[lang] OR Dutch[lang])

Search performed in 2017: ("Tegafur"[Mesh] OR "S 1 (combination)" [Supplementary Concept] OR "tegafur-gimeracil-oteracil" [Supplementary Concept] OR tegafur OR S1 OR S-1 OR "S 1" OR Teysuno) AND ("Dihydrouracil Dehydrogenase (NADP)"[Mesh] OR "Dihydropyrimidine Dehydrogenase Deficiency"[Mesh] OR "Dihydropyrimidine Dehydrogenase Deficiency" OR "Dihydropyrimidine Dehydrogenase" OR *DPYD* OR DPD) AND (English[lang] OR German[lang] OR Dutch[lang])

Supplementary Table 1. Literature review of *DPYD*-[5-FU/capecitabine] interactions to support the therapeutic dose guidelines to optimize dose

Reference	Code	Effect	Comments
ref. 1 – CAP/FU, mono/comb Henricks LM et al. Treatment algorithm for homozygous or compound heterozygous *DPYD* variant allele carriers with low-dose capecitabine. JCO Precis Oncol 2017 Oct 8.	Level of evidence score: 2 gene act. 1: Clinical Relevanc e Score A gene act. 0: Clinical Relevanc e Score A	5 patients, being either homozygous for a gene variant or having two different gene variants, received capecitabine or 5-FU treatment with doses based on the pre-treatment DPD activity in peripheral blood mononuclear cells. Pre-treatment DPD activity was also determined in a patient with genotype c.2846A>T/c.2846A>T, who did not receive treatment, because she was disease free after surgery. For 3 patients, the AUC of 5-FU after the first dose of capecitabine was determined, normalised to a dose of 850 mg/m^2 and compared to 22 patients from another study receiving combined chemotherapy with capecitabine 850 mg/m^2. Genotyping: - 2x c.1236A>G/c.1236A>G - 2x c.2846A>T/c.2846A>T - 1x *2A/*2A - 1 carrier of both c.1236A>G and c.2846A>T (either c.1236A>G/c.2846A>T (on separate alleles) or *1/(c.1236A>G+c.2846A>T) (variants on the same allele)) Results: - Of the four patients with gene activity score 1, the two patients with genotype c.1236A>G/c.1236A>G had respectively 79% and 42% of the normal DPD activity. The first was treated with 75% of the normal capecitabine dose in cycle 1 and with 100% in cycle 2. The second was treated with 50% of the normal 5-FU dose. The patients did not have severe toxicity on the reduced doses. The two patients with genotype c.2846A>T/c.2846A>T had respectively 29% and 10% of the normal DPD activity. The first was treated with 17% of the normal capecitabine dose (278 mg/m^2 once daily in combination with radiotherapy as neoadjuvant treatment) and the second was the patient who did not need treatment. The first patient tolerated treatment	Authors' 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.' Dose-corrected AUC versus gene activity 2: gene act. 1: 546% gene act. 0: 13812% Tolerated dose compared to gene activity 2: gene act. 1: 55% gene act. 0: 0.43%

table continues

		well without occurrence of severe toxicity and surgery was performed after treatment. The dose-corrected AUC of 5-FU in this patient was 866% of that of control patients. The mean DPD activity in these patients was 40%. There was a large variance in DPD activity between these patients (10-79%). - The patient with genotype *2A/*2A had undetectable DPD activity and tolerated monotherapy with 0.65% of the normal capecitabine dose (65 mg/m^2 every 5 days) for 1 month after which grade 2 diarrhoea developed. After a rest period of 3 weeks, treatment was restarted with the same dose, but every third gift was skipped (0.43% of the normal dose). The patient tolerated this dose also after addition of oxaliplatin and bevacizumab as originally planned and had stable metastatic colorectal carcinoma as best treatment response. The dose-corrected AUC of 5-FU in this patient was 13.812% of that of control patients. - The carrier of both c.1236A>G and c.2846A>T had 45% of the normal DPD activity, corresponding to gene activity score 1 (variants on different alleles). He was treated with 51% of the normal capecitabine dose in cycle 1 (daily dose of 900 mg/m^2 in combination with oxaliplatin), which was tolerated without toxicity. Increase to 71% of the planned dose (daily dose of 1250 mg/m^2) in cycle 2 resulted in grade 3 thrombocytopenia. The dose was reduced to 57% of the normal dose (1000 mg/m^2 daily), which was continued during cycle 3. However, because grade 2 thrombocytopenia developed after 8 days, the dose was reduced to 29% of the normal dose (500 mg/m^2 daily) for the rest of the cycle, resulting in platelets to increase to normal values. Progression of metastatic colorectal cancer was established after 3 cycles and capecitabine treatment was discontinued. The dose corrected AUC of 5-FU in this patient was 227% of that of control patients. NOTE: Patients were genotyped for *2A, *13, c.2846A>T and c.1236G>A.	DPD activity compared to gene activity 2: gene act. 1: 41% gene act. 0: 0%
ref. 2 – CAP, comb Henricks LM et al. Capecitabine-based treatment of a patient with a novel *DPYD*	Level of evidence score: 2 gene act. 0: Clinical Relevance Score A	A 59-year-old women with 0.5% of the normal DPD activity tolerated adjuvant chemotherapy with 0.8% of the normal capecitabine dose (77 mg/m^2 on days 1 and 6 of the first cycle and on days 1, 6 and 11 of the following cycles) in combination with oxaliplatin for eight cycles. Capecitabine-related toxicity like diarrhoea, hand-foot syndrome or leukopenia did not occur. However, sensory neuropathy developed during the first cycle, and became more severe (grade	Authors' conclusion: 'This case report demonstrates that a more comprehensive genotyping and phenotyping approach, combined with

table continues

genotype and complete dihydropyrimidine dehydrogenase deficiency. Int J Cancer. 2018 Jan 15;142(2):424-430.PubMed PMID: 28929491.		3) during the second cycle. Because this was most likely caused by oxaliplatin, the oxaliplatin dose was decreased to 75% from the third cycle onwards and discontinued after the sixth cycle. The dose-corrected AUC of 5-FU in this patient was 11.271% of that of control patients. Her genotype was *2A/(duplication of exon 17 and 18). NOTE: The patient was initially genotyped for *2A, *13, c.2846A>T and c.1236G>A. Additional gene variants were not found by sequencing of all 23 coding exons and flanking intronic regions, after which copy numbers of sequences were analysed.	pharmacokinetically-guided dose administration, enables save fluoropyrimidine treatment with adequate drug exposure in completely DPD deficient patients.' Dose-corrected AUC versus gene activity 2: gene act. 0: 11271%
ref. 3 – FU, mono/comb Meulendijks D et al. Pretreatment serum uracil concentration as a predictor of severe and fatal fluoropyrimidine-associated toxicity. Br J Cancer 2017;116:1415-24. PubMed PMID: 28427087.	Level of evidence score: 4 gene act. 1-1.5: CTC-AE 4	1606 *2A-negative patients from Deenen 2016 were genotyped for other gene variants. Toxicity was defined as toxicity grade ≥ 3, global toxicity as any toxicity, hospitalisation as toxicity related hospitalisation. Only outcomes during the first cycle of chemotherapy were included. ORs were adjusted for age, sex and treatment regimen. Genotyping: - 19 carriers of c.2846A>T - 3 carriers of *13 - 58 carriers of c.1236A>G Results: Result for carriers compared to non-carriers of the gene variant: gene variant / outcome / OR_{adj} (95% CI) c.2846A>T / global toxicity / NS, trend for an increase (p=0.095) gastrointestinal toxicity / NS haematological toxicity / NS, trend for an increase (p=0.066) hospitalisation / NS *13 / global toxicity / NS gastrointestinal toxicity / NS, trend for an increase (p=0.090) haematological toxicity / 24.9 (1.74-354) (S) hospitalisation / NS, trend for an	Authors' conclusion: 'None of the individual *DPYD* variants were found to be associated with global severe toxicity. For c.2846A>T and c.1679T>G combined, there was evidence for an association with global severe toxicity. In addition, *DPYD* c.1679T>G alone was associated with haematological toxicity.'

table continues

				increase (p=0.094)
		c.2846A >T and *13	global toxicity	3.0 (1.05-8.77) (S)
		c.1236A >G	global toxicity	NS
			gastrointestinal toxicity	NS
			haematological toxicity	NS
			hospitalisation	NS, trend for an increase (p=0.069)
		For the 3 gene variants combined, sensitivity was 6%, specificity 95%, positive predictive value 13% and negative predictive value 88% for prediction of global toxicity grade ≥ 3 in the first cycle. NOTE: No association was found for the gene variants *4 (84 carriers), except for a trend for gastrointestinal toxicity. However, most studies including a meta-analysis (Meulendijks 2015) do not show an association of this gene variant with toxicity. In addition, results regarding the effect on DPD activity are inconsistent.		
ref. 4 – FU/CAP, mono/comb Kodali S et al. Capecitabine-induced severe toxicity secondary to DPD deficiency and successful treatment with low dose 5-fluorouracil. J Gastrointest Cancer 2017;48:66-69. PubMed PMID: 26744322.	Level of evidence score: 2 gene act. 1: CTC-AE 4	A 51-year old male developed severe colitis with mucous stools (grade 4 toxicity) and neutropenic fever (neutrophils 0.18x10^9/L) on day 21 of neoadjuvant treatment with standard dose capecitabine (825 mg/m^2 twice daily) and radiotherapy. His genotype was *1/*2A. The patient tolerated adjuvant therapy with 5-FU 300 mg/m^2 per day as a continuous intravenous infusion (25% of the standard dose) and without bolus injections of 5-fluorouracil very well. Higher doses were not attempted, because they were judged not to influence recurrence or survival.		Authors' conclusion: 'The utility of pharmacokinetic-based dosing remains questionable as patients experienced toxicity even with 50% dose reduction of 5-FU, as recommended by current consortium guidelines. We therefore suggest that dosing of 5-FU should be customized in patients with DPD deficiency based on clinical judgment taking into account the severity of toxicity from initial exposure.'
ref. 5 – CAP, mono/comb Meulendijks D et al. Patients homozygous for *DPYD*	Level of evidence score: 2 gene act. 1: CTC-AE 2	Three patients treated with capecitabine containing chemotherapy were retrospectively determined to have genotype c.1236A>G/c.1236A>G. Gene variants *2A, *13 and c.2846A>T were not present in these patients. More than 4 weeks after the last treatment with fluoropyrimidines, DPD enzyme activity in		Authors' conclusion: 'The presented functional and clinical data indicate that the c.1129-5923 C>G variant is both functionally and

table continues

c.1129-5923C>G/ haplotype B3 have partial DPD deficiency and require a dose reduction when treated with fluoropyrimidines. Cancer Chemother Pharmacol 2016;78:875-80. PubMed PMID: 27544765.	peripheral blood mononuclear cells was determined and cDNA was analysed. Results: - A 47-year old female developed leukocytopenia grade 2 ($2.3x10^9$/L), neutropenia grade 2 ($1.3x10^9$/L), hand-foot syndrome grade 1, diarrhoea grade 1 and fatigue grade 1 on day 9 of neoadjuvant treatment with standard dose capecitabine (825 mg/m^2 twice daily) and radiotherapy. Because the symptoms intensified, the capecitabine dose was reduced by 40% on day 15. After dose reduction, treatment was well tolerated. Five days after a dose increase by 10%, she again developed leukopenia grade 2 ($2.5x10^9$/L) and neutropenia grade 1 ($1.5x10^9$/L). Despite this, treatment could be finished at reduced dose. The patient received surgery and was disease-free four years after treatment. The DPD activity of the patient was 41% of the normal DPD activity. - A 67-year old male developed fatigue grade 2 on day 7 of treatment with capecitabine 850 mg/m^2 on day 1-14 of the three-week cycle, docetaxel, oxaliplatin and bevacizumab. On day 11, the patient was hospitalised with neutropenia grade 2 ($1.3x10^9$/L) and fever grade 1 (38.7^{o}C, without apparent focus). After release from hospital, he refused further treatment. Because of disease progression, capecitabine 800 mg/m2 twice daily (64% of the standard dose) was started four months later as monotherapy. The patient again developed fatigue grade 2 and refused further treatment after cycle 1. The DPD activity of the patient was 55% of the normal DPD activity. - A 69-year old male tolerated 4 weeks of neoadjuvant treatment with standard dose capecitabine (825 mg/m^2 twice daily) and radiotherapy well. Treatment was completed without dose reductions or delays, and without adverse events and haematological changes. The patient had a relapse one year after surgery and died as a result of progressive disease before determination of DPD activity could be performed. cDNA analysis of the first two patients showed that they produced roughly equal amounts of wild type mRNA and aberrantly spliced mRNA with a premature stop codon. The authors indicate that the starting dose of capecitabine was relatively low in these patients (compared to the monotherapy dose of 1250 mg/m^2 twice daily). So, higher doses might have resulted in more pronounced toxicity. Amstutz 2009 describes a	clinically relevant, and support an upfront dose reduction of the fluoropyrimidine starting dose in patients carrying c.1129-5923C>G homozygously.' Tolerated dose versus gene activity 2: gene activity 1: 60% DPD activity versus gene activity 2: gene activity 1: 48%

table continues

		patient with genotype c.1236A>G/c.1236A>G, who developed fatal toxicity during the first cycle with full dose 5-FU plus cisplatin. NOTE: Patients were genotyped for c.1129-5923C>G and checked for the presence of c.1236G>A and c.959-51T>G, which are in complete linkage disequilibrium with c.1129-5923C>G in haplotype B3.	
ref. 6 – FU/CAP, mono/comb Lunenburg CA et al. Evaluation of clinical implementation of prospective *DPYD* genotyping in 5-fluorouracil- or capecitabine-treated patients. Pharmacogenomics 2016;17:721-9. PubMed PMID: 27181275.	Level of evidence score: 3 gene act. 1.5:CTC-AE 4(2)# gene act. 1:CTC-AE 4(2)# gene act. 0.5:CTC-AE 4(2)#	The results of routine prospective genotyping and genotype-guided dosing were retrospectively evaluated in patients receiving capecitabine or 5-fluorouracil, either as combined chemotherapy (different combinations) or as monotherapy (with or without radiotherapy). Genotyping was originally only for *2A (275 patients), but from approximately 30% of the total study time genotyping for *13 and 2846A>T was added (214 patients) and from 65% of the total study time genotyping for c.1236G>A was added (n = 109). Recommended dosing reductions were 50% of the normal dose per *2A- and *13-variant and 25% per c.1236A>G-variant. Recommended dosing reduction per c.2846A>T-variant was 50% (change to a recommendation of 25% reduction was only after the study), but was not applied. 14 patients with gene variants were identified. Due to the low number of patients with DPD variants the study was not powered to formally test the effect of genotype-guided dosing on fluoropyrimidine-induced toxicity and only explorative analyses could be performed. Genotyping: - 8x *1/c.1236A>G - 5x *1/*2A - 1 carrier of both *2A and c.2846A>T (either *2A/c.2846A>T (on separate alleles) or *1/(*2A+c.2846A>T) (variants on the same allele)) Results: - 8 patients (5x *1/c.1236A>G and 3x *1/*2A) received the recommended initial dose reduction and did not develop toxicity grade 3-4 in cycle 1. The dose of 4 patients was subsequently increased. Two patients (1x *1/c.1236A>G with a dose increase to 100% of the normal dose and 1x *1/*2A with a dose increase to 60% of the normal dose) did not develop toxicity grade 3-4. A patient with genotype *1/*2A developed diarrhoea grade 3 and enteritis after dose increase to 80% of the normal dose. Another patient with this genotype developed hand-foot-syndrome grade 2-3 after multiple cycles with the normal dose.	Authors' conclusion: 'Prospective *DPYD* screening can be implemented successfully in a real world clinical setting, is well accepted by physicians and results in low toxicity.'

table continues

		- 3 patients (1x *1/c.1236A>G and 2x *1/*2A) did not receive an initial dose reduction and developed toxicity grade 3-4 in cycle 1. For two of these patients, therapy was started before the genotype was known. For the third patient, the oncologist did not reduce the dose, because the dose in the chemotherapy regimen was already relatively low (capecitabine plus radiotherapy). For 1 patient with genotype *1/*2A, the dose was subsequently reduced to 50% of the normal dose and the patient did not develop toxicity grade 3-4 anymore. The other 2 patients quitted fluoropyrimidine therapy. - For the carrier of both *2A and c.2846A>T, there was no dose recommendation, because it was not known whether the variants were on different alleles or on the same allele. Because therapy had to be started before the DPD-activity would have been determined, the physician decided to use a 50% dose reduction, taking into account the results of genotyping and that this patient had tolerated 5-FU containing regimens before. Fluoropyrimidine therapy was stopped in this patient after the first cycle due to toxicity (≤ grade 3). - 2 patients (both with genotype *1/c.1236A>G) did not start fluoropyrimidine therapy.	
ref. 7 – FU, comb Lee AM et al. Association between *DPYD* c.1129-5923 C>G/hapB3 and severe toxicity to 5-fluorouracil-based chemotherapy in stage III colon cancer patients: NCCTG N0147 (Alliance). Pharmacogenet Genomics 2016;26:133-7. PubMed PMID: 26658227.	Level of evidence score: 3 gene act. 1-1.5: CTC-AE 4	A subset of patients from Lee 2014 was reanalysed: 1953 patients, negative for *2A, *13 and c.2846A>T, and treated with 12 cycles of adjuvant FOLFOX therapy (5-FU, folinic acid and oxaliplatin) with or without cetuximab. 62.9% of patients had any grade ≥ 3 adverse event, with 32.7% having any grade ≥ 3 adverse event common to 5-FU treatment. Adverse events classified as common to 5-FU treatment were fatigue, anorexia, dehydration, diarrhoea, stomatitis/mucositis, nausea/vomiting, leukopenia, neutropenia, febrile neutropenia, thrombocytopenia, and pain. Most frequent 5-FU adverse events included diarrhoea (12.5%), neutropenia (10.3%), pain (5.4%), fatigue (5.2%), nausea/vomiting (4.7%), and mucositis (4.1%). Results were adjusted for clinicopathological factors like age, sex, treatment, total number of treatment cycles and dose modifications. The latter two outcomes (higher percentage of patients with premature continuation and with dose modification) might be results of 5-FU adverse events instead of causes. Cetuximab increased the risk of 5-FU adverse events. Results were adjusted for this, but this indicates that adverse events common to 5-FU are not the same as 5-fluorouracil-induced adverse events. Genotyping:	Authors' conclusion: 'No significant associations were identified between c.1129 -5923 C>G/hapB3 and overall grade≥3 adverse event rate. Our results suggest that c.1129-5923 C>G/hapB3 have limited predictive value for severe toxicity to 5-FU-based combination chemotherapy.'

table continues

		- 1875x *1/*1 - 77x *1/c.1236A>G - 1x c.1236A>G/c.1236A>G Results: Risk of grade ≥ 3 adverse event for c.1236A>G/c.1236A>G versus *1/c.1236A>G versus *1/*1: any adverse event: NS, trend for an increase (p=0.082); OR_{adj} for (*1/c.1236A>G + c.1236A>G/c.1236A>G) compared to *1/*1 also showed a trend for an increase (NS, p=0.127). diarrhoea: NS neutropenia: S for an increase pain: NS fatigue: NS nausea/vomiting: NS stomatis/mucositis: NS dehydration: NS leukopenia: NS NOTE: Results were reported for 1129-5923C>G, which was in complete linkage disequilibrium with the also genotyped c.1236G>A.	
ref. 8 – FU/CAP, mono/comb Deenen MJ et al. Upfront genotyping of *DPYD*2A to individualize fluoropyrimidine therapy: a safety and cost analysis. J Clin Oncol 2016;34:227-34. PubMed PMID: 26573078.	Level of evidence score: 3 gene act. 1: Clinical Relevance Score A	1631 patients received genotype-guided therapy with capecitabine (90% of patients) or 5-FU (10% of patients), either as combined chemotherapy (different combinations) or as monotherapy (with or without radiotherapy). Genotyping was for *2A. For *1/*2A, dose reduction in the first two cycles was ≥ 50% and was followed by dose titration based on tolerance. Initial dose was not reduced for *1/*1. Patients with the *1/*2A genotype were compared with 48 patients with this genotype, treated with the full initial dose in published cohorts studies without genotype-guided dosing. Of these 48 patients, 79% was treated with 5-fluorouracil, 19% with capecitabine and 2% with tegafur combined with uracil. In addition, patients with the *1/*2A genotype were compared to patients with the *1/*1 genotype. For 16 *1/*2A-patients, 5-fluorouracil AUC in blood plasma after the first capecitabine dose was compared with that of 25 unselected patients from two studies (n = 11 and n = 14 per study). For 15 *1/*2A-patients, DPD enzyme activity in peripheral mononuclear blood cells was determined and compared with the mean Caucasian DPD enzyme activity (mainly *1/*1-patients). The study had 100% power to detect a reduction of the incidence of grade ≥ 3 toxicity in *2A-carriers from 85% to 20%.	Authors' conclusion: '*DPYD*2A genotype-guided dosing results in adequate systemic drug exposure and significantly improves safety of fluoropyrimidine therapy for the individual patient. On a population level, upfront genotyping seemed cost saving.' AUC versus gene activity 2: gene activity 1: 203%

table continues

The risk of grade ≥ 3 toxicity was higher in combination therapy than in monotherapy and chemo-radiotherapy regimens.
Genotyping:
- 1613x *1/*1
- 18x *1/*2A

Results:
Treatment characteristics of *1/*2A-patients:
- The initial dose varied from 29% to 60% of the full dose (median 46%). The final dose varied from 17% to 91% of the full dose. The median dose per treatment cycle was 48% (range 17% to 91%). All patients were treated with capecitabine.
- 5 patients developed toxicity grade ≥ 3 (first cycle 29% to 60% of the normal dose, final cycle 17% to 60% and maximum 29% to 67%)
- 2 patients developed toxicity grade 0 (first of the two cycles with 29% and second cycle with 59% of the nor-mal dose and all five cycles 48% of the normal dose, respectively)
- 11 patients developed toxicity grade 1 to 2 (first cycle 30% to 50% of the normal dose, final cycle 24% to 91% and maximum 46% to 91%)
- Toxicity was short in duration and well controlled using standard supportive care.
- For 6 patients, the dose was increased during treatment (dose in first cycle 29% to 47% of the normal dose; maxi-mum dose 46% to 91%).
In two of these patients (dose increase from 47% to 53% and from 44% to 67%, respectively), the dose was later reduced to the initial dose again because of toxicity.
- For 3 patients, the initial dose was still too high and had to be reduced further (initial dose 29% to 44% of the normal dose, final dose 17% to 24%).
- Of 4 evaluable patients, 2 achieved a partial response and 2 had stable disease.
In 4 of 5 patients with rectal cancer treated with chemo-radiotherapy, down staging of the tumour from pT3-4 to ypT0-2 was reached.

Percentage of *1/*2A patients with toxicity for reduced dosing compared to full dosing:

		value for full dosing
any grade ≥ 3 toxicity	x 0.38 (S) In addition, the observed toxicity was short in duration with reduced dosing and usually long-lasting with full dosing.	73%

table continues

		grade ≥ 3 haematological toxicity	x 0.26 (S)	66%
		grade ≥ 3 gastrointestinal toxicity	x 0.20 (S)	56%
		fluoropyrimidine-induced death	NS	10%
		Percentage of patients with toxicity for *1/*2A on reduced dosing compared to *1/*1 on full dosing:		value for *1/*1
		any toxicity grade ≥ 3	NS	23%
		any toxicity grade 1-2	NS	54%
		haematological toxicity grade ≥ 3	NS	10%
		haematological toxicity grade 1-2	NS	35%
		diarrhoea grade ≥ 3	NS	8%
		diarrhoea grade 1-2	NS	29%
		hand-foot syndrome grade ≥ 3	NS	5%
		hand-foot syndrome grade 1-2	NS	28%
		The authors indicate that the comparable toxicity burden suggests that *1/*2A is not underexposed when treated with a median dose of 48%.		
		Dose-normalised pharmacokinetics and DPD enzyme activity for *1/*2A compared to *1/*1:		value for *1/*1
		5-FU AUC normalised to a capecitabine dose of 1250 mg/m^2	x 2.03 (NS)	602 ng.h/ml
		DPD enzyme activity in peripheral mononuclear blood cells	x 0.64 (S)	9.9 nmol/ hr per mg protein
ref. 9 – FU/CAP, mono/comb Meulendijks D et al. Clinical relevance of *DPYD* variants c.1679T>G, c.1236G>A/HapB3, and c.1601G>A as predictors of severe fluoropyrimidine-associated	Level of evidence score: 4 gene act. 1: CTC-AE 4 gene act. 1-1.5: CTC-AE 4	Meta-analysis of 8 cohort studies with in total 7365 patients treated with 5-FU or capecitabine, either as combined chemotherapy (different combinations) or as monotherapy (with or without radiotherapy). Data on *13 were derived from 5 studies including a total of 5,616 patients and 11 carriers of *13. Data on c.1236G>A were derived from 6 studies including a total of 4,261 patients and 174 heterozygous carriers and 3 homozygous carriers of c.1236A>G. Data on *2A were derived from 7 studies including a total of 5.737 patients and 60 carriers of *2A. Data on c.2846A>T were derived from all 8 studies including a total of 7,318 patients and 85 carriers of c.2846A>T. 1 of the 8 studies in this meta-analysis is also included in the meta-analysis of Rosmarin 2014 (Rosmarin 2014). 2 of the 8 studies in this meta-analysis are also	Authors' conclusion: '*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

table continues

toxicity: a systematic review and meta-analysis of individual patient data. Lancet Oncol 2015;16:1639-50. PubMed PMID: 26603945.	included in the meta-analysis of Terrazzino 2013 (Morel 2006 and Deenen 2011). 5 of the 8 studies in this meta-analysis are also included separately in this risk analysis: Morel 2006, Deenen 2011, Lee 2014, Rosmarin 2014 and Meulendijks 2017. If possible, a RR was calculated for each study based on individual patient data and adjusted for age, sex, and treatment regimen. For 2 of the 5 studies for *13, it was not possible to use individual patient data. A random-effects model was used for the meta-analysis. Haematological toxicity included thrombocytopenia, neutropenia, leukocytopenia, and anaemia. Gastro-intestinal toxicity included diarrhoea, mucositis/stomatitis, and nausea/vomiting. Short timeframe was defined as shorter than the complete treatment duration, long timeframe as the whole treatment duration. In addition, a meta-analysis of 3 case-control studies with in total 799 patients was performed for c.1236G>A. One of these case-control studies is also included in the meta-analysis of Rosmarin 2014 (Schwab 2008) and two in the meta-analysis of Terrazzino 2013 (Schwab 2008 and Kleibl 2009). One of these case-control studies is also included separately in this risk analysis (Schwab 2008). Results: Risk of grade ≥ 3 toxicity for *1/*13 compared to *1/*1:	patients with cancer treated with fluoropyrimidines.'

	RR_{adj} (95% CI)	incidence for *1/*1 (% of patients)
any toxicity	4.40 (2.08-9.30) (S)	22%
haematological toxicity	9.76 (3.03-31.48) (S)	
gastrointestinal toxicity	5.72 (1.40-23.33) (S)	
hand-foot syndrome	- (RR could not be calculated due to an incidence of 0% in *1/*13)	

The heterogeneity between the studies was significant and substantial, possibly because of the small number of *1/*13.
There was no indication of publication bias.
The results for any toxicity were similar when patients carrying *2A and/or c.2846A>T were excluded from the meta-analysis. The association remained significant with p< 0.0167 after exclusion of any study from the meta-analysis,

table continues

except for Loganayagam 2013. After exclusion of Loganayagam 2013, the p-value was 0.0433.
The effect of *13 on risk of severe toxicity seemed similar in studies with long and short timeframes.
The sensitivity of *13 in prediction of grade ≥ 3 toxicity was 0.3% and the positive predictive value 46%.

Risk of grade ≥ 3 toxicity for (*1/c.1236A>G + c.1236A>G/c.1236A>G) compared to *1/*1:

	RR_{adj} (95% CI)	incidence for *1/*1 (% of patients)
any toxicity	1.59 (1.29-1.97) (S)	22%
haematological toxicity	2.07 (1.17-3.68) (S)	
gastrointestinal toxicity	2.04 (1.49-2.78) (S)	
hand-foot syndrome	NS (also for the sub-group treated with capecitabine)	

There was no significant heterogeneity between the studies.
There was no indication of publication bias.
The results for any toxicity were similar when patients carrying *2A and/or c.2846A>T were excluded from the meta-analysis. The association remained significant after exclusion of any study from the meta-analysis.
The effect of c.1236A>G on risk of severe toxicity seemed similar in studies with long and short timeframes.
The sensitivity of c.1236A>G in prediction of grade ≥ 3 toxicity was 6.4% and the positive predictive value 41%.
The meta-analysis of the case-control studies did not show a significant result, probably due to the smaller number of patients.
The authors reported to have treated 3 patients with genotype c.1236A>G/c.1236A>G safely with low dose capecitabine (825 mg/m^2 twice a day).

Risk of grade ≥ 3 toxicity for *2A-carriers compared to *1/*1:

	RR_{adj} (95% CI)	incidence for *1/*1 (% of patients)
any toxicity	2.85 (1.75-4.62) (S)	29%

The heterogeneity between the studies was significant and strong.

table continues

		There was no indication of publication bias. Risk of grade ≥ 3 toxicity for c.2846A>T-carriers compared to *1/*1: RR_{adj} (95% CI) — incidence for *1/*1 (% of patients) any toxicity — 3.02 (2.22-4.10) (S) — 25% The heterogeneity between the studies was significant and strong. There was no indication of publication bias. NOTE: c.1236G>A is in complete linkage disequilibrium with c.1129-5923C>G in haplotype B3. Studies analysing both gene variants were pooled. NOTE: Meta-analysis of 5 studies with in total 3900 patients, 182x *1/*4 and 2x *4/*4, showed no significant association between *4 and grade ≥ 3 toxicity. The only study that found a significant effect (Loganayagam 2013) was the cause of strong heterogeneity between the studies. In addition, results regarding the effect of *4 on DPD activity are inconsistent.	
ref. 10 – FU, comb Lee AM et al. *DPYD* variants as predictors of 5-fluorouracil toxicity in adjuvant colon cancer treatment (NCCTG N0147). J Natl Cancer Inst 2014;106:dju298. PubMed PMID: 25381393.	Level of evidence score: 3 gene act. 0.5-1: CTC-AE 4 gene act. 0.5 + gene act. 1.5: CTC-AE 4 gene act. 0.5:CTC-AE 5(2)#	2594 patients were treated with 12 cycles of adjuvant FOLFOX therapy (5-fluorouracil, folinic acid and oxaliplatin; 91.9% of patients) or FOLFIRI therapy (5-fluorouracil, folinic acid and irinotecan; 8.1% of patients) with or without cetuximab. Part of the patients received 6 cycles of FOLFOX followed by six cycles of FOLFIRI with or without cetuximab. 62.0% of patients had any grade ≥ 3 adverse event, with 33.1% having any grade ≥ 3 adverse event common to 5-fluorouracil treatment. Adverse events classified as common to 5-fluorouracil treatment were fatigue, anorexia, dehydration, diarrhoea, stomatitis/mucositis, nausea/vomiting, leukopenia, neutropenia, febrile neutropenia, thrombocytopenia, and pain. Most frequent 5-fluorouracil adverse events included diarrhoea (12.0%), neutropenia (11.7 %), nausea/vomiting (5.0%), fatigue (4.9%), and mucositis (4.2%). Follow-up for disease free survival was for 5 years. Results were adjusted for clinicopathological factors like age, sex, treatment, total number of treatment cycles and dose modifications. The latter two outcomes (higher percentage of patients with premature continuation and with dose modification) might be results of 5-fluorouracil adverse events instead of causes. Cetuximab increased the risk of 5-fluorouracil adverse events. OR's were adjusted for this, but other	Authors' conclusion: 'Statistically significant associations were found between *DPYD* variants (*DPYD**2A and 2846A>T) and increased incidence of grade 3 or greater 5FU-adverse events in patients treated with adjuvant 5-FU-based combination chemotherapy.'

table continues

outcomes were not. In addition, this indicates that adverse events common to 5-fluorouracil are not the same as 5-FU-induced adverse events.

Genotyping:
- 2532x *1/*1
- 24x *1/*2A
- 26x *1/c.2846A>T
- 1x *2A/c.2846A>T
- 1x *1/274C
- 5x *2A-genotyping failed
- 5x c.2846A>T-genotyping failed

Results:

Risk of grade ≥ 3 toxicity, premature treatment termination and disease free survival for *2A-carriers compared to non-carriers:

		incidence for non-carriers
any toxicity	OR_{adj} = 3.58 (95% CI: 1.01-12.64) (S)	62%
any 5-FU toxicity	OR_{adj} = 14.91 (95% CI: 4.26-52.18) (S)	33%
diarrhoea	NS	12%
neutropenia	x 5.7 (S)	11%
nausea/vomiting	x 4.2 (S)	4.8%
fatigue	NS	4.8%
stomatitis/mucositis	NS, trend for an increase, p=0.09	4.2%
dehydration	NS	2.3%
leukopenia	NS, trend for an increase, p=0.08	1.8%
febrile neutropenia	NS, trend for an increase, p=0.07	1.6%
anorexia	NS	1.5%
pain	NS	0.8%
thrombocytopenia	NS, trend for an increase, p=0.08	0.3%
premature treatment termination	x 1.7 (S)	26%
dose modification	NS	74%
disease free survival after 3 year	NS	73%

When restricting the analysis to Caucasians, sex or treatment, the association between *2A and grade ≥ 3 5-FU toxicity remained significant, whereas the association between *2A and grade ≥ 3 overall toxicity did not.

table continues

Risk of grade ≥ 3 toxicity, premature treatment termination and disease free survival for *c.2846A>T-carriers compared to non-carriers:

		incidence for non-carriers
any toxicity	OR_{adj} = 5.43 (95% CI: 1.52-19.43) (S)	62%
any 5-FU toxicity	OR_{adj} = 10.24 (95% CI: 3.57-29.40) (S)	33%
diarrhoea	x 2.8 (S)	12%
neutropenia	x 4.9 (S)	11%
nausea/vomiting	NS	5.0%
fatigue	NS	4.8%
stomatitis/mucositis	NS	4.1%
dehydration	x 5.0 (S)	2.2%
leukopenia	x 8.2 (S)	1.8%
febrile neutropenia	NS, trend for an increase, p=0.08	1.6%
anorexia	NS	1.5%
pain	NS	0.8%
thrombocytopenia	x 55.5 (S)	0.2%
premature treatment termination	NS	26%
dose modification	NS	74%
disease free survival after 3 year	NS	73%

When restricting the analysis to Caucasians, sex or treatment, the association between c.2846A>T and grade ≥ 3 5-FU toxicity remained significant. The association between c.2846A>T and grade ≥ 3 overall toxicity remained significant in the subgroups of Caucasians and males, but not in the subgroups of females, FOLFOX only and FOLFOX + cetuximab.

Other results:
- Because of its low frequency, a statistically significant association could not be demonstrated between *13 and either 5-FU or overall grade ≥ 3 toxicity (NS).
- The *2A/c.2846A>T-patient had a grade 5 adverse event. The patient was only able to receive one cycle of FOLFOX + cetuximab.
- The *1/274C-patient had no grade ≥ 3 adverse events.

table continues

		- The gene variants *2A, *13 and c.2846A>T together predicted 5-FU grade ≥ 3 toxicity with a sensitivity of 5.3%, specificity of 99.4%, positive predictive value of 81.8% and negative predictive value of 68%. The low sensitivity and negative predictive value might be attributed to the combination chemotherapy, which may add to the 5-FU toxicity. NOTE: Genotyping was for 25 gene variants of which only 4 (*2A, *13, c.2846A>T and 274G>C) were found in this population from the USA.	
ref. 11 – CAP/FU, comb Rosmarin D et al. Genetic markers of toxicity from capecitabine and other fluorouracil-based regimens: investigation in the QUASAR2 study, systematic review, and meta-analysis. J Clin Oncol 2014;32:1031-9. PubMed PMID: 24590654.	Level of evidence score: 4 gene act. 0-1,5: CTC-AE 4	After colorectal cancer excision, 927 patients received adjuvant therapy with capecitabine 1250 mg/m^2 twice daily on days 1-14 of a 3-week cycle either as monotherapy (n = 436) or in combination with bevacizumab (n = 491). Grade III-V toxicity comprised hand-foot syndrome (n = 206), diarrhoea (n = 97) and neutropenia (n = 19). Variant c.2846A>T: - Associated with grade III-V toxicity (OR = 9.35; 95% CI: 2.01-43.4) (S) - No association with grade III-V diarrhoea and grade III-V hand-foot syndrome (NS). Given the allele frequency found, this is apparently based on 5 defect alleles. Variants *2A, 496A>G, c.1236G>A: - No association with grade III-V toxicity, grade III-V diarrhoea and grade III-V hand-foot syndrome (NS). Given the allele frequency found, this is apparently based on 4 defect alleles for *2A, 83 for 496A>G and 18 for c.1236G>A. Variant c.2846A>T and/or *2A: - Associated with grade III-V toxicity (OR = 5.51; 95% CI: 1.95-15.5) (S) - No association with grade III-V diarrhoea and grade III-V hand-foot syndrome (NS) - Both patients who died were carriers of *2A or c.2846A>T Meta-analysis of 6 studies during which Caucasian patients received capecitabine or 5-FU-based therapy. Of these 6 studies, the study covered in the paragraph above and Schwab, 2008, were also included separately in this risk analysis. Variant *2A: - No association with grade III-V toxicity for capecitabine (2 studies, n = 1035) (NS) - No significant association with grade III-V toxicity for 5-FU infusion, but there was a trend (2 studies, n =	Authors' conclusion: "Global capecitabine toxicity (grades 0/1/2 v grades 3/4/5) was associated with the rare, functional *DPYD* alleles c.2846A>T>A and *2A (combined odds ratio, 5.51)."

table continues

		732) (NS; p=0.0075, whilst this should be less than 0.0048 due to multiple testing) - No significant association with grade III-V toxicity for 5-FU bolus injection, but increased risk of grade III-V neutropenia (OR = 12.9; 95% CI: 3.13-53.3) (1 study, n = 338) (S) Variant c.2846A>T: - No meta-analysis for capecitabine, 5-FU infusion and 5-FU bolus injection (1 study each time) Variant 496G>A: - No meta-analysis for capecitabine and 5-FU infusion (in both cases only 1 study) - No association with grade III-V toxicity for 5-FU bolus injection (2 studies, n = 379) (NS) Variant c.1236G>A: - No meta-analysis for capecitabine, 5-FU infusion and 5-FU bolus injection (1 study each time) Variant c.2846A>T and/or *2A: - No meta-analysis for capecitabine (only 1 study) - There was a significant association (p=0.05) with grade III-V toxicity for 5-FU infusion and 5-FU bolus injection (S) NOTE: No association was found for the gene variants *4, *5, *6 and *9A. However, associations with severe toxicity have never been found in studies concerning these gene variants.	
ref. 12 – FU/CAP, mono/comb Terrazzino S et al. *DPYD* IVS14+1 G>A and c.2846A>T genotyping for the prediction of severe fluoropyrimidine-related toxicity: a meta-analysis. Pharmacogenomics 2013;14:1255-72. PubMed PMID: 23930673.	Level of evidence score: 4 gene act. 1: CTC-AE 4 gene act. 1,5: CTC-AE 4	Meta-analysis of 15 studies investigating patients treated with fluorouracil, capecitabine or tegafur-uracil (1 study). Data on *2A (IVS14+1G>A) were derived from 15 studies including a total of 4,094 patients and 60 carriers of *2A. Data on c.2846A>T were derived from 7 studies including a total of 2,308 patients and 34 carriers of c.2846A>T. These 15 studies include 8 studies that have also been included separately in this risk analysis: Salgueiro 2004, Morel 2006, Largillier 2006, Boisdron-Celle 2007, Schwab 2008, Sulzyc-Bielicka 2008, Kristensen 2010 and Deenen 2011. *2A versus (no *2A): Increased risk of grade III-V toxicity (OR = 5.42; 95% CI: 2.79-10.52; increase in the percentage of patients with grade III-V toxicity from 39% to 68%) (S) Exclusion of each of the studies from the meta-analysis did not lead to substantially different results (OR = 4.05 - 7.32 (S)). The risk was increased in studies in which the percentage of patients with grade III-V toxicity was less than 40% (OR = 8.31; 95% CI: 3.63-19.06) (S).	Authors' conclusion: "The results of this meta-analysis confirm clinical validity of *DPYD* IVS14+1 G>A and 2846A>T as risk factors for the development of severe toxicities following fluoropyrimidine treatment."

table continues

	However, the increase was non-significant in studies including ≥40% of patients with toxicity. The results were similar if only prospective studies, only higher quality studies or only studies including ≥ 200 patients were analysed. In prospective studies, the risk also increased as the incidence of grade III-V toxicity decreased in the study. The risk was also increased when only studies investigating 5-FU-based therapy or 5-FU monotherapy were analysed. Increased risk of grade III-V haematological toxicity (OR = 15.77; 95% CI: 6.36-39.06) (S) Increased risk of grade III-V diarrhoea (OR = 5.54; 95% CI: 2.31-13.29) (S) Increased risk of grade III-V mucositis (OR = 7.48; 95% CI: 3.03-18.47) (S) *2A had a sensitivity of 5.2% (95% CI: 3.0-8.9) and a specificity of 99.2% (95% CI: 98.8-99.4) for predicting grade III-V toxicity (S) The sensitivity was 9.0% for studies that showed less than 40% grade III-V toxicity (95% CI: 5.7-13.9) (S). There was study heterogeneity in the overall group, but not in the group with less than 40% toxicity. *2A had a sensitivity of 13% (95% CI: 6.6-24.1) for predicting grade III-V haematological toxicity (S) *2A had a sensitivity of 5.6% (95% CI: 3.2-9.7) for predicting grade III-V diarrhoea (S) *2A had a sensitivity of 11.5% (95% CI: 6.2-20.5) for predicting grade III-V mucositis (S) c.2846A>T versus (no c.2846A>T): Increased risk of grade III-V toxicity (OR = 8.18; 95% CI: 2.65-25.25; increase in the percentage of patients with grade III-V toxicity from 34% to 71%) (S) Exclusion of each of the studies from the meta-analysis did not lead to substantially different results (OR = 6.20 - 12.88 (S)). The risk was increased in studies in which the percentage of patients with grade III-V toxicity was less than 40% (OR = 16.59; 95% CI: 5.06-54.43) (S). However, the increase was non-significant in studies including ≥40% of patients with toxicity. The results were similar if higher only quality studies or only studies including ≥ 200 patients were analysed. The risk was also increased when only prospective studies were analysed (OR = 18.14; 95% CI: 6.26-52.58) (S) or only studies investigating 5-FU-based therapy (OR = 21.38; 95% CI: 6.71-68.15) (S). There was moderate study heterogeneity in the overall group, but not in the low or high toxicity subgroups, among prospective studies or among those investigating 5-fluorouracil-based therapy. There may have been publication bias.	

table continues

		Increased risk of grade III-V diarrhoea (OR = 6.04; 95% CI: 1.77-20.66) (S) c.2846A>T had a sensitivity of 5.4% (95% CI: 1.7-16.1) and a specificity of 99.1% (95% CI: 98.7-99.4) for predicting grade III-V toxicity (S) The sensitivity was 11.2% for studies that showed less than 40% grade III-V toxicity (95% CI: 2.8-35.1) (S). There was heterogeneity between the studies. c.2846A>T had a sensitivity of 4.6% (95% CI: 2.2-9.4) for predicting grade III-V diarrhoea (S)	
ref. 13 – FU/CAP, comb Magnani E et al. Fluoropyrimidine toxicity in patients with dihydropyrimidine dehydrogenase splice site variant: the need for further revision of dose and schedule. Intern Emerg Med 2013;8:417-23. PubMed PMID: 23585145.	Level of evidence score: 2 gene act. 1: CTC-AE 4	3 patients with genotype *1/*2A with gastrointestinal or head and neck tumours received 5-FU or capecitabine-based therapy (adjuvant or metastatic therapy). A 4th patient with genotype *1/*2A was not given adjuvant therapy. A 43-year-old colon cancer patient was given adjuvant therapy with capecitabine/oxaliplatin and a 50% dose of capecitabine (500 mg/m^2 twice daily for 14 days, followed by a week-long rest period). The patient developed diarrhoea, grade 4 neutropenia and grade 3 thrombocytopenia after 19 days. The adjuvant therapy was discontinued. A 71-year-old colon cancer patient received the same adjuvant therapy including 40% of the normal capecitabine dose (400 mg/m^2 twice daily). After 1 day, the patient started vomiting and developed grade 3 abdominal pain. The adjuvant therapy was discontinued. A 68-year-old patient with metastatic maxillary sinus cancer initially received 5-FU/carboplatin/folinic acid with standard-dose 5-FU (3000 mg/m^2 continuous infusion + 400 mg/m^2 bolus every 3 weeks). After 15 days, he developed grade 4 neutropenia and thrombocytopenia, and grade 3 sepsis and ulceration of the palate. After recovery, the treatment was restarted at 44% of the original dose (1500 mg/m^2 by continuous infusion) and prophylactic growth factors. There was no toxicity for 2 cycles. In the third cycle, the dose was increased to 59% of the standard dose (2000 mg/m^2 bolus) and no growth factors were given. After 14 days, the patient developed grade 4 febrile neutropenia and grade 2 anaemia. He was henceforth given non-fluoropyrimidine-based therapy. The authors indicated that a 50% dose decrease in gene activity score 1 is not always adequate.	Authors' conclusion: "Our data suggest that greater dose reductions or alternative therapies are needed for patients with DPD IVS14+1 G>A mutations."
ref. 14 – FU, comb Vulsteke C et al. Genetic variability in the multidrug	Level of evidence score: 4 gene act. 1: Clinical	1012 breast cancer patients received neoadjuvant/adjuvant therapy with 5-FU, epirubicin and cyclophosphamide. The 5-FU dose was 500 mg/m^2 every 3 weeks with a maximum of 1000 mg (n=902) or 600 mg/m^2 with a maximum of 1200 mg (n = 110).	Authors' conclusion: "In our study, we did not observe any association with toxicity and IVS14+1 G>A. The absence of

table continues

resistance associated protein-1 (ABCC1/ MRP1) predicts hematological toxicity in breast cancer patients receiving (neo-)adjuvant chemotherapy with 5-fluorouracil, epirubicin and cyclophosphamide (FEC). Ann Oncol 2013;24:1513-25. PubMed PMID: 23396606.	Relevance Score AA	Variant *2A (c.1905+1G>A, rs3918290): No significant association with serious adverse events (febrile neutropenia, prolonged grade III-IV neutropenia or severe neutropenia, grade III-IV anaemia, grade III-IV thrombocytopenia or grade III-IV non-haematological toxicity) (NS) The authors indicated that the lack of association is likely due to the fact that 5-FU toxicity is not common among breast cancer patients treated with this combination therapy. The 5-FU dose in this combination therapy is much lower than the dose in combination therapies used for colorectal cancer. NOTE: Associations were also not found for gene variants *5 (1627A>G), *6 (2194G>A) and *9A (85T>C). However, associations with severe toxicity have never been found in studies concerning these gene variants.	a significant association with IVS14+1 G>A probably relates to the fact that 5-FU toxicity is not frequent in breast cancer patients treated with FEC due to a much lower 5-FU dose in breast compared with colorectal cancer patients."
ref. 15 – FU, mono/ comb van Kuilenburg AB et al. Evaluation of 5-fluorouracil pharmacokinetics in cancer patients with a c.1905+1 G>A mutation in *DPYD* by means of a Bayesian limited sampling strategy. Clin Pharmacokinet 2012;51:163-74. PubMed PMID: 22339448.	Level of evidence score: 3 gene act. 1: CTC-AE 5	Clinical aspects were determined in 20 patients who had been genotyped as *1/*2A beforehand and were treated with 5-FU. Kinetics were determined in 30 *1/*2A (c.1905+1G>A) and 18 *1/*1, who received a 5-FU bolus injection of 300 mg/m^2 and/or 450 mg/m^2. Treatment regimens were not given. *Clinical* - All 7 *1/*2A receiving a standard dose of 5-FU showed grade III-V toxicity, of which 3 showed grade IV neutropenia The severe toxicity occurred in the first cycle each time and 1 patient died. - Among 13 *1/*2A receiving low-dose 5-FU, 4 had grade III toxicity and none had grade IV toxicity The patients with grade III toxicity received on average 74% of the standard dose, and those with grade II or lower toxicity received 61% of the dose. *Kinetics* *1/*2A versus *1/*1: - The 5-FU AUC increased by 52% for the 300 mg/m^2 dose (from 6.0 to 9.1 mg.hour/L) and by 32% for the 450 mg/m^2 dose (from 13.4 to 17.7 mg.hour/L) (S) The dose-corrected AUC increased by 32% (from 0.026 to 0.034 mg.hour/L per mg/m^2; 45 and 25 patient/dose combinations respectively) (S). The AUC seems to be predictive of the first 2 hours after the injection and may therefore cause an	Authors' conclusion: "Profound differences in the elimination of 5FU could be detected between DPD-deficient patients and control patients. Furthermore, treatment of DPD-deficient patients with standard 5FU-containing chemotherapy was associated with severe (lethal) toxicity." Maximum clearance (V_{max} for 300 mg/m^2) versus EM: gene activity 1: 54% AUC_t versus EM: gene activity 1: 132%

table continues

		underestimate for *1/ *2A. The 5-FU concentration 1 hour after injection was around the detection limit for *1/*1. - The terminal half-life of 5-FU increased by 109% for the 300 mg/m^2 dose (from 0.128 to 0.268 hours) and by 69% for the 450 mg/m^2 dose (from 0.181 to 0.306 hours) (S) - The maximum enzymatic metabolic capacity (V_{max}) calculated in a multi-compartment model decreased by 46% for the 300 mg/m^2 dose (from 1749 to 942 mg/hour) and by 34% for the 450 mg/m^2 dose (from 1370 to 900 mg/hour) (S)	
ref. 16 – CAP, comb Deenen MJ et al. Relationship between single nucleotide polymorphisms and haplotypes in *DPYD* and toxicity and efficacy of capecitabine in advanced colorectal cancer. Clin Cancer Res 2011;17:3455-68. PubMed PMID: 21498394.	Level of evidence score: 4 gene act. 1: CTC-AE 5 (gene act. 1 + gene act. 1,5): CTC-AE 4 gene act. 1,5: CTC-AE 4	568 patients with advanced colorectal cancer were treated with capecitabine 1000 mg/m^2 twice daily for 14 days every 3 weeks, in combination with oxaliplatin and bevacizumab, with or without cetuximab. Oxaliplatin was discontinued from cycle 7 and the capecitabine dose increased to 1250 mg/m^2. Grade III-IV toxicity occurred in 85% of the patients. *1/*2A versus *1/*1: - Factor 3.0 increase in the percentage of patients with grade III-IV diarrhoea (from 24% to 71%) (S; strong association: false discovery rate < 0.3) The sensitivity of *2A for predicting grade III-IV diarrhoea was 4% and the specificity 100%. - No increase in the percentage of patients with grade II-III hand-foot syndrome and no significant increase in the percentage of patients with grade III-IV toxicity (NS) All 7 *1/*2A developed grade III-IV toxicity (including 3 women), and 1 patient died during the 3rd cycle. - Decrease in the cumulative dose over the first 6 cycles (S): the average dose decrease increased from 10% to 51% in the lowest-dose cycle and from 10% to 44% in cycle 6. - No difference in mortality or progression-free survival (NS) (*1/c.1236A>G + c.1236A>G/c.1236A>G) versus *1/*1: - Factor 2.2 increase in the percentage of patients with grade III-IV diarrhoea (from 23% to 50%) (S; strong association: false discovery rate < 0.3) The sensitivity of c.1236G>A for predicting grade III-IV diarrhoea was 10% and the specificity 97%. - No significant increase in the percentage of patients with grade II-III hand-foot syndrome or with grade III-IV toxicity (NS). - No significant increase in dose decreases (NS) - No difference in mortality or progression-free survival (NS) *1/c.2846A>T versus *1/*1:	Authors' conclusion: "Of the patients polymorphic for *DPYD* IVS14+1G>A, c.2846A>T, and c.1236G>A, 71% (5 of 7), 63% (5 of 8), and 50% (14 of 28) developed grade 3 to 4 diarrhoea, respectively, compared with 24% in the overall population. …… *DPYD* IVS14+1G>A and 2846A>T predict for severe toxicity to capecitabine, for which patients require dose reductions. ….. The data suggest that initial dose reductions of 50% in IVS14+1 G>A and 25% in c.2846A>T variant allele carriers with further dose titration would significantly reduce the total number of severe toxicity events, thereby separate validation is indicated."

table continues

	- Factor 2.6 increase in the percentage of patients with grade III-IV diarrhoea (from 24% to 62%) (S; medium association: false discovery rate 0.3-0.4) The sensitivity of c.2846A>T for predicting grade III-IV diarrhoea was 4% and the specificity 99%. - No significant increase in the percentage of patients with grade II-III hand-foot syndrome or with grade III-IV toxicity (NS). - Decrease in the cumulative dose over the first 6 cycles (S): the average dose decrease increased from 10% to 27% in the lowest-dose cycle and from 10% to 24% in cycle 6. - No difference in mortality or progression-free survival (NS) (*1/*6 + *6/*6) versus *1/*1: - Factor 1.8 increase in the percentage of patients with grade III-IV diarrhoea (from 23% to 41%) (S; medium association: false discovery rate 0.3-0.4) The sensitivity of *6 (2194G>A) for predicting grade III-IV diarrhoea was 12% and the specificity 95%. - No significant increase in the percentage of patients with grade II-III hand-foot syndrome or with grade III-IV toxicity (NS). - No significant increase in dose decreases (NS) - No difference in mortality or progression-free survival (NS) (*1/496G + 496G/496G) versus *1/*1: - Factor 1.4 increase in the percentage of patients with grade III-IV diarrhoea (from 23% to 33%) (S; weak association: false discovery rate < 0.3) The sensitivity of 496A>G for predicting grade III-IV diarrhoea was 24% and the specificity 84%. - Factor 1.3 increase in the percentage of patients with grade II-III hand-foot syndrome (from 41% to 53%) (S; weak association: false discovery rate < 0.3) The sensitivity of 496A>G for predicting grade II-III hand-foot syndrome was 22% and the specificity 85%. - No significant increase in the percentage of patients with grade III-IV toxicity (NS). - No significant increase in dose decreases (NS) - No difference in mortality or progression-free survival (NS) *13: - The percentage *1/*13 was 0% among 43 patients with grade IV-V toxicity or two forms of grade III-V toxicity and 1% in 99 randomly selected patients (NS) The authors indicated that the lack of association with grade III-IV toxicity for each of the investigated SNPs is likely caused by the high risk in the overall population.	

table continues

		NOTE: No associations were found for gene variants *4 (1601 G>A), *5 (1627A>G) and *9A (85T>C). However, associations with severe toxicity have never been found in studies concerning these gene variants.	
ref. 17 – FU/CAP, mono/comb Kristensen MH et al. Variants in the dihydropyrimidine dehydrogenase, methylenetetrahydrofolate reductase and thymidylate synthase genes predict early toxicity of 5-fluorouracil in colorectal cancer patients. J Int Med Res 2010;38:870-83. PubMed PMID: 20819423.	Level of evidence score: 3 gene act. 1,5: CTC-AE 4	68 patients with advanced colorectal cancer were given adjuvant or palliative treatment with fluoropyrimidine-based therapy. Therapy consisted of either a 5-FU bolus injection 500 mg/m^2 every 2 weeks plus folinic acid (n=24) or fluorouracil (400 mg/m^2 bolus plus 600 mg/m^2 by infusion every 2 weeks) plus folinic acid and oxaliplatin (n=27) or capecitabine 1250 mg/m^2 twice daily for 14 days every 3 weeks (n=17). There was no significant difference between incidences of grade I-IV toxicity in the first 2 cycles caused by the different chemotherapies. However, the proportion of grade III-IV toxicity did differ (67%, 33% and 0% respectively). Results: - Higher frequency of 1896C>T in the group with grade I-IV toxicity than in the group without toxicity (13% versus 2% 1896T heterozygotes; there were no homozygotes; RR = 6) (S) - Of the 4 1896T heterozygotes, 2 developed grade III-IV toxicity, 1 developed grade I toxicity and 1 did not develop toxicity; the number of patients with toxicity was 24, the number of patients without was 44. This is equivalent to 8.3% 1896T heterozygotes in the group with grade III-IV toxicity and 4.5% in the group with < grade III toxicity. This is equivalent to an RR of 1.8 for grade III-IV toxicity.	Authors' conclusion: "Patients with the genetic variant IVS14+1 G/A or c1896 C/T in the *DPYD* gene had a statistically significant increased risk of experiencing toxicity (RR 2 and 6, respectively), both having a high specificity (0.97 and 0.98, respectively) and low sensitivity (0.04 and 0.13, respectively). It is concluded that pre-treatment detection of genetic variants can help to predict early toxicity experienced by patients receiving 5-FU-based chemotherapy."
ref. 18 – FU/CAP, comb Gross E et al. Strong association of a common dihydropyrimidine dehydrogenase gene polymorphism with fluoropyrimidine-related toxicity in cancer patients. PLoS ONE 2008;3:e4003.	Level of evidence score: 3 gene act. 1,5: CTC-AE 5 gene act. 1: CTC-AE 4	128 Caucasian patients including 39 with poor tolerance to FU combination therapy (grade III or IV toxicity). 2 of the patients with poor tolerance died as a result of FU-associated toxicity. Independent group of 53 patients with poor tolerance to FU (n=39) or capecitabine combination therapy (n=14). The presence of variants was investigated by fully sequencing the DPD alleles. Variant 496A>G: Strongest association with grade III and IV toxicity: OR = 4.42 [95% CI = 2.12-9.23] for 92 patients with toxicity. The polymorphism attributable risk was 56.9%. The association was significant in patients with breast and gastro-oesophageal cancer (n=56 and n=158), but was non-significant in colon cancer patients n=128). 1 of the fatalities was heterozygous. All 3 homozygotes had grade III or IV toxicity. Grade III and IV toxicity (especially diarrhoea and hand-foot syndrome) also occurred in carriers using	Authors' conclusion: "Our results show compelling evidence that, at least in distinct tumour types, a common *DPYD* polymorphism strongly contributes to the occurrence of fluoropyrimidine-related drug adverse events. Carriers of this variant could benefit from individual dose adjustment of the fluoropyrimidine drug or alternate therapies."

table continues

		capecitabine-based chemotherapy. Chemotherapy was discontinued in 2 of these. The association seems stronger with combination therapy than with monotherapy. Variant IVS10-15T>C: Association with grade III and IV toxicity: OR = 3.38 [95% CI = 1.71-8.78] for 39 patients with toxicity. The association was significant in patients with breast and gastro-oesophageal cancer (n=46 and n=146), but was non-significant in colon cancer patients (n=58). Variant *2A (IVS14+1G>A): Low allele frequency in these groups (0.03 in patients with severe toxicity; 0 in healthy people and patients without severe toxicity) (NS difference). 16 other variants identified: No significant association with severe toxicity.	
ref. 19 – FU, mono Capitain O et al. The influence of fluorouracil outcome parameters on tolerance and efficacy in patients with advanced colorectal cancer. Pharmacogenomics J 2008;8:256-67.	Level of evidence score: 3 (gene act. 1 + gene act. 1,5):CTC-AE 4(2)[#]	76 French patients with advanced colon cancer received weekly or two-weekly FU plus folinic acid (initial FU dose 1200 and 2500 mg/m^2 respectively; by continuous infusion, two-weekly regimen partially using a bolus (400 mg/m^2); dose adjustments based on a target AUC of 25 mg.h/L; dose reduction of 10% in the event of significant grade II toxicity, discontinuation and dose decrease of 25% in the event of grade III toxicity and discontinuation of therapy in the event of grade IV toxicity), screening for *2A (IVS14+1G>A), c.2846A>T, *13 (1679 T>G) and 464T>A and for DPD-deficient patients and also for 19 other variants. - 11.8% of the patients (n=9) displayed abnormally low clearance of FU associated with abnormal dihydrouracil/uracil plasma ratio prior to therapy. An SNP was found in 3 of these (2x c.2846A>T, 1x *2A). - Despite pharmacological dose adjustments, the incidence of grade III and IV toxicity was higher in the group with reduced DPD activity (n=9) than in the group with normal DPD activity (33.3% versus 7.5%; S by 347%; OR = 6.20 [95% CI = 1.18-32.56]). - The incidence of grade III and IV toxicity was higher in the group with SNPs (n=3) than in the group without SNPs (66.7% versus 8.2%; S by 711%). - The authors indicated that the increased toxicity in DPD-deficient patients may have been prevented by reduced initial doses followed by pharmacokinetic dose adjustments.	Authors' conclusion: "Toxicity was linked to low UH2/U ratio, c.2846 A>T, IVS14+1 G>A for DPD."
ref. 20 – FU Sulzyc-Bielicka V et al.	Level of evidence score: 3	252 Polish colon cancer patients received FU chemotherapy and screening for *2A (IVS14+1G>A). - 1 patient was heterozygous. This patient was 1 of the 4 patients with grade III-IV neutropenia.	Authors' conclusion: "We conclude that IVS14 + 1G > A *DPYD* (*DPYD**2A) variant occurs in the Polish

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5-Fluorouracil toxicity-attributable IVS14 + 1G > A mutation of the dihydropyrimidine dehydrogenase gene in Polish colorectal cancer patients. Pharmacol Rep 2008;60:238-42.	gene act. 1:CTC-AE 4(2)[#]		population and is responsible for a significant proportion of life-threatening toxicity of 5-FU."
ref. 21 – FU, mono Schwab M et al. Role of genetic and nongenetic factors for fluorouracil treatment-related severe toxicity: a prospective clinical trial by the German 5-FU Toxicity Study Group. J Clin Oncol 2008;26:2131-8.	Level of evidence score: 3 gene act. 1: CTC-AE 4	683 German patients (670x *1/*1, 13x *1/*2A), of whom 110 with grade III/IV toxicity; FU monotherapy with folinic acid or levamisole; screening for *2A (IVS14+1G>A) and also sequencing of exons and exon/intron transitions in 28 patients with grade IV toxicity, grade III toxicity or grade 0-II toxicity. *1/*2A versus *1/*1: Increased risk of grade III/IV toxicity: OR = 4.67 [95% CI = 1.54-14.2]. Significantly increased risk of grade III/IV leukopenia and mucositis (OR = 10.19 [95% CI = 3.0-35.1] and OR = 5.8 [95% CI = 1.71-19.4] respectively), but not of grade III/IV diarrhoea. Significantly increased risk of grade III/IV toxicity in men (OR = 41.8 [95% CI = 9.2-190]), but not in women. The sensitivity of *2A genotyping for overall toxicity was 5.5% [95% CI = 0.02-0.11] with a positive predictive value of 0.46 [95% CI = 0.19-0.75]. Sequencing of 3x 28 patients with different toxicity classes: 12 additional SNPs, including 4 new ones. 5 variants (623G>A, *4 (1601G>A), *6 (2194G>A), c.2846 A>T and 2585G>C) further investigated in ≥ 250 patients. 2585G>C was found in 1 patient with grade IV mucositis, but not in other patients (NS). The percentage of patients with toxicity was increased for c.2846A>T (60% versus 16.1% in the overall population) (NS). All other variants did not show a significant association with toxicity.	Authors' conclusion: "*DPYD*, TYMS, and MTHFR play a limited role for FU related toxicity but a pronounced *DPYD* gene/sex-interaction increases prediction rate for male patients."

table continues

		Inclusion of the additional variants only led to a marginal improvement in the prediction of overall toxicity. The method of administration is an independent risk factor: the risk of grade III/IV toxicity was greater for the bolus Mayo regimen than for the high-dose infusion (OR=2.44 [95% CI 1.52-3.91]).	
ref. 22 – FU, comb Mercier C et al. Prospective phenotypic screening for DPD deficiency prior to 5-FU administration: decrease in toxicity, not in efficacy. J Clin Oncol 2008;26(May 20 suppl):abstr 14556. (meeting abstract)	Level of evidence score: 3	59 French patients with inoperable head and neck cancer; determination of DPD activity (dihydrouracil/uracil ratio) prior to FU combination therapy or radio-chemotherapy; mild DPD deficiency (dihydrouracil/uracil ratio < 0.5): FU dose was 80% of the standard dose, severe DPD deficiency (ratio < 0.33): FU dose was 50% of the standard dose, complete DPD deficiency: no FU. - 25% of the patients had mild and 22% severe DPD deficiency. - 12% of the patients with DPD deficiency and dose reduction showed severe toxicity. The incidence of severe toxicity was twofold lower in the overall group compared to the regimen without dose reduction. - There were no toxicity-induced fatalities. - The effectiveness was similar to the regimen without dose reduction (percentages of responders 64% and 81% for first-line chemotherapy and radio-chemotherapy and 50% and 38% for treatment for relapsed cancer).	Authors' conclusion: "5-FU dose tailoring based upon DPD status evaluation led to 2 fold decrease in occurrence of severe toxicities without impairing efficacy."
ref. 23 – FU, comb Jatoi A et al. Paclitaxel, carboplatin, 5-fluorouracil, and radiation for locally advanced esophageal cancer: phase II results of preliminary pharmacologic and molecular efforts to mitigate toxicity and predict outcomes: North Central Cancer	Level of evidence score: 3 gene act. 1: Clinical Relevance Score: AA	50 American patients with locally advanced oesophageal cancer (11x *1/*1, 1x *1/*2A, 16x *1/*5, 3x *1/*6, 13x *1/*9A, 4x *9A/*9A, 1x *5/*5) participating in a phase II study received FU 225 mg/m^2 per day by continuous infusion in combination with carboplatin, paclitaxel and radiotherapy; FU was temporarily discontinued in the event of FU-related grade III-IV toxicity, after which the dose was decreased by 20%; patients received median 81% and 66% of the standard FU dose during 1 and 2 cycles respectively; screening for *2A (IVS14+1G>A), *5 (1627A>G), *6 (2194G>A) and *9A (85T>C). - Almost all patients (94%) had at least 1 incident of grade III-IV toxicity, including 3 fatalities. - No significant associations of the polymorphisms with pathological complete response, time to progression/relapse of cancer, overall survival or grade III/IV toxicity. NB: *5, *6 and *9A do not have reduced DPD activity.	Authors' conclusion: "Genotyping for polymorphisms of dihydropyrimidine dehydrogenase, cytochrome P3A4, and glutathione-S-transferase did not predict tumour response or serious adverse events."

table continues

Treatment Group (N0044). Am J Clin Oncol 2007;30:507-13.			
ref. 24 – FU, comb Magné N et al. Dihydropyrimidine dehydrogenase activity and the IVS14+1G>A mutation in patients developing 5FU-related toxicity. Br J Clin Pharmacol 2007;64:237-40.	Level of evidence score: 3 gene act. 1:CTC-AE 4(2)#	131 French patients with poor tolerance to FU combination or monotherapy (grade II neurotoxicity or grade III-IV toxicity), including 9 fatalities, and 185 unselected patients; screening for DPD activity in peripheral mononuclear blood cells and for *2A (IVS14+1G>A). - 81% of the toxicity occurred during the 1st cycle of FU chemotherapy. - Inverse association between DPD activity and toxicity score (sum of the different toxicity grades per patient) (S). - Percentage of patients with clear or severe DPD deficiency was higher in the case group than in the control group (17% versus 2.7% and 6% versus 0% respectively). - Inverse association between lethal toxicity and DPD activity (S). - Inverse association between the severity of the individual types of toxicity (grade II central neurotoxicity; grade IV mucositis, diarrhoea, neutropenia or thrombocytopenia) and DPD activity (all five S). Median DPD activity was 1.6-3.2x lower in patients with severe toxicity. - Only 2 in 93 screened cases (2.2%) had *2A (both *1/*2A). Both had low DPD activity and high toxicity scores during the 1st cycle. Neither died.	Authors' conclusion: "Present data suggest that IVS14+1 mutation screening has limited effectiveness in identifying patients at risk for severe 5FU toxicity."
ref. 25 - FU/CAP, mono Saif MW et al. Dihydropyrimidine dehydrogenase deficiency (GPD) in GI malignancies: experience of 4-years. Pak J Med Sci Q 2007;23:832-9.	Level of evidence score: 2 gene act. 1: CTC-AE 4	23 patients with excessive toxicity on FU (n=8) or capecitabine therapy (n=15), including 16 Caucasians, 3 Afro-Americans and 3 South-Asians; screening for DPD activity in peripheral mononuclear blood cells and by genotyping. - 30% of the patients had DPD deficiency (n=7), including 3 who were treated with FU (500 mg/m^2 per week or 425 mg/m^2 per week) and folinic acid, 2 who were treated with capecitabine 1800 mg/m^2 and 2 who were treated with high-dose bolus FU (1400 mg/m^2) in combination with the uridine prodrug 2',3',5'-tri-O-acetyluridine. The deficiency was confirmed by genotyping in 1 patient: he was *1/*2A. - 28% of the DPD-deficient patients died due to toxicity (n=2), including 1 to capecitabine and 1 to high-dose bolus FU. - Re-challenge with capecitabine of a patient treated with FU/ folinic acid led to grade III hand-foot syndrome.	Authors' conclusion: "Screening patients for DPD deficiency prior to administration of 5-FU or capecitabine using 2-13C uracil breath test could potentially lower risk of toxicity."

table continues

ref. 26 – FU, mono Boisdron-Celle M et al. 5-Fluorouracil-related severe toxicity: a comparison of different methods for the pretherapeutic detection of dihydropyrimidine dehydrogenase deficiency. Cancer Lett 2007;249:271-82.	Level of evidence score: 3 gene act. 1: CTC-AE 4 gene act. 1,5: CTC-AE 4 gene act. 0,5:CTC-AE 5(2)#	252 French patients with advanced colon cancer (163x *1/*1, 6x *1/c.2846A>T, 1x *9A/c.2846A>T, 1x *1/*2A, 1x -1590C/*2A, 1x *2A/c.2846A>T+85C, 1x *1/-1590C, 67x *1/*9A, 1x -1590C/*9A, 10x *9A/*9A) received either FU 400 mg/m^2 bolus + 2500 mg/m^2 by 46-hour infusion every 2 weeks (n=168) or FU 1200 mg/m^2 by 4-hour infusion per week (n=84) (both regimens: plus folinic acid); dose adjustment from the second cycle based on the FU plasma concentration at the end of the previous infusion (C_{ss}); discontinuation of treatment in the event of grade IV toxicity; screening for *2A (IVS14+1G>A), c.2846A>T, *7 (295-298delTCAT), 1156G>T, *9A (85T>C), *9B (2657G>A), *10 (2983G>T), -1590T>C. (*1/*2A + -1590C/*2A) versus *1/*1: Clearance decreased by 80% (S; from 104.7 to 21.22 L/h per m^2) Increase in the percentage of patients with grade III-IV toxicity by 793% (S; from 5.6% to 50.0%). (*1/c.2846A>T + 1x *9A/c.2846A>T) versus *1/*1: Clearance decreased by 40% and 58% for the two-weekly and weekly regimens respectively (both S; from 136.0 to 81.2 L/h per m^2 and from 104.7 to 43.9 L/h per m^2). Increase in the percentage of patients with grade III-IV toxicity by 1175% (S; from 5.6% to 71.4%). *2A/c.2846A>T+85T versus *1/*1: Clearance decreased to almost 0 (NS; by almost 100%). Increase in the percentage of patients with grade III-IV toxicity by 1686% (NS; from 5.6% to 100%). The patient had grade IV multi-organ toxicity and died after 40 days in Intensive Care. (1x *9A + 2x *9A) versus *1/*1: No difference in clearance and incidence of toxicity (NS). 1x -1590C versus *1/*1: No difference in clearance and incidence of toxicity (NS). Analysis of relevant SNPs had a high specificity (98.3%), but a low sensitivity (47.1%) for detecting DPD deficiency.	Authors' conclusion: "Except in cases where alternative treatment is recommended because the 5-FU metabolism is close to zero, IVS14 + 1G>A or 2846A>T heterozygote are not strict contra-indications to 5-FU treatment, provided that the physician is aware of it and that added precautions are taken, such as an initial 5-FU dose reduction and an individual dose adjustment based on a close clinical and pharmacokinetic follow-up." "In the case of a homozygous status for a relevant SNP, with a uracil plasma level higher than 100 lg/L or a UH2/U ratio below 1, then fluoropyrimidine administration must be discussed and an alternative treatment proposed." Clearance versus gene activity 2: gene act.1.5: 55% gene act.1: 20% gene act.0.5: almost 0%
ref. 27 – FU, mono Cho HJ et al. Thymidylate synthase (TYMS) and	Level of evidence score: 3 gene act. 1,5:	21 Korean colon cancer patients with grade III-IV toxicity on FU therapy (500 mg/m^2 by continuous infusion on days 1-5, plus folinic acid) and 100 healthy volunteers; screening by sequencing all exons and flanking introns.	Authors' conclusion: "The findings, from Korean patients with colon cancer, suggest that polymorphisms of the *DPYD* gene are

table continues

dihydropyrimidine dehydrogenase (*DPYD*) polymorphisms in the Korean population for prediction of 5-fluorouracil-associated toxicity. Ther Drug Monit 2007;29:190-6.	Clinical Relevance Score: AA gene act. 1: Clinical Relevance Score: AA	- Very common variants (allele frequency 14-22%) in this Korean group were *5, 1737T>C and 1896T>C. No *2A was found. - The percentage of patients without SNPs was similar to that in healthy volunteers (9.5% versus 10%). - There was no significant correlation between specific genotypes and toxic response. NB: *5 does not have reduced DPD activity.	not associated with an in-creased risk for toxic response to 5-FU."
ref. 28 – CAP, comb Salgado J et al. Polymorphisms in the thymidylate synthase and dihydropyrimidine dehydrogenase genes predict response and toxicity to capecitabine-raltitrexed in colorectal cancer. Oncol Rep 2007;17:325-8.	Level of evidence score: 3 gene act. 1:CTC-AE 4(2)#	58 Spanish patients with advanced colon cancer received capecitabine (1000 mg/m^2 twice daily for 14 days) and raltitrexed every 3 weeks; screening for *2A (IVS14+1G>A). 1 patient was *1/*2A. This patient developed severe toxicity after the first cycle, after which FU was discontinued and more appropriate chemotherapy was started.	Authors' conclusion: "Considering the common use of fluoropyrimidines, genetic screening would be highly recommendable for the presence of the DPD gene mutation (IVS14+1G>A) related to toxicity, prior to 5-FU administration."
ref. 29 – FU, comb Morel A et al. Clinical relevance of different dihydropyrimidine dehydrogenase gene single nucleotide polymorphisms on 5-fluorouracil tolerance.	Level of evidence score: 3 gene act. 0-1,5: E gene act. 1:CTC-AE 5(2)# gene act. 0:CTC-AE 4(2)#	487 French patients (300x *1/*1, 10x *1/c.2846A>T, 8x *1/*2A, 1x -1590C/*2A, 1x *2A/*2A, 6x *1/-1590C, 144x *1/*9A, 15x *9A/*9A, 1x *1/*13) received FU monotherapy (n=168) or one of 4 different FU combination therapies (n=319); dose adjustment from the second cycle based on the FU plasma concentration at the end of the previous infusion (C_{ss}); discontinuation of treatment or continuation with individual dose adjustment in the event of grade III/IV toxicity; screening for 22 relevant SNPs, including 9 in all patients *2A (IVS14+1G>A), c.2846A>T, *7 (295-298delTCAT), 1156G>T, *9A (85T>C), *9B (2657G>A), *10 (2983G>T), -1590T>C and *13 (1679T>G)) in 171 patients with or without toxicity. 5 variants were found in the population.	Authors' conclusion: "Pretreatment detection of three *DPYD* SNPs could help to avoid serious toxic adverse events. This approach is suitable for clinical practice and should be compared or combined with pharmacologic approaches. In the case of dihydropyrimidine

table continues

Mol Cancer Ther 2006;5:2895-904.		(*1/*2A + *2A/*2A + *1/c.2846A>T + *1/*13) versus *1/*1: Clearance decreased by 43% (S; from 132.3 to 74.9 L/h per m^2) Increase in the percentage of patients with grade III-IV toxicity by 838% (S; from 6.6% to 61.9%). One *1/*2A patient died due to toxicity. The *2A/*2A patient developed grade IV diarrhoea, neutropenia and mucositis a few days after initiation of low-dose bolus FU in combination with epirubicin and cyclophosphamide. She was treated in Intensive Care for 15 days. Patients with SNPs: treatment was discontinued in 40% of the patients with severe toxicity and continued with a 25-50% dose reduction and pharmacokinetic follow-up in the other 60%. (*1/*2A + *1/*13) versus *1/*1: Clearance decreased by 54% (NS; from 132.5 to 60.8 L/h per m^2) *1/c.2846A>T versus *1/*1: Clearance decreased by 45% (NS; from 132.5 to 72.3 L/h per m^2) (*1/*9A + *9A/*9A + *1/-1590C) versus *1/*1: No difference in clearance (NS, increased by 3%). No significant difference in the percentage of patients with grade III-IV toxicity (NS). None of the homozygous patients had grade III/IV toxicity. The sensitivity and specificity of the analysis of the 3 most important SNPs for predicting toxicity were 0.31 and 0.98 respectively.	dehydrogenase deficiency, 5-FU administration often can be safely continued with an individual dose adjustment." Clearance versus gene activity 2: gene act.1.5: 55% gene act.1: 46%
ref. 30 – CAP, mono Largillier R et al. Pharmacogenetics of capecitabine in advanced breast cancer patients. Clin Cancer Res 2006;12:5496-502.	Level of evidence score: 3 gene act. 1:CTC-AE 5(2)[#]	105 French patients with advanced breast cancer received capecitabine monotherapy; screening for *2A (IVS14+1G>A). 1 patient was *1/*2A. This patient died due to haematological toxicity after treatment with capecitabine 1820 mg/m^2 per day for 12 days.	Authors' conclusion: "Our case report clearly identifies DPD deficiency as a source of life-threatening toxicity under capecitabine treatment."
ref. 31 – FU, mono Salgueiro N et al.	Level of evidence score: 3	73 Portuguese colon cancer patients (71x *1/*1, 1x *1/*2A, 1x *1/1845T), including 8 with grade III/IV toxicity; various FU regimens; sequencing of exon 14.	Authors' conclusion: "We conclude that mutations in exon 14 of *DPYD* gene are

table continues

Mutations in exon 14 of dihydropyrimidine dehydrogenase and 5-fluorouracil toxicity in Portuguese colorectal cancer patients. Genet Med 2004;6:102-7.	gene act. 1:CTC-AE 4	SNPs in exon 14 (n=2) versus no SNPs in exon 14: Increase in the percentage of patients with grade III-IV toxicity by 1076% (S; from 8.5% to 100%).	responsible for a significant proportion of life-threatening toxicity to 5-FU, and should therefore be excluded before its administration to cancer patients."
ref. 32 – FU Van Kuilenburg AB et al. High prevalence of the IVS14 + 1G>A mutation in the dihydropyrimidine dehydrogenase gene of patients with severe 5-fluorouracil-associated toxicity. Pharmacogenetics 2002;12:555-8.	Level of evidence score: 3 gene act. 1 + gene act. 0): CTC-AE 4	60 Dutch patients with grade III/IV toxicity on FU therapy (43x *1/*1, 16x *1/*2A, 1x *2A/*2A) and 54 controls, including 35 cancer patients; screening for DPD activity in peripheral mononuclear blood cells and for *2A. - 60% of the cases had reduced DPD activity (< 70% of the average activity in controls). - 29% of the cases had 1 or 2 *2A alleles. - Significantly higher *2A allele frequency in the cases than in the general population (S; increase by 1548% from 0.91% to 15%).	Authors' conclusion: "Our study demonstrates that a DPD deficiency is the major determinant of 5FU-associated toxicity. The apparently high prevalence of the IVS14 + 1G>A mutation warrants genetic screening for this mutation in cancer patients before the administration of 5FU."
ref. 33 – FU, mono Raida M et al. Prevalence of a common point mutation in the dihydropyrimidine dehydrogenase (DPD) gene within the 5'-splice donor site of intron 14 in patients with severe 5-	Level of evidence score: 3 gene act. 1:CTC-AE 5(2)[#] gene act. 0:CTC-AE 5(2)[#]	25 German patients (19x *1/*1, 5x *1/*2A, 1x *2A/*2A) with grade III/IV toxicity on FU monotherapy (n=20), FU chemo-radiotherapy (n=2) or FU combination therapy (n=3) and 851 controls, including 800 cancer patients; screening for *2A. - 24% of the cases had 1 or 2 *2A alleles. - Higher *2A allele frequency in the cases than in the controls (NS; increase by 2879% from 0.47% to 14%). - The homozygous patient and two heterozygous patients died due to toxicity.	Authors' conclusion: "Routine screening for the exon 14-skipping mutation and subsequent individual determination of the 5-FU pharmacokinetics of heterozygous patients provides a concept of individualized therapy and allows the avoidance of undesired treatment toxicity."

table continues

fluorouracil (5-FU)-related toxicity compared with controls. Clin Cancer Res 2001;7:2832-9.			
ref. 34 – FU, comb Yamaguchi K et al. Germline mutation of dihydropyrimidine dehydrogenase gene among a Japanese population in relation to toxicity to 5-fluorouracil. Jpn J Cancer Res 2001;92:337-42.	Level of evidence score: 3 (gene act. 2 + gene act. 1,5): Clinical Relevance Score: AA	69 Japanese patients (61x *1/*1, 4x *1/*9A; 1x *1/*5; 1x *1/74G, 1x *1/812delT, 1x *1/1714G); FU combination therapy or monotherapy (FU: either 800 mg/m^2 by 1-hour infusion or 500 mg/m^2 per day on days 1 and 5 by continuous infusion); screening by PCR and sequencing. - The percentage of patients with grade III/IV toxicity was lower among the 8 heterozygous patients than among the *1/*1 patients (NS; decrease by 18% to 0%). NB: *5 and *9A do not have reduced DPD activity.	Authors' conclusion: "Our observations of Japanese patients implied that the heterozygote is not associated with increased toxic response to 5FU."
ref. 35 – FU van Kuilenburg AB et al. Clinical implications of dihydropyrimidine dehydrogenase (DPD) deficiency in patients with severe 5-fluorouracil-associated toxicity: identification of new mutations in the DPD gene. Clin Cancer Res 2000;6:4705-12.	Level of evidence score: 3 (gene act. 1,5 + gene act. 1): CTC-AE 4	37 Dutch patients with grade III/IV toxicity on FU therapy and 22 controls; sequencing of introns and intron-exon transitions. - 59% of the cases had reduced DPD activity (< 70% of the average activity in controls). - Weak but significant correlation between DPD activity and time to toxicity. - Higher prevalence of grade IV neutropenia in patients with reduced DPD activity compared to those with normal DPD activity (S; increased by 323%, from 13% to 55%). No higher prevalence of other types of toxicity. - 79% of 14 patients with reduced DPD activity had 1 or 2 allele variants (3x *1/*1, 4x *1/*2A, 1x *2A/*9A, 1x *2A/*5, 1x *9A/496G, 1x *9A/496G/c.2846A>T, 1x *1/*5, 1x *5/*9A, 1x *6/*6). NB: *5, *6 and *9A do not have reduced DPD activity.	Authors' conclusion: "Our results demonstrated that at least 57% (8 of 14) of the patients with a reduced DPD activity have a molecular basis for their deficient phenotype."

table continues

ref. 36 – FU, cutaneous Johnson MR et al. Life-threatening toxicity in a dihydropyrimidine dehydrogenase-deficient patient after treatment with topical 5-fluorouracil. Clin Cancer Res 1999;5:2006-11.	Level of evidence score: 2 gene act. 0: CTC-AE 3	A 76-year-old white man developed severe stomatitis, severe inflammatory colitis, erythematous rash, neutropenia $0.6x10^9$/L and thrombocytopenia $57x10^9$/L one week after initiation of 5% FU cream twice daily on the scalp for the treatment of basal cell cancer. FU was discontinued and the patient made a gradual recovery over 3 weeks. The patient was *2A/*2A and had no detectable DPD enzyme activity in peripheral mononuclear blood cells. Assuming 10% cutaneous absorption, the authors estimate that application of 2 g of 5% FU cream leads to a total absorbed dose of ~20 mg/day (~0.33 mg/kg for this patient). This is much lower than the IV bolus FU dose of 500-550 mg/kg that is generally used for chemotherapy.	Authors' conclusion: "This study represents the first characterization of a DPD deficient patient who developed life-threatening toxicity after exposure to topical 5-FU. Considering the previously reported low cutaneous absorption rate (~10%) of topical 5-FU, we suggest that life-threatening toxicity in the population of patients receiving topical 5-FU will be limited to profoundly DPD-deficient patients (no measurable DPD enzyme activity)."
ref. 37 – FU SPC Fluorouracil PCH 15-10-12.	Level of evidence score: 0 gene act. 0-1,5: CTC-AE 4	Warning: There have been reports of increased 5-FU toxicity in patients with partially functional or non-functional dihydropyrimidine dehydrogenase (DPD). If appropriate, DPD enzyme activity should be determined prior to treatment with 5-fluoropyrimidines.	
ref. 38 – FU SPC Efudix (fluorouracil) crème 07-09-16.	Level of evidence score: 0 gene act. 0-1,5: CTC-AE 4	Warning: Individuals with a defective dihydropyrimidine dehydrogenase (DPD) enzyme may be susceptible to severe systemic toxicity on use of standard doses of Efudix due to an increased systemic 5-FU concentration. Evaluation of DPD activity may be considered in patients with confirmed or suspected systemic toxicity. Due to the relationship between DPD deficiency and systemic toxicity, individuals known to have DPD enzyme deficiency should be intensively monitored for systemic toxicity during Efudix treatment. Adverse events: Frequency not known: haematological conditions, such as pancytopenia, neutropenia, thrombocytopenia, leukocytosis; haemorrhagic diarrhoea, diarrhoea, vomiting, stomach pain, stomatitis, rash, nasal mucositis.* * Haematological conditions, stomatitis, rash, nasal mucositis (associated with systemic toxicity to medicinal products).	
ref. 39 - CAP	Level of evidence score: 0	Contraindications: Patients with known complete absence of dihydropyrimidine dehydrogenase (DPD) activity.	

table continues

SPC Xeloda (capecitabine) 26-07-16.	gene act. 0: CTC-AE 5 gene act. 0.5-1.5: CTC-AE 4	Warning: Rarely, unexpected, severe toxicity (e.g. stomatitis, diarrhoea, mucosal inflammation, neutropenia and neurotoxicity) associated with 5-FU has been attributed to a deficiency of DPD activity. Patients with low or absent DPD activity, an enzyme involved in 5-FU degradation, are at increased risk for severe, life-threatening, or fatal adverse reactions caused by 5-FU. Although DPD deficiency cannot be precisely defined, it is known that patients with certain homozygous or certain compound heterozygous mutations in the *DPYD* gene locus, which can cause complete or near complete absence of DPD enzymatic activity (as determined from laboratory assays), have the highest risk of life-threatening or fatal toxicity and should not be treated with Xeloda. No dose has been proven safe for patients with complete absence of DPD activity. For patients with partial DPD deficiency (such as those with heterozygous mutations in the *DPYD* gene) and where the benefits of Xeloda are considered to outweigh the risks (taking into account the suitability of an alternative non-fluoropyrimidine chemotherapeutic regimen), these patients must be treated with extreme caution and frequent monitoring with dose adjustment according to toxicity. There is insufficient data to recommend a specific dose in patients with partial DPD activity as measured by specific test. In patients with unrecognised DPD deficiency treated with capecitabine, life-threatening toxicities manifesting as acute overdose may occur. In the event of grade 2-4 acute toxicity, treatment must be discontinued immediately.
ref. 40 – FU SPC Fluorouracil 29-07-16 (USA) and other[a]	Level of evidence score: 0 gene act. 0: CTC-AE 5 gene act. 0.5-1.5: CTC-AE 5	Warning: Based on post-marketing reports, patients with certain homozygous or certain compound heterozygous mutations in the DPD gene that result in complete or near complete absence of DPD activity are at increased risk for acute early-onset of toxicity and severe, life-threatening, or fatal adverse reactions caused by 5-FU (e.g., mucositis, diarrhoea, neutropenia, and neurotoxicity). Patients with partial DPD activity may also have increased risk of severe, life-threatening, or fatal adverse reactions caused by 5-FU. Withhold or permanently discontinue 5-FU based on clinical assessment of the onset, duration and severity of the observed toxicities in patients with evidence of acute early-onset or unusually severe toxicity, which may indicate near complete or total absence of DPD activity. No 5-FU dose has been proven safe for patients with complete absence of DPD activity. There is insufficient data to recommend a specific dose in

table continues

		patients with partial DPD activity as measured by any specific test.
ref. 41 – FU SPC Carac (fluorouracil) cream 16-12-03 (USA).	Level of evidence score: 0 gene act. 0: CTC-AE 4	Contraindications: Carac should not be used in patients with dihydropyrimidine dehydrogenase (DPD) deficiency. DPD deficiency may lead to 5-FU entering the anabolic route, resulting in cytotoxic activity and possible toxicity. Warning: Patients should discontinue treatment with Carac if symptoms of DPD deficiency develop. Rare, unexpected systemic toxicity (e.g. stomatitis, diarrhoea, neutropenia and neurotoxicity) associated with parenteral administration of 5-FU has been attributed to DPD deficiency. A case of life-threatening systemic toxicity has been reported following topical use of 5% 5-FU by a patient with fully non-functional DPD. Symptoms included severe abdominal pain, haemorrhagic diarrhoea, vomiting, fever and chills. Physical examination showed stomatitis, erythematous rash, neutropenia, thrombocytopenia, inflammation of the oesophagus, stomach and small intestine. Although this patient had used 5% 5-FU cream, it is not known whether patients with severe DPD deficiency develop systemic toxicity in response to lower concentrations of topically administered 5-FU.

[#] For studies that did not show significant differences for intermediate metabolizers (IM) or poor metabolizers (PM) due to very low numbers of IM or PM in the study (<4), the effect for IM or PM was scored as if this concerned a case. This was indicated by placing the case code (2) behind the score.
[a] SPC Xeloda (capecitabine) 14-12-16 (USA).
Abbreviations: 5-FU: 5-fluorouracil; 95% CI: 95% confidence interval; CAP: capecitabine; Cl: clearance; comb: combination therapy (≥ 2 oncolytic drugs), C_{ss}: steady-state plasma concentration; DPD: dihydropyrimidine dehydrogenase; gene act.: gene activity score; gene activity score 2: two fully functional alleles (extensive metaboliser); gene activity score 1.5: one fully functional and one partially functional allele; gene activity score 1: one fully functional and one non-functional allele or two partially functional alleles; gene activity score 0.5: one non-functional and one partially functional allele; gene activity score 0: two non-functional alleles; mono: monotherapy (one oncolytic drug); NS: non-significant; RR: relative risk; S: significant; SNP: single nucleotide polymorphism.

Supplementary Table 2. Literature review of *DPYD*/[tegafur with DPD inhibitor] interactions to support the therapeutic dose guidelines to optimize dose

Reference	Code	Effect	Comments
ref. 1 Cubero DI et al. Tegafur-uracil is a safe alternative for the treatment of colorectal cancer in patients with partial dihydropyrimidine dehydrogenase deficiency: a proof of principle. Ther Adv Med Oncol 2012;4:167-72. PubMed PMID: 22754590.	Level of evidence score: 2 gene act. 1: AA	Four patients with colorectal cancer developed grade 3-4 toxicity after the first cycle of chemotherapy with 5-FU (intravenous bolus of 425 mg/m^2 on days 1 and 5, in combination with folinic acid). They were found to be *1/*2A. After recovery, treatment with tegafur-uracil in combination with folinic acid was initiated. A full dose (100%) was tegafur 100 mg/m^2 three times daily for 21 days followed by a week-long rest period. Doses were rounded down to multiples of 100 mg tegafur. Doses were guided by adverse events. The first patient received 60% in the first cycle, 80% in the second cycle, 100% in the third cycle and 90% in the fourth and fifth cycles of the full dose of tegafur without development of grade 3-4 toxicity. This patient had developed grade 4 mucositis, diarrhoea and myelotoxicity on 5-FU. The following 3 patients received 90% of the full dose of tegafur during 5 cycles without development of grade 3-4 toxicity in any of the cycles. Of the three patients, one developed grade 4 diarrhoea and grade 3 mucositis on 5-FU, the second grade 3 diarrhoea and myelotoxicity, and the third grade 3 mucositis, diarrhoea and myelotoxicity. The best response in the first and the last patient, who both had metastatic disease, was achieving stable disease. The second and third patients receiving adjuvant chemotherapy were disease-free two years after the therapy.	Authors' conclusion: "Here, we demonstrate a complete absence of severe toxicity in all patients and cycles analysed. We believe that UFT is a safe alternative for the treatment of patients with partial DPD deficiency."
ref. 2 Deenen MJ et al. Standard-dose tegafur combined with uracil is not safe treatment after severe toxicity from 5-fluoro-uracil or capecitabine. Ann Intern Med 2010;153:767-8. PubMed PMID: 21135311.	Level of evidence score: 2 gene act. 1: E gene act. 1,5: E	- One patient developed severe abdominal cramps, grade 4 diarrhoea, grade 4 neutropenia, dehydration and severe mucositis 10 days after initiation of capecitabine 1000 mg/m^2 BSA twice daily (in combination with oxaliplatin and bevacizumab). She recovered after discontinuation of capecitabine and 25 days at the hospital. A few months later she received tegafur-uracil 300 mg/m^2 per day in combination with folinic acid. After 10 days, she developed severe diarrhoea, mucositis, fever, dehydration and grade 4 neutropenia. She recovered after 25 days at the hospital. The patient was *1/*2A. - Three other patients requiring hospitalisation due to severe toxicity on 5-FU or capecitabine therapy also developed severe toxicity following treatment with standard-dose tegafur-uracil. The patients were *1/*2A, *1/c.2846A>T and *1/c.1236G>A respectively. The DPD activity was approximately 50% in the latter two patients. This confirms that	Authors' conclusion: "The standard dose of UFT is not safe after severe toxicity to 5-FU or capecitabine in DPD-deficient patients."

table continues

		they were heterozygous and did not have a second unknown non-functional allele. The authors stated that tegafur-uracil is probably not safe in patients with partial DPD deficiency due to the greater effect of the DPD inhibitor uracil in these patients. They referred to an article that showed that uracil increases the half-life of fluorouracil to a greater extent in DPD-deficient patients, which leads to an increased risk of toxicity. The authors also stated that the tegafur dose in tegafur-gimeracil-oteracil is 3x as low as in tegafur-uracil, while the DPD inhibitor is 200x more potent. However, 5-FU is still metabolised by DPD after administration of tegafur-gimeracil-oteracil. This means that DPD also remains essential for detoxification of 5-FU in this instance.
ref. 3 SPC Teysuno (tegafur/gimeracil/ oteracil) 05-04-17.	Level of evidence score: 0 gene act. 0: CTC-AE 4 gene act. 0,5-1,5: E	Contraindications: Known dihydropyrimidine dehydrogenase (DPD) deficiency. History of severe and unexpected reactions to fluoropyrimidine therapy. Pharmacodynamics: Mean 5-FU maximum plasma concentration (C_{max}) and area under the concentration-time curve (AUC) values were approximately 3-fold higher after Teysuno administration than after administration of tegafur alone, despite a 16-fold lower Teysuno dose (50 mg of tegafur) compared to tegafur alone (800 mg), and are attributed to inhibition of DPD by gimeracil. Maximum plasma uracil concentration was observed at 4 hours, with a return to baseline levels within approximately 48 hours after dosing, indicating the reversibility of DPD inhibition by gimeracil. In man, the apparent terminal elimination half-life (T1/2) of 5-FU observed after administration of Teysuno (containing tegafur, a 5-FU prodrug) was longer (approximately 1.6-1.9 hours) than that previously reported after intravenous administration of 5-FU (10 to 20 minutes). Following a single dose of Teysuno, T1/2 values ranged from 6.7 to 11.3 hours for tegafur, from 3.1 to 4.1 hours for gimeracil and from 1.8 to 9.5 hours for oteracil. Interactions: Sorivudine or its chemically related analogues such as brivudine irreversibly inhibit DPD, resulting in a significant increase in 5-FU exposure. This may lead to increased clinically significant fluoropyrimidine-related toxicities with potentially fatal outcomes.

Abbreviations: 5-FU: 5-fluorouracil; DPD: dihydropyrimidine dehydrogenase; gene act.: gene activity score; gene activity score 2: two fully functional alleles (extensive metaboliser); gene activity score 1.5: one fully functional and one partially functional allele; gene activity score 1: one fully functional and one non-functional allele or two partially functional alleles; gene activity score 0.5: one non-functional and one partially functional allele; gene activity score 0: two non-functional alleles.

Supplementary Table 3. Relationship between genotype result and predicted phenotype in patients carrying no variants or one or more variants leading to decreased DPD enzyme activity

Patients carrying no or one variant(s)		
Genotype result	**Genotype (given as functionality of both alleles)**	**Predicted Phenotype**
No aberrant variant (*1/*1)	Full functionality/ full functionality	Gene activity score 2 (100% of normal DPD enzyme activity)
Heterozygous for variant with reduced functionality (*1/c.2846A>T or *1/c.1236G>A)	Fully functionality/ reduced functionality	Gene activity score 1.5 (75% of normal DPD enzyme activity)
Heterozygous for variant with inactive functionality (*1/*2A or *1/*13)	Full functionality/ inactive functionality	Gene activity score 1 (50% of normal DPD enzyme activity)
Homozygous for variant with reduced functionality (c.2846A>T/c.2846A>T or c.1236G>A/c.1236G>A)	Reduced functionality/ reduced functionality	Gene activity score 1 (50% of normal DPD enzyme activity)
Homozygous for variant with inactive functionality (*2A/*2A or*13/*13)	Inactive functionality/ inactive functionality	Gene activity score 0 (0% of normal DPD enzyme activity)
Patients carrying two variants		
Genotype result	**Possible predicted phenotype**	**Reasoning**
Heterozygous for two different variants with reduced functionality (c.2846A>T/c.1236G>A or *1/c.2846A>T+c.1236G>A)	Gene activity score 1 to 1.5 (50% to 75% of normal DPD enzyme activity), phenotyping is required to quantify DPD enzyme activity	When two variants are located on different alleles the predicted gene activity score is 1. When two variants are located on the same allele the predicted gene activity score is dependent on the effect that the two variants have on each other. This effect is unknown. If one of the two variants has no additional effect on the functionality, then the activity of the allele is equal to that without the second variant, thus 0.5, and the gene activity score is 1.5. When the two variants act synergistic and the allele becomes fully inactive, then the activity ofthe allele is 0 and the gene activity score is 1. Since the c.2846A>T and c.1236G>A variants result in reduced DPD enzyme activity through different biological mechanisms (Asp949Val amino acid substitution and an mRNA splicing-defect, respectively), it is probable that they are independent of each other regarding their effect on the allele's functionality. This would result in an allele activity of 0.25 (each variant resulting in half of the allele functionality) and thus a gene activity score of 1.25. Unfortunately there is no recommendation available for gene activity score 1.25.

table continues

		However, other factors than genetic variants can also affect the DPD enzyme activity. For this reason, one should resort to the recommendation for the gene activity score of 1 when the measured DPD enzyme activity is approximately equal to 50% of normal DPD enzyme activity and to the recommendation for the gene activity score of 1.5 when the measured DPD enzyme activity is approximately equal to 75% of normal DPD enzyme activity. When the measured DPD enzyme activity is between 50% and 75% (e.g. 63%) one should resort to the recommendation for gene activity score 1. In this case, one should record a gene activity score of 1.25 in the patients' medical record.
Heterozygous for variants with reduced functionality or inactive functionality (*2A/c.2846A>Tc.2846A>T or *1/*2A+c.2846A>T; *13/c.2846A>Tc.2846A>T or *1/*13+c.2846A>T; *2A/c.1236G>A or *1/*2A+c.1236G>A; *13/c.1236G>A or *1/*13+c.1236G>A)	Gene activity score 0.5 or 1 (25% or 50% of normal DPD enzyme activity), phenotyping is required to quantify DPD enzyme activity	When two variants are located on different alleles the gene activity score is 0.5 (one allele with reduced functionality and one allele with inactive functionality). When two variants are located on the same allele, the gene activity score is 1 (one allele with full functionality and one allele with inactive functionality).
Heterozygous for two different variants with inactive functionality (*2A/*13 or *1/*2A+*13)	Gene activity score 0 or 1 (0% or 50% of normal DPD enzyme activity), phenotyping is required to quantify DPD enzyme activity	When two variants are located on different alleles the gene activity score is 0 (two alleles with inactive functionality). When two variants are located on the same allele the gene activity score is 1 (one allele with full functionality and one allele with inactive functionality).
Homozygous for one variant with reduced functionality and heterozygous for the other variant with reduced functionality	Gene activity score 0.5 to 1 (25% to 50% of normal DPD enzyme activity), phenotyping is required to quantify DPD enzyme activity	One of the alleles has an activity of 0.5. The activity of the other allele is unknown, but lies between 0 and 0.5 (see reasoning for heterozygous for two different alleles with reduced functionality). One should resort to the recommendation for gene activity score 0.5 when the measured
(c.2846A>T/c.2846A>T+ c.1236G>A or c.1236G>A/c.2846A>T+ c.1236G>A)		DPD enzyme activity is approximately 25% of normal DPD enzyme activity and to the recommendation of gene activity score 1 when the DPD enzyme activity is 50% of normal DPD enzyme activity. When the measured DPD enzyme activity is between 25% and 50% (e.g. 38%) one should resort to the recommendation for gene activity score 0.5. In this case, one should record a gene activity score of 0.75 in the patients' medical record.

table continues

Homozygous for a variant with reduced functionality and heterozygous for a variant with inactive functionality (c.2846A>Tc.2846A>T/*2A+ c.2846A>Tc.2846A>T or c.2846A>Tc.2846A>T/*13+ c.2846A>Tc.2846A>T or c.1236G>A/*2A+c.1236G>A or c.1236G>A/*13+ c.1236G>A)	Gene activity score 0.5	One of the alleles has an activity of 0.5, the activity of the other allele is 0. Therefore the gene activity score is 0.5.
Heterozygous for a variant with reduced functionality and homozygous for a variant with inactive functionality 2A/*2A+c.2846A>Tc.2846A>T or *2A/*2A+c.1236G>A or *13/*13+ c.2846A>T/c.2846A>T or *13/*13+c.1236G>A)	Gene activity score 0	Both alleles have an activity of 0. Therefore the gene activity score is 0.
Homozygous for a variant with inactive functionality and heterozygous for the other variant with inactive functionality (*2A/*2A+*13 or *13/*2A+*13)	Gene activity score 0	Both alleles have an activity of 0. Therefore the gene activity score is 0.
Homozygous for two different variants with reduced functionality (c.2846A>T+c.1236G>A/ c.2846A>T+c.1236G>A)	Gene activity score 0 to 1 (0% to 50% of normal DPD enzyme activity), phenotyping is required to quantify DPD enzyme activity	The activity of both alleles is unknown, but lies between 0 and 0.5 (see reasoning for heterozygous for two different reduced functionality alleles). One should resort to the recommendation for gene activity score 0 when the measured DPD enzyme activity is approximately 0% of normal DPD enzyme activity and the recommendation of gene activity score 1 when the DPD enzyme activity is 50% of normal DPD enzyme activity. When the measured DPD enzyme activity is between 0% and 50% (e.g. 25%) one should resort to the recommendation for gene activity score 0.5.
Homozygous for a variant with reduced functionality and a variant with inactive functionality +c.2846A>T/*2A+c.2846A>T or *13+c.2846A>T/*13+ c.2846A>T or *2A+c.1236G>A/*2A+ c.1236G>A or *13+c.1236G>A/*13+ c.1236G>A)	Gene activity score 0	Both alleles have an activity of 0. Therefore the gene activity score is 0.

table continues

Homozygous for two different variants with inactive functionality (*2A+*13/*2A+ *13)	Gene activity score 0	Both alleles have an activity of 0. Therefore the gene activity score is 0.
Patients carrying three or more variants		
Genotype result	**Reasoning for finding the possible predicted phenotype**	
Three or more variants	Since patients carrying three or more different variants are rare, only a general explanation of how to predict the phenotype is given. If one does encounter a patient carrying three or more variants, one must determine how these variants can be located among two alleles and determine if this leads to different predicted phenotypes. Since there are only two validated variants which result in a reduced functionality, an allele with three different variants will always have a variant with an inactive functionality and therefore the allele will have an activity of 0. The predicted allele activities for alleles with 0, 1 or 2 variants are indicated in the tables above. If all possible distributions of the variants across the alleles lead to the same gene activity score of the genotype (i.e. the sum of allele activities), then one can conclude this as the patient's gene activity score. If different distributions lead to genotypes with different gene activity scores, phenotyping is required to quantify DPD enzyme activity.	

Abbreviation: DPD: dihydropyrimidine dehydrogenase.

Supplementary Table 4. Genotype to predicted phenotype translation to be programmed into laboratory information system

Genotype	rs number variants	Nucleotide at position	Dose recommendation according to gene activity score
DPYD: WILDTYPE/WILDTYPE	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:T G:G A:A	GENE ACTIVITY SCORE 2
DPYD: WILDTYPE/*2A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A T:T G:G A:A	GENE ACTIVITY SCORE 1
DPYD: WILDTYPE/*13	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:G *13 G:G A:A	GENE ACTIVITY SCORE 1
DPYD: WILDTYPE/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:T G:G A:T c.2846A>T	GENE ACTIVITY SCORE 1,5
DPYD: WILDTYPE/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:T G:A c.1236G>A A:A	GENE ACTIVITY SCORE 1,5
DPYD: *2A/*2A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A *2A T:T G:G A:A	GENE ACTIVITY SCORE 0
DPYD: *13/*13	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G G:G *13 G:G A:A	GENE ACTIVITY SCORE 0
DPYD: c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:T G:G T:T c.2846A>T	GENE ACTIVITY SCORE 1
DPYD: c.1236G>A/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:T A:A c.1236G>A A:A	GENE ACTIVITY SCORE 1
DPYD: WILDTYPE/*2A WILDTYPE/*13	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A*2A T:G *13 G:G A:A	Unable to predict the gene activity score. Phenotyping should distinguish if both variants are present on separate alleles (GENE ACTIVITY SCORE 0) or on the same allele (GENE ACTIVITY SCORE 1).
DPYD: WILDTYPE/*2A WILDTYPE/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A T:T G:A c.1236G>A A:A	Unable to predict the gene activity score. Phenotyping should distinguish if both variants are present on separate alleles (GENE ACTIVITY SCORE 0.5) or on the same allele (GENE ACTIVITY SCORE 1).

table continues

DPYD: WILDTYPE/*2A WILDTYPE/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A T:T G:G A:T c.2846A>T	Unable to predict the gene activity score. Phenotyping should distinguish if both variants are present on separate alleles (GENE ACTIVITY SCORE 0.5) or on the same allele (GENE ACTIVITY SCORE 1).
DPYD: WILDTYPE/*13 WILDTYPE/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:G*13 G:A c.1236G>A A:A	Unable to predict the gene activity score. Phenotyping should distinguish if both variants are present on separate alleles (GENE ACTIVITY SCORE 0.5) or on the same allele (GENE ACTIVITY SCORE 1).
DPYD: WILDTYPE/*13 WILDTYPE/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:G*13 G:G A:T c.2846A>T	Unable to predict the gene activity score. Phenotyping should distinguish if both variants are present on separate alleles (GENE ACTIVITY SCORE 0.5) or on the same allele (GENE ACTIVITY SCORE 1).
DPYD: WILDTYPE/c.1236G>A WILDTYPE/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:T G:A c.1236G>A A:T c.2846A>T	Unable to predict the gene activity score. Phenotyping should distinguish if both variants are present on separate alleles (GENE ACTIVITY SCORE 1) or on the same allele (GENE ACTIVITY SCORE 1 to 1.5). When both variants are located on the same allele, it is not known whether the variants have an independent or synergistic effect or whether the second variant does not have an additional effect.
DPYD: c.1236G>A/c.1236G>A c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:T A:A c.1236G>A T:T c.2846A>T	Unable to predict the gene activity score (GENE ACTIVITY SCORE 0 to 1). Both variants are located on the same allele, but it is not known whether the variants have an independent or synergistic effect or whether the second variant does not have an additional effect.
DPYD: WILDTYPE/c.1236G>A c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:T G:A c.1236G>A T:T c.2846A>T	Unable to predict the gene activity score (GENE ACTIVITY SCORE 0.5 to 1). There is one allele with one variant and one allele with two variants, but it is not known whether the two variants on the same allele have an independent or synergistic effect or whether the second variant does not have an additional effect.
DPYD: c.1236G>A/c.1236G>A WILDTYPE/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:T A:A c.1236G>A A:T c.2846A>T	Unable to predict the gene activity score (GENE ACTIVITY SCORE 0.5 to 1). There is one allele with one variant and one allele with two variants, but it is not known whether the two variants on the same allele have an independent or synergistic effect or whether the second variant does not have an additional effect.
DPYD: *13/*13 c.1236G>A/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G G:G *13 A:A c.1236G>A A:A	GENE ACTIVITY SCORE 0

table continues

DPYD: *13/*13 WILDTYPE/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G G:G *13 G:A c.1236G>A A:A	GENE ACTIVITY SCORE 0
DPYD: *13/*13 c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G G:G *13 G:G T:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: *13/*13 WILDTYPE/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G G:G *13 G:G A:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: *13/*13 *2A/*2A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A*2A G:G *13 G:G A:A	GENE ACTIVITY SCORE 0
DPYD: *13/*13 WILDTYPE/*2A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A G:G *13 G:G A:A	GENE ACTIVITY SCORE 0
DPYD: *2A/*2A c.1236G>A/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A*2A T:T A:A c.1236G>A A:A	GENE ACTIVITY SCORE 0
DPYD: *2A/*2A WILDTYPE/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A*2A T:T G:A c.1236G>A A:A	GENE ACTIVITY SCORE 0
DPYD: *2A/*2A c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A*2A T:T G:G T:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: *2A/*2A WILDTYPE/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A*2A T:T G:G A:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: *2A/*2A WILDTYPE/*13	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A*2A T:G*13 G:G A:A	GENE ACTIVITY SCORE 0
DPYD: c.1236G>A/c.1236G>A WILDTYPE/*2A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A*2A T:T A:A c.1236G>A A:A	GENE ACTIVITY SCORE 0,5
DPYD: c.1236G>A/c.1236G>A WILDTYPE/*13	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:G*13 A:A c.1236G>A A:A	GENE ACTIVITY SCORE 0,5
DPYD: c.2846A>T/c.2846A>T WILDTYPE/*2A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A T:T G:G T:T c.2846A>T	GENE ACTIVITY SCORE 0,5

table continues

DPYD: c.2846A>T/c.2846A>T WILDTYPE/*13	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:G *13 G:G T:T c.2846A>T	GENE ACTIVITY SCORE 0,5
DPYD: *13/*13 c.1236G>A/c.1236G>A c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G G:G *13 A:A c.1236G>A T:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: *13/*13 wildtype/c.1236G>A c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G G:G *13 G:A c.1236G>A T:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: *13/*13 c.1236G>A/c.1236G>A wildtype/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G G:G*13 A:A c.1236G>A A:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: *13/*13 wildtype/c.1236G>A wildtype/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G G:G*13 G:A c.1236G>A A:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: wildtype/*13 c.1236G>A/c.1236G>A c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:G *13 A:A c.1236G>A T:T c.2846A>T	Unable to predict the gene activity score (GENE ACTIVITY SCORE 0 to 0.5). The activity of the allele with two variants (c.1236G>A and c.2846A>T) is not known, because it is not known whether the variants have an independent or synergistic effect or whether the second variant does not have an additional effect.
DPYD: wildtype/*13 wildtype/c.1236G>A c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:G *13 G:A c.1236G>A T:T c.2846A>T	Unable to predict the gene activity score. Phenotyping should distinguish if *13 and c.1236G>A are present on separate alleles (GENE ACTIVITY SCORE 0 to 0.5) or on the same allele (GENE ACTIVITY SCORE 0.5). When both variants are located on separate alleles, the activity of the allele with the two variants c.1236G>A and c.2846A>T is not known, because it is not known whether the variants have an independent or synergistic effect or whether the second variant does not have an additional effect.
DPYD: wildtype/*13 c.1236G>A/c.1236G>A wildtype/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:G *13 A:A c.1236G>A A:T c.2846A>T	Unable to predict the gene activity score. Phenotyping should distinguish if *13 and c.2846A>T are present on separate alleles (GENE ACTIVITY SCORE 0 to 0.5) or on the same allele (GENE ACTIVITY SCORE 0.5). When both variants are located on separate alleles, the activity of the allele with the two variants c.1236G>A and c.2846A>T is not known, because it is not known whether the variants have an independent or synergistic effect or whether the second variant does not have an additional effect.

table continues

DPYD: wildtype/*13 wildtype/c.1236G>A wildtype/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:G T:G*13 G:A c.1236G>A A:T c.2846A>T	Unable to predict the gene activity score (GENE ACTIVITY SCORE 0 to 1). Phenotyping should distinguish which variants are present on the same allele. The activity of an allele with the two variants c.1236G>A and c.2846A>T is not known, because it is not known whether the variants have an independent or synergistic effect or whether the second variant does not have an additional effect.
DPYD: *2A/*2A c.1236G>A/c.1236G>A c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A *2A T:T A:A c.1236G>A T:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: *2A/*2A wildtype/c.1236G>A c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A *2A T:T G:A c.1236G>A T:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: *2A/*2A c.1236G>A/c.1236G>A wildtype/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A *2A T:T A:A c.1236G>A A:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: *2A/*2A wildtype/c.1236G>A wildtype/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A *2A T:T G:A c.1236G>A A:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: *2A/*2A *13/*13 c.1236G>A/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A *2A G:G *13 A:A c.1236G>A A:A	GENE ACTIVITY SCORE 0
DPYD: *2A/*2A *13/*13 wildtype/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A *2A G:G *13 G:A c.1236G>A A:A	GENE ACTIVITY SCORE 0
DPYD: *2A/*2A *13/*13 c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A*2A G:G*13 G:G T:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: *2A/*2A *13/*13 wildtype/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A*2A G:G*13 G:G A:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: *2A/*2A wildtype/*13 c.1236G>A/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A*2A T:G*13 A:A c.1236G>A A:A	GENE ACTIVITY SCORE 0
DPYD: *2A/*2A wildtype/*13 wildtype/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A *2A T:G *13 G:A c.1236G>A A:A	GENE ACTIVITY SCORE 0
DPYD: *2A/*2A wildtype/*13 c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A *2A T:G *13 G:G T:T c.2846A>T	GENE ACTIVITY SCORE 0

table continues

DPYD: *2A/*2A wildtype/*13 wildtype/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	A:A *2A T:G *13 G:G A:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: wildtype/*2A c.1236G>A/c.1236G>A c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A T:T A:A c.1236G>A T:T c.2846A>T	Unable to predict the gene activity score (GENE ACTIVITY SCORE 0 to 0.5). The activity of the allele with two variants (c.1236G>A and c.2846A>T) is not known, because it is not known whether the variants have an independent or synergistic effect or whether the second variant does not have an additional effect.
DPYD: wildtype/*2A wildtype/c.1236G>A c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A T:T G:A c.1236G>A T:T c.2846A>T	Unable to predict the gene activity score. Phenotyping should distinguish if *2A and c.1236G>A are present on separate alleles (GENE ACTIVITY SCORE 0 to 0.5) or on the same allele (GENE ACTIVITY SCORE 0.5). When both variants are located on separate alleles, the activity of the allele with the two variants c.1236G>A and c.2846A>T is not known, because it is not known whether the variants have an independent or synergistic effect or whether the second variant does not have an additional effect.
DPYD: wildtype/*2A c.1236G>A/c.1236G>A wildtype/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A T:T A:A c.1236G>A A:T c.2846A>T	Unable to predict the gene activity score. Phenotyping should distinguish if *2A and c.2846A>T are present on separate alleles (GENE ACTIVITY SCORE 0 to 0.5) or on the same allele (GENE ACTIVITY SCORE 0.5). When both variants are located on separate alleles, the activity of the allele with the two variants c.1236G>A and c.2846A>T is not known, because it is not known whether the variants have an independent or synergistic effect or whether the second variant does not have an additional effect.
DPYD: wildtype/*2A wildtype/c.1236G>A wildtype/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A T:T G:A c.1236G>A A:T c.2846A>T	Unable to predict the gene activity score (GENE ACTIVITY SCORE 0 to 1). Phenotyping should distinguish which variants are present on the same allele. The activity of an allele with the two variants c.1236G>A and c.2846A>T is not known, because it is not known whether the variants have an independent or synergistic effect or whether the second variant does not have an additional effect.
DPYD: wildtype/*2A *13/*13 c.1236G>A/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A G:G *13 A:A A:A c.1236G>A	GENE ACTIVITY SCORE 0
DPYD: wildtype/*2A *13/*13 wildtype/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A G:G *13 G:A c.1236G>A A:A	GENE ACTIVITY SCORE 0

table continues

DPYD: wildtype/*2A *13/*13 c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A G:G *13 G:G T:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: wildtype/*2A *13/*13 wildtype/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A G:G *13 G:G A:T c.2846A>T	GENE ACTIVITY SCORE 0
DPYD: wildtype/*2A wildtype/*13 c.1236G>A/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A T:G *13 A:A c.1236G>A A:A	Unable to predict the gene activity score. Phenotyping should distinguish if *2A and *13 are present on separate alleles (GENE ACTIVITY SCORE 0) or on the same allele (GENE ACTIVITY SCORE 0.5).
DPYD: wildtype/*2A wildtype/*13 wildtype/c.1236G>A	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A T:G *13 G:A c.1236G>A A:A	Unable to predict the gene activity score (GENE ACTIVITY SCORE 0 to 1). Phenotyping should distinguish which variants are present on the same allele.
DPYD: wildtype/*2A wildtype/*13 c.2846A>T/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A T:G *13 G:G T:T c.2846A>T	Unable to predict the gene activity score. Phenotyping should distinguish if *2A and *13 are present on separate alleles (GENE ACTIVITY SCORE 0) or on the same allele (GENE ACTIVITY SCORE 0.5).
DPYD: wildtype/*2A wildtype/*13 wildtype/c.2846A>T	*DPYD*_rs3918290 *DPYD*_rs55886062 *DPYD*_rs56038477 *DPYD*_rs67376798	G:A *2A T:G *13 G:G A:T c.2846A>T	Unable to predict the gene activity score (GENE ACTIVITY SCORE 0 to 1). Phenotyping should distinguish which variants are present on the same allele.

[#]NOTE: In patients with two different gene variants, the gene activity score is dependent on location of the variants on the alleles. The variants can either be located on the same allele (resulting in one affected allele with reduced or absent DPD activity and one fully functional allele) or located on different alleles (resulting in two affected alleles).

Supplementary Table 5. Dutch Pharmacogenetics Working Group (DPWG) Guideline for *DPYD* and 5-FU/capecitabine: the therapeutic recommendation and its rationale, and the kinetic and clinical consequences for each aberrant gene activity score

Predicted phenotype: Gene activity score 0 **Ref.** [1-13]	
Therapeutic recommendation	SYSTEMIC ROUTE OF ADMINISTRATION: **Choose an alternative.** Tegafur is not an alternative, as this is also metabolised by DPD. **If an alternative is not available: determine the residual DPD activity in mononuclear cells from peripheral blood and adjust the initial dose accordingly.** A patient with 0.5% of normal DPD activity tolerated 0.8% of the standard dose (150 mg capecitabine every five days). A patient with undetectable DPD activity tolerated 0.43% of the standard dose (150 mg capecitabine every 5 days with every third dose skipped) The average Caucasian DPD activity is 9.9 nmol/hour per mg protein. Adjust the initial dose based on toxicity and efficacy. NOTE: If a patient carries two different genetic variations that lead to a non-functional DPD enzyme (e.g. *2A and *13), this recommendation only applies if the variations are on different alleles. If both variations are on the same allele, the patient is assigned a gene activity score of 1 and the recommendation for that gene activity score should be followed. These two situations can only be distinguished by determining the enzyme activity (phenotyping). CUTANEOUS ROUTE OF ADMINISTRATION: **Choose an alternative** NOTE: If a patient has two different genetic variations that lead to a non-functional DPD enzyme (e.g. *2A and *13), this recommendation only applies if the variations are on a different allele. If both variations are on the same allele, this patient is assigned a gene activity score of 1, for which no increased risk of severe, potentially fatal toxicity has been found with cutaneous use. These two situations can only be distinguished by determining the enzyme activity (phenotyping).
Rationale of the therapeutic recommendation	There are not enough data available to be able to make a substantiated recommendation on dose adjustments for patients assigned gene activity score 0. The recommendation for *1/*2A is a dose reduction by 50%. This would be equivalent to a dose reduction by 100% for *2A/*2A and therefore a dose reduction to 0%. This is equivalent to severe toxicity found in one patient with genotype *2A/*2A when using 5-FU cream on the scalp. Because of the indications that the tolerated dose is close to zero and the scarce data on tolerated doses in patients assigned a gene activity score of 0 (see below), an alternative is advised. The calculated dose reduction based on two patients is a reduction to 0.81% of the normal dose (0.72-0.89%; median 0.81%). However, this is based on too few patients to be used for a substantiated dose recommendation. In addition, in one of these patients, having undetectable DPD activity, the dose had to be reduced from 0.65% to 0.43% of the normal dose during treatment. However, there is a fairly good correlation between the residual DPD enzyme activity in peripheral blood mononuclear cells and the tolerated dose (Meulendijks 2016, Deenen 2016, Henricks 2017 JCO Precis Oncol and Henricks 2017 Int J Cancer). Therefore, if an alternative is not possible, adjusting the dose according to the residual DPD enzyme activity in peripheral blood mononuclear cells is advised. This strategy has been shown to be feasible in two patients with genotype *2A/*2A. A patient with 0.5% of the normal DPD activity tolerated 0.8% of the normal dose (150 mg capecitabine every five days) (Henricks 2017 Int J Cancer). A patient with undetectable DPD activity, tolerated 0.43% of the normal dose (150 mg capecitabine every five days with every third dose skipped) (Henricks 2017 JCO Precis Oncol).

table continues

Kinetic consequence	For two patients with genotype *2A/*2A the dose-corrected AUC of 5-FU increased by a factor 113 and 138 respectively after the first systemic capecitabine dose. Extrapolation of the decrease in clearance by 50% identified for *1/*2A would suggest a clearance of 0% for *2A/*2A (gene activity score 0). This is equivalent to severe toxicity found in one patient with *2A/*2A after using 5-FU cream on the scalp and the two previously described patients using very low tolerated systemic doses (0.8% and 0.43% of the standard dose).
Clinical consequence	SYSTEMIC ROUTE OF ADMINISTRATION: All patients assigned a gene activity score of 0 with known toxicity (n=2, both *2A/*2A), had grade III/IV toxicity and 50% died due to toxicity. Moreover, a patient with *2A/*2A developed severe toxicity after treatment with cutaneous 5-FU cream. CUTANEOUS ROUTE OF ADMINISTRATION: A patient with *2A/*2A developed severe toxicity after treatment with cutaneous 5-FU cream. All patients using systemic 5-FU assigned a gene activity score of 0 with known toxicity (n=2, both *2A/*2A), had grade III/IV toxicity and 50% died due to toxicity.
Predicted phenotype: Gene activity score 0.5 **Ref.** [3-5,8-12,14,15]	
Therapeutic recommendation	**Start with 25% of the standard dose or choose an alternative.** Adjustment of the initial dose should be guided by toxicity and effectiveness. Tegafur is not an alternative, as this is also metabolised by DPD. NOTE: This recommendation only applies if the two genetic variations are on a different allele. If both variations are on the same allele, this patient has gene activity score 1 and the recommendation for that gene activity score should be followed. These two situations can only be distinguished by determining the enzyme activity (phenotyping).
Rationale of the therapeutic recommendation	Clearance has only been determined for one patient assigned a gene activity score of 0.5 (Boisdron-Celle, 2007). The clearance found for this patient with genotype *2A/c.2846A>T was almost zero. Extrapolation of the required dose reduction by 50% for *1/*2A and the required dose reduction by 25% for *1/c.2846A>T and *1/c.1236G>A would, however, lead to a required dose reduction by 75% for *2A/c.2846A>T. The dose reductions for *1/*2A, *1/2486T and *1/c.1236G>A are based on more than one patient. Moreover, the Boisdron-Celle article found a much lower clearance for one patient with genotype *1/*2A than the weighted average for this genotype (reduction by 80% instead of by 50%). For this reason, the recommendation given is based on extrapolation and therefore constitutes a dose reduction to 25% of the normal dose. Instead of dose adjustment, physicians may also choose an alternative.
Kinetic consequence	Clearance decreased by almost 100% in one patient assigned a gene activity score of 0.5 (*2A/c.2846A>T). Extrapolation of the dose reductions identified for *1/*2A, *1/c.2846A>T and *1/c.1236G>A would, however, lead to a dose reduction by 75%.
Clinical consequence	Clinical consequences are only known for three patients (all genotype *2A/c.2846A>T). The first patient developed grade III/IV toxicity and died due to toxicity. The second patient developed grade V toxicity and tolerated only one cycle of FOLFOX plus cetuximab. The third patient received half of the standard dose, but despite this the fluoropyrimidine therapy was stopped after the first cycle due to side effects (≤ grade 3).
Predicted phenotype: Gene activity score 1 **Ref.** [1,3-6,8-12,14-39]	
Therapeutic recommendation	**Start with 50% of the standard dose or choose an alternative.** Adjustment of the initial dose should be guided by toxicity and effectiveness. Tegafur is not an alternative, as this is also metabolised by *DPD.*

table continues

	NOTE 1: The dose reduction described here is well substantiated for *1/*2A and c.1236G>A/c.1236G>A. The dose reduction for patients with c.2846A>T (c.2846A>T/c.2846A>T or c.1236G>A/c.2846A>T) is based on, among other factors, the dose reductions identified for *1/c.2846A>T. NOTE 2: If a patient has two different genetic variations that result in a partially functional DPD enzyme (e.g. c.2846A>T and c.1236G>A), this recommendation applies if the variations are on a different allele. If both variations are on the same allele, the gene activity score is between 1 and 1.5, depending on whether and how the two gene variations influence each other and on other factors that influence the DPD activity. Whether a gene activity score of 1 or 1.5 needs to be assigned in the case of two different genetic variations can only be determined by measuring the enzyme activity (phenotyping).
Rationale of the therapeutic recommendation	For 25 patients with genotype *1/*2A, one with genotype *1/*13, one with genotype c.2846A>T/c.2846A>T and one with genotype c.1236G>A/c.2846A>T, the weighted average of the dose adjustments calculated based on 5-FU clearance or AUC was a reduction to 45% (18-49%, median 33%). Because the relatively low median was caused by the low values found in the two smallest studies (n = 1 and n = 2 respectively), it was decided to base the dose recommendation on the weighted mean. The weighted mean of 45% was translated to 50% to be more achievable in clinical practice. This is similar to the dose reduction to 56% and 60% of the standard dose found by Deenen 2011 and Meulendijks 2016 when investigating patients with respectively *1/*2A and c.1236G>A/c.1236G>A in whom toxicity-guided dose adjustments were made. It is also similar to the mean tolerated dose of 55% found by Henricks 2017 JCO Precis Oncol for 2x c.1236G>A/c.1236G>A, 1x c.1236G>A/c.2846A>T and 1x c.2846A>T/c.2846A>T, although in this study a strongvariation between patients (and genotypes) was found. In addition, Deenen 2016 found no difference in toxicities between 18 patients with *1/*2A on an initial dose of maximally 50% of the standard dose and *1/*1-patients on the standard dose. Lunenburg 2016 found no grade ≥ 3 toxicity when treating three patients with *1/*2A with an initial dose of 50% of the standard dose. There are no data on clearance or AUC for c.1236G>A/c.1236G>A and only scarce data on clearance or AUC or on maximum tolerated dose in clinical practice for c.2846A>T/c.2846A>T and c.1236G>A/c.2846A>T. Deenen 2011 found a dose reduction to 74% of the standard dose for patients with *1/c.2846A>T when toxicity-guided dose adjustments were made. Extrapolation of the required dose reduction for *1/c.2846A>T would lead to a required dose reduction to 50% for c.2846A>T/c.2846A>T. This is equivalent to the dose reduction for *1/*2A and c.1236G>A/c.1236G>A, which are also in the gene activity score 1 group. Instead of dose adjustment, physicians may also choose an alternative.
Kinetic consequence	Increase in the AUC of 5-FUby 103% (16x *1/*2A), 127% (1x c.1236G>A/c.2846A>T) or 766% (1x c.2846A>T/c.2846A>T). 52-80% decrease in clearance. 69-109% increase in half-life.
Clinical consequence	7 of the 10 studies and two meta-analyses found an increased risk of grade ≥ 3 toxicity. Increased grade ≥ 3 toxicity: OR = 4.67-24.9; RR = 4.40-9.76. The highest ORs were found for haematological toxicity. There was a 74-793% increase in the percentage of patients with grade ≥ 3 toxicity. Out of 48 patients with genotype *1/*2A in published cohort studies, 73% developed grade ≥ 3 toxicity. The allele frequency of *2A in a group with grade III/IV toxicity was 1548-2879% higher. Toxicity generally occurred in the first cycle. Six patients died due to toxicity, including two that had used capecitabine. No association with grade ≥ 3 toxicity was found for breast cancer patients receiving adjuvant/neoadjuvant therapy with 5-FU, epirubicin and cyclophosphamide in a phase II study that showed 94% grade ≥ 3 toxicity and in a small study of 21 patients with grade ≥ 3 toxicity. 5-FU toxicity is not common in breast cancer patients treated with this combination therapy.

table continues

	A large study found that the *2A allele only increased the risk of grade ≥ 3 toxicity in men (OR = 41.8) and not in women. Other studies did not find any differences between men and women. When the dose was guided by toxicity, the average dose in the sixth cycle was 56% of the standard dose in seven patients with genotype *1/*2A. Dose reduction down to 40% or 50% of the standard dose was not adequate in two *1/*2A patients in another study. There was no difference in grade ≥ 3 toxicity between 18 patients with genotype *1/*2A at ≤ 50% of the standard dose and non-selected patients on the standard dose. In another study, four patients with genotype *1/*2A did not develop grade ≥ 3 toxicity at 50% of the standard dose. One of them had previously developed grade ≥ 3 toxicity during the first cycle at the standard dose. One of them tolerated a dose increase to 60%, the other two did not tolerate a dose increase to 80% and 100% respectively. Of the three patients with genotype c.1236G>A/c.1236G>A, one tolerated a standard dose. A second patient tolerated the treatment after dose reduction to 60% of the standard dose. Another study found a mean tolerated dose of 55% of the standard dose for 2x c.1236G>A/c.1236G>A, 1x c.1236G>A/c.2846A>T and 1x c.2846A>T/c.2846A>T, although in this study a strong variation between patients (and genotypes) was found (17-100% of the standard dose).
Predicted phenotype: Gene activity score 1.5 **Ref.** [3-5,8-12,14-16,20,21,25-27,33,38-41]	
Therapeutic recommendation	**Start with 75% of the standard dose or choose an alternative.** Adjustment of the initial dose should be guided by toxicity and effectiveness. Tegafur is not an alternative, as this is also metabolised by DPD.
Rationale of the therapeutic recommendation	For *1/c.2846A>T, the weighted average of the calculated dose adjustments was a reduction to 55%. However, Deenen 2011 investigated 8 patients with *1/c.2846A>T and found a toxicity-guided dose reduction to 74% of the standard dose. In addition, Lunenburg 2016 found no grade ≥ 3 toxicity when treating five patients with *1/c.1236G>A with an initial dose of 75% of the standard dose. As oncolytic under dosing should be avoided, the dose adjustment determined in clinical practice has been included in the recommendation. Instead of dose adjustment, physicians may also choose an alternative.
Kinetic consequence	40-58% decrease in clearance.
Clinical consequence	Four of the five studies and one meta-analysis found an increased risk of grade ≥ 3 toxicity. Increased grade ≥ 3 toxicity: OR = 4.42-9.35. The percentage of patients with grade ≥ 3 toxicity was 109-1175% higher. One patient (*1/496G) died due to toxicity. No association with grade ≥ 3 toxicity was found in one small study of 21 patients with grade ≥ 3 toxicity. When the dose for eight patients with genotype *1/c.2846A>T was guided by toxicity, the average dose in the sixth cycle was 76% of the standard dose. Five patients with genotype *1/c.1236G>A did not develop grade ≥ 3 toxicity at 75 % of the standard dose. The two patients for who the dose was then increased tolerated the standard dose. One patient with genotype *1/c.1236G>A, who was started at the standard dose, developed grade 3-4 toxicity in the first cycle.

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Supplementary Table 6. Dutch Pharmacogenetics Working Group (DPWG) Guideline for *DPYD* and tegafur with DPD inhibitors: the therapeutic recommendation and its rationale, and the kinetic and clinical consequences for each aberrant gene activity score

Predicted phenotype: Gene activity score 0 **Ref.** [1]	
Therapeutic recommendation	**Choose an alternative.** Do not choose 5-FU or capecitabine, as these are also metabolised by DPD. **If an alternative is not possible: start with a very low dose and adjust the initial dose based on toxicity and efficacy.** A substantiated recommendation for dose reduction cannot be made based on the literature. The recommendation for 5-FU and capecitabine is to determine the residual DPD activity in mononuclear cells from peripheral blood and to adjust the initial dose accordingly. A patient with 0.5% of the normal DPD activity tolerated 0.8% of the standard capecitabine dose (150 mg every 5 days). A patient with undetectable DPD activity tolerated 0.43% of the standard capecitabine dose (150 mg every five days with every third dose skipped) The average Caucasian DPD activity is 9.9 nmol/hour per mg protein. NOTE: If a patient carries two different gene variations that lead to a non-functional DPD enzyme (e.g. *2A and *13), this recommendation only applies if the variations are on different alleles. If both variations are on the same allele, this patient is assigned a gene activity score of 1 and the recommendation for that gene activity score should be followed. These two situations can only be distinguished by determining the enzyme activity (phenotyping).
Rationale of the therapeutic recommendation	There are no data available on the use of tegafur in combination with a DPD inhibitor for patients assigned a gene activity score of 0. The SPCs state that tegafur in combination with a DPD inhibitor is contraindicated in patients with dihydropyrimidine dehydrogenase deficiency, but do not substantiate this. However, two patients using standard doses of tegafur-uracil, who developed severe toxicity, were found to be assigned partially deficient phenotypes (gene activity scores of 1 and 1.5). The toxicity was similar to that found in patients treated with capecitabine or 5-FU, both of which are given without a DPD inhibitor. The DPD inhibitor is 200 times more potent in the tegafur-gimeracil-oteracil combination. However, 5-FU is still metabolised by DPD after administration of this combination and DPD is therefore also involved in 5-FU clearance. For 5-FU and capecitabine, the maximally tolerated dose of 50% of the normal dose for *1/*2A indicates that the maximally tolerated dose for *2A/*2A (gene activity score 0) is close to zero, as do the scarce data on tolerated doses in patients with gene activity score 0. For this reason, an alternative is advised. There is a fairly good correlation between the residual DPD enzyme activity in peripheral blood mononuclear cells and the tolerated 5-FU or capecitabine dose. Therefore, if an alternative is not available, adjusting the dose according to the residual DPD enzyme activity in peripheral blood mononuclear cells is advised. This strategy has been shown to be feasible for capecitabine in two patients with genotype *2A/*2A. A patient with 0.5% of the normal DPD activity tolerated 0.8% of the normal capecitabine dose (150 mg every five days). A patient with undetectable DPD activity, tolerated 0.43% of the normal capecitabine dose (150 mg every five days with every third dose skipped).This is why this strategy is also recommended for tegafur in case an alternative is not possible.

table continues

Kinetic consequence	Studies regarding the kinetic consequences are unavailable.
Clinical consequence	Studies regarding the clinical consequences are unavailable. The SmPC states that this combination is contraindicated in patients with DPD deficiency. This probably refers to gene activity score 0. No safe dose for 5-FU (the metabolite of tegafur) has been found for patients assigned a gene activity score of 0. In addition, four patients with a less deficient DPD activity (assigned a gene activity score of 1 or 1.5) had a comparable toxicity for treatment with tegafur/uracil as found for treatment with 5-FU or capecitabine.
Predicted phenotype: Gene activity score 0.5 **Ref.** [1]	
Therapeutic recommendation	**Choose an alternative or start with a low dose and adjust the initial dose based on toxicity and efficacy.** Do not choose 5-FU or capecitabine, as these are also metabolised by DPD. A substantiated recommendation for dose reduction cannot be made based on the literature. For 5-FU and capecitabine, starting with 25% of the standard dose is recommended. NOTE: This recommendation only applies if the two gene variations are on different alleles. If both variations are on the same allele, this patient is assigned a gene activity score of 1 and the recommendation for that gene activity score should be followed. These two situations can only be distinguished by determining the enzyme activity (phenotyping).
Rationale of the therapeutic recommendation	There are no data available on the use of tegafur in combination with a DPD inhibitor for gene activity score 0.5. The SPCs state that tegafur in combination with a DPD inhibitor is contraindicated in patients with a history of serious and unexpected reactions to fluoropyrimidine therapy, but do not substantiate this. However, two patients using standard doses of tegafur-uracil who developed severe toxicity were found to be assigned partially deficient phenotypes of gene activity scores of 1 and 1.5. The toxicity was similar to that found in patients treated with capecitabine or 5-FU, both of which are given without a DPD inhibitor. The recommendation for 5-FU and capecitabine in patients with gene activity score 0.5 is to reduce the dose to 25% of the standard dose or to choose an alternative. This is why a dose reduction or alternative is also recommended for tegafur.
Kinetic consequence	Studies regarding the kinetic consequences are unavailable.
Clinical consequence	Studies regarding the clinical consequences are unavailable. However, four patients with a less deficient DPD activity (assigned a gene activity score of 1 or 1.5) had a comparable toxicity for treatment with tegafur/uracil as found for treatment with 5-FU or capecitabine. In addition to this, four patients assigned a gene activity score of 1 could be treated with 90 % of the standard tegafur/uracil dose without grade 3-4 toxicity occurring.
Predicted phenotype: Gene activity score 1.0 **Ref.** [1-3]	
Therapeutic recommendation	**Choose an alternative or start with a low dose and adjust the initial dose based on toxicity and efficacy.** Do not choose 5-FU or capecitabine, as these are also metabolised by DPD. A substantiated recommendation for dose reduction cannot be made based on the literature. For 5-FUand capecitabine, starting with 50 % of the standard dose is recommended. NOTE: If a patient has two different gene variations that result in a partially functional DPD enzyme (e.g. c.2846A>T and c.1236G>A), this recommendation

table continues

	only applies if the variations are on different alleles. If both variations are on the same allele, the gene activity score assigned is between 1 and 1.5, depending on whether and how the two gene variations influence each other and on other factors that influence the DPD activity. Whether a gene activity score of 1 or 1.5 needs to be assigned in the case of two different genetic variations can only be determined by measuring the enzyme activity (phenotyping).
Rationale of the therapeutic recommendation	Treatment with tegafur in combination with the DPD inhibitor uracil in two patients with gene activity score 1 led to similar toxicity as found after treatment with 5-FU or capecitabine. However, four patients with an assigned gene activity score of 1 could be treated with 90% of the standard tegafur-uracil dose without grade 3-4 toxicity occurring. Similar to data found for 5-FU and capecitabine, treatment with a reduced dose of tegafur-uracil seems possible for patients who are assigned a gene activity score of 1. This is why a dose reduction or alternative is recommended.
Kinetic consequence	Studies regarding the kinetic consequences are unavailable.
Clinical consequence	In a study, two patients had a comparable toxicity for treatment with tegafur/uracil as found for treatment with 5-FU or capecitabine. In another study, four patients could be treated with 90 % of the standard tegafur/uracil dose without grade 3-4 toxicity occurring. All six patients had the genotype *1/*2A.
Predicted phenotype: Gene activity score 1.5 **Ref.** [1,3]	
Therapeutic recommendation	**Choose an alternative or start with a low dose and adjust the initial dose based on toxicity and efficacy.** Do not choose 5-FU or capecitabine, as these are also metabolised by DPD. A substantiated recommendation for dose reduction cannot be made based on the literature. For 5-FU and capecitabine, starting with 75 % of the normal dose is recommended.
Rationale of the therapeutic recommendation	Treatment with tegafur in combination with the DPD inhibitor uracil in two patients with gene activity score 1.5 led to similar toxicity as found after treatment with 5-FU or capecitabine. However, four patients with the more deficient phenotype (gene activity score 1) could be treated with 90% of the standard tegafur-uracil dose without grade 3-4 toxicity occurring. Similar to data found for 5-fluorouracil and capecitabine, treatment with a reduced dose of tegafur-uracil seems possible for patients with gene activity score 1 or higher. This is why a dose reduction or alternative is recommended.
Kinetic consequence	Studies regarding the kinetic consequences are unavailable.
Clinical consequence	Two patients with gene activity score 1.5 had a comparable toxicity for treatment with tegafur/uracil as found for treatment with 5-FU or capecitabine. Four patients with gene activity score 1 could be treated with 90 % of the standard tegafur/uracil dose without grade 3-4 toxicity occurring.

Abbreviations: Ref.: References; 5-FU: 5-fluorouracil; AUC: Area Under the Curve; DPD: dihydropyrimidine dehydrogenase; OR: Odds Ratio.

References

1. SPC Teysuno (tegafur/gimeracil/oteracil) 05 April 2017.
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Supplementary Table 7. Suggested clinical decision support texts for various health care professionals for 5-FU/capecitabine

DPD gene act. 0: 5-fluorouracil (5-FU)/capecitabine, SYSTEMIC

Pharmacist text / Hospital text / Prescriber text
Genetic variation increases the risk of severe, potentially fatal toxicity. A reduced conversion of 5-fluorouracil/ capecitabine to inactive metabolites means that the standard dose is a more than 100-fold overdose.

Recommendation:
- Choose an alternative
  Tegafur is not an alternative, as this is also metabolised by DPD.
- If an alternative is not possible:
  - Determine the residual DPD activity in mononuclear cells from peripheral blood and adjust the initial dose accordingly.
    A patient with 0.5% of the normal DPD activity tolerated 0.8% of the standard dose (150 mg capecitabine every 5 days). A patient with undetectable DPD activity tolerated 0.43% of the standard dose (150 mg capecitabine every 5 days with every third dose skipped)
    The average Caucasian DPD activity is 9.9 nmol/hour per mg protein.
  - Adjust the initial dose based on toxicity and efficacy.

NOTE: If a patient has two different genetic variations that lead to a non-functional DPD enzyme (e.g. *2A and *13), this recommendation only applies if the variations are on a different allele. If both variations are on the same allele, this patient has gene activity score 1 and the recommendation for that gene activity score should be followed. These two situations can only be distinguished by determining the enzyme activity (phenotyping).

Background information
Mechanism:
5-Fluorouracil and its prodrug capecitabine are mainly converted by dihydropyrimidine dehydrogenase (DPD) to inactive metabolites. Genetic variations result in reduced DPD activity and thereby to reduced conversion of 5-fluorouracil to inactive metabolites. As a result, the intracellular concentration of the active metabolite of 5-fluorouracil can increase, resulting in severe, potentially fatal toxicity.
For more information about the phenotype gene activity score 0: see the general background information about DPD on the KNMP Knowledge Bank or on www.knmp.nl (search for DPD).

Clinical consequences:
All patients with gene activity score 0 with known toxicity (n=2, both *2A/*2A), had grade III/IV toxicity and 50% died due to toxicity. Moreover, a patient with *2A/*2A developed severe toxicity after treatment with cutaneous 5-fluorouracil cream.

Kinetic consequences:
For 2 patients with genotype *2A/*2A the dose-corrected AUC of 5-fluorouracil increased by a factor 113 and 138 respectively after the first systemic capecitabine dose. Extrapolation of the decrease in clearance by 50% identified for *1/*2A would suggest a clearance of 0% for *2A/*2A (gene activity score 0). This is equivalent to severe toxicity found in one patient with *2A/*2A after using 5-fluorouracil cream on the scalp and the two previously described patients using very low tolerated systemic doses (0.8% and 0.43% of the standard dose).

Literature
1. Rosmarin D et al. Genetic markers of toxicity from capecitabine and other fluorouracil-based regimens: investigation in the QUASAR2 study, systematic review, and meta-analysis. J Clin Oncol 2014; 32:1031-9.
2. Deenen MJ et al. Relationship between single nucleotide polymorphisms and haplotypes in *DPYD* and toxicity and efficacy of capecitabine in advanced colorectal cancer. Clin Cancer Res 2011; 17:3455-68.
3. Gross E et al. Strong association of a common dihydropyrimidine dehydrogenase gene polymorphism with fluoropyrimidine-related toxicity in cancer patients. PLoS ONE 2008;3:e4003.
4. Boisdron-Celle M et al. 5-Fluorouracil-related severe toxicity: a comparison of different methods for the pretherapeutic detection of dihydropyrimidine dehydrogenase deficiency. Cancer Lett 2007;249:271-82.

5. Morel A et al. Clinical relevance of different dihydropyrimidine dehydrogenase gene single nucleotide polymorphisms on 5-fluorouracil tolerance. Mol Cancer Ther 2006;5:2895-904.
6. Van Kuilenburg AB et al. High prevalence of the IVS14 + 1G>A mutation in the dihydropyrimidine dehydrogenase gene of patients with severe 5-fluorouracil-associated toxicity. Pharmacogenetics 2002;12:555-8.
7. Raida M et al. Prevalence of a common point mutation in the dihydropyrimidine dehydrogenase (DPD) gene within the 5'-splice donor site of intron 14 in patients with severe 5-fluorouracil (5-FU)- related toxicity compared with controls. Clin Cancer Res 2001;7:2832-9.
8. van Kuilenburg AB et al. Clinical implications of dihydropyrimidine dehydrogenase (DPD) deficiency in patients with severe 5-fluorouracil-associated toxicity: identification of new mutations in the DPD gene. Clin Cancer Res 2000;6:4705-12.
9. Johnson MR et al. Life-threatening toxicity in a dihydropyrimidine dehydrogenase-deficient patient after treatment with topical 5-fluorouracil. Clin Cancer Res 1999;5:2006-11.
10. SPC Carac cream (VS), Efudix crème, Fluorouracil P and Xeloda.

DPD gene act. 0: 5-fluorouracil (5-FU) CUTANEOUS

Pharmacist text/ Hospital text / Prescriber text
Genetic variation increases the risk of severe, potentially fatal toxicity. A reduced conversion of 5-fluorouracil/ capecitabine to inactive metabolites means that the normal dose is an overdose.

Recommendation:
- Choose an alternative
  NOTE: If a patient has two different genetic variations that lead to a non-functional DPD enzyme (e.g. *2A and *13), this recommendation only applies if the variations are on a different allele. If both variations are on the same allele, this patient has gene activity score 1, for which no increased risk of severe, potentially fatal toxicity has been found with cutaneous use. These two situations can only be distinguished by determining the enzyme activity (phenotyping).

Background information
Mechanism:
5-Fluorouracil is mainly converted by dihydropyrimidine dehydrogenase (DPD) to inactive metabolites. Genetic variations result in reduced DPD activity and thereby to reduced conversion of 5-fluorouracil to inactive metabolites. As a result, the intracellular concentration of the active metabolite of 5-fluorouracil can increase, resulting in severe, potentially fatal toxicity.
For more information about the phenotype gene activity score 0: see the general background information about DPD on the KNMP Knowledge Bank or on www.knmp.nl (search for DPD).

Clinical consequences:
A patient with *2A/*2A developed severe toxicity after treatment with cutaneous 5-fluorouracil cream. All patients using systemic 5-fluorouracil with gene activity score 0 with known toxicity (n=2, both *2A/*2A), had grade III/IV toxicity and 50% died due to toxicity.

Kinetic consequences:
For 2 patients with genotype *2A/*2A the dose-corrected AUC of 5-fluorouracil increased by a factor 113 and 138 respectively after the first systemic capecitabine dose.
Extrapolation of the decrease in clearance by 50% identified for *1/*2A would suggest a clearance of 0% for *2A/*2A (gene activity score 0). This is equivalent to severe toxicity found in one patient with *2A/*2A after using 5-fluorouracil cream on the scalp and the two previously described patients using very low tolerated systemic doses (0.8% and 0.43% of the standard dose).

Literature
1. Rosmarin D et al. Genetic markers of toxicity from capecitabine and other fluorouracil-based regimens: investigation in the QUASAR2 study, systematic review, and meta-analysis. J Clin Oncol 2014; 32:1031-9.
2. Gross E et al. Strong association of a common dihydropyrimidine dehydrogenase gene polymorphism with fluoropyrimidine-related toxicity in cancer patients. PLoS ONE 2008;3:e4003.
3. Boisdron-Celle M et al. 5-Fluorouracil-related severe toxicity: a comparison of different methods for the pretherapeutic detection of dihydropyrimidine dehydrogenase deficiency. Cancer Lett 2007;249:271-82.
4. Morel A et al. Clinical relevance of different dihydropyrimidine dehydrogenase gene single nucleotide polymorphisms on 5-fluorouracil tolerance. Mol Cancer Ther 2006;5:2895-904.
5. Van Kuilenburg AB et al. High prevalence of the IVS14 + 1G>A mutation in the dihydropyrimidine dehydrogenase gene of patients with severe 5-fluorouracil-associated toxicity. Pharmacogenetics 2002;12:555-8.
6. Raida M et al. Prevalence of a common point mutation in the dihydropyrimidine dehydrogenase (DPD) gene within the 5'-splice donor site of intron 14 in patients with severe 5-fluorouracil (5-FU)- related toxicity compared with controls. Clin Cancer Res 2001;7:2832-9.
7. van Kuilenburg AB et al. Clinical implications of dihydropyrimidine dehydrogenase (DPD) deficiency in patients with severe 5-fluorouracil-associated toxicity: identification of new mutations in the DPD gene. Clin Cancer Res 2000;6:4705-12.
8. Johnson MR et al. Life-threatening toxicity in a dihydropyrimidine dehydrogenase-deficient patient after treatment with topical 5-fluorouracil. Clin Cancer Res 1999;5:2006-11.
9. SPC Carac cream (VS) en Efudix crème.

DPD gene act. 0.5: 5-fluorouracil (5-FU)/capecitabine

Pharmacist text / Hospital text / Prescriber text
Genetic variation increases the risk of severe, potentially fatal toxicity. A reduced conversion of 5-fluorouracil/ capecitabine to inactive metabolites means that the normal dose is an overdose.

Recommendation:
- Start with 25% of the standard dose or choose an alternative.
  Adjustment of the initial dose should be guided by toxicity and effectiveness.
  Tegafur is not an alternative, as this is also metabolised by DPD.
  NOTE: This recommendation only applies if the two genetic variations are on a different allele. If both variations are on the same allele, this patient has gene activity score 1 and the recommendation for that gene activity score should be followed. These two situations can only be distinguished by determining the enzyme activity (phenotyping).

Background information
Mechanism:
5-Fluorouracil and its prodrug capecitabine are mainly converted by dihydropyrimidine dehydrogenase (DPD) to inactive metabolites. Genetic variations result in reduced DPD activity and thereby to reduced conversion of 5-fluorouracil to inactive metabolites. As a result, the intracellular concentration of the active metabolite of 5-fluorouracil can increase, resulting in severe, potentially fatal toxicity.
For more information about the phenotype gene activity score 0.5: see the general background information about DPD on the KNMP Knowledge Bank or on www.knmp.nl (search for DPD).

Clinical consequences:
Clinical consequences are only known for 3 patients (all genotype *2A/2846T). The first patient developed grade III/IV toxicity and died due to toxicity. The second patient developed grade V toxicity and tolerated only one cycle of FOLFOX plus cetuximab. The third patient received half the standard dose, but despite this the fluoropyrimidine therapy was stopped after the first cycle due to side effects (≤ grade 3).

Kinetic consequences:
Clearance decreased by almost 100% in one patient with gene activity score 0.5 (*2A/2846T). Extrapolation of the dose reductions identified for *1/*2A, *1/2846T and *1/1236A would, however, lead to a dose reduction by 75%.

Literature
1. Deenen MJ et al. Relationship between single nucleotide polymorphisms and haplotypes in *DPYD* and toxicity and efficacy of capecitabine in advanced colorectal cancer. Clin Cancer Res 2011; 17:3455-68.
2. Boisdron-Celle M et al. 5-Fluorouracil-related severe toxicity: a comparison of different methods for the pretherapeutic detection of dihydropyrimidine dehydrogenase deficiency. Cancer Lett 2007;249:271-82.
3. Morel A et al. Clinical relevance of different dihydropyrimidine dehydrogenase gene single nucleotide polymorphisms on 5-fluorouracil tolerance. Mol Cancer Ther 2006;5:2895-904.
4. SPC Efudix crème and Fluorouracil PCH.

DPD gene act. 1: 5-fluorouracil (5-FU)/capecitabine

Pharmacist text / Hospital text / Prescriber text
Genetic variation increases the risk of severe, potentially fatal toxicity. A reduced conversion of 5-fluorouracil/capecitabine to inactive metabolites means that the normal dose is an overdose.

Recommendation:
- Start with 50% of the standard dose or choose an alternative.
  Adjustment of the initial dose should be guided by toxicity and effectiveness.
  Tegafur is not an alternative, as this is also metabolised by DPD.
  NB1: The dose reduction described here is well substantiated for *1/*2A and 1236A/1236A. The dose reduction for patients with 2846T (2846T/2846T or 1236A/2846T) is based on, among other factors, the dose reductions identified for *1/2846T.
  NB2: If a patient has two different genetic variations that result in a partially functional DPD enzyme (e.g. 2846T and 1236A), this recommendation applies if the variations are on a different allele. If both variations are on the same allele, the gene activity score is between 1 and 1.5, depending on whether and how the two gene variations influence each other and on other factors that influence the DPD activity. Whether a gene activity score of 1 or 1.5 needs to be assigned in the case of two different genetic variations can only be determined by measuring the enzyme activity (phenotyping).

Background information
Mechanism:
5-Fluorouracil and its prodrug capecitabine are mainly converted by dihydropyrimidine dehydrogenase (DPD) to inactive metabolites. Genetic variations result in reduced DPD activity and thereby to reduced conversion of 5-fluorouracil to inactive metabolites. As a result, the intracellular concentration of the active metabolite of 5-fluorouracil can increase, resulting in severe, potentially fatal toxicity.
For more information about the phenotype gene activity score 1: see the general background information about DPD on the KNMP Knowledge Bank or on www.knmp.nl (search for DPD).

Clinical consequences:
7 of the 10 studies and two meta-analyses found an increased risk of grade ≥ 3 toxicity. Increased grade ≥ 3 toxicity: OR = 4.67-24.9; RR = 4.40-9.76. The highest ORs were found for haematological toxicity. There was a 74-793% increase in the percentage of patients with grade ≥ 3 toxicity. Out of 48 patients with genotype *1/*2A in published cohort studies, 73% developed grade ≥ 3 toxicity. The allele frequency of *2A in a group with grade III/IV toxicity was 1548-2879% higher. Toxicity generally occurred in the first cycle. Six patients died due to toxicity, including two that had used capecitabine.
No association with grade ≥ 3 toxicity was found for breast cancer patients receiving adjuvant/neoadjuvant therapy with 5-fluorouracil, epirubicin and cyclophosphamide in a phase II study that showed 94% grade ≥ 3 toxicity and in a small study of 21 patients with grade ≥ 3 toxicity. 5-Fluorouracil toxicity is not common in breast cancer patients treated with this combination therapy.
A large study found that the *2A allele only increased the risk of grade ≥ 3 toxicity in men (OR = 41.8) and not in women. Other studies did not find any differences between men and women.
When the dose was guided by toxicity, the average dose in the sixth cycle was 56% of the standard dose in 7 *1/*2A. Dose reduction down to 40% or 50% of the standard dose was not adequate in two *1/*2A patients in another study. There was no difference in grade ≥ 3 toxicity between 18 *1/*2A at ≤ 50% of the standard

dose and non-selected patients on the standard dose. In another study, 4 *1/*2A did not develop grade ≥ 3 toxicity at 50% of the standard dose. One of them had previously developed grade ≥ 3 toxicity during the first cycle at the standard dose. One of them tolerated a dose increase to 60%, the other two did not tolerate a dose increase to 80% and 100% respectively. Of the 3 patients with genotype 1236A/1236A, one tolerated a standard dose. A second patient tolerated the treatment after dose reduction to 60% of the standard dose. Another study found a mean tolerated dose of 55% of the standard dose for 2x 1236A/1236A, 1x 1236A/2846T and 1x 2846T/2846T, although in this study a strong variation between patients (and genotypes) was found (17-100% of the standard dose).

Kinetic consequences:
Increase in the AUC of 5-fluorouracil by 103% (16x *1/*2A), 127% (1x 1236A/2846T) or 766% (1x 2846T/2846T).

52-80% decrease in clearance.
69-109% increase in half-life.

Literature

1. Rosmarin D et al. Genetic markers of toxicity from capecitabine and other fluorouracil-based regimens: investigation in the QUASAR2 study, systematic review, and meta-analysis. J Clin Oncol 2014; 32:1031-9.
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24. SPCs Efudix crème and Fluorouracil PCH.

DPD gene act. 1.5: 5-fluorouracil (5-FU)/capecitabine

Pharmacist text / Hospital text / Prescriber text
Genetic variation increases the risk of severe, potentially fatal toxicity. A reduced conversion of 5-fluorouracil/ capecitabine to inactive metabolites means that the normal dose is an overdose.

Recommendation:

- Start with 75% of the standard dose or choose an alternative.
  Adjustment of the initial dose should be guided by toxicity and effectiveness.
  Tegafur is not an alternative, as this is also metabolised by DPD.

Background information
Mechanism:
5-Fluorouracil and its prodrug capecitabine are mainly converted by dihydropyrimidine dehydrogenase (DPD) to inactive metabolites. Genetic variations result in reduced DPD activity and thereby to reduced conversion of 5-fluorouracil to inactive metabolites. As a result, the intracellular concentration of the active metabolite of 5-fluorouracil can increase, resulting in severe, potentially fatal toxicity.
For more information about the phenotype gene activity score 1.5: see the general background information about DPD on the KNMP Knowledge Bank or on www.knmp.nl (search for DPD).

Clinical consequences:
4 of the 5 studies and one meta-analysis found an increased risk of grade ≥ 3 toxicity. Increased grade ≥ 3 toxicity: OR = 4.42-9.35. The percentage of patients with grade ≥ 3 toxicity was 109-1175% higher. One patient (*1/496G) died due to toxicity.
No association with grade ≥ 3 toxicity was found in one small study of 21 patients with grade ≥ 3 toxicity.
When the dose for 8 *1/2846T was guided by toxicity, the average dose in the sixth cycle was 76% of the standard dose. 5 patients with genotype *1/1236A did not develop grade ≥ 3 toxicity at 75 % of the standard dose. The two patients for who the dose was then increased tolerated the standard dose. One patient with genotype *1/1236A, who was started at the standard dose, developed grade 3-4 toxicity in the first cycle.

Kinetic consequences:
40-58% decrease in clearance.

Literature
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12. SPCs Efudix crème and Fluorouracil PCH.

Supplementary Table 8. Suggested clinical decision support texts for health care professionals for tegafur with DPD inhibitors

DPD gene act. 0: tegafur
Pharmacist text / Hospital text / Prescriber text

Genetic variation increases the risk of severe, possibly fatal toxicity. A reduced conversion of tegafur to inactive metabolites means that the normal dose is an overdose.

Recommendation:
- Choose an alternative
  Do not choose 5-fluorouracil or capecitabine, as these are also metabolised by DPD.
- If an alternative is not possible: start with a very low dose and adjust the initial dose based on toxicity and efficacy.
  A substantiated recommendation for dose reduction cannot be made based on the literature. The recommendation for 5-fluorouracil and capecitabine is to determine the residual DPD activity in mononuclear cells from peripheral blood and to adjust the initial dose accordingly. A patient with 0.5% of the normal DPD activity tolerated 0.8% of the standard capecitabine dose (150 mg every 5 days).
  A patient with undetectable DPD activity tolerated 0.43% of the standard capecitabine dose (150 mg every 5 days with every third dose skipped)
  The average Caucasian DPD activity is 9.9 nmol/hour per mg protein.

NOTE: If a patient has two different gene variations that lead to a non-functional DPD enzyme (e.g. *2A and *13), this recommendation only applies if the variations are on a different allele. If both variations are on the same allele, this patient has gene activity score 1 and the recommendation for that gene activity score should be followed. These two situations can only be distinguished by determining the enzyme activity (phenotyping).

Background information

Mechanism:
Tegafur is mainly converted by CYP2A6 to 5-fluorouracil. 5-Fluorouracil is mainly (> 80 %) converted by dihydropyrimidine dehydrogenase (DPD) to inactive metabolites. Genetic variations result in reduced DPD activity and thereby to reduced conversion of 5-fluorouracil to inactive metabolites. As a result, the intracellular concentration of the active metabolite of 5-fluorouracil can increase, resulting in severe, potentially fatal toxicity. Tegafur is used in combination with the DPD inhibitor gimeracil (molar ratio 1:0.4) and was used in combination with the DPD inhibitor uracil (molar ratio 1:4). Both DPD inhibitors exhibit competitive inhibition of DPD. This is why efficacy is achieved at lower concentrations of the metabolites formed by DPD, which seem to contribute to the toxicity. Inhibition by DPD inhibitors is reversible and reduces over time.
For more information about the phenotype gene activity score 0: see the general background information about DPD on the KNMP Knowledge Bank or on www.knmp.nl (search for “DPD”).

Clinical consequences:
There are no studies into the clinical consequences of tegafur in combination with a DPD inhibitor for gene activity score 0. The SmPC states that this combination is contra-indicated in patients with DPD deficiency. This probably refers to gene activity score 0. No safe dose has been found for gene activity score 0 for 5-fluorouracil (the metabolite of tegafur). In addition to this, four patients with a less strongly reduced DPD activity (gene activity score 1 or 1.5) had a comparable toxicity for treatment with tegafur/uracil as found for treatment with 5-fluorouracil or capecitabine.

Kinetic consequences:
There are no studies into the kinetic consequences.

Literature
1. Deenen MJ et al. Standard-dose tegafur combined with uracil is not safe treatment after severe toxicity from 5-fluorouracil or capecitabine. Ann Intern Med 2010;153:767-8.
2. SPC Teysuno.

DPD gene act. 0.5: tegafur

Pharmacist text / Hospital text / Prescriber text
Genetic variation increases the risk of severe, possibly fatal toxicity. A reduced conversion of tegafur to inactive metabolites means that the normal dose is an overdose.

Recommendation:
- Choose an alternative or start with a low dose and adjust the initial dose based on toxicity and efficacy
  5-fluorouracil and capecitabine are not alternatives, as these are also metabolised by DPD.
  It is not possible to offer substantiated advice for dose reduction based on the literature. For 5-fluorouracil and capecitabine, starting with 25% of the standard dose is recommended.
  NOTE: This recommendation only applies if the two gene variations are on a different allele. If both variations are on the same allele, this patient has gene activity score 1 and the recommendation for that gene activity score should be followed. These two situations can only be distinguished by determining the enzyme activity (phenotyping).

Background information

Mechanism:
Tegafur is mainly converted by CYP2A6 to 5-fluorouracil. 5-Fluorouracil is mainly (> 80 %) converted by dihydropyrimidine dehydrogenase (DPD) to inactive metabolites. Genetic variations result in reduced DPD activity and thereby to reduced conversion of 5-fluorouracil to inactive metabolites. As a result, the intracellular concentration of the active metabolite of 5-fluorouracil can increase, resulting in severe, potentially fatal toxicity. Tegafur is used in combination with the DPD inhibitor gimeracil (molar ratio 1:0.4) and was used in combination with the DPD inhibitor uracil (molar ratio 1:4). Both DPD inhibitors exhibit competitive inhibition of DPD. This is why efficacy is achieved at lower concentrations of the metabolites formed by DPD, which seem to contribute to the toxicity. Inhibition by DPD inhibitors is reversible and reduces over time.
For more information about the phenotype gene activity score 0.5: see the general background information about DPD on the KNMP Knowledge Bank or on www.knmp.nl (search for “DPD”).

Clinical consequences:
There are no studies into the clinical consequences of tegafur in combination with a DPD inhibitor for gene activity score 0.5. However, four patients with a less strongly reduced DPD activity (gene activity score 1 or 1.5) had a comparable toxicity for treatment with tegafur/uracil as found for treatment with 5-fluorouracil or capecitabine. In addition to this, four patients with gene activity score 1 could be treated with 90 % of the standard tegafur/uracil dose without grade 3-4 toxicity occurring.

Kinetic consequences:
There are no studies into the kinetic consequences.

Literature
1. Cubero DI et al. Tegafur-uracil is a safe alternative for the treatment of colorectal cancer in patients with partial dihydropyrimidine dehydrogenase deficiency: a proof of principle. Ther Adv Med Oncol 2012;4:167-72.
2. Deenen MJ et al. Standard-dose tegafur combined with uracil is not safe treatment after severe toxicity from 5-fluorouracil or capecitabine. Ann Intern Med 2010;153:767-8.
3. SPC Teysuno.

DPD gene act. 1.0: tegafur

Pharmacist text / Hospital text / Prescriber text
Genetic variation increases the risk of severe, possibly fatal toxicity. A reduced conversion of tegafur into inactive metabolites means that the normal dose is an overdose.

Recommendation:
- Choose an alternative or start with a low dose and adjust the initial dose based on toxicity and efficacy
  5-Fluorouracil and capecitabine are not alternatives, as these are also metabolised by DPD.
  It is not possible to offer substantiated advice for dose reduction based on the literature. For 5-fluorouracil and capecitabine, starting with 50 % of the standard dose is recommended.
  NOTE: If a patient has two different gene variations that result in a partially functional DPD enzyme (e.g. 2846T and 1236A), this recommendation only applies if the variations are on a different allele. If both variations are on the same allele, the gene activity score is between 1 and 1.5, depending on whether and how the two gene variations influence each other and on other factors that influence the DPD activity. Whether a gene activity score of 1 or 1.5 needs to be assigned in the case of two different genetic variations can only be determined by measuring the enzyme activity (phenotyping).

Background information

Mechanism:
Tegafur is mainly converted by CYP2A6 to 5-fluorouracil. 5-Fluorouracil is mainly (> 80 %) converted by dihydropyrimidine dehydrogenase (DPD) to inactive metabolites. Genetic variations result in reduced DPD activity and thereby to reduced conversion of 5-fluorouracil to inactive metabolites. As a result, the intracellular concentration of the active metabolite of 5-fluorouracil can increase, resulting in severe, potentially fatal toxicity. Tegafur is used in combination with the DPD inhibitor gimeracil (molar ratio 1:0.4) and was used in combination with the DPD inhibitor uracil (molar ratio 1:4). Both DPD inhibitors exhibit competitive inhibition of DPD. This is why efficacy is achieved at lower concentrations of the metabolites formed by DPD, which seem to contribute to the toxicity. Inhibition by DPD inhibitors is reversible and reduces over time.
For more information about the phenotype gene activity score 1: see the general background information about DPD on the KNMP Knowledge Bank or on www.knmp.nl (search for “DPD”).

Clinical consequences:
In a study, two patients had a comparable toxicity for treatment with tegafur/uracil as found for treatment with 5-fluorouracil or capecitabine. In another study, four patients could be treated with 90 % of the standard tegafur/uracil dose without grade 3-4 toxicity occurring. All six patients had the genotype *1/*2A.

Kinetic consequences:
There are no studies into the kinetic consequences.

Literature
1. Cubero DI et al. Tegafur-uracil is a safe alternative for the treatment of colorectal cancer in patients with partial dihydropyrimidine dehydrogenase deficiency: a proof of principle. Ther Adv Med Oncol 2012;4:167-72.
2. Deenen MJ et al. Standard-dose tegafur combined with uracil is not safe treatment after severe toxicity from 5-fluorouracil or capecitabine. Ann Intern Med 2010;153:767-8.
3. SPC Teysuno.

DPD gene act. 1.5: tegafur

Pharmacist text / Hospital text / Prescriber text
Genetic variation increases the risk of severe, possibly fatal toxicity. A reduced conversion of tegafur into inactive metabolites means that the normal dose is an overdose.

Recommendation:
- Choose an alternative or start with a low dose and adjust the initial dose based on toxicity and efficacy
5-Fluorouracil and capecitabine are not alternatives, as these are also metabolised by DPD.
It is not possible to offer substantiated advice for dose reduction based on the literature. For 5-fluorouracil and capecitabine, starting with 75 % of the normal dose is recommended.

Background information

Mechanism:
Tegafur is mainly converted by CYP2A6 to 5-fluorouracil. 5-Fluorouracil is mainly (> 80 %) converted by dihydropyrimidine dehydrogenase (DPD) to inactive metabolites. Genetic variations result in reduced DPD activity and thereby to reduced conversion of 5-fluorouracil to inactive metabolites. As a result, the intracellular concentration of the active metabolite of 5-fluorouracil can increase, resulting in severe, potentially fatal toxicity. Tegafur is used in combination with the DPD inhibitor gimeracil (molar ratio 1:0.4) and was used in combination with the DPD inhibitor uracil (molar ratio 1:4). Both DPD inhibitors exhibit competitive inhibition of DPD. This is why efficacy is achieved at lower concentrations of the metabolites formed by DPD, which seem to contribute to the toxicity. Inhibition by DPD inhibitors is reversible and reduces over time.
For more information about the phenotype gene activity score 1.5: see the general background information about DPD on the KNMP Knowledge Bank or on www.knmp.nl (search for “DPD”).

Clinical consequences:
Two patients with gene activity score 1.5 had a comparable toxicity for treatment with tegafur/uracil as found for treatment with 5-fluorouracil or capecitabine. Four patients with gene activity score 1 could be treated with 90 % of the standard tegafur/uracil dose without grade 3-4 toxicity occurring.

Kinetic consequences:
There are no studies into the kinetic consequences.

Literature
1. Cubero DI et al. Tegafur-uracil is a safe alternative for the treatment of colorectal cancer in patients with partial dihydropyrimidine dehydrogenase deficiency: a proof of principle. Ther Adv Med Oncol 2012;4:167-72.
2. Deenen MJ et al. Standard-dose tegafur combined with uracil is not safe treatment after severe toxicity from 5-fluorouracil or capecitabine. Ann Intern Med 2010;153:767-8.
3. SPC Teysuno.

Supplementary Table 9. The clinical implication score of *DPYD*-fluoropyrimidines is "essential", based on the criteria and corresponding scores given by the DPWG

Clinical Implication Score Criteria	Possible score	Given score
Clinical effect associated with gene/drug interaction		
3 (D) ≤ CTCAE Grade ≤4 (E)	+	
4 (E) < CTCAE Grade ≤5 (F)	++	++[a]
Increased efficacy	+	
Level of evidence supporting the associated clinical effect		
One study with level of evidence score 3	+	
At least two studies with level of evidence score 3	++	
Three or more studies with level of evidence score 3	+++	+++[b]
Effectiveness of the intervention		
Number needed to genotype (NNG)		
100 < NNG ≤ 1000	+	
10 < NNG ≤ 100	++	++[c]
NNG ≤ 10	+++	
PGx information in the drug-label		
Recommendation to genotype	+	
At least one genotype/phenotype mentioned as a contraindication	+	+[d]
Total Score	9+	8+
Corresponding Clinical Implication Score[e]		**Essential**

[a] Patients assigned to be DPD deficient but have received normal doses of fluoropyrimidines been associated with CTCAE grade 5 toxicity;

[b] Eight studies of sufficient quality have shown an association with CTCAE grade 5 toxicity (references in Supplementary Table 1: 10, 15, 16, 18, 26, 29, 30 and 33);

[c] The NNG was calculated using the "Calculations of the number of adverse events prevented with an effective pre-emptive genotyping program".[1] The pooled odds ratios and relative risks for*2A, 1236A, 2846T and *13 was 5.2, extracted from meta-analyses Meulendijks et al., Terrazzino et al., and Rosmarin et al.[2-4] The calculated NNG was 53.9;

[d] 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.[5] Similar information on DPD deficiency is provided in the United States by the Food and Drug Administration (FDA) for capecitabine.[6] Comparable reports are made in SPCs of 5-FU;[7,8]

[e] essential, beneficial, potentially beneficial or not required.

Abbreviations: DPD: dihydropyrimidine dehydrogenase; CTCAE: Common terminology criteria for adverse events.

References

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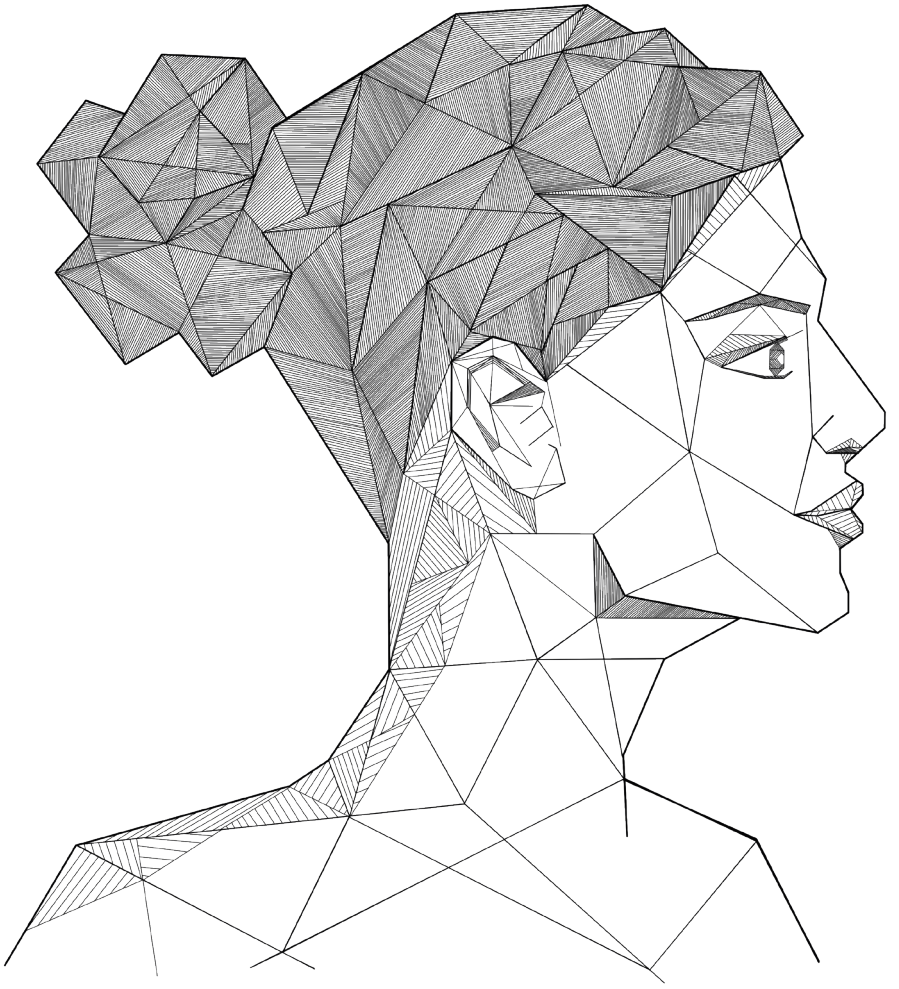

CHAPTER 5

DPYD genotype-guided dose individualisation of fluoropyrimidine therapy in patients with cancer: a prospective safety analysis

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Abstract

Fluoropyrimidine treatment can result in severe toxicity in up to 30% of patients and is often the result of reduced activity of the key metabolic enzyme dihydropyrimidine dehydrogenase (DPD), mostly caused by genetic *DPYD* variants. In a prospective clinical trial, we investigated whether upfront screening for four *DPYD* variants and *DPYD*-guided dose individualization can reduce fluoropyrimidine-induced toxicity.

Prospective genotyping of *DPYD**2A, c.2846A>T, c.1679T>G, and c.1236G>A was performed in adult cancer patients for which fluoropyrimidine-based chemotherapy was considered in their best interest. All patients about to start with a fluoropyrimidine regimen (capecitabine or 5-fluorouracil as single agent or in combination with other chemotherapeutic agents and/or radiotherapy) could be included in the study. 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), *DPYD* wild-type patients were treated according to standard of care. The primary endpoint of the study was the incidence of severe (CTC-AE grade≥3) overall fluoropyrimidine-related toxicity. This toxicity incidence was compared between *DPYD* variant allele carriers and *DPYD* wild-type patients in the study in an intention-to-treat analysis, and relative risks for severe toxicity were compared between the current study and a historical cohort of *DPYD* variant allele carriers treated with full dose fluoropyrimidine-based therapy (derived from a previously published meta-analysis). This trial is registered under clinicaltrials.gov identifier NCT02324452 and is completed.

In total, 1,103 evaluable patients were enrolled, of whom 85 *DPYD* variant carriers (7.7%). Overall grade≥3 toxicity was higher in *DPYD* variant carriers than in wild-type patients (39% vs 23%, p=0.0013). The relative risk (RR) for grade≥3 toxicity was 1.31 (95% confidence interval [95%CI]:0.63–2.73) for genotype-guided dosing vs 2.87(95%CI:2.14–3.86) in the historical cohort for *DPYD**2A, no toxicity vs 4.30(95%CI:2.10–8.80) in c.1679T>G, 2.00(95%CI:1.19–3.34) vs 3.11(95%CI:2.25–4.28) for c.2846A>T, and 1.69(95%CI:1.18–2.42) vs 1.72(95%CI: 1.22–2.42) for c.1236G>A.

Upfront *DPYD* genotyping was feasible in routine clinical practice, and improved patient safety of fluoropyrimidine treatment. For *DPYD**2A and c.1679T>G carriers, a 50% initial dose reduction seems adequate. For c.1236G>A and c.2846A>T carriers, a larger dose reduction of 50% (instead of 25%) needs to be investigated. As fluoropyrimidines are among the most commonly used anticancer agents, the findings of this study are of high clinical importance, as they endorse implementing *DPYD* genotype-guided dosing as the new standard of care.

Acknowledgments

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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 experience 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 in addition puts a heavy burden on health-care costs.

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 (e.g. a ~50% reduced DPD activity compared to normal) is present in 3—5% of the Western population. 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, IVS14+1G>A), c.2846A>T (rs67376798, D949V), c.1679T>G (rs55886062, *DPYD**13, I560S), and c.1236G>A (rs56038477, E412E, 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 without affecting treatment efficacy. In a previous study prospective genotyping and dose-individualization for one *DPYD* variant, *DPYD**2A, in a cohort of 1,631 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%) comparable to that in *DPYD**2A wild-type patients treated with a standard fluoropyrimidine dose (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 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 participants

This study was a prospective multicenter clinical trial in which 17 hospitals in the Netherlands participated. 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 adult cancer patients (≥18 years) intended to start with a fluoropyrimidine-based anticancer therapy, either as single agent or in combination with other chemotherapeutic agents and/or radiotherapy. Patients with all tumor types for which fluoropyrimidine-based therapy was considered in their best interest could be included. Prior chemotherapy was allowed, except for prior use of fluoropyrimidines. 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 (Supplementary methods). There were no restrictions on comorbidities, except for diseases expected to interfere with study or the patient's safety. Full inclusion and exclusion criteria can be found in the Supplementary methods.

Procedures

Treatment

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 a 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 anticancer agents or radiotherapy were left unchanged at start of treatment. Homozygous or compound heterozygous *DPYD* variant allele carriers were excluded from the study and could be 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.

Assessments

Toxicity was graded by participating centers according to the National Cancer Institute common terminology criteria for adverse events (CTC-AE),[18] and severe toxicity was defined as grade 3 or higher. Patients were followed for toxicity during the entire treatment period and until toxicity was resolved. Toxicity scored by the treating physician or qualified nurse practitioner as possibly, probably or definitely related to fluoropyrimidine-treatment was considered treatment-related toxicity (definitions in the Supplementary methods). Toxicity-related hospitalization and treatment discontinuation due to adverse events were also investigated. Standard laboratory assessments were performed prior to start of treatment

and each new cycle according to routine clinical care, for evaluation of treatment safety.

DPYD genotyping
Genotyping of 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 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 the Supplementary methods). 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 literature.[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.[21]

Outcomes
The primary endpoint of the study was the frequency of severe overall fluoropyrimidine-related toxicity across the entire treatment duration. A comparison was made between the incidence of severe toxicity in *DPYD* variant allele carriers treated with reduced dose and in wild-type patients treated with standard dose in this study. In addition to this, the relative risk for severe toxicity of these *DPYD* variant allele carriers treated with reduced dose compared to non-carriers in the study was calculated. A comparison between this calculated relative risk and a similarly calculated relative risk for *DPYD* variant allele carriers treated with full dose in a historical cohort derived from a previously published meta-analysis[10] was made. Secondary endpoints included pharmacokinetics of capecitabine and 5-FU in *DPYD* variant allele carriers and measurements of DPD enzyme activity. Another secondary endpoint was a cost analysis on individualized dosing based on upfront *DPYD* genotyping, of which results will be reported separately.

Statistical analysis
The sample size was based on a one stage A'Hern (phase II) design[22] and 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. This resulted in a required sample size of eleven variant carriers. To reach this number of variant carriers, we used a single *DPYD* variant (c.2846A>T, assumed variant frequency of 1%) to calculate the total sample size, resulting in

a total expected sample size of 1,100 evaluable patients. Detailed information on the sample size calculation can be found in the Supplementary methods. Patients were considered evaluable when meeting the inclusion and exclusion criteria, and if they received at least one fluoropyrimidine drug administration.

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 (Fisher's exact test was chosen when the smallest cell count was 5 or lower; for this test the double one-tailed exact probability was reported). Baseline characteristics between *DPYD* variant allele carriers and wild-type patients in the study were compared using either χ^2 test, Fisher's exact test or Kruskal-Wallis rank sum test depending on the type of variable. 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 on an intention-to-treat population were performed using SPSS (version 23.0) and R (version 3.1.2). This study is registered with ClinicalTrials.gov, number NCT02324452.

Results

Patient and treatment characteristics

Between April 30th, 2015 and December 21st, 2017, a total of 1,181 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 1,103 evaluable patients, of whom 85 were 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 in the Supplementary Table 1. The most common tumor type was colorectal cancer (64%). In total, 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 (Supplementary results). One of these patients, (c.2846A>T carrier) was treated by mistake with a full capecitabine dose for the first two cycles, which resulted in fatal fluoropyrimidine-related toxicity. Although dosing recommendations were not followed in these four patients, all results were included in the analysis (intention-to-treat analysis).

Doses were escalated during treatment in eleven out of 85 *DPYD* variant allele carriers (13%). In five of these patients (two *DPYD**2A and three c.1236G>A carriers) the higher dose was not well tolerated, leading to a dose reduction. Also, one patient (c.2846A>T carrier) discontinued treatment after the dose escalation due to toxicity. Five patients (one c.2846A>T, one c.1236G>A, one c.1679T>G, and two *DPYD**2A carriers) were able to

continue treatment with the escalated dose.

The median follow-up period (similar to the entire treatment duration or when toxicity was resolved) was 71 days (interquartile range [IQR]: 36—161 days). For wild-type patients median follow-up was 69 days (IQR 36—161 days) and for *DPYD* variant allele carriers 90 days (IQR 35—168 days).

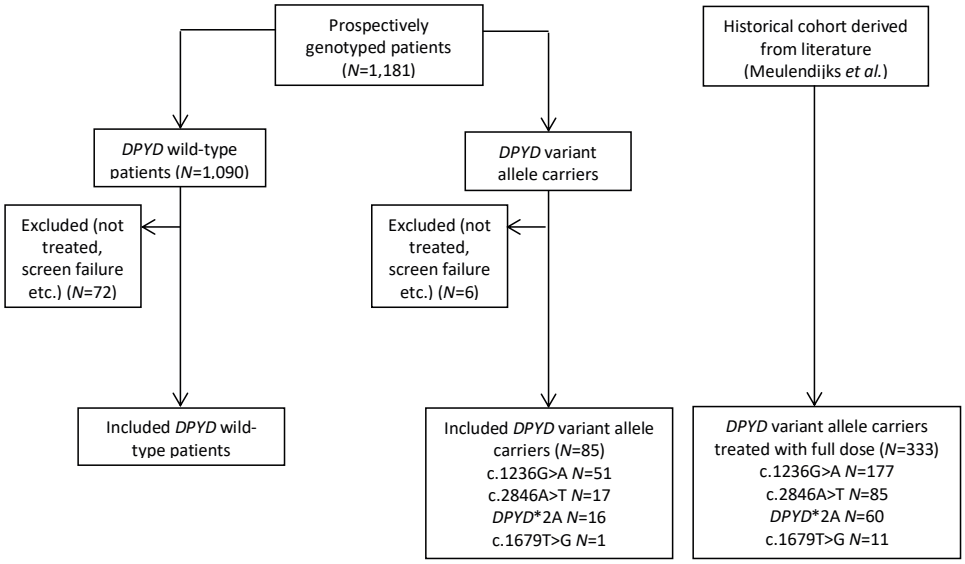

Figure 1. Consort diagram of included patients

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=0.0013). The incidence of grade ≥4 toxicity was low but was comparable between both groups as well (four out of 85 (5%) for *DPYD* variant allele carriers vs 29 out of 1,018 3% for wild-type patients, p=0.49, Table 2).

The percentage of toxicity in *DPYD* variant allele carriers was mainly driven by the two most common variants, who also had higher toxicity frequencies. In total, 20 out of 51 c.1236G>A carriers experienced severe toxicity (39%) and eight out of 17 c.2846A>T carriers (47%). For *DPYD**2A carriers, five out of 16 patients (31%) experienced severe toxicity. The single c.1679T>G carrier, who did receive reduced-dose treatment, tolerated the treatment well and did not experience severe treatment-related toxicity over the course of treatment (three cycles).

For 16 out of 85 *DPYD* variant allele carriers (19%) fluoropyrimidine-related toxicity resulted in hospitalization, compared to 140 out of 1,018 wild-type patients (14%, p=0.26). Median

duration of hospitalization was five days for both *DPYD* variant allele carriers and wild-type patients (IQR 3—7 days, and 3—10 days, respectively). For 15 out of 85 *DPYD* variant allele carriers (18%) fluoropyrimidine treatment was stopped due to fluoropyrimidine-related toxicity, compared to 175 out of 1,018 wild-type patients (17%), which was comparable between both groups (p=1.0).

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.[4,5]

Table 1. Demographic and clinical characteristics of patients

Characteristic	***DPYD* variant allele carriers**	**Wild-type patients**	**Total**	**P-value**[a]
	***N*=85**	***N*=1,018**	***N*=1,103**	
Sex				
Male	48 (56%)	545 (54%)	593 (54%)	0.68
Female	37 (44%)	473 (46%)	510 (46%)	
Age				
Median [IQR]	63 [54—71]	64 [56—71]	64 [56—71]	0.61
Ethnic origin				
Caucasian	84 (99%)	964 (95%)	1,048 (95%)	0.61
African	0	19 (2%)	19 (2%)	
Asian	1 (1%)	23 (2%)	24 (2%)	
Other[b]	0	12 (1%)	12 (1%)	
Tumor type				
Non-metastatic CRC	32 (38%)	440 (43%)	472 (43%)	0.48
Metastatic CRC	24 (28%)	208 (20%)	232 (21%)	
BC	10 (12%)	131 (13%)	141 (13%)	
GC	6 (7%)	57 (6%)	63 (6%)	
Other[c]	13 (15%)	182 (18%)	195 (18%)	
Type of treatment regimen				
CAP mono	14 (16%)	191 (19%)	205 (19%)	0.40
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%)	0.40
5-FU other	5 (6%)	75 (7%)	80 (7%)	
BSA				
Median [IQR]	1.9 [1.8—2.1]	1.9 [1.8—2.1]	1.9 [1.8—2.1]	0.60

table continues

Characteristic	*DPYD* variant allele carriers *N*=85	Wild-type patients *N*=1,018	Total *N*=1,103	P-value[a]
WHO performance status				
0	39 (46%)	515 (51%)	554 (50%)	0.68
1	36 (42%)	412 (40%)	448 (41%)	
2	4 (5%)	38 (4%)	42 (4%)	
NS [d]	6 (7%)	53 (5%)	59 (5%)	
Number of treatment cycles				
Median [IQR]	4 [1—8]	3 [1—8]	3 [1—8]	0.97
DPYD status				
Wild-type	0	1,018 (100%)	1,018 (92%)	NA
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] P-value comparing *DPYD* variant allele carriers to *DPYD* wild-type patients. A Kruskal-Wallis rank sum test was used for age, BSA, and number of treatment cycles, a Fisher's exact test was used for ethnic origin and WHO performance status and a χ^2 test for sex, tumor type, and treatment regimen;

[b] Other ethnic origins included Hispanic descent, mixed-racial parentage and unknown ethnic origin;

[c] 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;

[d] 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; IQR: interquartile range; NA: not applicable; 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	c.1236G>A	c.2846A>T	*DPYD**2A	c.1679T>G
	N=85	*N*=1,018		*N*=51	*N*=17	*N*=16	*N*=1
Relative dose intensity whole treatment *Mean [range]*[c]	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]*[c]	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[d]	33 (39%)	231 (23%)	0.0013[a]	20 (39%)	8 (47%)	5 (31%)	0
Grade≥3 gastrointestinal toxicity	17 (20%)	86 (8%)	0.00089[a]	11 (22%)	4 (24%)	2 (13%)	0
Grade≥3 hematological toxicity	13 (15%)	65 (6%)	0.0043[a]	7 (14%)	4 (24%)	2 (13%)	0
Grade 3 hand-foot syndrome[e]	1 (1%)	36 (4%)	0.41[b]	0	1 (6%)	0	0
Grade≥3 cardiac toxicity	1 (1%)	9 (1%)	1.0[b]	1 (2%)	0	0	0
Grade≥3 other treatment-related toxicity	9 (11%)	78 (8%)	0.45[a]	7 (14%)	1 (6%)	1 (6%)	0
Overall grade ≥4 toxicity[d]	4 (5%)	29 (3%)	0.49[b]	3 (6%)	1 (6%)	0	0
Grade≥4 gastrointestinal toxicity	1 (1%)	8 (1%)	1.0[b]	1 (2%)	0	0	0
Grade≥4 hematological toxicity	1 (1%)	12 (1%)	1.0[b]	1 (2%)	0	0	0
Grade≥4 cardiac toxicity	0	1 (0%)	NA	0	0	0	0
Grade≥4 other treatment-related toxicity	3 (4%)	9 (1%)	0.12[b]	2 (4%)	1 (6%)	0	0
Fluoropyrimidine-related hospitalization	16 (19%)	140 (14%)	0.26[a]	10 (20%)	4 (24%)	2 (13%)	0
Stop of FP due to adverse events	15 (18%)	175 (17%)	1.0[a]	8 (16%)	3 (18%)	4 (25%)	0
Fluoropyrimidine-related death	1 (1%)[f]	3 (0%)	0.55[b]	0	1 (6%)[f]	0	0

[a] P-value determined with χ^2 test, with Yates' continuity correction;
[b] P-value determined with Fisher's exact test with one-sided probability (with the p-value multiplied by two);
[c] 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 alone and for the entire treatment duration;
[d] Overall toxicity includes all toxicities evaluated as possibly, probably or definitely related to fluoropyrimidine-treatment;

[e] Defined as palmar-plantar erythrodysesthesia syndrome by the common terminology criteria for adverse events (CTC-AE) version 4.03;[18]

[f] 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; FP: fluoropyrimidines; NA: not applicable.

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

As another primary comparison, the relative risk for severe toxicity of *DPYD* variant allele carriers with genotype-guided dosing was compared with the corresponding relative risk for severe toxicity of *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 for severe toxicity for each *DPYD* variant obtained in the meta-analysis[10] are described in Table 3 (incidences of toxicity can be found in the Supplementary Table 2) and were compared to calculated relative risks in the current study. This analysis showed that genotype-guided dosing reduced the relative risk for severe toxicity in *DPYD**2A carriers from 2.87 (95% confidence interval [95%CI]: 2.14—3.86)[10] when treated with full dose to 1.31 (95%CI: 0.63—2.73) when treated with individualized dose, thus showing a clinically relevant reduction of toxicity risk.

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

***DPYD* variant**	***DPYD* variant carriers treated with reduced dose (this study)**	***DPYD* variant carriers treated with full dose (meta-analysis)**
	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	1.31 (0.63–2.73)	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 a 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.

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. The risk for c.1236G>A in the historical cohort was 1.72 (95%CI: 1.22—2.42),[10] and in our study it was 1.69 (95%CI:

1.18—2.42), showing that the 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),[10] 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.

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] suggesting that mean drug exposure of all combined *DPYD* variant allele carriers treated with a reduced dose was 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 the Supplementary Table 3.

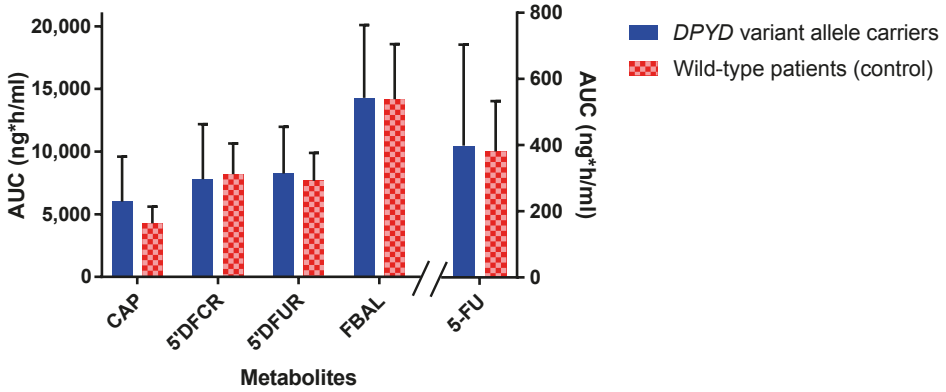

Figure 2. Pharmacokinetics of *DPYD*-guided capecitabine dosing

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.

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 (with standard deviation) in *DPYD* wild-type patients was 9.4 (3.6) nmol/(mg*h), similar to as previously published.[23] 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. No correlation between DPD enzyme activity and the occurrence of severe fluoropyrimidine-related toxicity in *DPYD* variant allele carrying patients was seen (Figure 3 and Supplementary Table 4).

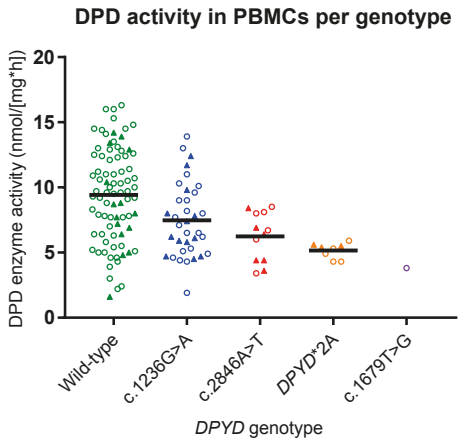

Figure 3. DPD enzyme activity in *DPYD* variant allele carriers and wild-type patients

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=0.0050), c.2846A>T (6.2 (1.9) nmol/[mg*h], p=0.0034), and *DPYD**2A (5.2 (0.6) nmol/[mg*h], p=0.0012). 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]). Patients with grade ≥3 fluoropyrimidine-related toxicity are depicted by closed triangles, patients without grade <3 toxicity by open circles; wild-type patients are treated with standard fluoropyrimidine doses, *DPYD* variant allele carriers with initially reduced doses according to protocol.

Abbreviations: DPD: dihydropyrimidine dehydrogenase; PBMCs: peripheral blood mononuclear cells.

Discussion

This is, to our knowledge, 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 carriers, was

safe in the single c.1679T>G carrier, and moderately decreased the toxicity risk in 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 is able to improve patient safety, as toxicity risk was reduced for three of the four variants in our study. Although sample sizes of variant allele carriers were modest and not all reductions in toxicity risk were statistically significant, these findings imply high clinical relevance. Also, implementation of *DPYD* genotype-guided dosing resulted in similar frequencies of toxicity-related hospitalization and discontinuation of treatment due to fluoropyrimidine-related toxicity 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%; drastically 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, while a relative risk for severe toxicity of 4.30 has been reported in literature, we showed that this patient did not experience severe toxicity in a completed treatment with 50% reduced dose. The DPD enzyme activity was about 50% decreased as well in this patient, which is in line with expectations based on previous studies.[24]

For carriers of the c.1236G>A and c.2846A>T variant, risk of severe toxicity remained relatively high despite dose individualization based on our dosing recommendations (25% reduction). 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 are considered to 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) mentions that evidence is limited regarding the optimal degree of dose reduction for the decreased function variants c.1236G>A and c.2846A>T, and a 25% dosing recommendation is mainly based on one small retrospective study. Therefore, they advise a 25%—50% dose reduction in heterozygous c.1236G>A and c.2846A>T carriers.[13] 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 *DPYD* wild-type controls. Exposure to 5-FU in the variant allele carriers was at least equal to levels observed in wild-type patients receiving standard dose, which is circumstantial evidence that the applied genotype-guided dose-reduction will not result in under-treatment. However, these pharmacokinetic results need to be interpreted with caution for some reasons. In patients with reduced DPD activity, 5-FU metabolism is affected, with 5-FU being the third metabolite derived from the parent compound capecitabine, which limits the interpretation of 5-FU exposure. Furthermore, pharmacokinetics of capecitabine and its metabolites exhibit a high inter-individual variability in exposure –even in wild-type patients– and are therefore difficult to interpret. In addition, based on the limited

number of patients with a *DPYD* variant of whom we also obtained pharmacokinetic data (Supplementary Table 3) firm conclusions on the basis of pharmacokinetic measurements alone cannot be drawn.

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 needs 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 is important to ensure an adequate and safe dose for all patients. Therefore, we recommend a more cautious initial dose reduction of 50%, followed by close monitoring and individual dose titration.

The mean value for c.2846A>T DPD enzyme activity was approximately 35% reduced compared to normal. These DPD activity measurements show that 25% dose reduction might not be sufficient for most of the patients, and this 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 as well.

In this study, initially reduced doses were escalated in eleven out of 85 (13%) *DPYD* variant allele carriers, although only five patients were able to tolerate this escalated dose. In *DPYD* wild-type patients dose escalations are uncommon in clinical practice (3% in our study, mostly patients who started with an initially reduced dose as a precaution measure).

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 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 limitation of this study is that a historical cohort of *DPYD* variant allele carriers treated with full dose was used as control, and no direct comparison was made with a control cohort within the study. Inherently to this chosen design, differences between the study populations could have influenced the observed toxicity outcomes. However, this study design was chosen as a randomized clinical trial is considered unethical in this context, since it is known that *DPYD* variant allele carriers are at increased risk of severe toxicity when treated with a full dose of fluoropyrimidines.[25] A previously performed clinical study was stopped prematurely as a patient in the arm without dose individualization died due to treatment-related toxicity.[26]

This study focused on toxicity and did not evaluate survival or other effectiveness outcomes, as this was considered not feasible due to the large variation in tumor types and treatment regimens. We did, however, perform pharmacokinetic measurements, which suggest that applied dose reductions in *DPYD* variant allele carriers did not result in under-dosing.

The four *DPYD* variants investigated in this study are especially relevant to Caucasian populations. For ethnicities other than Caucasians, more research on the frequency and clinical relevance of these and other *DPYD* variants is recommended.[27] In our current study, homozygous and compound heterozygous *DPYD* variant allele carriers were not included and

were treated with individualized fluoropyrimidine dosing or alternative treatment outside this study.[17] However, for this group of patients *DPYD* genotype-guided dosing is of even greater importance than for heterozygous *DPYD* variant allele carriers, as these patients in general have less remaining DPD activity or even complete absence of DPD activity, and a full fluoropyrimidine dose, when not identified as DPD deficient patients, is therefore likely to be fatal.

Although our study revealed that the applied approach of genotype-guided adaptive dosing significantly reduced severe fluoropyrimidine-induced toxicity and prevented treatment related death, additional methods should be explored and prospectively tested to further reduce treatment related toxicity not only in poor metabolizers, but also in *DPYD* wild-type patients.

In conclusion, we showed safety of patients treated with fluoropyrimidines was improved by dose individualization based on *DPYD* genotype. Dose reduction of 50% in heterozygous *DPYD**2A and c.1679T>G carriers reduced toxicity risk markedly. The applied dose reductions of 25% in heterozygous c.1236G>A and c.2846A>T carriers appear to be insufficient 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 and c.1236G>A carriers with subsequent individual dose titrations should therefore be considered.

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SUPPLEMENT CHAPTER 5

DPYD genotype-guided dose individualisation of fluoropyrimidine therapy in patients with cancer: a prospective safety analysis

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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 polymorphic *DPYD* genotype or compound heterozygous *DPYD* genotype.

Toxicity assessments

For causality assessment of toxicity the following definitions were used:

- Possible: the event follows a reasonable temporal sequence from the time of drug administration, but could have been produced by other factors such as the patient's clinical state, other therapeutic interventions or concomitant drugs.
- Probable: the event follows a reasonable temporal sequence from the time of drug administration, and follows a known response pattern to the study drug. The toxicity cannot be reasonably explained by other factors such as the patient's clinical state, therapeutic interventions or concomitant drugs.
- Definite: the event follows a reasonable temporal sequence from the time of drug administration, and follows a known response pattern to the study drug, cannot be reasonably explained by other factors such as the patient's condition, therapeutic interventions or concomitant drugs; AND occurs immediately following study drug administration, improves on stopping the drug, or reappears on re-exposure.

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 A'Hern (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 eleven *DPYD* variant allele carriers would give a one-sided type I error probability α of 2.93% 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 1,100 treated patients. To account for a proportion of patients not evaluable for the study, the target accrual was set at 1,250 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 lithium heparin tubes at nine different time points up to eight hours after capecitabine intake (pre-dose, 0.25, 0.5, 1, 2, 3, 4, 6, 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. To a sample volume of 300 µl of plasma, 900 µl of methanol-acetonitrile (50:50 v/v) was added to precipitate the plasma proteins. Samples were vortex-mixed for 10 seconds, shaken for 10 minutes at 1,250 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 (150 x 2.1 mm ID, 1.8 µlm particles) 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 applied: 100% A from 0—2.5 minutes, an increase from 0% to 90% B from 2.5—7.5 minutes, and 100% A from 7.5—9 minutes. For detection an API5500 triple quadruple mass spectrometer (Sciex) equipped with a turbo ionspray interphase was used, using optimized mass transitions *m/z* 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.

Data sharing statement

Data collected in the study, including individual participant data, will not be made available to others, except to researchers involved in the study. However, upon request, data sharing for additional research is possible and will be supported. Requests will be judged on scientific and clinical rationale and may need to be reviewed by an authorized institutional review

board (IRB) prior to data sharing. The study protocol of this study is publicly available (as online supplement available with this publication).

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 3. In 24 out 26 patients (92%) pharmacokinetic sampling was performed at day 1 of cycle 1. In two patients this was done at day 1 of another 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.

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

Characteristics	*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 [IQR]	63 [54—71]	62 [52—71]	62 [53—72]	64 [58—70]	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 [IQR]	1.9 [1.8—2.1]	1.9 [1.7—2.1]	2.0 [1.7—2.1]	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 [IQR]	4 [1—8]	4 [2—8]	3 [1—7]	3 [1—7]	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; IQR: interquartile range; NS: not specified.

Supplementary Table 2. Incidences of severe toxicity in *DPYD* variant allele carriers in this study and the historical cohort

***DPYD* variant**	***DPYD* variant carriers treated with reduced dose (this study)**	***DPYD* variant carriers treated with full dose (meta-analysis)**
	***N* of patients with overall grade ≥3 toxicity / total *N* of patients with this variant (%)**	***N* of patients with overall grade ≥3 toxicity / total *N* of patients with this variant (%)**
c.1236G>A	20 / 51 (39%)	65 / 177 (37%)
c.2846A>T	8 / 17 (47%)	53 / 85 (62%)
*DPYD**2A	5 / 16 (31%)	43 / 60 (72%)
c.1679T>G	0 / 1 (0%)	6 / 11 (55%)

Supplementary Table 3. Pharmacokinetic parameters of capecitabine and metabolites in *DPYD* variant allele carriers and controls

Metabolite	Mean $AUC_{0-\infty}$ (ng*h/ml) [CV%][a] *DPYD* variant allele carriers (*N*=26)	Wild-type control patients (*N*=23)[b]	Mean $T_{1/2}$ (h) [CV%] *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] c.1236G>A (*N*=16)	c.2846A>T (*N*=5)	*DPYD**2A (*N*=4)	c.1679T>G (*N*=1)	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)
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 4. DPD enzyme activity in patients with and without severe toxicity

***DPYD* genotype**	**Patients without severe toxicity[a]**		**Patients with severe toxicity[a]**		**P-value[b]**
	Mean activity (SD)	***N* of patients**	**Mean activity (SD)**	***N* of patients**	
Wild-type	9.6 (3.6)	67	8.7 (3.7)	15	0.36
c.1236G>A	7.6 (3.0)	22	7.3 (2.6)	13	0.79
c.2846A>T	6.8 (1.9)	6	5.7 (1.8)	6	0.33
*DPYD**2A	4.9 (0.7)	5	5.5 (1.1)	3	0.22
c.1679T>G	NA	1	NA	0	NA

[a] Severe toxicity is defined as CTC-AE grade 3 or higher;

[b] P-value determined with *t*-test.

Abbreviations: CTC-AE: common terminology criteria for adverse events; NA: not applicable.

Supplementary Table 5. Overview of participating centers in this study

Center	Principal investigator	Number of eligible patients included
Erasmus Medical Center, Rotterdam, the Netherlands	Prof. Ron H.J. Mathijssen, MD	264
The Netherlands Cancer Institute, Amsterdam, the Netherlands	Prof. Jan H.M. Schellens, MD	210
Catharina Hospital, Eindhoven, the Netherlands	Geert-Jan Creemers, MD	118
Leiden University Medical Center, Leiden, the Netherlands	Prof. Hans Gelderblom, MD	93
Hospital Gelderse Vallei, Ede, the Netherlands	Arnold Baars, MD	88
Reinier de Graaf Hospital, Delft, the Netherlands[a]	Vincent O. Dezentjé, MD / Annelie J.E. Vulink, MD	79
Haaglanden Medical Center, the Hague, the Netherlands	Frank J.F. Jeurissen, MD	46
Deventer Hospital, Deventer, the Netherlands	Alexander L.T. Imholz, MD	41
Haga Hospital, the Hague, the Netherlands[a]	Prof. Johanna E.A. Portielje, MD / Danny Houtsma, MD	35
Maastricht University Medical Center, Maastricht, the Netherlands	Rob L.H. Jansen, MD	28
Franciscus Gasthuis and Vlietland, Rotterdam, the Netherlands	Paul Hamberg, MD	24
Amphia Hospital, Breda, the Netherlands	Albert J. ten Tije, MD	20
Bravis Hospital, Roosendaal, the Netherlands	Helga J. Droogendijk, MD	17
University Medical Center, Utrecht, the Netherlands	Prof. Miriam Koopman, MD	14
Wilhelmina Hospital, Assen, the Netherlands	Peter Nieboer, MD	13
Laurentius Hospital, Roermond, the Netherlands	Marlène H.W. van de Poel, MD	9
Canisius-Wilhelmina Hospital, the Netherlands	Caroline M.P.W. Mandigers, MD	4

[a] In these centers the principal investigator was switched during the study.

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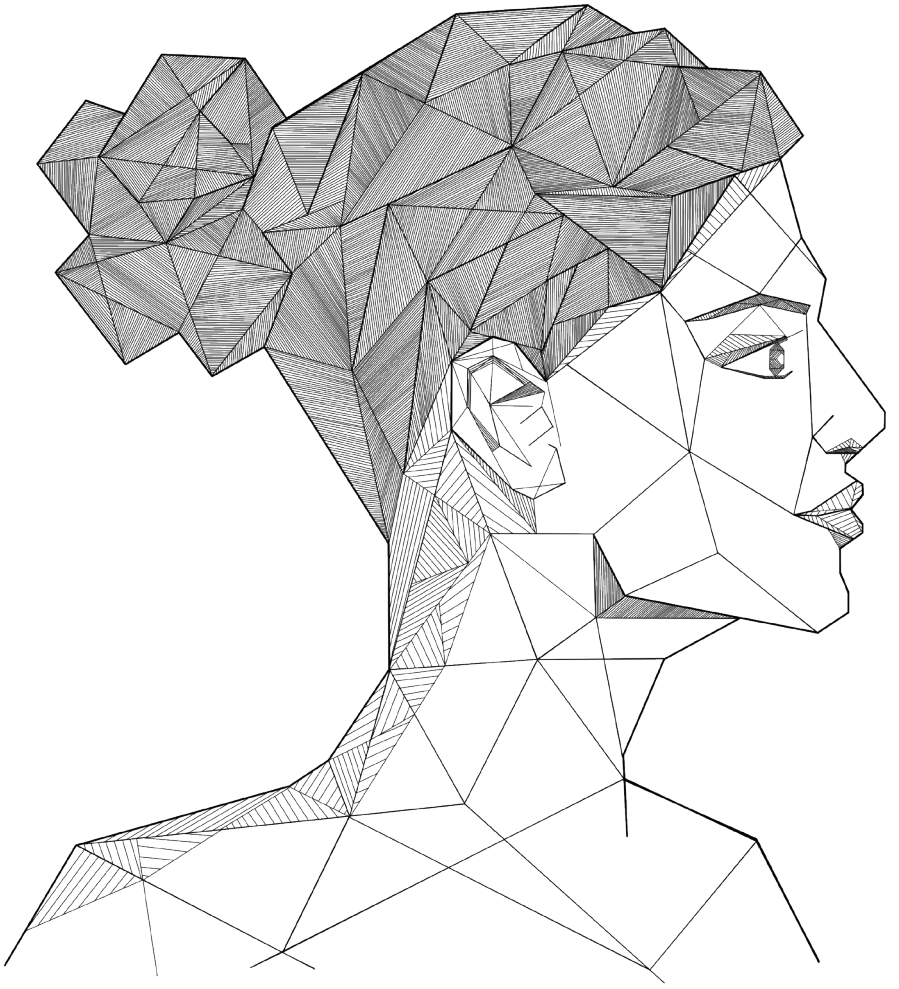

CHAPTER 6

A cost analysis of upfront *DPYD* genotype-guided dose individualization in fluoropyrimidine-based anticancer therapy

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**Contributed equally*

Abstract
Fluoropyrimidine therapy including capecitabine or 5-fluorouracil can result in severe treatment-related toxicity in up to 30% of patients. Toxicity is often related to reduced activity of dihydropyrimidine dehydrogenase (DPD), the main metabolic fluoropyrimidine enzyme, primarily caused by genetic *DPYD* polymorphisms. In a large prospective study, it was concluded that upfront *DPYD*-guided dose individualization is able to improve safety of fluoropyrimidine-based therapy. In our current analysis, we evaluated whether this strategy is cost-saving.

A cost-minimization analysis from a health care payer perspective was performed as part of the prospective clinical trial (NCT02324452) in which patients prior to start of fluoropyrimidine-based therapy were screened for the *DPYD* variants *DPYD**2A, c.2846A>T, c.1679T>G, and c.1236G>A, and received an initial dose reduction of 25% (c.2846A>T, c.1236G>A) or 50% (*DPYD**2A, c.1679T>G). Data on treatment, toxicity, hospitalization and other toxicity-related interventions were collected. The model compared prospective screening for these *DPYD* variants with no *DPYD* screening. One-way and probabilistic sensitivity analyses were also performed.

Expected total costs of the screening strategy were €2,599 per patient, compared to €2,650 for non-screening, resulting in a net cost-saving of €51 per patient. Results of the probabilistic sensitivity and one-way sensitivity analysis demonstrated that the screening strategy was very likely to be cost-saving or worst case cost-neutral.

Upfront *DPYD*-guided dose individualization, improving patient safety, is cost-saving or cost neutral, but is not expected to yield additional costs. These results endorse implementing *DPYD* screening before start of fluoropyrimidine treatment as standard of care.

Acknowledgements
All 17 participating centers are acknowledged for their contribution to patient inclusion. We thank dr. Maarten Deenen for providing his previously developed cost model (Deenen *et al.* J Clin Oncol 2016) and his input to the study.

Introduction

The class of fluoropyrimidine anticancer drugs includes 5-fluorouracil (5-FU) and its oral prodrug capecitabine. These drugs are used by approximately two million patients yearly worldwide,[1] and are the cornerstone of chemotherapeutic treatment for several solid tumor types, including colorectal, breast, gastric and head- and neck cancer. While fluoropyrimidine drugs are highly valuable treatment options, severe and potential fatal fluoropyrimidine-related toxicity remains a major clinical limitation. Around 15—30% of the patients develop severe treatment-related toxicity,[2,3] usually associated with interruption or discontinuation of therapy and often hospitalization, resulting in increased health care costs.

During the last decades it has become clear that safety of patients treated with fluoropyrimidine-based anticancer therapy is strongly affected by inter-individual variability in the enzyme dihydropyrimidine dehydrogenase (DPD), which is the main metabolic enzyme of fluoropyrimidines. The DPD enzyme is present in the liver and inactivates over 80% of 5-FU.[4] DPD enzyme activity varies widely between patients, with an estimated 3 to 8% of the population having a reduced DPD activity.[5,6] DPD deficiency results in reduced 5-FU clearance, and as a direct consequence, highly increased risk of severe treatment-related toxicity when DPD-deficient patients are treated with standard doses of a fluoropyrimidine drug.[7]

DPD deficiency can be caused by genetic polymorphisms in *DPYD*, the gene encoding DPD. Currently, four *DPYD* variants are considered as being clinically relevant and dosing recommendations are provided for these variants: *DPYD**2A, c.1679T>G, c.2846A>T and c.1236G>A).[8,9] Upfront genotyping followed by a fluoropyrimidine dose reduction in carriers in any of these four variants has proven a useful strategy to improve patient safety.[10,11] However, this strategy has not yet been universally implemented in daily clinical care.

One of the potential barriers that can make physicians reluctant to implement upfront *DPYD* screening as a routine test, is uncertainty on the cost-effectiveness of a *DPYD* screening strategy.[12] Deenen *et al.* previously showed that upfront screening for one *DPYD* variant, *DPYD**2A, is cost-saving, as average total medical costs in the screening arm were €2,772 per patient and therefore lower than the non-screening arm, for which the average total medical costs were €2,817 per patient. This shows that the reduction in toxicity-related costs outweighs the screening costs.[10] In our current study, we aimed to investigate the medical costs associated with upfront screening for the four *DPYD* variants currently considered clinically relevant and dose individualization in heterozygous carriers of a *DPYD* variant, therefore evaluating the net cost effects of this expanded *DPYD* genotyping strategy.

Patients and methods

Study design and participants

The cost analysis was performed as part of a recently published clinical trial.[11] This was a multicenter study in which 17 hospitals in the Netherlands participated (NCT02324452). Study approval was obtained 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 inclusion in the study.

The study population consisted of patients treated 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. Before start of fluoropyrimidine therapy, patients were genotyped for four *DPYD* variants (*DPYD**2A, c.1679T>G, c.2846A>T and c.1236G>A). 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.[9,13] To achieve maximal safe exposure, dose escalation was allowed after the first two cycles, provided that treatment was well tolerated and was left at the discretion of the physician. The dose of other chemotherapeutic agents or radiotherapy was left unchanged at the start of treatment. Homozygous or compound heterozygous *DPYD* variant allele carriers were not included in the study. Non-carriers of the above mentioned *DPYD* variants were considered wild-type patients in this study, and were treated according to existing standard of care.

Toxicity was graded by participating centers according to the National Cancer Institute common terminology criteria for adverse events (CTC-AE),[14] 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. Toxicity-related hospitalization and treatment discontinuation due to adverse events were also investigated.

The primary end point of the prospective 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 wild-type patients treated with standard dose in this study, and also with *DPYD* variant allele carriers treated with full dose in a historical cohort derived from a previously published meta-analysis.[8] Secondary endpoints of the prospective study included a cost analysis of individualized dosing based on upfront genotypic assessment, and pharmacokinetics of capecitabine and 5-FU in *DPYD* variant allele carriers.

Cost analysis

To compare the prospective screening for four *DPYD* variants (screening strategy) with no *DPYD* screening (non-screening strategy), a cost analysis model was composed. This analysis consisted of a cost-minimization analysis using a decision analytical model from a health care payer perspective.

A previously published model by Deenen *et al.*[10] was used and updated with data from the current study and current prices. Estimated parameters incorporated in the model were derived from data of the present trial and relevant data from literature.[15,16] Interventions for treatment-related toxicity were prospectively collected for all patients during the trial. An overview of the decision tree is depicted in 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 and 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. Cost-saving was calculated as the difference between the net direct costs of the *DPYD* screening strategy versus the non-screening strategy.

To examine the effects on variations in parameter values, one-way and probabilistic sensitivity analyses were performed. 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 1,000 simulations (Monte Carlo). 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.

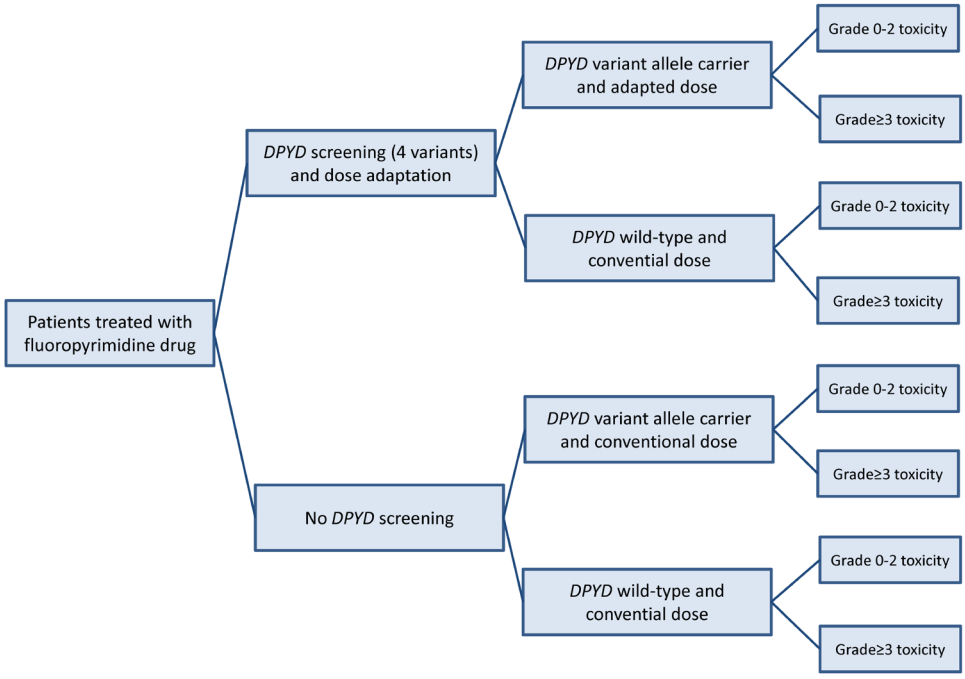

Figure 1. Decision tree for cost analysis

Results

Patient characteristics and toxicity incidence

The study was open for inclusion between April 30th, 2015 and December 21st, 2017. In this period, a total of 1,103 evaluable patients were enrolled in this study, of whom 85 heterozygous *DPYD* variant allele carriers (7.7%) and 1,018 wild-type patients (92.3%). The group of *DPYD* variant allele carriers included 51 c.1236G>A carriers, 17 c.2846A>T

carriers, 16 *DPYD**2A carriers and one c.1679T>G carrier. Details on patient characteristics, treatment and toxicity incidence are published separately.[11] In short, 33 out of 85 *DPYD* variant allele carriers (39%) experienced grade ≥3 treatment-related toxicity, while this was significantly lower in the group of wild-type patients with 231 out of 1,018 patients (23%) experiencing severe toxicity (p=0.001). Compared to the historical cohort of *DPYD* variant allele carriers treated with full dose, *DPYD* genotype-guided dosing markedly decreased the risk of severe fluoropyrimidine-related toxicity for three out of four variants (*DPYD**2A, c.1679T>G and c.2846A>T; Figure 2). No reduction in severe treatment-related toxicity was shown for c.1236G>A.

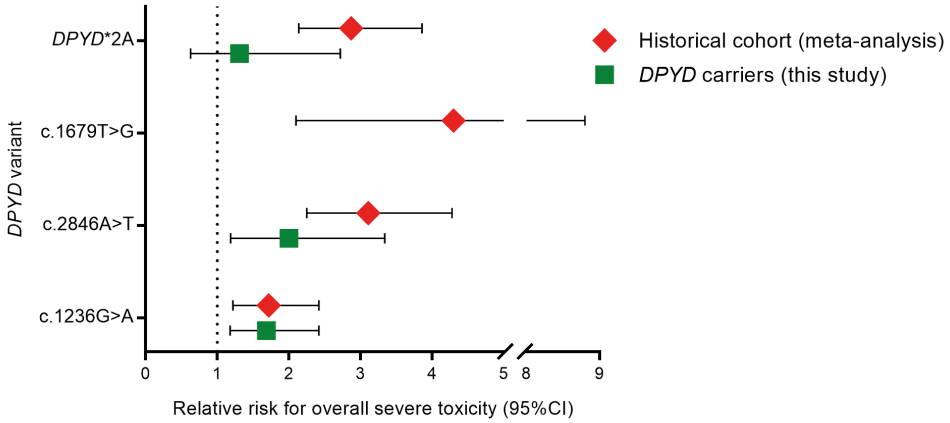

Figure 2. Relative risk for severe treatment-related toxicity of *DPYD* variant allele carriers receiving dose-reduction (this study) and *DPYD* variant allele carriers treated with full dose (historical cohort)
The relative risk for overall grade ≥3 fluoropyrimidine-related toxicity compared to non-carriers of this variant was calculated with data from this study[11] and for the historical cohort with data derived from a previously published random-effects meta-analysis.[8] Unadjusted relative risks for the meta-analysis are depicted, as the relative risk in the current study was also calculated as an unadjusted value. For c.1679T>G no relative risk could be calculated in this study, as only one patient who carried c.1679T>G was present. This patient did not experience severe toxicity.
Abbreviations: 95%CI: 95% confidence interval.

Cost analysis

All parameter estimates used in the model are provided in Table 1. In the cost analysis the expected total costs for the screening strategy were €2,599 per patient, compared to €2,650 per patient for the non-screening strategy, resulting in a net cost-saving of €51 per patient treated.

Results of the one-way sensitivity analysis are depicted in Figure 3, demonstrating that the frequency of the *DPYD* variant allele genotype had the largest influence on outcome of the cost analysis, 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 Figure 4. Average cost-savings from the simulation in the probabilistic sensitivity analysis were €52 per patient (95%-interval range -€38 to €176). Average gain in safety was 0.89% (95%-interval range -0.04% to 1.79%). 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.

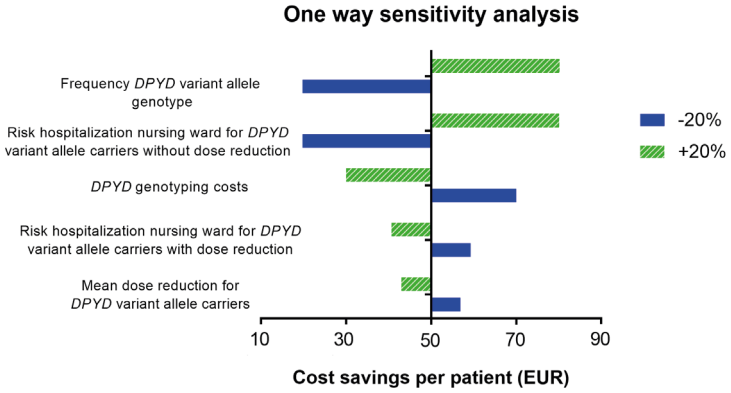

Figure 3. One-way sensitivity analysis of upfront *DPYD* genotyping versus non-screening

All parameters were individually varied by ±20% (-20% depicted in blue, +20% depicted in green), effects of which cost-savings are indicated by horizontal bars. The vertical line indicates the baseline costs savings of €50.

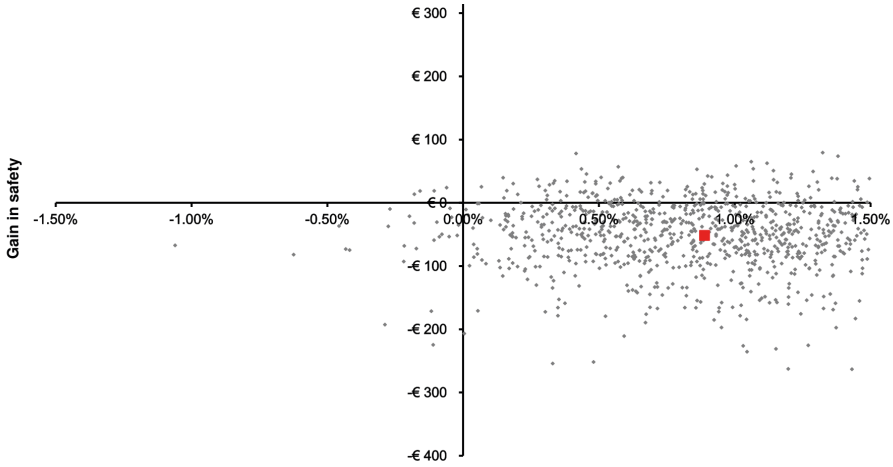

Figure 4. Probabilistic sensitivity analysis of the cost analysis

For this sensitivity analysis, all parameters were varied simultaneously by running 1,000 Monte Carlo simulations. The red square indicates the observed values.

Table 1. Cost and probability parameters used in the cost analysis

Probabilities and other parameters				
Variable	**Baseline value**	**Standard error[a]**	**Sensitivity range[b]**	**Reference**
Frequency *DPYD* genotype				
DPYD wild-type	0.9229	0.0080	Fixed	This study[11]
DPYD variant allele carrier	0.0771	0.0080	0.0617—0.0925	This study[11]
Risk severe toxicity				
DPYD wild-type	0.2269	Fixed	Fixed	This study[11]
DPYD variant allele carrier, reduced dose	0.3882	0.0526	0.3106—0.4658	This study[11]
DPYD variant allele carrier, standard dose	0.5015	0.0274	0.4012—0.6018	Meta-analysis[8]
DPYD wild-type				
Hospitalization nursing ward	0.1356	Fixed	Fixed	This study[11]
Mean duration (days)	7.9855	Fixed	Fixed	This study[11]
Hospitalization ICU	0.0088	Fixed	Fixed	This study[11]
Mean duration (days)	3.1111	Fixed	Fixed	This study[11]
DPYD variant allele carrier, reduced dose				
Hospitalization nursing ward	0.1647	0.0400	0.1318—0.1976	This study[11]
Mean duration (days)	5.7857	1.3350	4.6286—6.9428	This study[11]
Hospitalization ICU	0.0235	0.0163	0.0188—0.0282	This study[11]
Mean duration (days)	1.0000	0.1000	0.8000—1.2000	This study[11]
DPYD variant allele carrier, standard dose				
Hospitalization nursing ward	0.2350	0.0422	0.1880—0.2820	Analysis on previous study[10,20]
Mean duration (days)	13.1000	3.0000	10.4800—15.7200	Analysis on previous study[10,20]
Hospitalization ICU	0.0310	0.0172	0.0248—0.0372	Analysis on previous study[10,20]
Mean duration (days)	7.0000	3.0000	5.6000—8.4000	Analysis on previous study[10,20]
Mean number of cycles				
Capecitabine	5.0208	0.1567	4.0166—6.0250	This study[11]
5-FU	5.0426	0.3639	4.0341—6.0511	This study[11]
Type of fluoropyrimidine drug				
Capecitabine	0.83	Fixed	Fixed	This study[11]
5-FU	0.17	Fixed	Fixed	This study[11]
Mean dose intensity for *DPYD* variant allele carriers	0.6910	0.0124	0.5528—0.8292	This study[11]

table continues

Cost parameters (expressed in €)				
Variable	**Baseline value**	**Standard error[a]**	**Sensitivity range[b]**	**Reference**
DPYD genotyping costs	100	Fixed	80—120	This study[11]
Hospitalization nursing ward (per day)	636	Fixed	Fixed	Guideline[15]
Hospitalization ICU (per day)	2,015	Fixed	Fixed	Guideline[15]
Additional costs for interventions related to toxicity (expect hospitalization)				
Grade 0-2	86	Fixed	Fixed	This study[11]
Grade ≥3	234	Fixed	Fixed	This study[11]
Treatment costs capecitabine (per cycle)				
Capecitabine medication	144.06	30	Fixed	This study[11] / Price info drugs[16]
Medical doctor visit	132	Fixed	Fixed	Guideline[15]
Treatment costs 5-FU per cycle				
5-FU medication + pharmacy preparation	59.29	20	Fixed	This study / Price info drugs[16]
Administration at day care	276	Fixed	Fixed	Guideline[15]
Medical doctor visit	132	Fixed	Fixed	Guideline[15]

[a] The standard error was 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.

Discussion

The cost analysis performed in this study showed that prospective *DPYD* screening for these four variants and dose individualization is cost-saving. This confirms that upfront *DPYD* screening does not result in an increase in healthcare costs, while it can significantly improve patient safety and prevent toxicity-related deaths, as shown previously.[11] Results of the probabilistic sensitivity analysis and one-way sensitivity demonstrated that, even when varying parameters in the model, the screening strategy is unlikely to result in an increase in costs.

However, the net saving for the screening strategy in our cost analysis was with €51 relatively small. One of the determinants for this finding is that in our clinical study patients carrying a *DPYD* variant were still at increased risk of developing severe treatment-related toxicity, compared to wild-type patients (39% versus 23%, p=0.001).[11] The higher incidence of toxicity in *DPYD* variant allele carriers was mainly driven by carriers of the variants c.1236G>A and c.2846A>T. For these two variants a 25% dose reduction was applied in the study, which was concluded to be probably insufficient to reduce the incidence of toxicity to the background incidence in wild-type patients.

Our results are in line with four previous studies investigating costs of *DPYD* genotyping and toxicity.[10,17] Deenen *et al.* previously confirmed that upfront screening for one *DPYD* variant (*DPYD**2A) is cost-saving.[10] Another study, by Cortejoso *et al.* investigated screening for three variants (*DPYD**2A, c.2846A>T, c.1679T>G) and compared genotyping costs and costs for treating severe neutropenia in a retrospective analysis. Occurrence of severe neutropenia resulted in average costs for treatment for this side effect of €3,044 per patient (drug and hospitalization costs). Genotyping costs for the three *DPYD* variants were only €6.40 per patient (approximately 16 times less expensive than in our study). The authors calculated that *DPYD* genotyping would be cost-effective, provided that at least 2.1 cases of severe neutropenia per 1,000 treated patients are prevented by upfront genotyping of the three variants.[17] This was, however, not validated in a prospective setting.

The third study, by Murphy *et al.*, investigated the cost implications for reactive *DPYD* screening (i.e. screening patients for *DPYD* variants after experiencing severe toxicity) versus prospective screening.[18] In a period of three years, all patients experiencing severe (grade ≥3) fluoropyrimidine-related toxicity in an Irish hospital were screened for four *DPYD* variants (*DPYD**2A, c.2846A>T, c.1679T>G and c.1601G>A). Genotyping costs if prospective *DPYD* screening for all patients would have been performed were calculated. Total costs of hospitalization for five *DPYD* variant allele carriers (identified after experiencing severe toxicity) were €232,061, while prospectively testing would have cost in total €23,718 for the 134 included patients (€177 per patient), showing that hospitalization costs are significantly higher than costs for prospective *DPYD* screening.[18] The main difference between their study and our study was that the study by Murphy *et al.* did not collect data on the prospective *DPYD* screening strategy, but only on reactive *DPYD* screening.

The fourth study was a retrospective study as well, performed by Toffoli *et al*.[19] Toxicity-related costs on 550 colorectal cancer patients were investigated and genotyping of the same four variants as in our study was performed, but this was done retrospectively and not used for dose adjustments. This showed that average costs for treatment of toxicity were

higher in *DPYD* variant allele carriers (€2,972) than in non-carriers (€825), p<0.0001.[19]

To conclude, in addition to the important finding that upfront *DPYD* genotype-guided dose individualization is able to markedly increase patient safety, this study now confirms that this upfront *DPYD* screening strategy does not result in an increase in direct medical costs. This further endorses that *DPYD* genotyping should be implemented as routine clinical care.

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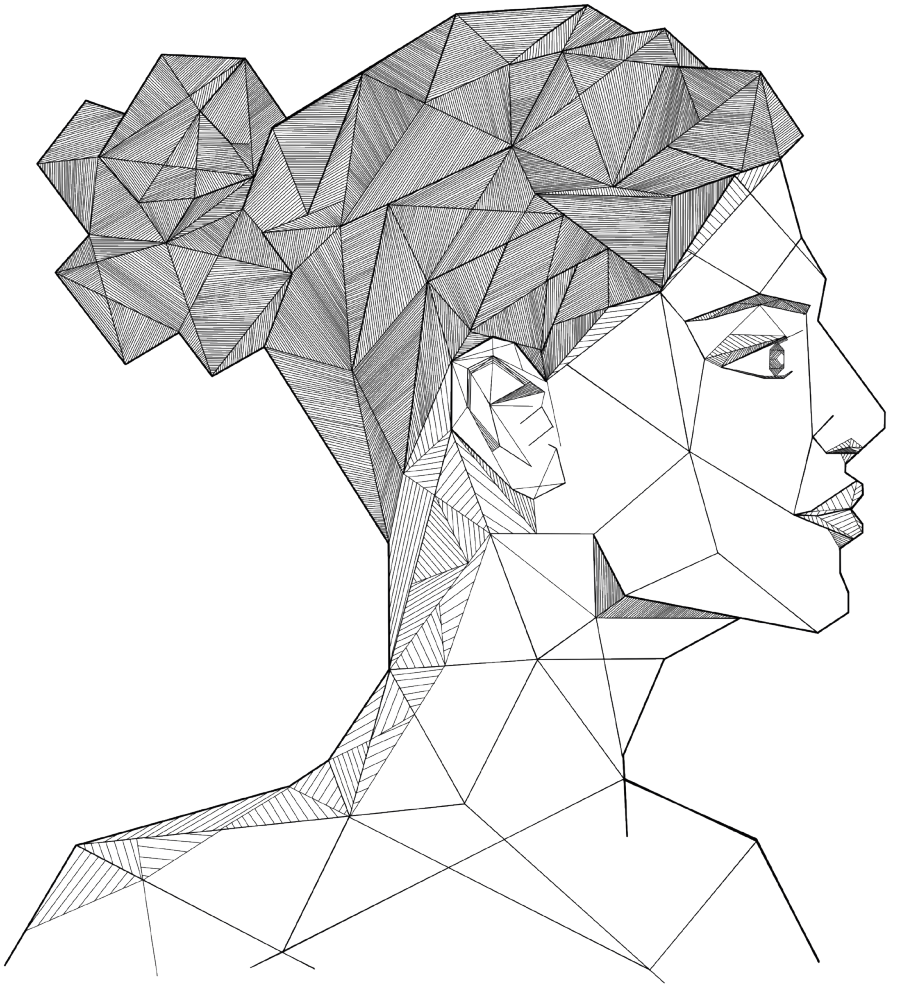

CHAPTER 7

Standard fluoropyrimidine dosages in chemoradiation therapy result in an increased risk of severe toxicity in *DPYD* variant allele carriers

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Abstract

Prospective *DPYD* genotyping prevents severe fluoropyrimidine-induced toxicity by decreasing dosages in *DPYD* variant allele carriers. Fluoropyrimidine dosages in chemoradiation therapy (CRT) are lower compared to other fluoropyrimidine-containing regimens. Pharmacogenetic guidelines do not distinguish between regimens, leaving physicians in doubt to apply dose reductions. Our aim was to investigate severe toxicity in *DPYD* variant allele carriers receiving CRT.

Medical records of 828 patients who received fluoropyrimidine-based CRT were reviewed from three centers. Severe (grade ≥3) toxicity in *DPYD* variant allele carriers receiving upfront fluoropyrimidine dose reductions according to pharmacogenetic dosing guidelines and *DPYD* variant allele carriers not receiving fluoropyrimidine dose reductions was compared with *DPYD* wild-type patients receiving standard dose of fluoropyrimidines in CRT.

DPYD variant allele carriers treated with standard dosages (*N*=34) showed an increased risk of severe gastrointestinal (adjusted OR: 2.58, 95% confidence interval [95%CI]: 1.02—6.53, p=0.045) or severe haematological (adjusted OR: 4.19, 95%CI: 1.32—13.25, p=0.015) toxicity compared with wild-type patients (*N*=771). *DPYD* variant allele carriers who received dose reductions (*N*=22) showed a comparable frequency of severe gastrointestinal toxicity compared with wild-type patients, but more (not statistically significant) severe haematological toxicity. Hospitalisations for all *DPYD* variant allele carriers were comparable, independent of dose adjustments; however, the mean duration of hospitalisation was significantly shorter in the dose reduction group (p=0.010).

Standard fluoropyrimidine dosages in CRT resulted in an increased risk of severe toxicity in *DPYD* variant allele carriers. We advise to apply fluoropyrimidine dose reductions according to current guidelines in *DPYD* variant allele carriers starting CRT.

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Introduction

Fluoropyrimidines, such as 5-fluorouracil (5-FU) and capecitabine, are the backbone of chemotherapy regimens for solid tumours such as colorectal and breast cancer.[1-3] Since the 90's, 5-FU has been in use in neoadjuvant chemoradiation therapy (CRT) for patients with stages 2-3 rectal cancer.[4,5] Fluoropyrimidines affect nucleotide metabolism and inhibit the repair of radiation-induced DNA damage in patients and act as a radiation sensitiser.[6] Fluoropyrimidines in combination with radiotherapy are used at lower dosages than those in other treatment regimens. An example; for patients with advanced colorectal cancer capecitabine, dosages are usually 1,250 mg/m^2 bid (twice daily) for two weeks followed by one week rest, repeated every three weeks.[7] In combination with radiotherapy, a continuous regimen is preferred to optimise radio-sensitisation. The maximum tolerated dose of capecitabine was 825 mg/m^2 bid for patients with rectal cancer.[8,9]

Adverse events are well known in fluoropyrimidine treatment and differ between treatment regimens. Severe (grade ≥3) side-effects in stage 3 or 4 colorectal cancer patients treated with capecitabine monotherapy dosed 1,250 mg/m^2 bid in three-week cycles, were hand-foot syndrome (~18%), diarrhoea (~14%), stomatitis (~3%), vomiting (~3%) and neutropenia (~3%).[10-12] Severe side-effects in locally advanced rectal cancer patients treated with CRT, including 825 mg/m^2 capecitabine continuously for five weeks, were grade ≥3 radiation dermatitis (~9%), diarrhoea (~2-7%), fatigue (~2%), neutropenia (~2%) and anaemia (~2%).[13,14]

Over 80% of 5-FU is degraded into inactive metabolites by the key enzyme dihydropyrimidine dehydrogenase (DPD).[15] DPD is encoded by the gene *DPYD*. DPD and variants in *DPYD* are associated with the onset of severe fluoropyrimidine-induced toxicity. To prevent severe fluoropyrimidine-induced toxicity prospective *DPYD* genotyping is increasingly used in clinical practice, followed by dose reductions in patients who carry a *DPYD* variant. For four variants (*DPYD**2A, c.1905+1G>A, rs3918290; *DPYD**13, c.1679T>G, rs55886062; c2846A>T, rs67376798; c.1236G>A/HapB3, rs56038477) individual dosing guidelines are currently given by the Dutch Pharmacogenetics Working Group and Clinical Pharmacogenetics Implementation Consortium.[16,17] Dosing guidelines advise that *DPYD* variant allele carriers should receive a percentage of the standard dose, for example 50 or 75%, depending on the specific variant.[18] These guidelines do not distinguish between treatment regimens in which different fluoropyrimidine dosages are given. Because fluoropyrimidine dosages in CRT regimens are lower than those in other treatment regimens, it is questioned if dose adjustments in dosing guidelines should be applied in patients receiving fluoropyrimidines in CRT. The objective of this study was to investigate the frequency of severe treatment-related toxicity in *DPYD* variant allele carriers receiving reduced or standard fluoropyrimidine dosages in CRT, to determine whether dose reductions are required.

Methods

Study population

The study population consisted of three combined databases. All patients were treated with fluoropyrimidine-based CRT according to the various tumour types and were genotyped for the aforementioned four variants in *DPYD.*

At the Netherlands Cancer Institute (NKI), Amsterdam, the Netherlands, a prospective clinical trial was executed in which patients were prospectively genotyped for *DPYD**2A followed by dose reductions of ≥50% in *DPYD**2A carriers (NCT00838370).[19] The trial was approved by the institutional review board (IRB) of all participating institutes, and all *DPYD**2A carriers provided written informed consent before study registration. The patients were retrospectively genotyped for the three other variants (*DPYD**13, c.2846A>T, c.1236G>A). A total of 497 patients received CRT and were selected for the present study. Two patients had missing genotypes and were excluded. Radiation dose in Gray (Gy) and fractions (Fr) given to the patient could be collected retrospectively for 425 patients.

At Leiden University Medical Center (LUMC), Leiden, the Netherlands, a retrospective database was created for the purpose of this study. The study was reviewed and approved by the IRB. All patients scheduled to start fluoropyrimidine-based CRT between April 2013 and September 2017 were evaluated. In total, 253 patients started therapy. In April 2013, only *DPYD**2A was genotyped; *DPYD**13 and c.2846A>T were added to the genotyping panel in October, and c.1236G>A was added in May 2014. Some patients were prospectively genotyped for *DPYD**2A alone (*N*=20) or *DPYD**2A, *DPYD**13 and c.2846A>T (*N*=35). Missing genotypes were determined retrospectively. Thirteen patients could not be genotyped and were excluded. Data were collected from the electronic patient files. Ten percent of the data was checked by an independent data manager. Ten percent of toxicity data was checked by an oncologist and radiation oncologist. Limited discrepancies were discussed and similar errors were searched and corrected.

At CRO-Aviano National Cancer Institute, Northern Italy, 207 patients were enrolled in a study from December 1993 to April 2016. All procedures were reviewed and approved by the IRB and patients signed written informed consent for research purposes. Ninety-five patients were included in the present study of whom additional chemotherapy treatment details could be collected. Sixteen patients were prospectively tested for *DPYD**2A, *DPYD**13 and c.2846A>T, and 79 patients were tested after start of treatment. Missing genotypes of c.1236G>A were determined retrospectively. Two patients had incomplete genotype data and were excluded.

Groups

All included patients in the combined database were grouped into wild-types receiving standard fluoropyrimidine dosages in CRT, *DPYD* variant allele carriers receiving standard fluoropyrimidine dosages in CRT or *DPYD* variant allele carriers receiving upfront reduced fluoropyrimidine dosages in CRT. *DPYD* variant allele carriers are heterozygous or homozygous for a *DPYD* variant (*DPYD**2A, *DPYD**13, c.2846A>T or c.1236G>A). Initial dose reductions (25 or 50%) were applied corresponding to pharmacogenetic guidelines.[16,17]

Toxicity

Treatment-related toxicity data were scored prospectively according to the National Cancer Institute common terminology criteria for adverse events (CTC-AE) v3.0[20] for the NKI and CRO databases, and retrospectively using CTC-AE v4.03[21] for the LUMC database. It was not possible to determine missing toxicities retrospectively. In CRT a continuous regimen

is used, and there are no cycles; therefore, the highest toxicity grade over the entire treatment period was used. Gastrointestinal toxicity included diarrhoea, mucositis, nausea and vomiting (nausea or vomiting were not scored by all databases). Haematological toxicity included leukopenia, thrombocytopenia and neutropenia.

Statistics

To study the association between study groups and severe gastrointestinal or haematological toxicity multivariable logistic regression models with grouped diagnosis as covariate were estimated. Gastrointestinal and haematological toxicity outcomes were dichotomised (grades 0—2 versus grades 3—5). Diagnoses were grouped according to tumour location, either pelvic or non-pelvic region (grouped diagnosis). Differences in baseline characteristics between study groups were tested using Pearson Chi-square or Kruskal Wallis tests. Owing to the retrospective character of this study, there was no protocol on how to deal with additional dose adjustments during treatment in the analysis. A Mann–Whitney U test was applied to compare duration of hospitalisation between *DPYD* variant allele carriers who received dose reductions or standard dosages. P-values of <0.05 were considered statistically significant. Statistical analyses were performed using SPSS (v23, Chicago, IL, USA).

Results

Study population

The combined database of 828 patients was divided into three study groups. Seven hundred seventy-one patients were wild-types, 34 patients were *DPYD* variant allele carriers who received standard fluoropyrimidine dosages in CRT and 23 patients were *DPYD* variant allele carriers who received upfront reduced (50 or 75%) fluoropyrimidine dosages in CRT. Baseline characteristics per database and study group are shown in Tables 1 and 2. Each original database included patients in each study group, described in Table 2. Cancer of the rectum was the most present in 71.7% of the patients. 86.6% of the patients received capecitabine. Baseline characteristics between study groups showed no significant differences.

In one *DPYD* *2A carrier, dose reductions were applied during treatment but not at the first drug administration. In three *DPYD**2A carriers initial reduced dosages were increased during treatment. Three out of four patients had a total dose intensity of approximately 50% (according to current dosing guidelines). The fourth patient was excluded from statistical analyses. These four patients were described in Table 2.

Toxicity

Toxicity of patients from this study treated with comparable treatment schedules was similar to toxicity of rectal cancer patients described in literature (Supplementary Table 1). Differences in toxicity between databases were observed. Grade 2 radiation dermatitis and grade 2 'other toxicity' were very high in the LUMC and CRO database, respectively, resulting in a high overall toxicity percentage in these databases (Supplementary Table 2). Toxicity separated per study group is shown in Table 3.

Percentages of severe gastrointestinal and haematological toxicity were 8 and 2.9% for wild-types, 17.6 and 11.8% for *DPYD* variant allele carriers treated with a standard dose,

Table 1. Baseline characteristics of patients from three original databases and of the combined database (total)

Characteristics	DB#1:NKI (*N*=495) *N* (%)	DB#2:LUMC (*N*=240) *N* (%)	DB#3:CRO (*N*=93) *N* (%)	TOTAL (*N*=828) *N* (%)
Sex, male	283 (57.2)	122 (50.8)	60 (64.5)	465 (56.2)
Age, median [range]	62 [32-86]	65 [23-86]	63 [33-88]	63 [23-88]
BSA, median [range]	1.9 [1.38-2.71]	1.89 [1.39-2.54]	1.85 [1.4-2.2]	1.9 [1.38-2.71]
Diagnosis				
Rectum cancer	344 (69.5)	157 (65.4)	93 (100)	594 (71.7)
Anus cancer	80 (16.2)	36 (15.0)	-	116 (14)
Vulva/vagina cancer	1 (0.2)	17 (7.1)	-	18 (2.2)
Pancreas cancer	-	5 (2.1)	-	5 (0.6)
Upper GI cancer	54 (10.9)	10 (4.2)	-	64 (7.7)
Other cancers	16 (3.2)	15 (6.3)	-	31 (3.7)
Grouped diagnosis				
Pelvic region cancer[a]	432 (87.3)	223 (92.9)	93 (100)	748 (90.7)
Non-pelvic region cancer[b]	60 (12.1)	17 (7.1)	-	77 (9.3)
Other cancers	3 (0.6)	-	-	3 (0.4)
Treatment type				
Capecitabine	442 (89.3)	183 (76.3)	92 (98.9)	717 (86.6)
5-FU	53 (10.7)	57 (23.8)	1 (1.1)	111 (13.4)
Treatment date [range]	[01/2007-02/2012]	[12/2012-09/2017]	[04/2006-04/2016]	[05/2006-09/2017]
Radiotherapy				
Gy: median [range]	50 [20-78][c]	50 [7.2-69.4]	55 [31.5-55.2]	50 [7.2-78][c]
Fr: median [range]	25 [5-39][c]	25 [4-38]	25 [15-28][d]	25 [4-39][e]
DPYD carriers total	36 (7.3)	18 (7.5)	3 (3.2)	57 (6.9)
*DPYD*2A*	7 (1.4)	6 (2.5)	-	13 (1.6)
*DPYD*13*	1 (0.2)	-	-	1 (0.1)
c.2846A>T	9 (1.8)	-	1 (1.1)	10 (1.2)
c.1236G>A	17 (3.4)	12 (5)	2 (2.2)	31 (3.7)
c.1236G>A homozygote	2 (0.4)	-	-	2 (0.2)

[a] Included are cancers of the colon sigmoidal, rectum, anus, vulva, vagina, cervix, uterus, endometrium, bladder, urethra, prostate and double tumours with one tumour in the pelvic area;

[b] Included are cancers of the breast, stomach, oesophagus, pancreas, skin, tongue;

[c] Seventy-one patients have missing data;

[d] One patient has missing data;

[e] Seventy-two patients have missing data.

Abbreviations: BSA: body surface area; CRO: Aviano National Cancer Institute; DB: database; *DPYD*: gene encoding dihydropyrimidine dehydrogenase; 5-FU: 5-fluorouracil; Fr: fractions; GI: gastro-intestinal tract; Gy: gray; LUMC: Leiden University Medical Center; NKI: Netherlands Cancer Institute.

and 9.1 and 9.1% for *DPYD* variant allele carriers who received a reduced dose, respectively (Figure 1, Table 3). *DPYD* variant allele carriers treated with a standard dose had a significantly increased risk to develop severe gastrointestinal toxicity (adjusted OR: 2.58, 95% confidence interval [95%CI]: 1.023—6.534, p=0.045) and severe haematological toxicity (adjusted OR:4.19, 95%CI: 1.323—13.253, p=0.015) compared with *DPYD* wild-type patients treated with standard dose. No statistical significant difference was found for the risk of developing severe gastrointestinal toxicity (adjusted OR: 1.10, 95%CI: 0.250—4.804, p=0.904) or severe haematological toxicity (adjusted OR: 3.88, 95%CI: 0.837—18.016, p=0.083) in *DPYD* variant allele carriers who received an initially reduced dose compared with wild-types. Grouped diagnosis was not significantly associated with the development of severe gastrointestinal toxicity (adjusted OR: 0.26, 95%CI: 0.061—1.069), while it was for severe haematological toxicity (adjusted OR: 4.21, 95%CI: 1.760—10.053, p=0.001), with more toxicity in pelvic malignancies.

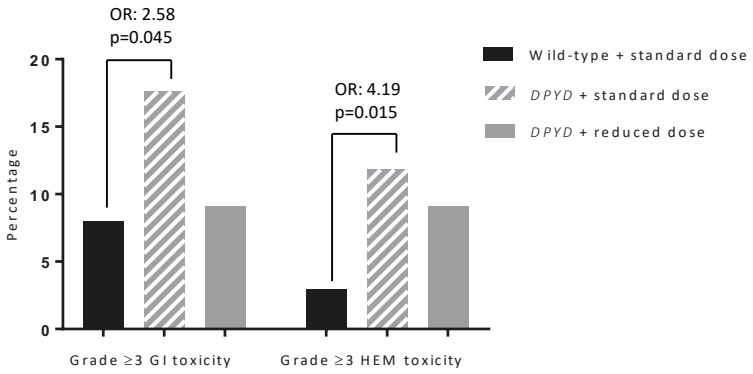

Figure 1. Percentages of severe toxicity

Shown are the percentages of severe gastrointestinal and severe haematological toxicity of *DPYD* variant allele carriers with and without fluoropyrimidine dose reductions and wild-type patients in chemoradiation treatment.

Abbreviations: OR: adjusted odds ratio; *DPYD*: gene encoding dihydropyrimidine dehydrogenase; GI= gastrointestinal; HEM: haematological.

Included in Table 3 are any changes applied in chemotherapy during treatment due to adverse events, such as dose interruptions. Compared with wild-type patients, *DPYD* variant allele carriers had more dose reductions during treatment, stopped treatment prematurely and were hospitalised more often, regardless of any received dose reductions. However, the mean duration of hospitalisation of *DPYD* variant allele carriers who received a dose reduction was notably shorter (4 days) compared with the *DPYD* variant allele carriers treated with a standard dose (23 days, p=0.010).

Table 2. Baseline characteristics per study group

No significant differences between study groups in baseline characteristics were found. Differences in median dose intensity, treatment date and *DPYD* carriers were not tested. All original databases were able to include patients in each study group. Of the 34 *DPYD* variant allele carriers who received standard fluoropyrimidine dosages in CRT, 29 patients were included from NKI, three patients from LUMC (2x *DPYD**2A, 1x c.1236G>A) and two c.1236G>A carriers from the CRO database. Of the 23 *DPYD* variant allele carriers who received upfront dose reductions in CRT, 15 patients were included from LUMC, seven *DPYD**2A carriers from NKI and one c.2846A>T carrier from the CRO database.

Characteristics	**WT + standard[a] (*N*=771) *N* (%)**	***DPYD* + standard[b] (*N*=34) *N* (%)**	***DPYD* + reduced[c] (*N*=23) *N* (%)**
Sex, male	432 (56)	20 (58.8)	13 (56.5)
Age, median [range]	63 [23-88]	64 [45-79]	66 [50-78]
BSA, median [range]	1.89 [1.38-2.71]	1.93 [1.51-2.34]	2 [1.50-2.44]
Diagnosis			
Rectum cancer	554 (71.9)	22 (64.7)	18 (78.3)
Anus cancer	106 (13.7)	7 (20.6)	3 (13.0)
Vulva/vagina cancer	18 (2.3)	-	-
Pancreas cancer	5 (0.6)	-	-
Upper GI cancer	58 (7.5)	5 (14.7)	1 (4.3)
Other cancers	30 (3.9)	-	1 (4.3)
Grouped diagnosis			
Pelvic region cancer[d]	697 (90.8)	29 (85.3)	22 (95.7)
Non-pelvic region cancer[e]	71 (9.2)	5 (14.7)	1 (4.3)
Other cancers	3 (0.4)	-	-
Treatment type			
Capecitabine	668 (86.6)	29 (85.3)	20 (87)
5-FU	103 (13.4)	5 (14.7)	3 (13)
Median dose intensity[f]	97%	91%	61%
Treatment date [range]	[05/2006-09/2017]	[02/2008-10/2014]	[12/2007-08/2017]
Radiotherapy			
Gy: median [range]	50 [7.2-73.6]	50 [36-64.8]	50 [45-78]
Fr: median [range]	25 [4-39]	25 [18-36]	25 [23-39]
DPYD carriers			
*DPYD*2A*	-	2 (5.9)	11 (47.8)
*DPYD*13*	-	1 (2.9)	-
c.2846A>T	-	9 (26.5)	1 (4.3)
c.1236G>A	-	20 (58.8)	11 (47.8)
c.1236G>A homozygote	-	2 (5.9)	-

[a] Wild-type patients receiving standard fluoropyrimidine dosages in chemoradiation therapy;

[b] *DPYD* variant allele carriers receiving standard fluoropyrimidine dosages in chemoradiation therapy;

[c] *DPYD* variant allele carriers receiving initially reduced fluoropyrimidine dosages according to current

guidelines compared with standard fluoropyrimidine dosages used in chemoradiation therapy. One *DPYD**2A variant carrier started intravenous 5-FU therapy at a 100% dose before the genotype result became available. When the genotype was known, the administration of 5-FU was prematurely stopped after 2 instead of 4 days. In the second cycle a 50% dose reduction over 4 days was applied. The overall dose intensity of this patient was 49%. In three *DPYD**2A carriers initial reduced dosages were increased during treatment. One patient was included in the clinical trial (NCT00838370) before existence of dosing guidelines and started with 30% of the standard total dose, which was increased to 46%. One patient went from 50 to 60% of the standard total dose and for another patient the dose was increased from 50 to 83%. The latter patient was excluded from statistical analyses, due to the substantial dose increase. The c.2846A>T variant carrier who received a dose reduction, was treated with a 60% dose;

[d] Included are cancers of the colon sigmoidal, rectum, anus, vulva, vagina, cervix, uterus, endometrium, bladder, urethra, prostate and double tumours with one tumour in the pelvic area;

[e] Included are cancers of the breast, stomach, oesophagus, pancreas, skin and tongue;

[f] Dose intensity was calculated by dividing the received amount of mg of chemotherapy by the initial scheduled amount of mg of chemotherapy.

Abbreviations: BSA: body surface area; *DPYD:* gene encoding dihydropyrimidine dehydrogenase; 5-FU: 5-fluorouracil; Fr: fractions; GI: gastro-intestinal tract; Gy: gray; WT: wild-type patients.

Table 3. Toxicity of patients per study group

Shown per study group are percentages of several types of (grouped) toxicity after chemoradiation therapy and actions following toxicity. *P*-values are shown for executed statistical tests.

Type of event	WT + standard[a] (*N*=771) *N* (%)	*DPYD* + standard[b] (*N*=34) *N* (%)	*DPYD* + reduced[c] (*N*=22) *N* (%)
Grade 2 Diarrhoea	122 (15.8)	5 (14.7)	3 (13.6)
Grade ≥3 Diarrhoea	58 (7.5)	6 (17.6)	2 (9.1)
Grade 2 Mucositis	51 (6.6)	2 (5.9)	2 (9.1)
Grade ≥3 Mucositis	13 (1.7)	-	-
Grade 2 Nausea[d]	13 (4.2)	2 (40)	1 (6.7)
Grade ≥3 Nausea[d]	2 (0.6)	1 (20)	-
Grade 2 Vomiting[e]	12 (5.4)	2 (66.7)	1 (7.1)
Grade ≥3 Vomiting[e]	1 (0.5)	1 (33.3)	-
Grade 2 Neutropenia	8 (1)	1 (2.9)	1 (4.5)
Grade ≥3 Neutropenia	12 (1.6)	2 (5.9)	2 (9.1)
Grade 2 Leukocytopenia	60 (7.8)	7 (20.6)	2 (9.1)
Grade ≥3 Leukocytopenia	17 (2.2)	4 (11.8)	2 (9.1)
Grade 2 Thrombocytopenia	6 (0.8)	-	1 (4.5)
Grade ≥3 Thrombocytopenia	5 (0.6)	-	-
Grade 2 Anaemia[d]	25 (8)	1 (20)	2 (13.3)
Grade ≥3 Anaemia[d]	1 (0.3)	1 (20)	-
Grade 2 Radiation dermatitis[d]	77 (24.7)	1 (20)	5 (33.3)
Grade ≥3 Radiation dermatitis[d]	13 (4.2)	-	-
Grade 2 HFS	19 (2.5)	-	1 (4.5)
Grade 3 HFS	5 (0.6)	-	-

table continues

Type of event	WT + standard[a] (*N*=771) *N* (%)	*DPYD* + standard[b] (*N*=34) *N* (%)	*DPYD* + reduced[c] (*N*=22) *N* (%)
Grade 2 Cardio toxicity	21 (2.7)	-	-
Grade ≥3 Cardio toxicity	11 (1.4)	-	-
Grade 2 Fatigue[e]	28 (12.6)	1 (33.3)	4 (28.6)
Grade ≥3 Fatigue[e]	2 (0.9)	2 (66.7)	-
Grouped type of events			
Grade 2 GI toxicity[f]	138 (17.9)	5(14.7)	6 (27.3)
Grade ≥3 GI toxicity[f]	62 (8)	6 (17.6) p=0.045[g]	2 (9.1) p=0.904[g]
Grade 2 HEM toxicity[h]	62 (8)	7 (20.6)	2 (9.1)
Grade ≥3 HEM toxicity[h]	22 (2.9)	4 (11.8) p=0.015[g]	2 (9.1) p=0.083[g]
Grade 2 Overall toxicity[i]	252 (32.7)	7 (20.6)	7 (31.8)
Grade ≥3 Overall toxicity[i]	105 (13.6)	8 (23.5)	5 (22.7)
Actions			
Chemotherapy changes			
Dose reductions	34 (4.4)	4 (11.8)[j]	2 (9.1)[k]
Dose increases	4 (0.5)	-	2 (9.1)[l]
Interruptions	38 (4.9)	-	1 (4.5)
Prematurely stopped	76 (9.9)	6 (17.6)	4 (18.2)
Treatment-related hospitalization	60 (7.8)	6 (17.6)	4 (18.2)
Days of hospitalization, mean [range]	13 [1—76]	23 [6—36]	4 [2—5] p=0.010[m]

[a] Wild-type patients receiving standard fluoropyrimidine dosages in chemoradiation therapy;
[b] *DPYD* variant allele carriers receiving standard fluoropyrimidine dosages in chemoradiation therapy;
[c] *DPYD* variant allele carriers receiving initially reduced fluoropyrimidine dosages according to current guidelines compared with standard fluoropyrimidine dosages used in chemoradiation therapy;
[d] Data of 332 patients in total, data of 5 patients in the group of *DPYD* variant allele carriers treated with a standard dose and data of 15 patients in the group of *DPYD* variant allele carriers who received dose reductions;
[e] Data of 239 patients in total, data of 3 patients in the group of *DPYD* variant allele carriers treated with a standard dose and data of 14 patients in the group of *DPYD* variant allele carriers who received dose reductions;
[f] GI toxicity includes diarrhoea, mucositis, nausea, vomiting;
[g] *P*-values shown are compared with wild-type patients;
[h] HEM toxicity includes neutropenia, thrombocytopenia, leukocytopenia;
[i] Overall toxicity includes diarrhoea, mucositis, nausea, vomiting, neutropenia, thrombocytopenia, leukocytopenia, anaemia, radiation dermatitis, HFS, cardio toxicity, fatigue and other toxicity;
[j] Dosages were reduced from 100 to 60-77%;
[k] Dosages were reduced from 70 to 45% and 100 to 50% (applying dosing guidelines 2 days after start of therapy);
[l] Dosages were increased from 30 to 46% and from 50 to 60%;
[m] *P*-values shown are compared with *DPYD* variant allele carriers who received a standard dose.
Abbreviations: DPYD: gene encoding dihydropyrimidine dehydrogenase; GI: gastrointestinal; HFS: hand-foot syndrome; HEM: haematological; WT: wild-type patients.

Case description

To illustrate the importance of dose reductions in *DPYD* variant allele carriers, we have shown the course of one *DPYD**2A carrier in Figure 2. This patient was excluded from the statistical analyses due to a substantially increased dose during treatment. Being one of the first *DPYD* variant allele carriers who received 50% dosed CRT, it was decided that the fluoropyrimidine dose would be titrated up to 100% if the patient would have no side-effects after two weeks. However, diarrhoea grade 1—2 was present, and the dose was increased to 83%. After four weeks, severe toxicity (diarrhoea, vomiting, nausea grade 3 and dermatitis grade 2) occurred and chemotherapy, and later radiotherapy, was stopped prematurely. The patient was hospitalised for 31 days, of which three days at the intensive care unit. After hospitalisation, the patient had to recover completely from toxicity for 39 days in a nursing home (rehabilitation). Although it cannot be excluded that toxicity would have evolved in the severity as was now shown at an 83% dose level when treated entirely with a 50% dose level, it is clear that the dose increase was most likely a reason for the development of severe toxicity.

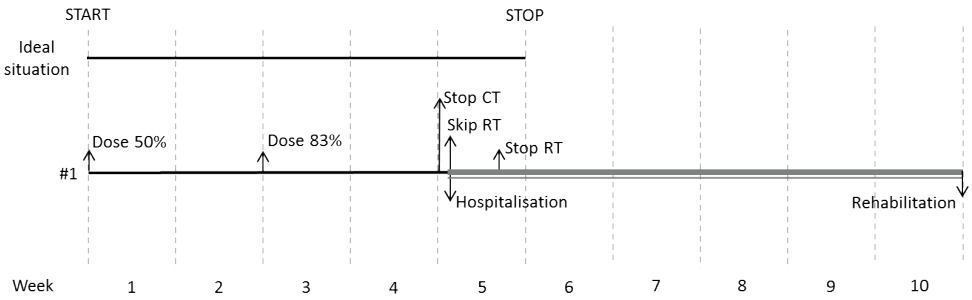

Figure 2. Course of treatment and toxicity

Shown is the course of an ideal treatment, and the treatment and toxicity for one patient (#1). The patient is a carrier of the *DPYD**2A variant and started therapy on a 50% dose. After two weeks, the dose was increased to 83%. Thereafter, the patient developed severe toxicity, and therapy was discontinued. The patient was hospitalised for 31 days (including three days at the intensive care unit) and had to recover completely from toxicity for 39 days in a nursing home (rehabilitation).
Abbreviations: CT: chemotherapy; RT: radiotherapy.

Discussion

Fluoropyrimidine dosages are lower in CRT compared with other fluoropyrimidine treatment regimens, and it is unclear if pharmacogenetic dose adjustments should be made for *DPYD* variant allele carriers receiving CRT. Dose titration in CRT is more difficult compared with other treatment regimens where the schedule contains so-called stop weeks. To our knowledge, this is the first study specifically investigating *DPYD* pharmacogenetics of fluoropyrimidines in CRT. *DPYD* variant allele carriers treated with standard fluoropyrimidine dosages in CRT showed a significantly increased risk to develop severe toxicity compared with wild-type patients. This indicates the need for pharmacogenetic dose reductions in CRT, despite the

lower standard dosages.

Although over 800 patients are considered, the number of patients with a *DPYD* variant remains limited due to the low prevalence of *DPYD* variants. We were unable to show that the risk of toxicity in *DPYD* variant allele carriers who received dose reductions was equivalent to the risk of wild-type patients. Also, 85% of the *DPYD* variant allele carriers treated with a standard dose were carriers of the c.1236G>A and c.2846A>T variants. *DPYD**2A and *DPYD**13 carriers have a higher risk of toxicity when treated with standard dosages compared with c.1236G>A and c.2846A>T carriers. Therefore, it is possible that more toxicity could have occurred in this group if *DPYD* variants would have been equally distributed, increasing the difference in toxicity compared with the other study groups. Moreover, in the *DPYD* group with initial dose reductions, *DPYD* variants and corresponding dose reductions (25 versus 50%) were equally distributed.

Noteworthy, the number of hospitalisations due to toxicity was similar in both groups of *DPYD* variant allele carriers, yet the duration of hospitalisation was significantly shorter in *DPYD* variant allele carriers treated with a reduced fluoropyrimidine dose. A possible explanation for this could be that treating physicians are alarmed of a potentially increased risk of toxicity because of DPD deficiency and more rapidly decide to hospitalise a patient in response to signs of potential toxicity. A second explanation is that *DPYD* variant allele carriers who received dose reductions recovered faster of toxicity.

In two *DPYD* variant allele carriers who received initially reduced dosages and did not experience (severe) toxicity, the dose was increased during treatment. This shows that physicians might still have fear of underdosing patients and reducing efficacy of the treatment.

Grouped diagnosis was significantly associated to severe haematological toxicity, with more severe toxicity in pelvic malignancies. A possible explanation may be that more bone marrow is exposed to radiation in the pelvic area compared with other areas, increasing the chance of myelosuppression.

With over 800 patients included, this study provides a large amount of toxicity data of wild-type patients and *DPYD* variant allele carriers receiving CRT. However, our study has several limitations. First, three databases were combined and were partly retrospective, possibly introducing bias. However, each database included patients in each study group, limiting bias. General differences in scoring toxicity per database could exist; however, criteria for toxicity grades are well marked and should therefore be limited. One database used the new version of CTC-AE; however, updates did not influence the grading of toxicity of interest for this study.

Second, not all databases contained the full toxicity spectrum of interest in this study (e.g. nausea, vomiting, radiation dermatitis, fatigue); therefore, overall toxicity consisted of different toxicities per original database and was not used as a primary end-point.

And third, pharmacokinetic sampling was not executed in this study, which could have shown that dose reductions in *DPYD* variant allele carriers result in equivalent fluoropyrimidine metabolite plasma levels compared with wild-types treated with standard dosages, as was done previously for *DPYD**2A variant allele carriers.[19]

Conclusions

Our study is the first to show that *DPYD* variant allele carriers have an increased risk of severe toxicity when treated with standard dosages in CRT, indicating that dose reductions are necessary in these patients as well. The present study provides the only evidence at this time, and based on these data we advise that fluoropyrimidine dose reductions should also be applied in *DPYD* variant allele carriers who will start CRT to prevent severe fluoropyrimidine-induced toxicity.

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SUPPLEMENT CHAPTER 7

Standard fluoropyrimidine dosages in chemoradiation therapy result in an increased risk of severe toxicity in *DPYD* variant allele carriers

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Supplementary table 1. Toxicity of study patients compared to patients from literature
Selected studies from literature had relatively large patient cohorts and reported toxicity extensively.

Type of event	Study[a] (*N*=674) %	Literature[1-3] %
Grade 2 Diarrhoea	17.4	3—20
Grade ≥3 Diarrhoea	8.6	2—7
Grade 2 Nausea[b]	3.8	2—10
Grade ≥3 Nausea[b]	1.1	-
Grade 2 Mucositis	2.1	7
Grade ≥3 Mucositis	0.7	-
Grade 2 Radiation dermatitis[b]	15.8	15—25
Grade ≥3 Radiation dermatitis[b]	2.3	0—9
Grade 2 Fatigue[c]	9.3	11
Grade ≥3 Fatigue[c]	1.7	2
Grade 2 HFS	3	3—4
Grade 3 HFS	0.6	-
Grade 2 Neutropenia	0.9	5—8
Grade ≥3 Neutropenia	0.7	1—2
Grade 2 Leukocytopenia	8.5	19—20
Grade ≥3 Leukocytopenia	1.6	1—2
Grade 2 Thrombocytopenia	0.3	-
Grade ≥3 Thrombocytopenia	0.3	-
Grade 2 Anaemia[b]	5.6	7—20
Grade ≥3 Anaemia[b]	0.4	-

[a] Selected from this study are all patients with a similar capecitabine schedule to referenced locally advanced rectal patients from literature (825 mg/m2 twice daily, for 5 weeks with or without weekends);

[b] Data of 266 patients;

[c] Data of 172 patients.

Abbreviations: HFS: hand-foot syndrome.

Supplementary table 2. Toxicity of patients from three original databases and of the combined database (total)

Type of event	DB#1:NKI (*N*=495) *N* (%)	DB#2:LUMC (*N*=239) *N* (%)	DB#3:CRO (*N*=93) *N* (%)	TOTAL (*N*=827) *N* (%)
Grade 2 Diarrhoea	56 (11.3)	50 (20.9)	24 (25.8)	130 (15.7)
Grade ≥3 Diarrhoea	43 (8.7)	19 (7.9)	4 (4.3)	66 (8)
Grade 2 Mucositis	15 (3)	5 (2.1)	1 (1.1)	21 (2.5)
Grade ≥3 Mucositis	3 (0.6)	4 (1.7)	-	7 (0.8)
Grade 2 Nausea	*ND*	14 (5.9)	2 (2.2)	16 (4.8)[a]
Grade ≥3 Nausea	*ND*	3 (1.3)	-	3 (0.9)[a]
Grade 2 Vomiting	*ND*	15 (6.3)	*ND*	15 (6.3)[b]
Grade ≥3 Vomiting	*ND*	2 (0.8)	*ND*	2 (0.8)[b]
Grade 2 Neutropenia	5 (1)	5 (2.1)	-	10 (1.2)
Grade ≥3 Neutropenia	10 (2)	5 (2.1)	1 (1.1)	16 (1.9)
Grade 2 Leukocytopenia	27 (5.5)	24 (10)	18 (19.4)	69 (8.3)
Grade ≥3 Leukocytopenia	10 (2)	7 (2.9)	6 (6.5)	23 (2.8)
Grade 2 Thrombocytopenia	3 (0.6)	3 (1.3)	1 (1.1)	7 (0.8)
Grade ≥3 Thrombocytopenia	2 (0.4)	3 (1.3)	-	5 (0.6)
Grade 2 Anaemia	*ND*	26 (10.9)	2 (2.2)	28 (8.4)[a]
Grade ≥3 Anaemia	*ND*	2 (0.8)	-	2 (0.6)[a]
Grade 2 Radiation dermatitis	*ND*	80 (33.5)	3 (3.2)	83 (25)[a]
Grade ≥3 Radiation dermatitis	*ND*	9 (3.8)	4 (4.3)	13 (3.9)[a]
Grade 2 HFS	12 (2.4)	7 (2.9)	1 (1.1)	20 (2.4)
Grade 3 HFS	4 (0.8)	1 (0.4)	-	5 (0.6)
Grade 2 Cardio toxicity	10 (2)	7 (2.9)	4 (4.3)	21 (2.5)
Grade ≥3 Cardio toxicity	5 (1)	1 (0.4)	5 (5.4)	11 (1.3)
Grade 2 Fatigue	*ND*	33 (13.8)	*ND*	33 (13.8)[b]
Grade ≥3 Fatigue	*ND*	4 (1.7)	*ND*	4 (1.7)[b]
Grade 2 Other toxicity[c]	*ND*	13 (5.4)	38 (40.9)	51 (15.4)[a]
Grade ≥3 Other toxicity[c]	*ND*	7 (2.9)	6 (6.5)	13 (3.9)[a]
Grouped type of events				
Grade 2 GI toxicity[d]	67 (13.5)	56 (23.4)	26 (28)	149 (18)
Grade ≥3 GI toxicity[d]	44 (8.9)	22 (9.2)	4 (4.3)	70 (8.5)
Grade 2 HEM toxicity[e]	29 (5.9)	24 (10)	18 (19.4)	71 (8.6)
Grade ≥3 HEM toxicity[e]	12 (2.4)	10 (4.2)	6 (6.5)	28 (3.4)
Grade 2 Overall toxicity[f]	93 (18.8)	118 (49.4)	55 (59.1)	266 (32.2)
Grade ≥3 Overall toxicity[f]	61 (12.3)	38 (15.9)	19 (20.4)	118 (14.3)

[a] Data of 332 patients;

[b] Data of 239 patients;

[c] Other toxicity includes anal pain, chronic enteritis, cystitis (genitourinary tract), dizziness, dysgeusia, enterocolitis, fever, headache, multi-organ failure, papulopustular rash, proctitis, radio-enteritis, skin hyperpigmentation, stomatitis, thromboembolic event, ulcer of the small intestine;

[d] GI toxicity includes diarrhoea, mucositis, nausea, vomiting;

[e] HEM toxicity includes neutropenia, thrombocytopenia, leukocytopenia;
[f] Overall toxicity includes diarrhoea, mucositis, nausea, vomiting, neutropenia, thrombocytopenia, leukocytopenia, anaemia, radiation dermatitis, HFS, cardio toxicity, fatigue and other toxicity.
Abbreviations: CRO: Aviano National Cancer Institute; DB: database; GI: gastrointestinal; HFS: hand-foot syndrome; HEM: haematological; LUMC: Leiden University Medical Center; ND: not defined in database; NKI: Netherlands Cancer Institute.

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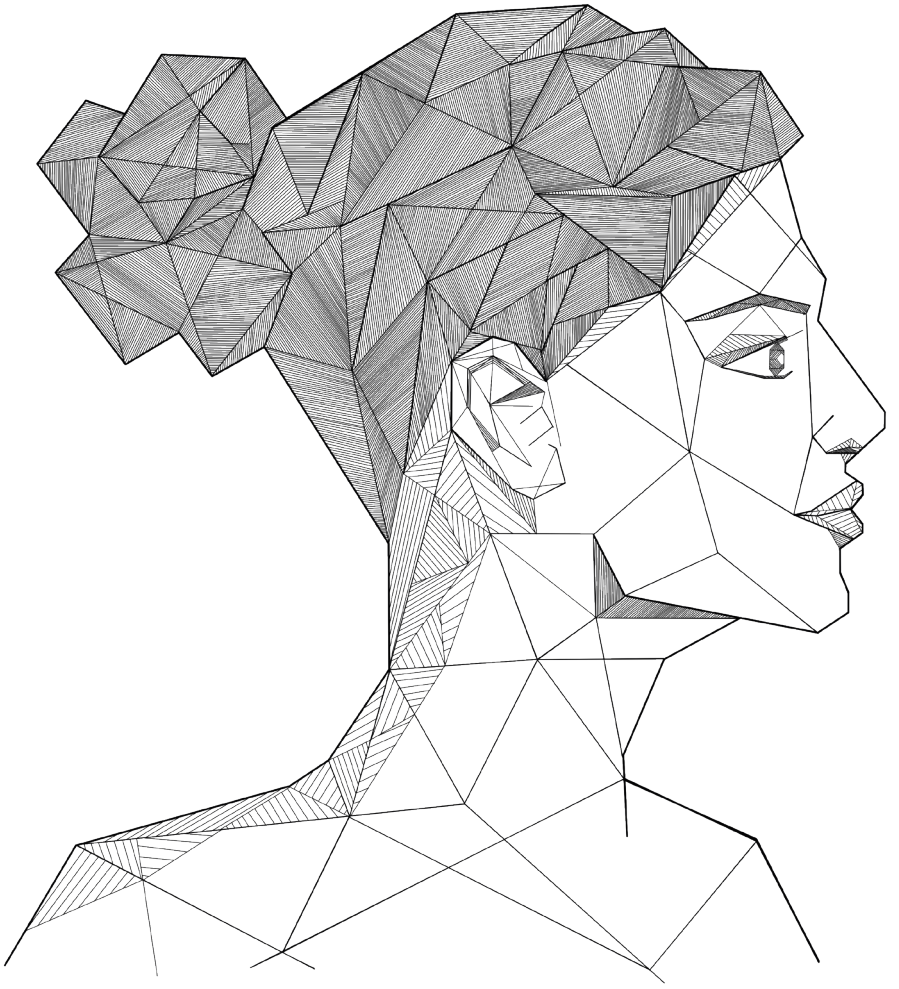

CHAPTER 8

Evaluation of clinical implementation of prospective *DPYD* genotyping in 5-fluorouracil or capecitabine treated patients

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Abstract
Fluoropyrimidines are commonly used anti-cancer drugs, but lead to severe toxicity in 10—30% of patients. Prospective *DPYD* screening identifies patients at risk for toxicity and leads to a safer treatment with fluoropyrimidines. This study evaluated the routinely application of prospective *DPYD* screening at the Leiden University Medical Center.

Prospective *DPYD* screening as part of routine patient care was evaluated by retrospectively screening databases and patient files to determine genotype, treatment, dose recommendations and dose adjustments.

86,9% of all patients with a first fluoropyrimidine prescription were screened. Fourteen out of 275 patients (5.1%) carried a *DPYD* variant and received a 25—50% dose reduction recommendation. None of the patients with a *DPYD* variant treated with a reduced dose developed toxicities.

Prospective *DPYD* screening can be implemented successfully in a real world clinical setting, is well accepted by physicians and results in low toxicity.

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All medical oncologists and fellows working at the LUMC department of Medical Oncology during the observation period of the study are kindly thanked.

Introduction

Fluoropyrimidines such as 5-fluorouracil (5-FU) and its oral prodrug capecitabine are the cornerstone anticancer drugs for several types of cancer such as colorectal cancer, head–neck cancer and breast cancer. Approximately 10–30% of the patients receiving 5-FU or capecitabine experience severe (grade ≥3) toxicity, such as diarrhea, mucositis and hand-foot syndrome.[1] 5-FU is extensively metabolized (>80%) by the liver enzyme dihydropyrimidine dehydrogenase (DPD). DPD is encoded by the gene *DPYD* for which more than 160 genetic variants are known, some of them being pathogenic by reducing enzyme function.[2,3] There is a strong correlation between reduced DPD activity and increased risk for severe and potentially lethal toxicity following treatment with a normal dose of 5-FU.[4-7] Toxicity occurred in 73% of *DPYD**2A carriers, compared with 23% of wild-types.[8] Several meta-analyses have consistently shown that *DPYD**2A, c.2846A>T, *DPYD**13 and c.1236C>G/HapB3 are associated with toxicity.[1,6,9] Although the sensitivity of *DPYD* genotyping is low (<14.5% for *DPYD**2A and c.2846A>T combined), prospective screening for genetic variants in *DPYD* is a well-known strategy to detect patients who have reduced DPD enzyme activity (DPD deficient).[8,10,11] Patients with no or reduced DPD enzyme activity can be treated more safely when applying a 25–50% dose reduction of 5-FU or capecitabine, or using an alternative drug.[10,12,13] Recently it was shown that prospective screening for *DPYD**2A followed by a 50% dose reduction significantly reduces the number of severe toxicities and is cost-effective.[8] Several pharmacogenetic guidelines are available that provide dose recommendations when a reduced function *DPYD* variant is present. The pharmacogenetic guidelines of the Dutch Pharmacogenetic Working Group (DPWG), recommend a 25–50% dose reduction of 5-FU or capecitabine for the first treatment cycle followed by dose titration guided upon toxicity during subsequent cycles for patients with a variant in *DPYD* (*DPYD**2A, *DPYD**13, c.2846A>T or c.1236G>A). A minimum of 50% reduction or alternative therapy is advised for homozygous patients, depending on the variant.[14] The Clinical Pharmacogenetics Implementation Consortium (CPIC)[15,16] recommends a 50% dose reduction of 5-FU or capecitabine for patients with *DPYD**2A, *DPYD**13 and c.2846A>T and alternative therapy for patients who are homozygous for these variants. While these guidelines are very useful for dose adjustments in patients with a genetic variant, they do not advocate prospective *DPYD* testing prior to initiation of therapy.

At Leiden University Medical Center (LUMC; Leiden, The Netherlands), a routine *DPYD* screening program prior to prescribing 5-FU or capecitabine was initiated in April 2013. In this retrospective study we evaluated the physician's acceptance of prospective *DPYD* screening for patients who were prescribed 5-FU or capecitabine in LUMC and the adherence of the recommended dose reduction.

Methods

Setting

At LUMC all patients with an indication for a fluoropyrimidine containing therapy were routinely screened for *DPYD* variants by the laboratory of the department of Clinical Pharmacy and Toxicology (CPT) using two independent techniques (TaqMan® Genotyping SNP assay from Thermo Fisher Scientific [MA USA], and a home-brew pyrosequencing (PSQ),

described previously).[17] Within LUMC the Electronic Medication Record (EMR) system EZIS (version 5.2, Chipsoft) is used, which can be consulted by physicians, pharmacists and nurses. *DPYD* genotyping results are communicated electronically by the responsible pharmacist into the EMR and are visible for other users of the EMR.

The prospective screening program was initiated on 15 April 2013. During a kick-off meeting attended by medical oncologists and fellows, the staff was informed and agreed on the prospective program. New medical oncologists and fellows were informed about the prospective screening program during the regular introduction program for new staff members. Genotyping was performed three times per week (Monday, Wednesday and Friday) in order to minimize the lag time between sampling and test. This resulted in a turnaround time of 2 days, allowing rapid start of treatment if needed. Ethical approval by the Institutional Review Board of LUMC was not required for the current study as it evaluates standard care. Patient data from the EMR was handled following the Codes of Proper Use and Proper Conduct in the Self-Regulatory Codes of Conduct.[18]

Study end points

Three study end points were evaluated to determine the successfulness of the screening program that was introduced at LUMC. We evaluated:

- The 'implementation', in other words, requests of the *DPYD* tests as standard care in daily practice;
- The proportion of test results with a dose recommendation provided by the pharmacist;
- The follow-up of the dose recommendations by oncologists, calculated as the number of follow-ups of dose recommendations by prescribers, excluding the patients in which a follow-up was not possible (e.g., no therapy).

Study procedures

The implementation, or routinely application of the prospective (pretreatment) *DPYD* screening in daily practice was evaluated by determining the proportion of patients who were screened for *DPYD* variants when an incident prescription for 5-FU or capecitabine was given. The data were extracted from two electronic databases. The first database contains data of all patients who are genotyped for *DPYD* variants. The second database (EMR EZIS) contains individual patient medical records. This system is also used by oncologists to electronically prescribe 5-FU and capecitabine. Prescription data prior to the start of the study was studied as well, to ascertain that 5-FU or capecitabine prescription was indeed the first prescription for the patient. The patient identification number was used to connect data from both databases. Discrepancies between information in the queried databases were resolved by manually checking the individual electronic patient records to identify the reason of their absence in one of the two searches. After connecting the data from both databases, all patient data were anonymized. All manual changes (additional information, removal of duplicates, among others) to the queries were double checked by the two first authors (CL and MvS).

To evaluate the follow up of the recommended dose reductions by the oncologists, medical records of patients carrying a variant in *DPYD* were inventoried as to determine

if the oncologist followed the dose advice. The genotyping data of the laboratory of CPT was used to determine the patients carrying a *DPYD* variant. Prospective execution of the genotyping could be determined by comparing the genotyping date and start date of the therapy. Regular drug regimens and notations of dose reductions in the medical records were searched to check applied dose reductions.

After completion of the study, an explorative analysis was executed in order to describe the course of toxicity in relation to the provided dose recommendations. In order to perform this analysis, toxicity information regarding the 5-FU or capecitabine therapy was retrieved from the EMR for patients with a *DPYD* variant. Toxicity was scored by the oncologists using the National Cancer Institute common terminology criteria for adverse events (CTC-AE), version 4.03.[19]

Results

The implementation of the prospective screening program for DPYD

The prospective *DPYD* screening program was implemented on 15 April 2013 (study start date) at LUMC. From this date until 13 December 2014 (study end date) 540 patients were genotyped for *DPYD* variants at LUMC. Initially, patients were screened only for the presence of the *DPYD**2A variant. Later on *DPYD**13, c.2846A>T and c.1236G>A were added to the *DPYD* screening. An overview is shown in Table 1. After removal of duplicate or invalid records, 529 evaluable genotyped patients remained. Of these 529 patients, 275 patients were patients treated at the LUMC and 254 patients were treated at other hospitals, but genotyped as a service provided by the department of CPT of the LUMC. The dose reductions that were advised for each individual *DPYD* variant are displayed in Table 1.

Table 1. Recommended reductions of initial 5-fluorouracil or capecitabine dose

Advice given by CPIC and DPWG guidelines at the time the variant was added to the routine screening.

***DPYD* variant**	**Initial dose reduction (%)**	**Inclusion in screening program**	**Patients screened**
*DPYD**2A (c.1905+1G>A)	50	April 15th, 2013	529
*DPYD**13 (c.1679T>G)	50	October 10th, 2013	440
c.2846A>T	50 → 25[a]	October 10th, 2013	440
c.1236G>A	25	May 28th, 2014	254

[a] The dose reduction advice for c.2846A>T has been updated to 25% in February 2015.

A total of 2,498 records of 5-FU or capecitabine prescriptions prior to 31 December 2014 were found. After removal of duplicates, invalid records (e.g., incomplete data) or patients not meeting eligibility criteria (e.g., prescription prior to April 2013), 337 patients remained who were prescribed 5-FU (16%) or capecitabine (84%) for the first time at LUMC within the study period.

Genotyped patients were compared with patients who were prescribed 5-FU or capecitabine, resulting in 236 matching patients. Thirty-nine patients were genotyped for *DPYD*, but were not prescribed 5-FU or capecitabine. Also, 101 patients were prescribed

5-FU or capecitabine, but were not genotyped for *DPYD* variants (Figure 1).

Two patients, who received 5-FU or capecitabine and were genotyped, were excluded because their medical records revealed they had received 5-FU or capecitabine prior to 15 April 2013. Of the 39 patients who were genotyped without receiving 5-FU or capecitabine therapy, 33 patients eventually did not start their therapy, although there was an intention to treat at the time of requesting the screening test. Six patients started their therapy after 31 December 2014 and were therefore not identified by the search. Of the 101 patients with a 5-FU or capecitabine prescription and no *DPYD*-genotyping record, the medical records were screened resulting in a legitimate reason not to genotype in 60 cases (Table 2). Legitimate reasons included; any notes on prior treatment with 5-FU or capecitabine (e.g., outside LUMC) or invalid patient files (e.g., no medical dossier found for the oncology department). For 41 patients who had a prescription for newly 5-FU or capecitabine no reason was found to neglect genotyping. After data cleaning, 314 patients with a newly 5-FU or capecitabine prescription remained in the dataset and 273 of these patients were genotyped as depicted in Figure 1. The clinical acceptance of the prospective *DPYD* screening program is displayed as percentage per month in Figure 2. The average clinical acceptance was 86.9%.

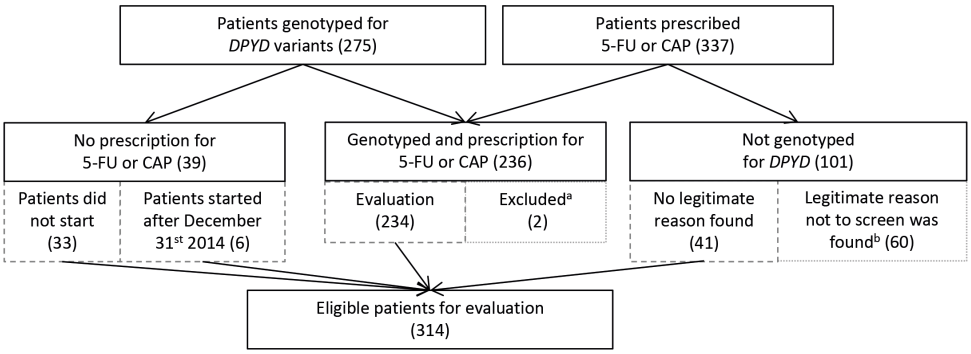

Figure 1. Patient selection

Flowchart following the results from the two searches. Patients could be both genotyped and prescribed 5-FU or capecitabine, or only genotyped, or only prescribed 5-FU or capecitabine. If the intention to treat was present, patients should have been genotyped and these patients are 'eligible for evaluation'.

[a] These two patients were excluded because their medical records revealed they had received 5-FU or capecitabine prior to April 15th 2013;

[b] Legitimate reasons were: e.g., any notes on prior treatment with 5-FU or capecitabine (e.g., outside LUMC) or invalid patient files (e.g., no medical dossier found for the oncology department).

Abbreviations: 5-FU: 5-fluorouracil, CAP: capecitabine.

Table 2. Excluded patients

Patients (*N*=60) with legitimate reasons not to screen were excluded from analysis.

Patients (*N*)	Reason not to perform *DPYD* genotyping
8	5-FU or CAP therapy started just prior to the start date of 15 April 2013
30	5-FU or CAP was used before April 2013 without problems and would start again after 15 April 2013
20	No medical dossier at the Medical Oncology department was found, therefore the patient was not treated at the LUMC
2	These dossiers were fake patients used for education purposes

Abbreviations: 5-FU: 5-fluorouracil; CAP: capecitabine.

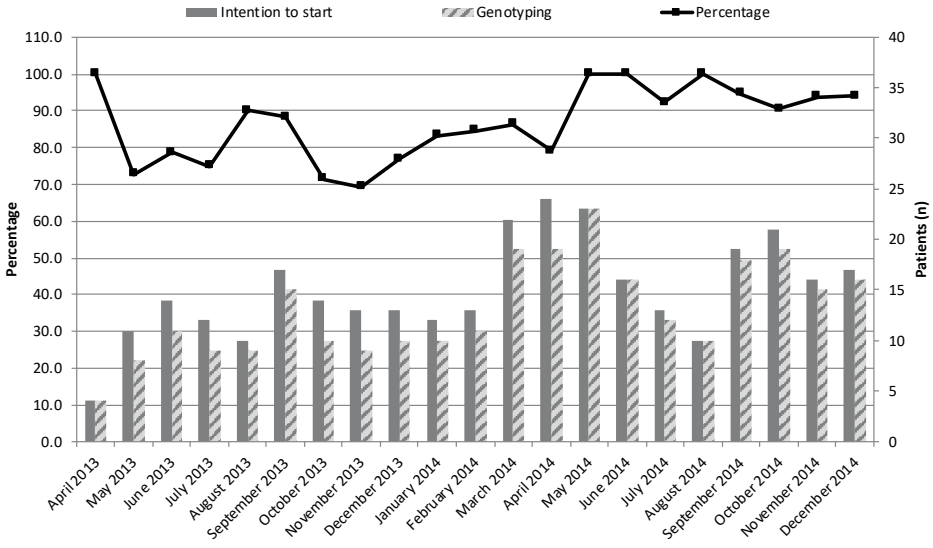

Figure 2. Proportion of eligible patients that were genotyped

The figure shows the eligible patients for evaluation per month in actual patient numbers. If the intention to treat with 5-FU or capecitabine was present, patients were eligible. Also the actual patient numbers of the genotyped patients per month are shown and the calculated percentage which represents the clinical acceptance, or how well implemented the prospective *DPYD* screening is.

Table 3. *DPYD* variants

***DPYD* variant**	**SNPs (*N*)**	**Tested patients (*N*)**	**LUMC (%)**	**Literature (%)**	**Ref.**
*DPYD**2A (c.1905+1G>A)	6	275	2.2	~1.0–1.8	[10,20]
*DPYD**13 (c.1679T>G)	0	214	0	~0.1	[12]
c.2846A>T	1	214	0.5	~1.0–1.4	[10,12]
c.1236G>A	8	109	7.3	~2.6–4.9	[10,21]
Total	15 (*N*=14)	275	5.1	4.7–8.2	

DPYD variants found in LUMC patients and these numbers compared with frequencies in the literature.

The follow-up of the dose recommendations by oncologists

Dose reduction was advised after the first administration of 5-FU or capecitabine (post-dose) for two patients. The medical record of the first patient showed that the initial screening result became available after the start of therapy. Dose adjustments could not be applied, toxicity occurred and the advised dose reduction was applied in the second cycle (Table 4, patient 12). The other patient was screened after start of therapy, but stopped therapy completely due to toxicity, thus applying a dose reduction was not applicable. For this patient the reason not to screen prospectively was absent in the medical record (Table 4, patient 2).

For eleven patients a dose reduction was recommended prior to the start of therapy (prospective). This resulted in an initial dose reduction in eight of 11 patients. For one patient the recommend dose reduction was not applied and full dose was given (Table 4, patient 13). In two patients the recommended dose reduction could not be applied since they did not start therapy. One patient did not start therapy due to renal failure and the presence of a *DPYD* variant (Table 4, patient 14), and one patient refused to start therapy (Table 4, patient 5). Also one patient was genotyped prospectively, but received a recommendation for phenotyping due to compound heterozygosity (Table 4, patient 9). This patient started treatment with a 50% reduced dose at the oncologists discretion. An overview of the above mentioned data are displayed in Table 4. The adherence to the dose recommendations (pre- and post-dose) is 90% (9 out of 10).

Analysis of results on clinical outcomes

The explorative analysis showed that the prospective dose recommendations given, resulted in initial dose reductions in eight patients. None of these eight patients developed severe toxicity (grade ≥3) during the first cycle. After the first or second cycle it was possible to increase the dosages, guided by toxicity. Dosages were increased in four patients (from 50% up to 60, 80 and 100%, and from 75 to 100%, respectively, all receiving capecitabine). However, this led to the development of severe toxicity in two *DPYD**2A carrying patients (80% capecitabine led to diarrhea grade 3 followed by 31 days of hospitalization and 100% capecitabine led to hand-foot syndrome grade 3). Toxicity data can be found in Table 4.

In one patient with a *DPYD**2A variant who received capecitabine in combination with radiotherapy, the dose recommendation was not followed by the physician and this patient experienced diarrhea (grade 4), enteritis and leukopenia, for which hospitalization of 18

days was required and capecitabine therapy was permanently terminated (Table 4, patient 13).

Discussion

In this study, the successfulness of routine application of a prospective *DPYD* screening program followed by pharmacogenetically guided dose recommendations was studied. The percentage of patients in which screening was performed was relatively high: 86.9% of all eligible (newly prescribed 5-FU or capecitabine) patients. In the study period, 13.1% of the patients were not screened prior to receiving 5-FU or capecitabine therapy, which on average comes down to one patient per month. Follow-up of dose recommendations given by the pharmacist were applied in all cases except one, resulting in a high acceptance.

Our study has several limitations. Due to the retrospective design of our study, available data may not always have been fully complete. For example for some patients, it was not possible to retrieve why *DPYD* screening was not requested or whether a patient actually started fluoropyrimidine therapy. In addition, the study was performed with data obtained in a real world clinical setting instead of a regulated and controlled case report form. We had to manually check patient files to obtain specific information and not all physicians may have systematically annotated CTC-AE grading continuously to describe toxicity. Due to the low number of *DPYD* variant carriers our study was not powered to formally test the effect of *DPYD* screening on fluoropyrimidine-induced toxicity and only explorative analyses could be performed.

In this study, we determined the level of routine application of *DPYD* screening in daily practice, which increased at the end of the study period to 90–100%. This might indicate that prescribers were undergoing a learning or acceptance curve following the initial start, and were getting used to apply *DPYD* genotyping increasingly in their daily routine.

We believe patients do not need to be genotyped if previous 5-FU or capecitabine usage without toxicity is known or if patients were genotyped (*DPYD*) or phenotyped (DPD) previously. However, within the 41 (13.1%) remaining patients legitimate reasons can still exist (e.g., well-tolerated treatment before 2013 with 5-FU or capecitabine), but might not have been filed in the medical record. Therefore we can conclude the 90–100% (≤ 1 patient not tested per month) rate was an effective prospective *DPYD* screening implementation. Disputable is, if this clinical acceptance can become 100% continuously. In order to support the clinical implementation, the use of a clinical decision support system might be suitable. In LUMC a clinical decision support system entitled adverse drug event alerting system (ADEAS) is used in daily practice in the hospital pharmacy of LUMC.[22] This system is used by hospital pharmacists to systematically select patients at risk of possible adverse drug events. It retrieves data from several information systems, and uses clinical rules to select the patient at risk of adverse drug events.

As mentioned before, sensitivity of genotyping is relatively low (<14.5% for *DPYD**2A and c.2846A>T combined).[11] Even if all patients with a *DPYD* variant are identified and treated with an appropriately reduced dose, not all fluoropyrimidine-related toxicity can be prevented. Adding a DPD phenotyping test may increase sensitivity, but is expensive and logistically challenging to implement in clinical practice.[13] SNPs located in other genes than *DPYD*

(e.g., *TYMS*) have been associated with fluoropyrimidine-induced toxicity with conflicting results. However, testing for these SNPs holds the potential to increase sensitivity.[23] Even though *DPYD* screening cannot prevent all fluoropyrimidine-related toxicity, we feel that the available evidence strongly supports implementation in clinical practice and can prevent fluoropyrimidine-induced deaths.[8,11,24]

The presence of one of the four *DPYD* variants that were pre-emptively tested resulted in a recommendation to the oncologist to reduce the initial dose of 5-FU or capecitabine by 25–50% depending on the identified variant. In February 2015 the recommended dose reduction for c.2846A>T was changed from 50 to 25%, following the updated guidelines of the DPWG.[25,26]

One patient (Table 4, patient 13) received full capecitabine dose, since the treating oncologist argued that she was afraid of under dosing the patient as the dosage of capecitabine in chemoradiation schemes is already lower compared with other treatments and there is less opportunity to increase the dose in subsequent treatment cycles. The patient developed severe toxicity illustrating that the recommended dose reductions should also be applied to lower capecitabine doses used in chemoradiation, despite lack of published data about capecitabine toxicity during chemoradiation therapy.

Conclusion

This study for the first time shows that systematic prospective *DPYD* screening can be implemented successfully in real world daily clinical practice. The applied 25–50% dose reduction for patients with a *DPYD* variant resulted in absence of toxicity. However, a more active follow-up of adherence to provided dose recommendations might improve patient safety even further.

Table 4. *DPYD* variant carrying patients
Listed for LUMC patients who carry a *DPYD* variant are genotyping data, therapy details and toxicity data.

pt#	Cancer type	Therapy	*DPYD* variant	Pro-spective screen-ing?	Initial dose adjust-ment?[a]	Toxicity (grade 3–4)?	Toxicity specifications	Hospital admissions?	Second dose adjustment?	Toxicity (grade 3–4)?	Toxicity specifica-tions	Hospital admissions?
1	Colo-rectal	CAPOX	c.1236G>A	YES	YES	NO	N/A	N/A	YES (to 100%)	NO	N/A	N/A
2	Mouth	TPF + RT	c.1236G>A	NO[b]	NO	YES	Diarrhoea IV + Neutropenia/ Thrombocyto-penia III	YES (6+16 days)	N/A (Quit af-ter 2nd cycle)	N/A	N/A	N/A
3	Colon (met.)	CAPOX + BEV	c.1236G>A	YES	YES	NO	N/A	N/A	NO	N/A	N/A	N/A
4	Anus	5-FU + RT	c.1236G>A	YES	YES	NO	N/A	N/A	NO	N/A	N/A	N/A
5	Colon	N/A	c.1236G>A	YES	DNS[c]	N/A	N/A	N/A	N/A	N/A	N/A	N/A
6	Pharynx	5-FU + RT	c.1236G>A	YES	YES	NO	N/A	N/A	NO	N/A	N/A	N/A
7	Pancreas	CAP	c.1236G>A	YES	YES	NO	N/A	N/A	N/A (Quit)	N/A	N/A	N/A
8	Rectal	CAP + RT	*DPYD**2A	YES	YES	NO	N/A	N/A	YES (to ±80%)	YES	Diarrhoea III + Enteritis	YES (31 days)
9	Mamma (met.)	CAPOX	*DPYD**2A + c.2846A>T	YES	No dose recomm.[d]	NO	N/A	N/A	N/A (Quit)	N/A	N/A	N/A
10	Mamma (met.)	CAPOX	*DPYD**2A	YES	YES	NO	N/A	N/A	YES (to 100%)	YES (not in first cycles)	HFS II–III	NO (switch to Paxclitaxel, after 8 cycles)

table continues

pt#	Cancer type	Therapy	*DPYD* variant	Pro-spective screen-ing?	Initial dose adjust-ment?[a]	Toxicity (grade 3–4)?	Toxicity specifications	Hospital admissions?	Second dose adjustment?	Toxicity (grade 3–4)?	Toxicity specifica-tions	Hospital admissions?
11	Rectal	CAPOX	*DPYD**2A	YES	YES	NO	N/A	N/A	YES (to 60%)	NO	N/A	N/A
12	Gastric (met.)	EOX	*DPYD**2A	NO[e]	NO	YES	Diarrhoea III	NO	YES (to 50%)	NO	N/A	N/A
13	Rectal	CAP + RT	*DPYD**2A	YES	NO	YES	Diarrhoea IV + Enteritis + Leukopenia	YES (18 days)	N/A (Quit after TOX in first cycle)	N/A	N/A	N/A
14	Rectum	N/A	c.1236G>A	YES	DNS [f]	N/A	N/A	N/A	N/A	N/A	N/A	N/A

[a] Initial dose adjustment is the dose adjustment made prior to the first dose of 5-FU or CAP;
[b] Genotyping was performed on November 7th 2014, while therapy started on November 5th, 2014;
[c] Patient did not start therapy on its own wish;
[d] For this patient no dose reduction advice was given because this patient was compound heterozygous (carrying two variants), and it was not possible to predict the remaining DPD enzyme activity with the current information. The advice given was to test the actual DPD enzyme activity with another method;
[e] Both genotyping and start of therapy where on January 24th, 2014. Therefore the result of the genotyping was not awaited;
[f] Patient did not start therapy due to renal failure and presence of the *DPYD* variant.
Abbreviations: RT: radiotherapy; CAPOX: Capecitabine + Oxaliplatin; TPF: Docetaxel + Cisplatin + 5-fluorouracil; CAPOX + BEV: Capecitabine + Oxaliplatin + Bevacizumab; 5-FU: 5-fluorouracil; CAP: Capecitabine; EOX: Epirubicin + Oxaliplatin + Capecitabine; DNS: Did not start; dose recomm.: dose recommendation.

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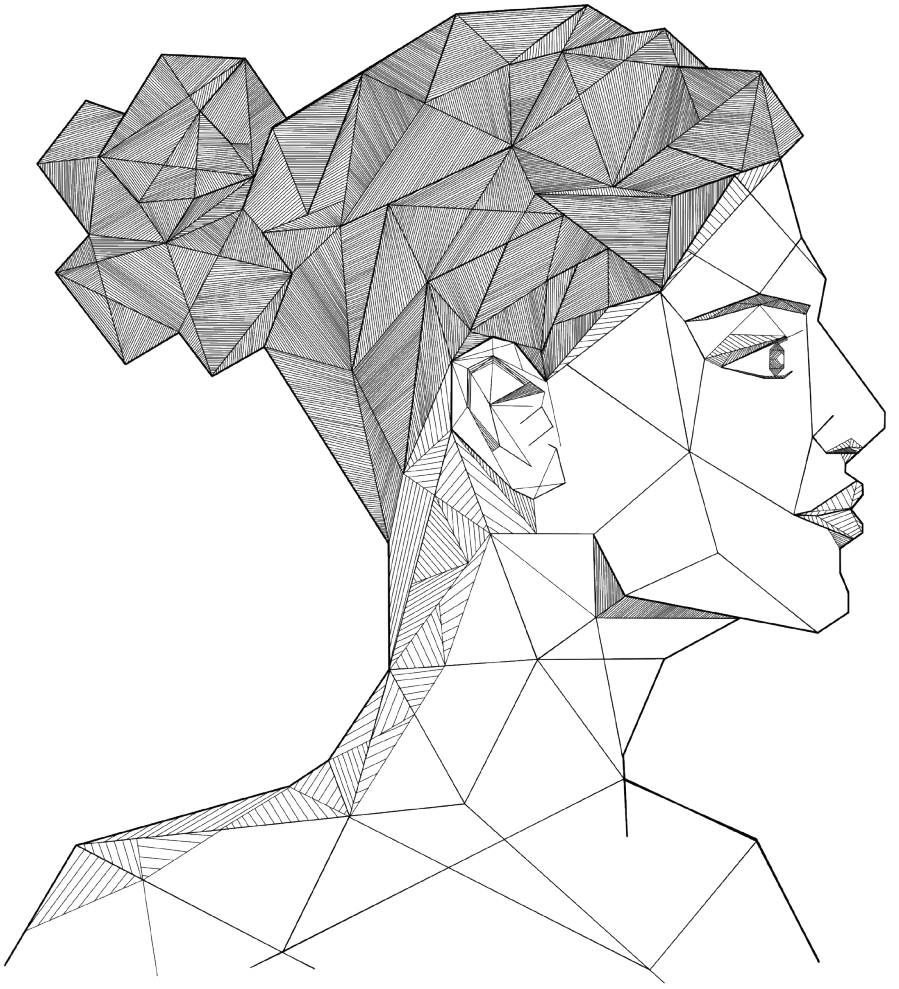

CHAPTER 9

Confirmation practice in pharmacogenetic testing; how good is good enough?

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Abstract
Pharmacogenetic testing is increasingly implemented in routine diagnostics. However, quality control measures, in particular confirmation practices e.g. the use of two independent genotyping techniques, are subject of debate and there are no clear guidelines. The aim of the current paper is to discuss the current practice in confirmation testing in the field of pharmacogenetics and draw attention to this situation. *DPYD* genotyping is used as a case example to highlight the importance of assigning the correct genotype. Current confirmation practices in laboratories were explored through a survey. Substantial heterogeneity was observed with 54% of the laboratories applying different forms of confirmation practice. Finally, we evaluated over ten years of genotyping results from two large genotyping facilities, which both use a second, independent genotyping technique. Discrepancies between tests were identified in nine patients (0.01%), possibly due to allele dropout. We feel that a second, independent technique is useful for genetic tests with a high clinical impact, such as *DPYD* testing. Guidelines can help to align confirmatory laboratory practices for pharmacogenetics, which may need to be specified per gene and test.

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Introduction

Over the past ten years, our knowledge of pharmacogenetics (PGx) has increased significantly. With decreasing assay costs, availability of PGx dosing guidelines and inclusion of PGx information in drug labels PGx testing has become an attractive strategy for routine diagnostics.[1] For some diseases and drugs (pharmaco)genetic testing to predict therapeutic response is already widely accepted in clinical practice (e.g. lung cancer and *EGFR* status) or even mandatory (e.g. abacavir and *HLA-B**5701 allele carriers).[2] For a limited number of (pharmaco)genetic tests approval of the Food and Drug Administration (FDA) is available, e.g. *CYP2D6* (Luminex) and INFINITI *CYP2C19* assays, possibly increasing its use in clinical care.[3-5] The Roche AmpliChip for cytochrome p450 *CYP2D6* and *CYP2C19* (Roche Molecular Diagnostics, Pleasanton, CA, USA) was the first FDA approved (December 24th, 2004) and commercially available PGx test.[6] If no FDA-approved assay is available, laboratory developed tests (LDTs) can be used.[7]

Many laboratories use LDTs. It is important to have quality assurance of the PGx test results from these LDTs, which can be achieved by participating in a proficiency testing program. Proficiency testing programs are regulated by independent organizations, such as the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC),[8] the German Reference Institute for Bioanalytics (RfB),[9] the European Molecular Genetics Quality Network (EMQN) in the UK,[10] or the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML).[11] In addition, the Genetic Testing Reference Material Coordination Program (GeT-RM) was set-up to guard quality assurance, assay development, validation and proficiency testing.[12] Another less commonly applied quality control measure used by laboratories to ensure quality of PGx test results is confirmation practice, e.g. the use of two independent genotyping techniques. However, these measures have disadvantages, such as increased costs and labour, and are subject of debate. It is yet unknown if differences in laboratory practices exist as there are no clear guidelines on this particular quality control aspect.

The aim of the current paper is to discuss the current practice in confirmation testing in PGx and draw attention to this situation. We first assess current confirmation practices to assure the validity of PGx test results, by means of a questionnaire using *DPYD* genotyping as an impactful case. Secondly, we evaluate genotyping results from Leiden University Medical Center (LUMC) and Erasmus Medical Center Rotterdam (Erasmus MC), where two independent genotyping methods are applied to confirm results.

Importance of analytical validity and assigning the correct genotype

The number of executed PGx tests is rapidly increasing, partly due to incorporation of PGx information in drug labels —currently over 260— some of them strongly suggesting or demanding a priori PGx testing (e.g. abacavir, clopidogrel, eliglustat).[13] For some other diseases or drugs, which have a (pharmaco)genetic application available, the use in clinical practice remains limited and is subject to debate (e.g. bupropion, tamoxifen).[1,14] By contrast to many other clinical laboratory tests, a (pharmaco)genetic test is usually performed only once in the lifespan of a patient. As a result, it is of utmost importance that the correct result is reported. Consequences of a false positive or false negative result could be

fatal, as is explained in the following example of *DPYD* genotyping for fluoropyrimidines (5-fluorouracil/5-FU, and capecitabine).[15] There is compelling evidence on the reduction of severe fluoropyrimidine-induced toxicity when using prospective PGx for four *DPYD* variants, and dosing recommendations for these four *DPYD* variants have been published by the Clinical Pharmacogenetics Implementation Consortium (CPIC) and the Dutch Pharmacogenetics Working Group (DPWG).[16-19] Despite this, clinical implementation is not yet part of routine clinical care in many hospitals.[20] When exposed to standard dosages of fluoropyrimidines, carriers of a *DPYD* variant are at high risk for severe, or even fatal, toxicity. Despite the low frequency of *DPYD* variants, prospective genotyping of *DPYD* variants in all patients prior to initiating fluoropyrimidine treatment was shown to be cost-saving.[21] Thus, it is safer, but not more expensive to genotype patients. Misclassification of the *DPYD* genotype can result in suboptimal therapy (false positive) or even have lethal consequences from fluoropyrimidine treatment in standard dosages (false negative). In addition, therapeutic drug monitoring (TDM) could be used to monitor the 5-FU dose during treatment, but is rarely executed. For capecitabine, the oral pro-drug of 5-FU, TDM protocols need to be developed. This particular example shows the clinical importance and substantial consequences of PGx testing and illustrates why it is of utmost importance to report the correct result.

The dilemma

Laboratories apply different genotyping techniques to generate PGx results. Sanger sequencing remains the gold standard for DNA sequencing,[22] even though this can be prone to errors.[23] In general, PCR-based assays (including Sanger sequencing) are considered a robust methodology with reliable results. Each assay is subjected to extensive validation by the company or laboratory to reduce the risk of a priori errors. However, after the implementation of a test in clinical practice, it is still possible to have false positive or false negative results, e.g. due to allele dropout.[24] Allele dropout can be caused by a newly acquired variant located at the site of a primer, causing the binding of this primer to fail. A genetic variant located on that DNA strand will not be genotyped, and the patient is misclassified as homozygous carrier of the variant on the other strand.

To mitigate the risk of allele dropout a laboratory can use a second, independent method that uses different primers to confirm results. However, this results in increased costs, labour and turn-around-time. Should laboratories execute a second method to confirm results, or not? The dilemma of the quality control aspect of PGx testing is based on the probability of a genotyping error to occur, the level of increased effort and costs to detect the error and the consequence of not detecting the error. A genotyping error, e.g. due to allele dropout, can be detected by a second, independent genotyping assay, which is the most adequate, but comprehensive, available method. Abolishing a second method or repetition can thus save both time and costs, possibly increasing the likeliness of use of PGx testing since cost-effectiveness is often reported as a barrier for implementing PGx testing.[15] The consequence of an error in PGx can be substantial, yet it is unrealistic to aim to never have an incorrect result. This dilemma is why differences in confirmation practices in laboratories could exist and why guidelines are required to align laboratory practices. These differences could be overcome by clear guidelines from regulatory authorities, however,

notifications from regulatory authorities are also not conclusive about this dilemma. In January 2017, the FDA discussed that regulatory aspects on the quality control of LDTs are still under debate.[25] In Europe, guidelines on good pharmacogenomics practice (GPP) by the European Medicines Agency (EMA) issued in September 2018 include a chapter on quality aspects on PGx analyses. They describe the importance of proper validation prior to using genetic tests in clinical trials or a diagnostic setting and the detection of respective allele-drop-outs, as primer-based technologies are prone for these artefacts. However, no specific standpoint is taken regarding the use of a second, independent technique.[26] Also, the *In Vitro* Diagnostic Regulation (IVDR) of the European Parliament and of the Council on *in vitro* diagnostic medical devices has recently been updated and will come into force in 2022. Yet, these guidelines do not explicitly state what actions to guarantee quality are required in the laboratory.

Confirmation practice

Current confirmation practice in laboratories

In order to investigate the consequences of the lack of clear guidelines we assessed the current confirmation practices of laboratories. A short questionnaire comprising three general questions on *DPYD* genotyping and confirmation practices in the laboratory was sent to laboratories in Europe and the Netherlands participating in the proficiency testing program of the RfB and SKML, respectively. Details on the set-up of the questionnaire can be found in the Supplementary Material. Out of the 475 laboratories, 35 completed the questionnaire. One laboratory participated in both the European (RfB) and Dutch (SKML) questionnaire. 28 laboratories executed genotyping tests. Of all laboratory techniques, the TaqMan assay and melting curve analyses were most frequently used. A large variation between laboratories in confirmation practice was observed. Almost half of the laboratories did not execute a second test (either independent or repetition).

Two independent genotyping methods as confirmation practice

In addition, we assessed the impact of confirmation methods in PGx. At LUMC and the IFCC PGx reference laboratory at Erasmus MC, the most elaborate confirmation method, executing two independent genotyping tests using two different platforms, are used. We evaluated over ten years of aggregated genotyping data of these two large genotyping laboratories performing duplicate analyses on two independent platforms. Details of the two laboratories can be found in the Supplementary Material. In total, 89,842 duplicate tests were executed for patient care in over ten years of genotyping. Nine discrepancies (0.01%) between tests were observed. One discrepancy in *CYP3A5**3 was the result of chimerism due to allogeneic hematopoietic stem cell transplantations, which resulted in the determination of the genotype of both patient and donor.[27,28] Four discrepancies in *CYP3A5**3, one discrepancy in *DPYD**13 and three discrepancies in *CYP2D6**6 were identified, possibly due to allele dropout. The probability of finding a discrepant result when using two independent techniques according to our data was calculated to be 0.01%.

Discussion

The topic of confirmatory testing in the rapidly growing field of PGx deserves attention. At this moment, there are no clear guidelines on the required confirmation practice aspects of PGx testing. Should laboratories execute a second method to confirm results, or not? The FDA is in debate on this dilemma and the current guidelines of the EMA are not very precise on the use of confirmation methods. Our supporting data show that there is great heterogeneity between laboratories in confirmation practice. Discrepant results were identified between two tests in about 0.01% of samples.

Our data show a substantial variation of approaches for *DPYD* genotyping used in laboratories across Europe as well as a limited use of second, independent techniques as a confirmation method to assure the correctness of genotyping results. Almost half of the responders do not apply any of the suggested confirmation or replication methods, and implies the need for centrally organized guidelines. We selected *DPYD* as an example for its clinical relevance, as a false negative result or misclassification can have a fatal outcome. The number of centres which routinely test for *DPYD* is relatively low and it is possible that a questionnaire focussing on a gene that is more commonly tested would have resulted in a higher response rate. However we do not expect major differences in confirmation practice between genes within a laboratory.

To assess the usefulness of applying two independent genotyping techniques for confirmatory testing we evaluated genotyping results of almost 90,000 samples tested in two laboratories in over ten years of genotyping. We identified nine discrepant results (0.01%) between the two independent genotyping techniques. One discrepant result was caused by chimerism following allogeneic hematopoietic stem cell transplantations, and is thus not due to analytical failure. To prevent this particular type of error, a check-box for "transplantation patient" was added to the genotyping request form. Two other stem cell transplantation patients were correctly genotyped after the check-box was added. For the other eight samples, misclassification due to allele dropout was the most probable cause of the discrepancies. In this study, a frequency of 0.01% of misclassification was shown, whereas previous publications show higher frequencies of misclassification (0.27% in 365 patients, Scantamburlo *et al*.[29] and 0.44% in 30,769 genotypes, Blais *et al*.).[24] A difference in discrepant results between the two genotyping centres was identified and might be explained by the different genotyping techniques used in each centre, as the call rate and accuracy of the techniques can be different. Additionally, *CYP2D6* data of one centre was not included, as this centre did not use a second, independent genotyping platform to confirm genotyping results for *CYP2D6*. *CYP2D6* is a highly polymorphic gene and *CYP2D6*-assays could be more prone to allele dropout.

Another important aspect to consider is that allele dropout is test specific: it depends on the positions of variants and the primer positions of the assay. Therefore, caution should be taken in generalizing our results. Specific quality control analyses per assay may be warranted. One could envision for example a minimum amount of samples to be tested to show that allele dropout for that particular assay and primers is low, possibly as a requirement for diagnostic companies to demonstrate. This brings along a second important consideration, which is that the sensitivity of detecting allele dropouts is directly

proportional to the amount of heterozygotes present. In other words, discrepancies for *CYP2D6**4 (allele frequency 23%)[30] will be detected much earlier than discrepancies for, e.g. *CYP2D6**7 (allele frequency 0.05%).[30] In this aspect, the determined discrepancy rate of 0.01% might actually be higher for specific variants. In addition, the tests in this study were mainly executed in patients with a Caucasian ethnic background. As frequencies of genetic variants can vary between different ethnic populations, results could be different in another population.

The large number of genotyping test results is a strength of this study. However, specific allele dropout will depend on the number of samples with a particular variant. The low discrepancy rate shows high concordance and robustness of the methods used. As described before, the consequence of a misclassified genotype can be substantial, resulting in either underdosing or overdosing, sub sequentially leading to inefficacy or, potentially lethal, toxicity (e.g. *DPYD* genotyping). We expect that next generation sequencing (NGS) might replace some of the current assays in the upcoming years. NGS is also subject to allele dropout as it is PCR based, but possibly less compared to current techniques. This is caused by the fact that NGS has multiple coverage depth of the same variants, thus a failed reaction of one primer will not directly results in a misclassification of the variant.

Differences exist between laboratories in which *DPYD* variants are genotyped, or they might not genotype for *DPYD* variants at all. This could have great impact on patient care as DPD phenotypes might be predicted differently between laboratories. The impact could be greater compared to the impact due to differences between laboratories in confirmation practice as quality control of these tests. This also accounts for other variants in other genes, and for the fact that not all associated variants per gene are discovered yet. Besides assay errors, human errors (switch of samples) might also occur. However, this discussion is out of the scope of this paper, were we focus on the dilemma of confirmation practice.

Conclusions

We have shown substantial variability between laboratories in the use of a second confirmatory technique for PGx testing. The risk of a discrepancy may differ between assays and the clinical implications will depend on the gene tested. Therefore we feel that a second, independent technique is useful for genetic tests with a high clinical impact, such as *DPYD* testing. Guidelines can help to align confirmatory laboratory practices for PGx, however, they may need to be specified per gene and per test.

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SUPPLEMENT CHAPTER 9

Confirmation practice in pharmacogenetic testing; how good is good enough?

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Current confirmation practice

Laboratories participating in the largest European proficiency testing program organized by the RfB received the questionnaire by hard-copy. Laboratories in the Netherlands participating in the national proficiency testing program organized by SKML were contacted via e-mail with the questionnaire. Questionnaires were sent via RfB and SKML via their respective subscriber lists. Responding to the invitation was completely voluntary, results were collected by RfB (hard-copy) and SKML (e-mail) and were processed anonymously. Data were collected in Microsoft Excel 2010. The questionnaire contained the three following general questions;

1) Which SNPs in *DPYD* are currently tested in your lab?
2) Which techniques are used for these SNPs?
3) How is quality controlled? By using:
*two independent techniques, *two technicians using the same technique, *one technician using the same technique on two independent days, *using two independent samples per patient, *other; namely:, *none

The third question was used to identify which confirmation or replication methods for the current genotyping are used.

Two independent genotyping methods as confirmation practice

Two large genotyping laboratories (LUMC and Erasmus MC) performed two independent genotyping tests for over ten years.

At LUMC, both a commercially available TaqMan assay and a home-brew pyrosequencing method were employed to determine the patient's genotype. The results of each independent method were checked by two independent technicians, and approved by a hospital pharmacist. When results between two methods were concordant, results were added to the digital system. Results and additional notes on results are then automatically available for physicians to consult. From January 2009 results were recorded electronically in a Global Laboratory Information Management System (GLIMS, version 8.10.6, MIPS©). For *CYP2D6*, the FDA-approved AmpliChip (Hoffmann-La Roche, Basle, Switzerland)[1] was used, until discontinuation of the AmpliChip by Roche forced switching to the GenoChip *CYP2D6* macroarray (PharmGenomics GmbH, Mainz, Germany) in April 2015.[2] No second independent genotyping platform was used for the AmpliChip and GenoChip variants.

At Erasmus MC, commercially available TaqMan assays and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) were used, in addition to AmpliChip, *CYP2D6* kit (Luminex Corporation, Austin, TX, USA), *CYP2C19* INFINITI (AutoGenomics, Carlsbad, CA, USA), DMET (Affymetrix/Thermo Fisher Scientific, Waltham, MA, USA). The results of each method were checked by two independent technicians, and approved by a clinical chemist. Results were recorded in the electronic hospital information systems EZIS and HiX (ChipSoft©) from April 2005. For *CYP2D6*, the FDA-approved AmpliChip was used, until discontinuation of the AmpliChip by Roche forced switching to the FDA-approved Luminex *CYP2D6* Assay[3] and CE-IVD marked INFINITI CYP450 2D6-BC Assay.[4]

The AmpliChip included 25 SNPs in *CYP2D6,* copy number variation in *CYP2D6,* and three SNPs in *CYP2C19*. The GenoChip is a macroarray for eleven SNPs and a deletion in

CYP2D6 and gene duplication. The Luminex and INFINITI assays include 15 SNPs and gene duplications. At Eramsus MC Taqman or PCR-RFLP were used to determine 15 and 25 variants from the Luminex/INFINITI assays and AmpliChip assay respectively. *CYP2D6* and *CYP2C19* are involved in different drug metabolisms.[5] The other routinely tested SNPs are SNPs in genes coding for enzymes involved in drug metabolism from the cytochrome P450 (*CYP2C19**17, *CYP2C19**2, *CYP2C19*3*, *CYP2C9**2, *CYP2C9**3, *CYP3A4**22, *CYP3A5**3, *CYP3A5**6), SNPs correlated to fluoropyrimidine-induced toxicity (*DPYD**2A, *DPYD**13, c.2846A>T, c.1236G>A),[6] simvastatin-induced toxicity (*SLCO1B1* rs4149056),[7] calcineurin inhibitors pharmacokinetics (*ABCB1-c1236t, ABCB1-g2677t, ABCB1-t129c, ABCB1-t3435c*),[8] warfarin-induced toxicity (*VKORC1* rs9934438)[9] and thiopurines-induced toxicity (*TPMT**2, *TPMT**3A/*3B/*3C).[10] All results of which a second independent genotyping test was executed were reviewed. For Erasmus MC *CYP2D6* genotypes were taken into account. Mismatches between two tests were directly investigated upon discovery and summarized in this study.

Supplementary Results

Current confirmation practice

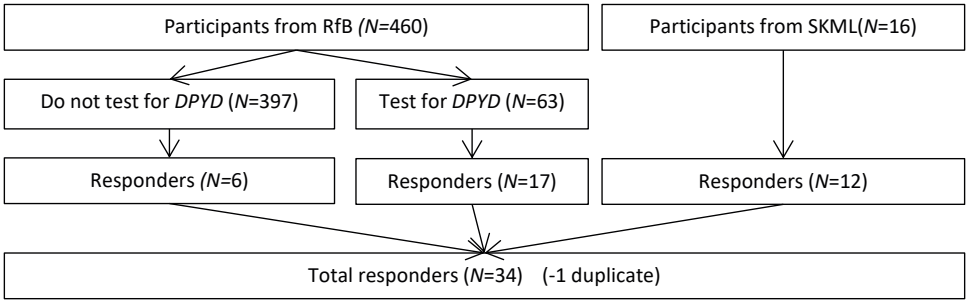
The 460 laboratories participating in the proficiency testing program organized by RfB received the hard-copy questionnaire focusing on *DPYD*. In addition, the questionnaire was also sent by e-mail to 16 Dutch laboratories participating in the proficiency testing program of SKML. In total, we received 35 completed questionnaires, of which six laboratories do not execute genotyping assays. Of 63 of the 460 European laboratories it is known that they test for *DPYD* and these laboratories were more likely to reply to our questionnaire compared to laboratories not testing for *DPYD.* Response rate in European laboratories testing for *DPYD* was 27% (17 out of 63) compared to 75% response (twelve out of 16) in Dutch laboratories testing for *DPYD*. One laboratory responded to both Dutch and European questionnaires, and was only taken into account once. Supplemental Figure 1 shows the flowchart of invited laboratories and their responses.

The first question was asked to determine which *DPYD* SNPs are currently tested in different laboratories. 16 out of 22, and eleven out of twelve responders in Europe and the Netherlands execute *DPYD* genotyping, respectively. Of all centres genotyping for *DPYD*, only one centre did not test for the most-described *DPYD* variant in literature: *DPYD**2A. Five centres genotyped only one variant (*DPYD**2A), whereas the other centres combined multiple SNPs in their genotyping program. The four variants for which dosing guidelines are available, were most frequently tested. The different genotyped *DPYD* variants against the total number of responders are shown in Supplemental Figure 2.

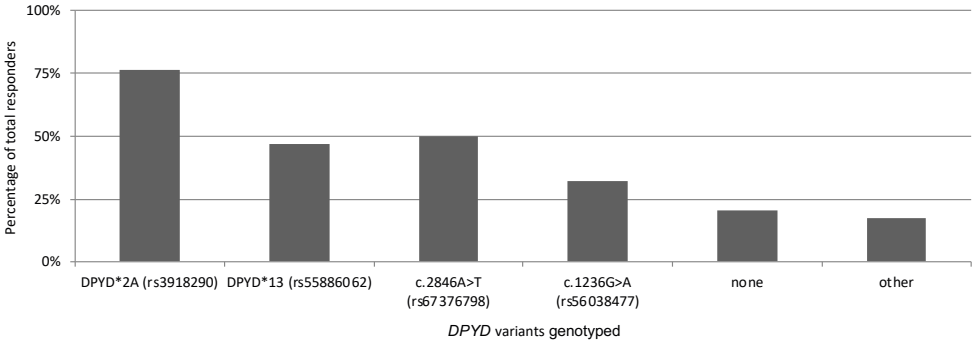
The second question aimed at gaining insight into which techniques were used for genotyping. Only answers of laboratories testing for *DPYD* (*N*=27) were included in the evaluation of questions 2 and 3. Multiple answers could be given to questions 2 and 3. Of all laboratory techniques, the TaqMan assay and melting curve analyses were most frequently used. There were no considerable frequency differences of the applied techniques between Europe and the Netherlands. An overview of all applied genotyping techniques is shown in Supplemental Figure 3.

A large variation in confirmation practice was observed (Supplemental Figure 4). The specific answer 'one technician using the same technique on two independent days' was only given by European laboratories.

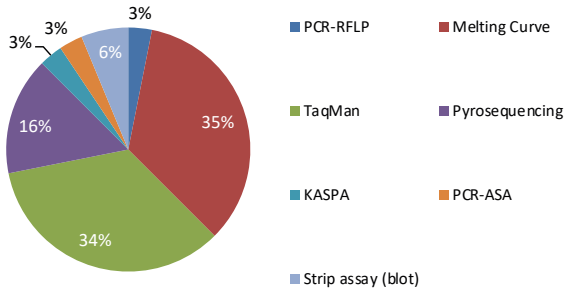
Answers from 'other' included e.g. "use of a heterozygous positive control DNA" or "two persons judge the result", which we consider as part of the genetic analysis or regular checks, not confirmation methods. Supplemental Figure 4 shows the diversity in answers.



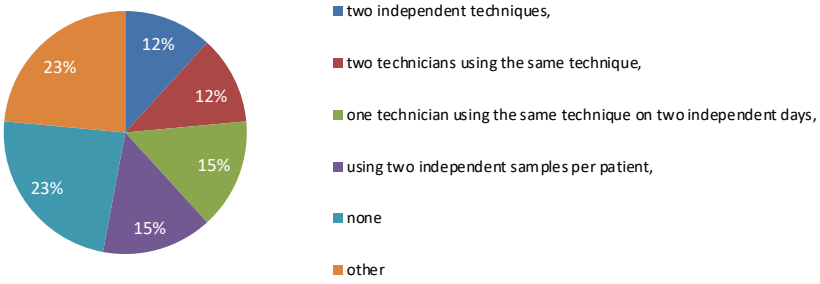
Supplemental Figure 1. Invited laboratories and responders
Overview of invited laboratories and responders to the questionnaire.



Supplemental Figure 2. Different *DPYD* SNPs tested in PGx
Overview of 34 responders, shown as percentages. 'Other' includes: c.496A>G (rs2297595), *DPYD**4 (rs1801158), *DPYD**6 (rs1801160), c.1129-5923C>G (rs75017182), *DPYD**7 (rs72549309), Y186C (rs115232898).



Supplemental Figure 3. Different genotyping techniques used in PGx
Overview of 32 answers from 27 responders, shown as percentages.
Abbreviations: PCR: Polymerase Chain Reaction; RFLP: Restriction Fragment Length Polymorphism; Melting Curve: Melting curve analyses, including High Resolution Melt, 'LightCycler', 'Rotorgene Melt Curve' and 'LightSnip assay'; ASA: allele-specific amplification.



Supplemental Figure 4. Confirmation practice in PGx
Overview of 34 answers from 27 responders, shown as percentages. 'Other' includes: "two technicians judge the results", "new sample is requested with divergent result", "two persons judge", "authorized by Clinical Chemistry", "phenotyping if necessary", "use of synthetic controls", "use of internal sequenced controls", "use of heterozygous positive control DNA". In this study these are not considered a (cost inducing) confirmation or replication method.

Two independent genotyping methods as confirmation practice
Since 2005 (Erasmus MC) and 2010 (LUMC) PGx tests are executed as a part of routine diagnostics in clinical care. Digitally stored results were included in the analysis. For LUMC and Erasmus MC data on 16,932 and 72,910 SNP tests were available, making the sample size a total of 89,842 tests. Results are shown for each SNP and separated per laboratory in Supplemental Table 1. Results of *CYP2D6* are shown in Supplemental Table 2.

At LUMC, in 2011 a single discrepancy was observed as previously described.[11,12] Briefly; the TaqMan assay identified a patient as *CYP3A5**3/*3, while results from PSQ showed that the patient was *CYP3A5**1/*3. A second blood sample was obtained and genotyped. Results from the second analysis (both Taqman as PSQ) classified the patient as *CYP3A5**1/*3. However, the PSQ results showed some inconsistencies, making the results questionable.

After further investigations and consult with the attending nephrologist, it appeared that this patient had a history with allogeneic hematopoietic stem cell transplantations. Saliva samples from both patient and stem cell donor were obtained and genotyped, which showed a *CYP3A5**1/*3 genotype for the patient and *CYP3A5**3/*3 genotype for the donor. At Erasmus MC, in 2011 a discrepancy for *CYP3A5**3 was found. Thereafter, 300 patients were genotyped at Erasmus MC in a single run, using TaqMan. Comparing the outcome with PCR-RFLP revealed three discrepancies. Upon sequencing, the TaqMan assay appeared to have been wrongly addressing the wild type status, possibly due to allele dropout caused by a variant located at one of the TaqMan primers.

At Erasmus MC, in 2016 a *DPYD* result was found discrepant between PCR-RFLP and TaqMan-based validated assays, PCR-RFLP giving heterozygosity *1/*13, indicating a 50% dose reduction, whereas the other assay indicated wild type for the four SNPs tested (regular starting dose). Both methods were repeated, confirming the earlier results. Direct sequencing revealed that wild type was the correct outcome. This was the only discrepant finding for *DPYD.*

At Erasmus MC, the genotype of three patients showed discrepancies for *CYP2D6**6*.* The AmpliChip assay showed wildtype for *CYP2D6**6, whereas TaqMan determined *CYP2D6**6. The tests were repeated, and sequencing of the samples revealed the correct genotype (*CYP2D6**6). Like the *CYP3A5*3* discrepancies, a possible explanation was a variant located at one of the AmpliChip primers, resulting in allele dropout and thereby misclassification, missing the *CYP2D6**6 allele. Previously, another discrepancy for *CYP2D6*6*, possibly caused by allele dropout, was described by Rasmussen *et al.*[13] We are not aware of publications on other discrepancies of *CYP3A5*3* or *DPYD**13.

In total, eight discrepancies were found at Erasmus MC in 72,910 SNPs tested in duplo (0.01%). For LUMC, one discrepancy was found in 16,932 SNPs tested in duplo (0.006%), which could be attributed to chimerism in a patient with previous stem cell transplantation. In total, the probability of finding a discrepant result when using two independent techniques thus calculates to be 0.01%.

Supplemental Table 1. Genotyping results

Shown are the results per variant distributed on carrier status and the calculated minor allele frequencies. In total, 89,842 variants were reviewed in this study, for which a second independent genotyping platform was used (16,932 LUMC + 24,705 Erasmus MC + 48,205 *CYP2D6* in Supplemental Table 2).

Variant				Results LUMC				Results Erasmus MC				Results Total						
Gene	**Location**	**Rs number**	*****	**WT**	**HET**	**HM**	**MAF**	**WT**	**HET**	**HM**	**MAF**	**WT**	**HET**	**HM**	**% WT**	**%HET**	**%HM**	**MAF**
CYP2C19	c.−806C>T	rs12248560	*17	170	74	15	20% “	1223	618	90	21% “	1393	692	105	64%	32%	5%	21% “
CYP2C19	c.681G>A	rs4244285	*2	120	54	6	18% “	1399	474	58	15%	1519	528	64	72%	25%	3%	16%
CYP2C9	c.430C>T	rs1799853	*2	70	23	1	13% “	990	240	22	11% “	1060	263	23	79%	20%	2%	11% “
CYP2C9	c.42614A>C	rs1057910	*3	83	11	0	6% “	1105	143	4	6% “	1188	154	4	88%	11%	0%	6% “
CYP3A4	c.522-191C>T	rs35599367	*22	52	2	0	2% “	1155	118	3	5% “	1207	120	3	91%	9%	0%	5% “
CYP3A5	c.6986A>G	rs776746	*3	82	324	1087	84%	15	64	263	86%	97	388	1350	5%	21%	74%	84%
CYP3A5	14690G>A	rs10264272	*6	1448	34	1	1% “	337	5	0	1% “	1785	39	1	98%	2%	0%	1% “
DPYD	c.1905+1G>A	rs3918290	*2A	3160	56	0	1% “	2348	45	1	1% “	5508	101	1	98%	2%	0%	1% “
DPYD	c.1679T>G	rs55886062	*13	3112	4	0	0% “	2426	2	0	0% “	5538	6	0	100%	0%	0%	0% “
DPYD	c.2846A>T	rs67376798		3080	36	0	1% “	2401	24	2	1%	5481	60	2	99%	1%	0%	1%
DPYD	c.1236G>A	rs56038477		2805	132	1	2% “	2361	64	2	1%	5166	196	3	96%	4%	0%	2% “
SLCO1B1	c.521T>C	rs4149056		13	5	0	14% “	120	85	6	23%	133	90	6	58%	39%	3%	22%
TPMT	c.238G>C	rs1800462	*2	271	1	0	0% “	2021	21	0	1% “	2292	22	0	99%	1%	0%	0% “
TPMT	c.460G>A	rs1800460	*3A, *3B	283	21	1	4% “	1872	158	11	4%	2155	179	12	92%	8%	1%	4%
TPMT	c.719A>G	rs1142345	*3A, *3C	247	21	4	5%	1836	191	15	5%	2083	212	19	90%	9%	1%	5%
VKORC1	c.1173C > T / -1639G>A	rs9934438	*2	4	5	1	35% “	128	118	53	37%	132	123	54	43%	40%	17%	37%
ABCB1	c.1236C>T	rs1128503		0	2	0	50% “	6	12	5	48% “	6	14	5	24%	56%	20%	48% “
ABCB1	c.2677G>T>A	rs2032582		0	2	0	50% “	6	12	5	48% “	6	14	5	24%	56%	20%	48% “
ABCB1	c.-129T>C	rs3213619		4	2	0	17% “					4	2	0	67%	33%	0%	17% “
ABCB1	c.-3435T>C	rs1045642		1	1	0	25% “	6	14	2	41% “	7	15	2	29%	63%	8%	40% “
TOTAL				15005	810	1117		21755	2408	542		36760	3218	1659	88%	8%	4%	8%

Abbreviations: WT: wildtype; HET: heterozygous carrier; HM: homozygous carrier; MAF: minor allele frequency; *: nomenclature; “: indicates Hardy Weinberg Equilibrium.

Supplemental Table 2.Genotyping results for *CYP2D6*

Shown are the results on *CYP2D6* from Erasmus MC. Not all variants from the AmpliChip and INFINITY/Luminex were determined using a second independent genotyping platform.

Gene	Technique	# of assays	# of variants	WT[a]	HET	VAR	MUL-VAR	% WT	%HET	%VAR+MUL-VAR	MAF
CYP2D6	AmpliChip	652	25	2500	255	520	111	74%	8%	19%	11%
CYP2D6	INFINITY/Luminex	2127	15	4530	852	1744	303	61%	11%	28%	17%
TOTAL		48205									

[a] for *CYP2D6* wildtypes, heterozygous and homozygous carriers are classified differently. For example, a *1/*2 *CYP2D6* carrier has the *2 variant, but is classified as wildtype. In this table we show wild-types, heterozygous carriers (1 SNP), variant carriers (2 SNPs) and multi-variant carriers (3 SNPs or more).

Abbreviations: # of assays: number of assays executed over time; # of variants: number of variants per assay that were confirmed with a second independent genotyping platform; WT: wildtype; HET: heterozygous carrier; VAR: variant carriers; MUL-VAR: multi-variant carriers; MAF: minor allele frequency.

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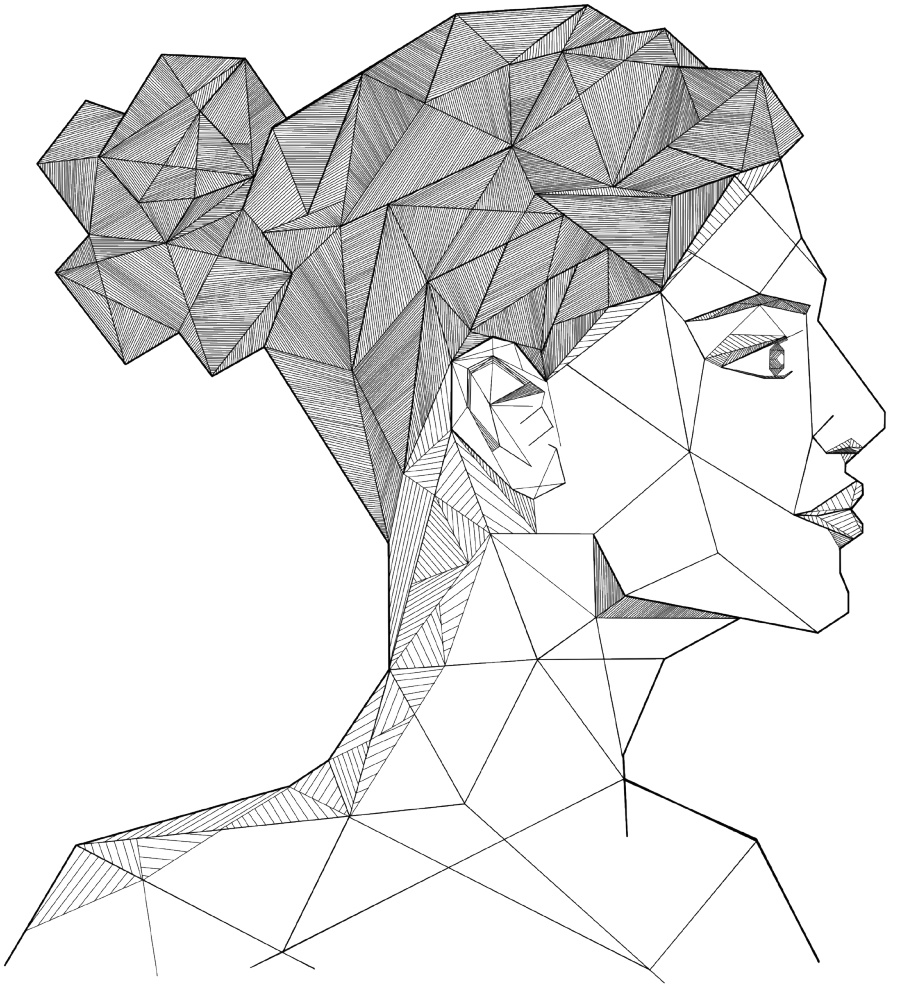
BEYOND CURRENT *DPYD* PHARMACOGENETICS



CHAPTER 10

Comparison of four phenotyping assays for predicting dihydropyrimidine dehydrogenase (DPD) deficiency and severe fluoropyrimidine-induced toxicity: a clinical study

Manuscript in preparation

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Abstract

Fluoropyrimidines are widely used anticancer drugs. Prospective *DPYD* (encoding dihydropyrimidine dehydrogenase, DPD, the key metabolic enzyme for degradation of fluoropyrimidines) genotyping followed by dose adjustments in *DPYD* variant allele carriers reduces severe fluoropyrimidine-induced toxicity. However, when using this approach still ~20% of patients experience severe toxicity. We evaluated four DPD phenotyping assays, aiming to determine which is most suitable for identifying patients at risk for severe fluoropyrimidine-induced toxicity, and identifying DPD deficient patients.

Study participants underwent testing of two, three or four DPD phenotyping assays before starting fluoropyrimidine-based therapy; the endogenous dihydrouracil/uracil (DHU/U) ratio, endogenous uracil levels, the oral uracil loading dose, and the 2-^{13}C-uracil breath test. Phenotyping results were associated with the onset of severe toxicity and DPD deficiency according to the DPD enzyme activity measurement in peripheral blood mononuclear cells. Sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV) and F1-score (harmonic mean of sensitivity and PPV) were calculated per phenotyping assay as predictive measures for severe (grade ≥3) fluoropyrimidine-induced toxicity and DPD deficiency.

In total, 1,037 patients participated in this study. Of these, 1,037, 92 and 82 patients underwent two, three or four DPD phenotyping assays, respectively. Two phenotyping assays were analysed on their performance for the prediction of severe fluoropyrimidine-induced toxicity. No differences were identified between wild-type patients who did or did not experience severe toxicity in the mean endogenous DHU/U ratio or mean endogenous uracil levels. The F1-scores of both assays were 10 and 24%, respectively. In the comparison of phenotyping assays in performance for prediction of DPD deficiency, four phenotyping assays were analysed in both wild-type patients and *DPYD* variant allele carriers. The highest F1-score of the phenotyping assays in predicting DPD deficiency was 40% for the oral uracil loading dose.

All four investigated DPD phenotyping assays in this study have been favourably evaluated as predictive test for the occurrence of severe fluoropyrimidine-induced toxicity in previous studies. However, in a first-time prospective head-to-head comparison study we could not show associations with the onset of severe fluoropyrimidine-induced toxicity or DPD deficiency. In order to determine the true clinical value of DPD phenotyping assays, additional research is required.

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 5-FU has 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 these patients.[1,2] With over two million patients treated with fluoropyrimidines each year worldwide, many patients are at risk of developing severe toxicity.[3]

Abundant research has been carried out on dihydropyrimidine dehydrogenase (DPD), the key metabolic enzyme of fluoropyrimidines, and the gene *DPYD* encoding DPD. Low DPD activity itself and several *DPYD* variants resulting in low DPD activity have both individually 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, can reduce the risk for severe toxicity. This was shown for prospective genotyping of *DPYD**2A, c.1679T>G, c.2846A>T and c.1236G>A/HapB3, followed by initially reduced dosages in *DPYD* variant allele carriers.[7,8] However, genotyping to predict severe fluoropyrimidine-induced toxicity has an inherently limited sensitivity, as other genetic and also non-genetic factors are known to play a role in the variability in DPD activity and the onset of severe fluoropyrimidine-induced toxicity. Phenotyping of the DPD enzyme, as a way to determine the DPD activity, can potentially better predict severe fluoropyrimidine-induced toxicity as it takes both pharmacogenetic and other factors influencing DPD activity into account.

A well-established method to determine DPD activity is measurement of DPD enzyme activity in peripheral blood mononuclear cells (PBMCs). The activity in PBMCs is well-correlated to the DPD enzyme activity in the liver, and reference values have been established.[6,9] However, the method is not widely used since feasibility in clinical practice remains challenging due to substantial costs, complex sample logistics and specific equipment required for the radio assay. In addition, the results are influenced by the distribution of blood cells (e.g. monocytes, granulocytes) in the sample,[10] and there is a substantial intra patient variability (up to 25%) in DPD enzyme activity, possibly caused by circadian rhythm.[11,12]

Several DPD phenotyping assays have previously been investigated, focussing on the metabolism of uracil (U) and dihydrouracil (DHU), the endogenous substrate and product of DPD, respectively. Two of these assays are the determination of the endogenous uracil levels and the DHU/U ratio. Several studies have shown an association between the pre-treatment endogenous DHU/U ratio in plasma and 5-FU pharmacokinetics,[13-16] and also with severe fluoropyrimidine-induced toxicity.[15,17-19] In addition, Meulendijks *et al.* have recently shown that high pre-treatment serum uracil concentrations were also strongly related to severe and fatal fluoropyrimidine-induced toxicity.[20] Another DPD phenotyping assay for estimating the *in vivo* DPD activity is the oral uracil loading dose assay.[21,22] In this assay, a high dose of uracil is administered orally, and uracil and DHU levels are measured using a limited sampling strategy.[21] In this way, the DPD enzyme function is utilized to the full capacity. In case of reduced uracil conversion, also partially DPD deficient patients can be identified. Finally, the *in vivo* DPD activity can also be determined using the 2-^{13}C-uracil breath test.[23]

This assay uses a personalized dose of 2-^{13}C uracil, 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.[23]

Several of these assays have been evaluated in head-to-head comparisons to DPD enzyme activity measurements in PBMCs in healthy volunteers, or patients selected after experiencing severe toxicity.[11,21,23-27] However, when using an enriched patient cohort for severe toxicity, calculated assay characteristics such as sensitivity or specificity will be biased and not representative for routine clinical care. Strengths and weaknesses of these assays have been reviewed independently of each other;[28,29] however, a head-to-head comparison in clinical practice has never been investigated. Therefore, in this prospective study, we evaluated four DPD phenotyping assays in patients prior to treatment with fluoropyrimidines in order to determine the association with the onset of severe fluoropyrimidine-induced toxicity and detecting DPD deficiency, defined as DPD enzyme activity in PBMCs below the cut-off value.

Materials and methods

Study design and patients

This study was a pre-planned analysis in a large prospective multi-centre clinical trial (clinicaltrials.gov identifier NCT02324452, here referred to as main study cohort or clinical trial) improving the safety of fluoropyrimidines by prospective *DPYD* genotyping.[7] Two out of four phenotyping assays (endogenous DHU/U ratio and endogenous uracil levels) were executed in patients recruited in the seventeen Dutch hospitals participating in the clinical trial. All four DPD phenotyping assays were executed in three hospitals, and three out of four assays were executed in another hospital (excluding the 2-^{13}C-uracil breath test). Patient recruitment for this study was open from 30 April, 2015, until 21 December, 2017. Ethical approval of this study was granted by the medical ethical committee of The Netherlands Cancer Institute, Amsterdam, the Netherlands. All patients provided written informed consent before enrollment in this study.

All assays were executed before start of fluoropyrimidine therapy. 92 patients were asked to participate in all four phenotyping assays, which made intra-patient comparisons possible. Results of the DPD phenotyping assays were determined after start of treatment and were not used for dose individualization. Dose adjustments of the fluoropyrimidine drug were done based on *DPYD* genotype only as per protocol of the clinical trial.

Toxicity was graded according to the National Cancer Institute common terminology criteria for adverse events (CTCAE; version 4.03)[30] and severe toxicity was defined as CTCAE grade ≥3. Only toxicity defined by the treating physician as definitely, probably and possibly related to fluoropyrimidine treatment was taken into account. Patients were followed for toxicity during the entire treatment period and were evaluated for toxicity if they received at least one fluoropyrimidine drug administration. The endpoints of this study were the association of each DPD phenotyping assay with the onset of severe fluoropyrimidine-induced toxicity and DPD deficiency, defined as low DPD activity levels in PBMCs (≤5.9 nmol/[mg*h]).[6]

Inclusion and exclusion criteria for this study were the same as in the clinical trial; eligibility to start with fluoropyrimidine-based therapy, 18 years or older, an adequate performance

status, adequate renal and liver biochemistry and haematological values, and no prior treatment with fluoropyrimidines.

Study procedures

One blood draw for the endogenous DHU/U ratio and endogenous uracil level assays was taken prior to start of treatment. For patients participating in three or four DPD phenotyping assays the study scheme was as follows. During two random days prior to start of fluoropyrimidine-based treatment, all three or four phenotyping assays were performed in each patient (study scheme in Figure 1). On the first day, blood draws for the DPD enzyme activity in PBMCs and two DPD phenotyping assays (endogenous DHU/U ratio and endogenous uracil levels) were taken prior to 9 am. Immediately thereafter, the third phenotyping assay (oral uracil loading dose of 1000 mg uracil) was performed. The U/DHU ratio was assessed at 120 minutes after administration of uracil. At least one day later, but prior to start of fluoropyrimidines, the fourth phenotyping assay (2-^{13}C-uracil breath test with 6 mg/kg 2-^{13}C-uracil) was performed including blood draws for ^{13}C-uracil and ^{13}C-dihydrouracil plasma measurements. The DOB_{50} value from breath samples was correlated to ^{13}C-dihydrouracil plasma levels and the ^{13}C-DHU/U ratio. The oral uracil loading dose and 2-^{13}C-uracil breath test were performed on two separate days to exclude any interference, as uracil was administered orally for 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.[31]

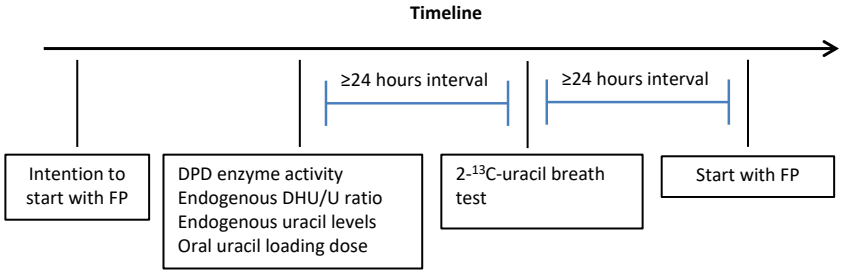

Figure 1. Study scheme

The study scheme per patient. Minimum interval between the tests and between tests and start of fluoropyrimidine-therapy was 24 hours. There was no predefined maximum number of days between assays as patients usually started relatively quickly with therapy when the decision to start was made. *Abbreviations:* FP: fluoropyrimidines; DHU: dihydrouracil; U: uracil.

DPD phenotyping assays

Patients underwent the DPD phenotyping assays in the hospital of their recruitment. Protocols for each DPD phenotyping assay were made available and discussed with executive personnel. In four hospitals, trained personnel was available to execute three or four DPD phenotyping assays. Each DPD phenotyping assay is described in more detail in the

supplementary material. In addition, the time of last food intake prior to the blood draw for the endogenous DHU/U ratio was collected in all patients.

Sample size calculation and statistical analyses

The sample size for comparison of the four phenotyping assays was based on the co-primary aim (the association between the result of a phenotyping assay and severe fluoropyrimidine-induced toxicity). It was calculated to be 240 patients (see detailed description in the supplementary material). The association between the result of a phenotyping assay and DPD deficiency (as determined by the DPD enzyme activity in PBMCs) was investigated as secondary aim.

Patient characteristics or toxicity differences between patient groups were tested using χ^2 test or non-parametric test. The DPD phenotyping assays were executed for the first time in the participating centres, i.e. in a research setting and were not cross-validated. To investigate the effect of centres on the outcome per assay, a mixed model analysis was executed to demonstrate the general reliability of the assay. A univariate analysis of variance was done to compare outcomes per centre. In this analysis, the centre with the highest recruitment rate was chosen as the reference centre. *DPYD* variant allele carriers were excluded in these analyses, as they are underwent a per protocol dose adjustment and hence are at lower risk for toxicity. Age, gender and baseline BSA were additionally taken into account as possible covariates to minimize the risk of biased results of the analyses. When age, gender or baseline BSA were associated with the outcome of the phenotyping assay, the distribution of the covariate was checked between centres using Chi-Square tests or univariate analysis of variance. The possible clinical consequence of the divergent results was discussed per assay and data could be excluded from further analyses.

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 the onset of severe toxicity or DPD deficiency were determined. *DPYD* variant allele carriers who received dose reductions based on their identified genotype could only be included in an analysis for association with DPD activity, not for the association with onset of severe toxicity. 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, 1,037 patients participated in the phenotyping study of which 1,037, 92 and 82 patients underwent two, three and four phenotyping assays, respectively. Patient and treatment characteristics of the 92 patients were similar to those of the main study (*N*=1,103), with the exception that the 92 patients were slightly younger (median age of 60 versus 64 years, p=0.011, Table 1). Details on fluoropyrimidine-induced toxicity are depicted in Supplementary Table 1. In total, 19 out of 92 patients (21%) experienced severe fluoropyrimidine-induced toxicity, which is comparable to the main study in which 264 out of 1,103 patients (24%) experienced severe toxicity (p=0.477).

Phenotyping assays

General performance of assays

For the DPD enzyme activity measurements, the variance was estimated to be 8.6 ± 1.4 nmol/(mg*h). For centre as covariate, the variance was estimated to be 6.5 ± 5.7 nmol/(mg*h). The deviations between centres in general (intra class variation) was 43.0%, therefore the general reliability of the DPD enzyme activity measurements was 57%. For the endogenous DHU/U ratio, endogenous uracil levels, 2-^{13}C-uracil breath test and the oral uracil loading dose assay the general reliabilities were 74.1%, 92.9%, 73.5%, and 94.3%, respectively.

Endogenous DHU/U ratio

The endogenous DHU/U ratio was determined in 1,037 patients. Results of wild-type patients (non-carriers of the four *DPYD* variants, *N*=955) were compared between seventeen study centres. The endogenous DHU/U ratio differed significantly in nine study centres compared to the reference centre (lowest divergent mean DHU/U ratio 5.9, to the highest divergent mean DHU/U ratio 13.9, p<0.001). It appeared that age was significantly associated with the outcome of the DHU/U ratio (p<0.001). Age was differently distributed between the centres (p<0.001). The lowest statistically divergent mean DHU/U ratio was not as low as the suggested DHU/U ratio cut-off value (4.31)[20] for DPD deficient patients, therefore no patients were excluded. The median, interquartile range (IQR) and standard deviation (SD) of each assay are shown in Table 2.

Endogenous uracil levels

Endogenous uracil levels were determined in 1,037 patients. Results of wild-type patients (*N*=955) were compared between seventeen study centres. The endogenous uracil levels differed significantly in four study centres compared to the reference centre (lowest divergent mean uracil level 8.3 ng/ml, to the highest divergent mean uracil level 18.8 ng/ml, p<0.001). It appeared that gender was significantly associated with the outcome of the uracil levels (p=0.030), with lower uracil levels in females. Males and females were significant differently distributed between the centres (p=0.046). The divergent results were substantially higher, even higher than the previously suggested cut-off value (13.9 ng/ml)[20] for DPD deficient patients, therefore the data were considered unreliable and patients recruited in these centres (*N*=172) were excluded from further analyses. 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 1), therefore time of food intake was not taken into account as covariate in further analyses.

DPD enzyme activity

The DPD enzyme activity in PBMCs was determined in 92 patients. Results of 82 wild-type patients were compared between study centres. The mean DPD enzyme activity was significantly lower in one of the four participating centres (5.23 nmol/(mg*h) versus 10.89 nmol/(mg*h) in the reference centre, p<0.001). These results were substantially lower, even lower than the cut-off value (≤5.9 nmol/[mg*h])[6] for DPD deficiency, therefore the data were considered unreliable and patients recruited in this centre (*N*=19) were excluded from further analyses in which DPD deficiency was taken into account.

Table 1. Baseline characteristics of patients who underwent three or four DPD phenotyping assays and from the main study cohort

Characteristic	Phenotyping assays (*N*=92)	Main study cohort (*N*=1,103)	P-value[a]
Sex			
Male	56 (61%)	593 (54%)	0.189
Female	36 (39%)	510 (46%)	
Age			
Median [IQR]	60 [53-67]	64 [56-71]	0.011
Ethnic origin			
Caucasian	87 (95%)	1048 (95%)	0.786
African descent	1 (1%)	19 (2%)	
Asian	2 (2%)	24 (2%)	
Other	2 (2%)	12 (1%)	
Tumour type			ND
Non-metastatic CRC	38 (41%)	472 (43%)	
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			0.854
CAP mono	12 (13%)	205 (19%)	
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			0.207
Median [IQR]	2.0 [1.79-2.10] (*N*=91)	1.92 [1.77-2.10] (*N*=1075)	
WHO performance status			0.151
0	49 (53%)	554 (50%)	
1	41 (45%)	448 (40%)	
2	1 (1%)	42 (4%)	
NS	1 (1%)	59 (5%)	
Number of treatment cycles			0.987
Median [IQR]	3 [1-6]	3 [1-8]	
DPYD status			0.281
Wild-type	82 (89%)	1018 (92%)	
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 a comparison of 92 patients who underwent three or four DPD phenotyping

assays to patients from the main study cohort. We used a non-parametric test for independent samples to compare medians of age, BSA and number of treatment cycles; and a χ^2 test for gender, ethnic origin, treatment regimen and WHO status.
Abbreviations: IQR: interquartile range; CRC: colorectal cancer; BC: breast cancer; GC: gastric cancer; CAP mono: capecitabine monotherapy (with or without bevacizumab); CAP + RT: capecitabine combined with radiotherapy (with or without mitomycin); CAPOX: capecitabine combined with oxaliplatin (with or without bevacizumab); CAP other: capecitabine combined with other anticancer drugs; 5-FU mono: 5-fluorouracil monotherapy; 5-FU + RT: 5-fluorouracil combined with radiotherapy (with or without mitomycin); FOLFOX: 5-fluorouracil combined with oxaliplatin and leucovorin (with or without bevacizumab); 5-FU other: 5-fluorouracil combined with other anticancer drugs; BSA: body surface area; WHO: world health organisation; NS: not specified, either WHO 0, 1 or 2; ND: not done; *DPYD*; gene encoding dihydropyrimidine dehydrogenase.

Table 2. Overview of assay measurements and outcomes
DPD enzyme activity assay and four phenotyping assays are shown, including the number of patients per assay, the number of patients excluded from further analyses due to divergent results of the participating centre in which the assays were executed. After exclusion of these patients, the calculated medians, SD, and the IQR are shown for these patients. The median and SD are also shown for wild-type patients only and *DPYD* variant allele carriers only.

Phenotyping assay	*N* of patients	Patients excluded?	All patients			Wild-type patients		*DPYD* variant allele carriers	
			Median ± SD	IQR	*N* of patients	Median ± SD	*N* of patients	Median ± SD	*N* of patients
DPD enzyme activity	92	Yes (*N*=19)	10.3 ± 3.0 nmol/(mg*h)	8.0-12.9 nmol/(mg*h)	73	10.4 ± 3.0 nmol/(mg*h)	65	9.2 ± 3.1 nmol/(mg*h)	8[a]
Endogenous DHU/U ratio	1037	No	8.7 ± 3.9	6.6-11.6	1037	9.0 ± 3.9	955	7.2 ± 4.0	82
Endogenous uracil levels	1037	Yes (*N*=172)	10.2 ± 8.3 ng/ml	8.0-13.3 ng/ml	865	10.0 ± 8.4 ng/ml	794	13.3 ± 7.3 ng/ml	71
Oral uracil loading dose[b]	92	No	0.72 ± 0.86	0.33-1.17	92	0.58 ± 0.74	82	1.69 ± 1.23	10
2-^{13}C-uracil breath test[c]	82	No	158.9 ± 33.9 ‰	144.2-182.0 ‰	82	161.0 ± 34.0 ‰	74	145.8 ± 28.3 ‰	8

[a] Of these patients, six were carrier of the c.1236G>A variant, one was carrier of the c.2846A>T variant and one was a *DPYD**2A carrier;
[b] Results represent the U/DHU-ratio at 120 minutes;
[c] Results represent the DOB_{50}.
Abbreviations: SD: standard deviation; IQR: interquartile range; U: uracil; DHU: dihydrouracil; DOB_{50}: delta-over-baseline ratio at 50 minutes.

Oral uracil loading dose
The oral uracil loading dose assay was performed in 92 patients. Results of 82 wild-type patients were compared between study centres. The mean U/DHU ratio was significantly lower in one centre (0.622) compared to the reference centre (1.03, p=0.046). It appeared that baseline BSA was significantly associated with the outcome of the U/DHU ratio (p=0.008), a higher baseline BSA was related to a lower U/DHU ratio. Baseline BSA was not differently distributed between the centres (p=0.637). The different mean U/DHU ratio of one centre was far from the cut-off U/DHU ratio (2.4)[21] of DPD deficient patients, therefore no patients were excluded from further analyses.

2-^{13}C-uracil breath test
The 2-^{13}C-uracil breath test was determined in 82 patients.[23] On average, 488 mg 2-^{13}C-uracil was administered, ranging from 312 to 840 mg (6 mg/kg dose). Results of 74 wild-type patients were compared between study centres. The mean delta-over-baseline ratio at t=50 minutes (DOB_{50}) was significantly lower in one centre (137.7 ‰) compared to the other two centres (173.5 ‰ and 168.4 ‰, p<0.009). It appeared that gender was significantly associated with the outcome of the DOB_{50} (p=0.003), with higher DOB_{50} values in females. Males and females were not significant differently distributed between the centres (p=0.263). The significantly different mean DOB_{50} was not as low as the DOB_{50} cut-off value (128.9 ‰)[25] for DPD deficient patients, therefore no patients were excluded. A significant correlation between the DOB_{50} determined in breath samples and the ^{13}C-dihydrouracil plasma levels (r^2=0.178, p<0.001) could be demonstrated, not for the ^{13}C-DHU/U ratio (r^2=0.014, p=0.29). Results are shown in Supplementary Figure 2.

Association with onset of severe toxicity
Clinical validity parameters, i.e. sensitivity, specificity, NPV, PPV and F1-score of the endogenous DHU/U ratio and the endogenous uracil levels for their association with the onset of severe fluoropyrimidine-induced toxicity were calculated and shown in Table 3. The endogenous uracil levels have the highest F1-score of 24%. No significant difference was identified between the median endogenous DHU/U ratio or endogenous uracil level between patients who experienced severe toxicity or not (Figure 2). For the oral uracil loading dose and 2-^{13}C-uracil breath test too few patients were enrolled, therefore the association with the onset of severe toxicity was investigated in an explorative way only for these two phenotyping assays (Supplementary Table 2, Supplementary Figure 3). Yet, the data show similar results in clinical validity parameters and also no difference between medians of patients who experienced severe toxicity or not.

Association with DPD deficiency
DPD deficiency, defined as low DPD activity levels in PBMCs (≤5.9 nmol/[mg*h]),[6] was identified in 7 out of 73 patients (9.6%) or 6 out of 64 patients (9.4%). Clinical validity parameters for association with DPD deficiency are shown in Table 4. High specificity and NPV values were identified, but low sensitivity and PPV values. The oral uracil loading dose has the highest F1-score of 40%. The endogenous uracil levels have the highest sensitivity of 43%.

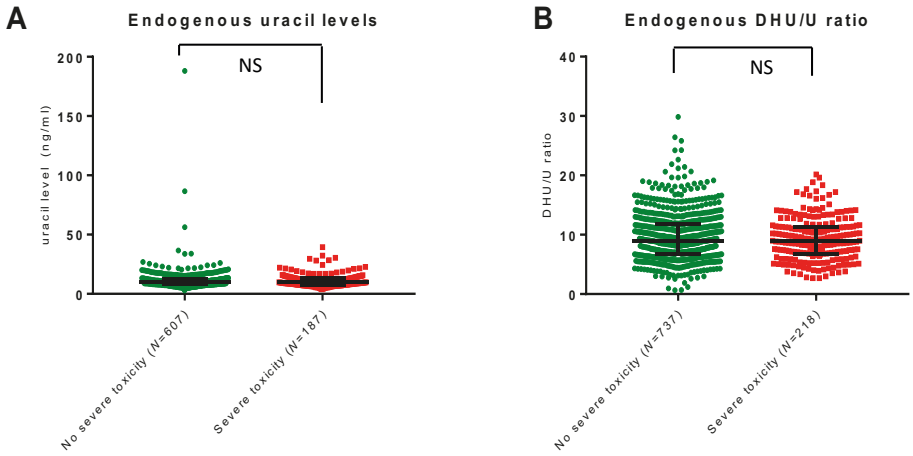

Figure 2. Results of phenotyping assays separated by the occurrence of severe fluoropyrimidine-induced toxicity

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 the analysis as they received initial dose reductions based on their genotype result. For the endogenous uracil levels, 161 wild-type patients were excluded due to divergent phenotyping assay results of the centre in which these patients were recruited.

Abbreviations: U: uracil; DHU: dihydrouracil; NS: not significant p-value.

Table 3. Comparison of phenotyping assays in performance for prediction of severe fluoropyrimidine-induced toxicity

Clinical validity parameters for the prediction of severe (grade ≥3) fluoropyrimidine-induced toxicity are shown for the endogenous DHU/U ratio and endogenous uracil levels. *DPYD* variant allele carriers were excluded, since *DPYD* variant allele carriers received an initial dose reduction based on their genotype, and therefore bias could exist in the onset of severe fluoropyrimidine-induced toxicity. For the endogenous uracil levels, 161 wild-type patients were excluded due to divergent phenotyping assay results of the centre in which these patients were recruited.

Assay	*N* of patients	Median (IQR)	Cut-off for DPD deficiency	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)	F1-score[a] (%)
Endogenous DHU/U ratio	955	9.0 (6.8-11.6)	≤4.31[b]	6	95	77	26	10
Endogenous uracil levels	794	10.0 (7.8-12.9) ng/ml	≥13.9 ng/ml[20]	21	82	77	27	24

[a] The F1-score represents the harmonic mean of sensitivity and PPV;

[b] This cut-off value is determined by calculating the 6% lower limit of the data, as was described by Meulendijks *et al.* [20]

Abbreviations: IQR: interquartile range; DPD: dihydropyrimidine dehydrogenase; NPV: negative predictive value; PPV: positive predictive value; DHU: dihydrouracil; U: uracil.

Discussion

Despite recent advances by applying prospective *DPYD* genotyping, ~20% of patients treated with fluoropyrimidines still suffer from severe toxicity.[7] These patients are wild-type for the four genotyped *DPYD* variants, yet could still be DPD deficient due to currently untested variants. Therefore, it is of great importance to explore the clinical value of DPD phenotyping assays in order to potentially further reduce the risk of severe fluoropyrimidine-induced toxicity. In this study, we conducted two DPD phenotyping assays in 1,037 patients and 82 patients underwent all four DPD phenotyping assays, in order to rule out inter-individual variation. To the best of our knowledge, this is the first study with this unique design, taking into account that our patient cohort was not selected based on –or enriched for– (severe) toxicity, but represents a patient cohort more representative of routine clinical care. However, in the analyses with severe toxicity we excluded *DPYD* variant allele carriers, thus relatively more wild-type patients were included. Still, some wild-type patients are DPD deficient, indicating that we were able to calculate assay performance measures, such as sensitivity and specificity, for the onset of severe toxicity. In the comparison of DPD deficiency, *DPYD* variant allele carriers were not excluded.

Table 4. Comparison of phenotyping assays in performance for prediction of DPD deficiency

Per phenotyping assay clinical validity parameters are shown for the prediction of DPD deficiency. DPD deficiency is defined as a DPD enzyme activity in PBMCs ≤5.9 nmol/(mg*h), and was identified in 7 out of 73 patients (9.6%) and 6 out of 64 patients (9.4%). The results of the DPD enzyme activity were substantially divergent in one centre. Therefore, these results were considered unreliable and could not be compared to results of the phenotyping assays in predicting DPD deficiency. 19 patients were excluded.

Assay	*N* of patients	Median (IQR)	Cut-off for DPD deficiency	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)	F1-score[a] (%)
Endogenous DHU/U ratio	73	8.3 (6.4-11.1)	≤4.31[b]	14	97	91	33	20
Endogenous uracil levels	73	11.8 (8.9-14.9) ng/ml	≥13.9 ng/ml[20]	43	73	92	14	21
2-^{13}C-uracil breath test	64[c]	DOB_{50}: 159.0 (140.3-181.7) ‰	DOB_{50} ≤128.9 ‰[23,25]	33	86	93	20	25
Oral uracil loading dose	73	U/DHU-ratio at 120 min: 0.61 (0.31-1.15)	U/DHU-ratio at 120 min ≥2.4[21]	29	98	93	67	40

[a] The F1-score represents the harmonic mean of sensitivity and PPV;

[b] This cut-off value is determined by calculating the 6% lower limit of the data, as was described by Meulendijks *et al*.[20];

[c] The 2-^{13}C-uracil breath test was executed in 64 out of 73 patients.

Abbreviations: IQR: interquartile range; DPD: dihydropyrimidine dehydrogenase; NPV: negative predictive value; PPV: positive predictive value; DHU: dihydrouracil; U: uracil; NA: not applicable; DOB_{50}: delta-over-baseline ratio at 50 minutes.

The goal of this study was to explore the clinical value of DPD phenotyping assays to identify DPD deficient patients with an increased risk for severe fluoropyrimidine-induced toxicity.

Previously, high endogenous uracil levels have been associated to the onset of severe toxicity.[20] In our study, there was no difference between the mean endogenous DHU/U ratio or mean endogenous uracil levels between wild-type patients for four *DPYD* variants, who experienced severe toxicity or not. Possibly, when including *DPYD* variant allele carriers a difference would have been visible. Yet, we aimed to identify DPD deficient patients in addition to *DPYD* variant allele carriers who are DPD deficient. In terms of clinical validity parameters, our results for the endogenous uracil levels (sensitivity 21%, specificity 82%, NPV 77%, PPV 27%) were only slightly different from previously published parameters to predict severe fluoropyrimidine-induced toxicity (sensitivity 18%, specificity 95%, NPV 90%, PPV 35%).[20] Taking the limited number of patients for two out of four phenotyping assays into account, none of the phenotyping assays investigated in this study showed a combination of both high PPV and NPV parameters, which could predict the clinical value of a test. Of note, sensitivity and PPV of an assay will remain limited even though there is a high odds ratio, if e.g. adverse events are frequent and deficient patients are rare.[32] This is also the case for DPD deficiency and severe fluoropyrimidine-induced toxicity, and we identified low clinical validity parameters.

Our study is the first study in which several phenotyping assays were compared head-to-head in the same patients. For two out of four assays (endogenous DHU/U ratio and endogenous uracil levels), we recruited over 1,000 patients representative of routine clinical care patients. However, our study has some limitations. The 92 patients who underwent three or four phenotyping assays were a little younger compared to patients from the main study cohort, possibly due to the higher patient burden to participate in multiple DPD phenotyping assays. However, we feel this difference is not clinically relevant and it did not influence the occurrence of severe fluoropyrimidine-induced toxicity in these patients.

Secondly, we identified variation in the results of the phenotyping assays, either possibly caused by differences between centres or baseline characteristics (i.e. age, gender or BSA). Per assay, we have examined divergent results and we have corrected for these variations by excluding patients. While we now attributed the identified variation to differences between study centres, these divergent results might also be caused by already existing fluctuation in phenotyping results due to the character of the assay and measurement method. In addition, variation in the clock time of sampling may have affected uracil levels as the metabolizing enzyme DPD shows significant circadian variation.[11] Variation in phenotyping results might also be caused by a different distribution of DPD deficient patients between centres.

Furthermore, predefined cut-off values per phenotyping assay derived from literature were used to be able to divide patients in DPD deficient and non-DPD deficient patients and calculate clinical validity parameters. Cut-off values are also necessary for clinical use, as it would be difficult to determine which patients would require an initial dose reduction without the use of cut-off values. However, the use of cut-off values limits the interpretation of the data of each phenotyping assay. In addition, DPD deficiency itself does not follow a cut-off at a certain point as its severity varies in gradation between completely DPD deficient, partially DPD deficient or non-DPD deficient. Therefore, it would be better not to use cut-off

values in DPD phenotyping assays, but to analyse the results without categorizing patients.

We discussed differences in DPD phenotyping assays, yet the endpoint toxicity can also influence the results. Variation in the outcome of severe toxicity might be caused by different types of treatment regimens between patients, which we did not correct for. As explained for DPD deficiency, analysing the data by categorizing patients also applies to the categorization of toxicity into severe (grade 3-5) and non-severe (grade 0-2) toxicity, where grade 2 toxicity is a grey area in the assessment of toxicity.

Despite our unique data set, we were unable to show that any of the phenotyping assays was associated with DPD deficiency or the onset of severe fluoropyrimidine-induced toxicity very well. The latter is possibly due to the fact that only ~30-50% of severe fluoropyrimidine-induced toxicity can initially be explained by DPD deficiency.[33] Previously it was described that clinical validity and utility were not yet determined for all phenotyping assays,[29] yet with this study we were unable to fully complement this lack of evidence.

Conclusion

We compared four DPD phenotyping assays (the endogenous dihydrouracil/uracil (DHU/U) ratio, endogenous uracil levels, the oral uracil loading dose, and the 2-^{13}C-uracil breath test) in a first-time head-to-head comparison study. None of the phenotyping assays were associated with DPD deficiency or the onset of severe fluoropyrimidine-induced toxicity very well. In order to determine the clinical value of DPD phenotyping assays additional research is required.

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SUPPLEMENT CHAPTER 10

Comparison of four phenotyping assays for predicting dihydropyrimidine dehydrogenase (DPD) deficiency and severe fluoropyrimidine-induced toxicity: a clinical study

Manuscript in preparation

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Supplementary material

Calculation of sample size

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.

Methods assays

DPD enzyme activity[1,2]

The DPD enzyme activity in PBMCs was determined using a validated radio-assay, which is based on conversion of the radiolabelled probe 4-^{14}C thymine to 4-^{14}C dihydrothymine.[1] As this method is considered the gold standard in DPD measurements in the Netherlands, four phenotyping assays were correlated to this assay. 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 (*N*=4), whole blood was either shipped overnight to the Academic Medical Center in Amsterdam for further processing, or was processed at the hospital of blood draw (*N*=3) as described before, to isolate PBMCs.[1] After processing, isolated PMBCs were kept at -80°C before measurement of DPD activity at the Academic Medical Center in Amsterdam.

Endogenous DHU/U ratio and endogenous uracil levels[3,4]

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 in Amsterdam. In patients who participated in three or four DPD phenotyping assays, a 4 ml blood (heparin tube) was drawn between 8 and 9 am, after overnight fasting, and centrifuged at 4°C at 1500g for 10 minutes. Plasma was kept at -80°C until measurement. In patients from the clinical trial, blood to determine uracil and DHU levels 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.[5]

Oral uracil loading dose[6,7]

Previously, a loading dose of 500 mg/m^2 uracil was used in this assay. To increase feasibility, a standardized dose of 1,000 mg uracil was administered. Patients had to fast overnight for a minimum of eight hours. Food and drinks had to be abstained for the duration of the assay. Uracil was dissolved in warm water and administered between 8 and 9 am, to minimize effects of circadian rhythm. Four ml blood (EDTA tube) was taken at 60 and 120 minutes 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 1,500g for ten minutes. 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 test[8-10]

A personalized dose of 6 mg/kg 2-^{13}C uracil was administered to patients after overnight fasting (minimum eight 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 minutes after administration of uracil. Blood samples were centrifuged immediately at 4°C at 1,500g for ten minutes. 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.

Supplementary results

Supplementary Table 1. Toxicity data of patients who underwent three or four DPD phenotyping assays and the main study cohort

Type of event	Phenotyping assays (*N*=92)	Main study cohort (*N*=1,103)	P-value[a]
Overall grade ≥3 toxicity	19 (21%)	264 (24%)	0.477
Grade ≥3 gastrointestinal toxicity	6 (7%)	103 (9%)	0.367
Grade ≥3 hematological toxicity	10 (11%)	78 (7%)	0.180
Grade 3 hand-foot syndrome	4 (4%)	37 (3%)	0.389
Grade ≥3 cardiological toxicity	0	10 (1%)	0.447
Grade ≥3 other treatment-related toxicity	3 (3%)	87 (8%)	0.106
Fluoropyrimidine-related hospitalization	7 (8%)	156 (14%)	0.079
Stop of fluoropyrimidines due to adverse events	20 (22%)	190 (17%)	0.133
Fluoropyrimidine-related death	0	2 (0%)	ND

[a] All p-values represent a comparison of 92 patients who underwent three or four DPD phenotyping assays to patients from the main study cohort. We used χ^2 test or Fisher exact test.

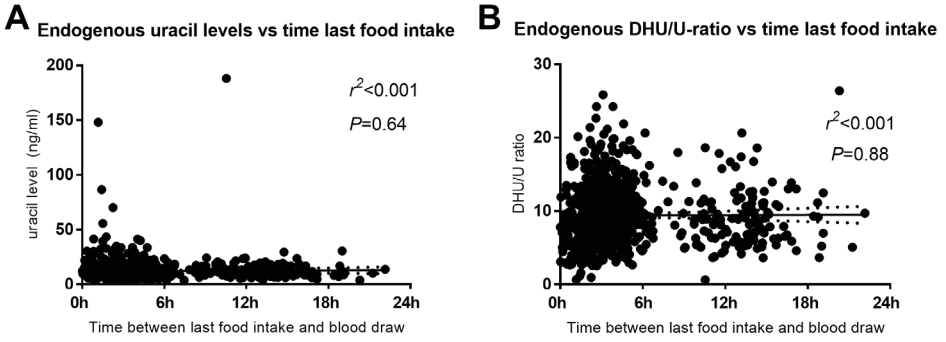
Abbreviations: ND: not done.

Supplementary Table 2. Comparison of phenotyping assays in performance for prediction of severe fluoropyrimidine-induced toxicity

Clinical validity parameters for the prediction of severe fluoropyrimidine-induced toxicity are shown for the 2-^{13}C-uracil breath test and uracil loading assay. *DPYD* variant allele carriers were excluded, since *DPYD* variant allele carriers received an initial dose reduction based on their genotype, and therefore bias could develop in the onset of severe fluoropyrimidine-induced toxicity.

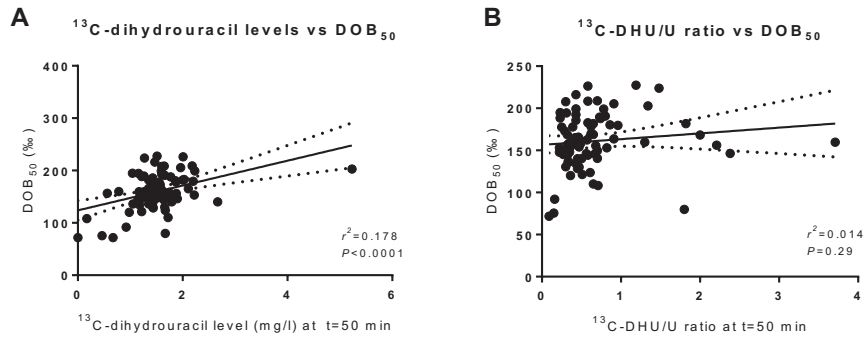
Assay	*N* of patients	Median (IQR)	Cut-off for DPD deficiency	Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)	F1-score[a] (%)
2-^{13}C-uracil breath test	74	DOB_{50}: 161 (145.6-186.3) ‰	DOB_{50} ≤128.9 ‰[8,9]	27	89	88	30	29
Oral uracil loading dose	82	U/DHU ratio at 120 min: 0.58 (0.31-1.09)	U/DHU-ratio at 120 min ≥2.4[6]	7	97	82	33	11

[a] The F1-score represents the harmonic mean of sensitivity and PPV.



Supplementary Figure 1. Endogenous uracil levels and endogenous DHU/U ratio plotted against the time between the blood draw and last food intake

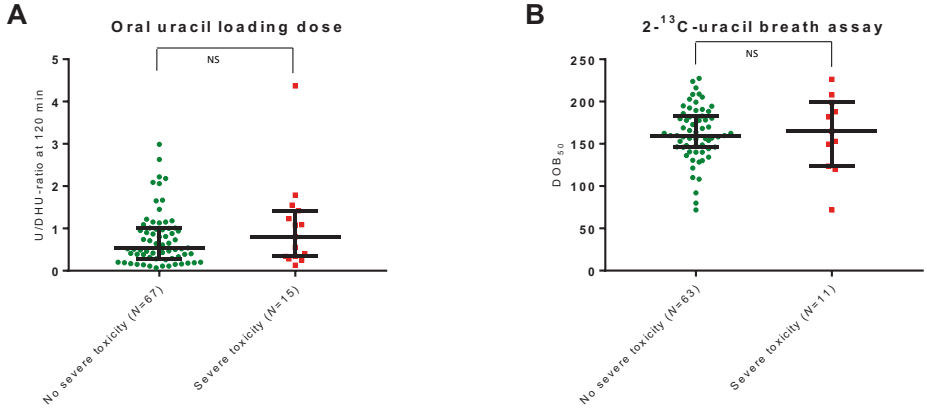
Abbreviations: DHU: dihydrouracil; U: uracil; vs: versus.



Supplementary Figure 2. Correlation between breath samples and plasma samples of the 2-^{13}C-uracil breath test

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 test was evaluated by estimating Pearson's correlations coefficients.

Abbreviations: vs: versus; DOB_{50}: delta-over-baseline ratio at 50 minutes; DHU: dihydrouracil; U: uracil.



Supplementary Figure 3. Results of 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 the analysis as they received initial dose reductions based on their genotype result.

Abbreviations: U: uracil; DHU: dihydrouracil; DOB_{50}: delta-over-baseline ratio at 50 minutes; NS: not significant p-value.

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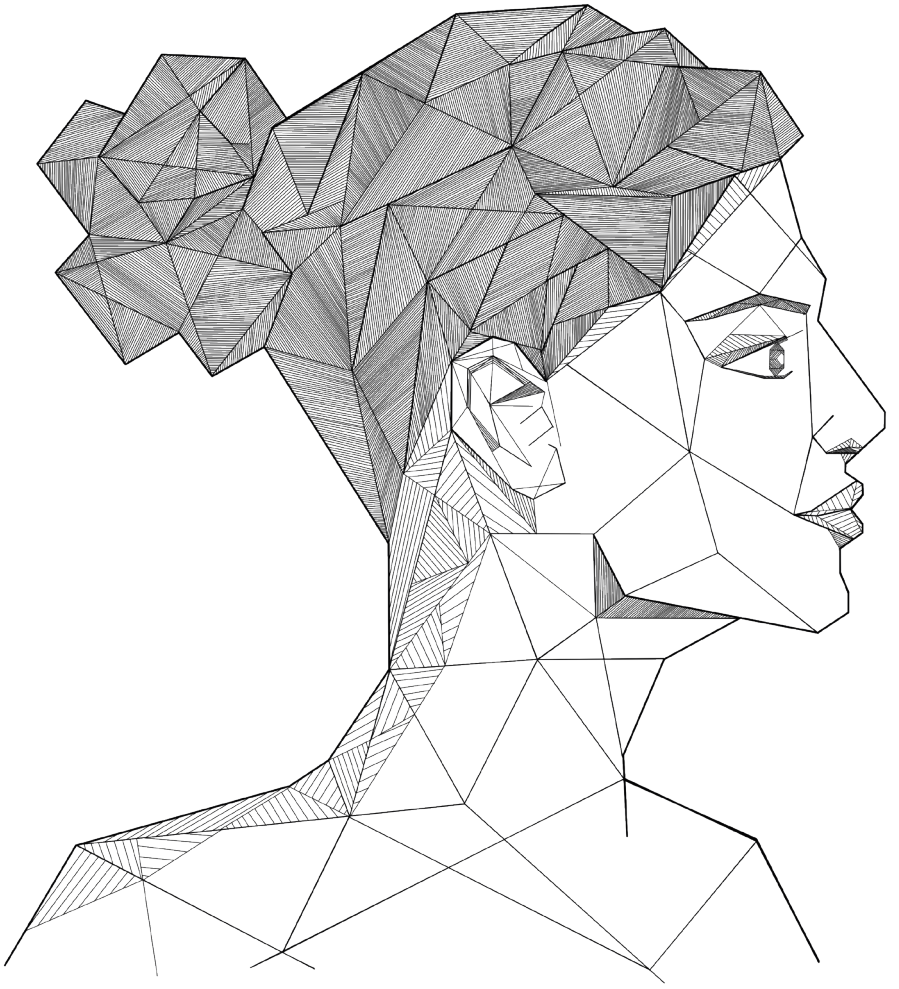

CHAPTER 11

Diagnostic and therapeutic strategies for fluoropyrimidine treatment of patients carrying multiple *DPYD* variants

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Abstract

DPYD genotyping prior to fluoropyrimidine treatment is increasingly implemented in clinical care. Without phasing information (i.e. allelic location of variants), current genotype-based dosing guidelines cannot be applied to patients carrying multiple *DPYD* variants. The primary aim of this study is to examine diagnostic and therapeutic strategies for fluoropyrimidine treatment of patients carrying multiple *DPYD* variants.

A case series of patients carrying multiple *DPYD* variants is presented. Different genotyping techniques were used to determine phasing information. Phenotyping was performed by DPD enzyme activity measurements. Publicly available databases were queried to explore the frequency and phasing of variants of patients carrying multiple *DPYD* variants.

Four out of seven patients carrying multiple *DPYD* variants received a full dose of fluoropyrimidines and experienced severe toxicity. Phasing information could be retrieved for four patients. In three patients, variants were located on two different alleles, i.e. in trans. Recommended dose reductions based on the phased genotype differed from the phenotype-derived dose reductions in three out of four cases. Data from publicly available databases show that the frequency of patients carrying multiple *DPYD* variants is low (<0.2%), but higher than the frequency of the commonly tested *DPYD**13 variant (0.1%).

Patients carrying multiple *DPYD* variants are at high risk of developing severe toxicity. Additional analyses are required to determine the correct dose of fluoropyrimidine treatment. In patients carrying multiple *DPYD* variants, we recommend that a DPD phenotyping assay be carried out to determine a safe starting dose.

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Introduction

Fluoropyrimidines (including 5-fluorouracil (5-FU) and capecitabine) are the cornerstone of treatment for various types of cancer, and are used by millions of patients worldwide each year.[1-3] However, up to one-third of treated patients experience severe toxicity (common terminology criteria for adverse events (CTC-AE) grade ≥3), such as diarrhea, hand-foot syndrome or mucositis upon treatment with fluoropyrimidines.[4,5] These adverse events can lead to mortality in approximately 1% of patients who experience severe toxicity.[4,6] Dihydropyrimidine dehydrogenase (DPD) is the key enzyme in the metabolism of 5-FU and its decreased activity is strongly associated with toxicity.[7,8] Variants in *DPYD*, the gene encoding DPD, can lead to decreased DPD enzyme activity.[9-12] Prospective *DPYD* genotyping of four main *DPYD* variants followed by dose reductions in patients carrying any of these four *DPYD* variants is safe, cost-effective and feasible in clinical practice.[13-15] These *DPYD* variants are *DPYD**2A, rs3918290, c.1905+1G>A, IVS14+1G>A; *DPYD**13, rs55886062, c.1679T>G, I560S; c.1236G>A/HapB3, rs56038477, E412E; and c.2846A>T, rs67376798, D949V. For these four variants, convincing evidence has been provided warranting implementation in clinical practice.[4,5,12,15-17]

An increasing number of hospitals apply prospective *DPYD* genotyping when treating patients with fluoropyrimidines.[18] Individual dosing guidelines for the abovementioned four *DPYD* variants are provided by the Dutch Pharmacogenetics Working Group (DPWG) and the Clinical Pharmacogenetics Implementation Consortium (CPIC).[19,20] Dosing guidelines are based on the expected remaining DPD enzyme activity and can be applied to patients who are heterozygous carriers of a single *DPYD* variant. For homozygous *DPYD* variant allele carriers (two identical variants) and compound heterozygous *DPYD* variant allele carriers (two or more different variants), dosing guidelines are not yet available (or treatment with an alternative drug is advised), although safe treatment with low-dose fluoropyrimidines in these homozygous *DPYD* patients was demonstrated by a recent case series.[21]

Patients who carry multiple variants (compound heterozygous) can carry the variants on a single allele (in *cis*) or on different alleles (in *trans*). In the first case, one functionally active allele remains, whereas in the latter case, both alleles are affected, which may result in a proportionally decreased enzyme activity (Figure 1). With currently used genotyping techniques, the allelic location of variants (phasing) cannot be determined. This uncertainty hampers adequate interpretation of the pharmacogenetic test result in compound heterozygous patients and makes it impossible to give an appropriate dose recommendation based on the genotype alone. Since it is likely that in the future, even more *DPYD* variants will be tested, the probability of finding compound heterozygous *DPYD* variant allele carriers will increase. The aims of this study are to examine diagnostic and therapeutic strategies for fluoropyrimidine treatment of patients carrying multiple *DPYD* variants and the frequency and phasing of variants of compound heterozygous *DPYD* patients in publicly available databases.

Methods

In this study, we present seven compound heterozygous *DPYD* variant allele carriers as clinical cases. In addition, we have performed *in silico* research in publicly available databases.

Patients

Data and DNA from patient cases carrying multiple *DPYD* variants were collected. Patients were identified either after development of severe toxicity from fluoropyrimidine-containing therapy, by additional retrospective genotyping in a clinical trial (clinicaltrials.gov identifier NCT00838370),[13] or prior to treatment in routine clinical care. The study was reviewed and approved by the institutional review board of the Leiden University Medical Center (LUMC, G18.15). Patient data from the electronic medical records was handled following the codes of proper use and proper conduct in the self-regulatory codes of conduct.[22] Toxicity to fluoropyrimidine-containing therapy was graded by the treating physicians using the National Cancer Institute CTC-AE version 4.03,[23] and severe fluoropyrimidine-induced toxicity was defined as CTC-AE grade ≥3. In some cases, additional patient material to determine the phasing of the *DPYD* variants was collected. In these cases, additional patient consent was obtained.

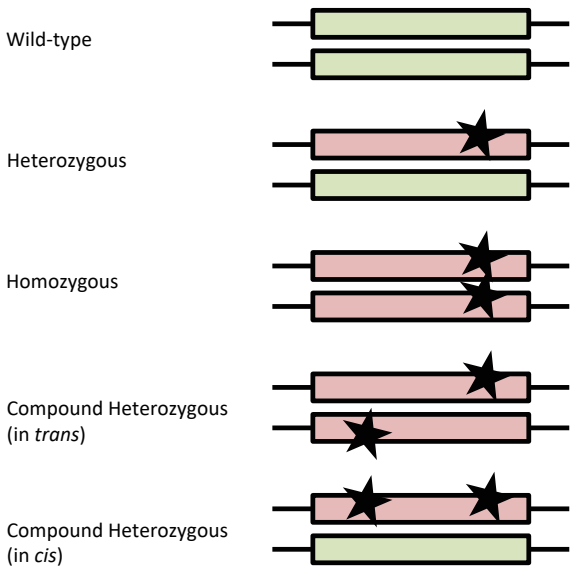

Figure 1. Illustration of zygosity and clinical interpretations

A wild-type patient carries no variants, resulting in normal activity alleles (green). A heterozygous patient carries one variant, resulting in one reduced or inactive allele (red) and one active allele (green). A partly reduced enzyme activity is expected, since there is still one active allele left. For homozygous patients, both variants result in a reduced or inactive allele (red). Depending on the effect of the variants on the protein, a reduced or absent enzyme activity is expected. Compound heterozygous patients can carry variants on different alleles (in *trans*) or on one allele (in *cis*), resulting in differences in enzyme function, either like that of a heterozygous patient or a homozygous patient. Stars represent variants; boxes represent alleles.

Dihydropyrimidine dehydrogenase enzyme activity measurements

For all patients, DPD enzyme activity was determined. This could be either prior to treatment or retrospectively after the occurrence of severe toxicity. DPD enzyme activity measurement in peripheral blood mononuclear cells (PBMCs)[24,25] was used as a reference to assess DPD activity, and has been used previously to determine dosages in *DPYD* variant carrying patients.[21,26] A validated method[27] was used, containing radiolabeled thymine as a substrate and consisting of high-performance liquid chromatography (HPLC) with online radioisotope detection using liquid scintillation counting. Normal values for healthy volunteers are 9.9±2.8 nmol/(mg*h), for DPD deficient patients are 4.8±1.7 nmol/(mg*h), and reference values range from 5.9 to 14 nmol/(mg*h).[28] Dose reductions based on DPD enzyme activity were performed in a one-to-one ratio, as was previously described by Henricks *et al.*[21] Thereafter, toxicity-guided dosing was used.

Molecular methods for estimation of phasing

In regard to the size of the *DPYD* gene, the location of the variants, and the material available (DNA, RNA) from the patients, three molecular methods to determine the phasing of the variants could be used in this study. In four patients, we could execute one or more of these methods. These methods are explained and illustrated in the Supplementary Material (Figure S1). Details on these techniques have been published elsewhere.[29-31]

Frequencies of compound heterozygous DPYD carriers

To investigate the incidence of compound heterozygous *DPYD* variant allele carriers (of the four genotyped *DPYD* variants) large databases were queried.[32,33] The incidence was calculated using minor allele frequencies (MAFs) of each variant identified in the databases separately. Since the determined variants are not in the same haplotype, it was assumed that the inheritance of these individual *DPYD* variants is independent. All genotypes from the databases were calculated to be in Hardy-Weinberg equilibrium, except for *DPYD**2A and c.1236G>A for the Exome Aggregation Consortium (ExAC)[32] and Genome Aggregation Database (gnomAD)[33] due to a slight overrepresentation of homozygous cases. The calculated frequencies were compared to frequencies from databases in which phasing could be determined.

Exome Aggregation Consortium and Genome Aggregation Database

Both the ExAC[32] and gnomAD[33] databases collect exome sequencing data and aggregate the data for public use. The ExAC dataset (v.0.3.1) contains sequenced data of 60,706 unrelated individuals. The gnomAD dataset (v.2.0) contains sequenced data of 123,136 exomes and 15,496 genomes from unrelated individuals. In ExAC, 2,791 *DPYD* variants, and in gnomAD, 2,190 *DPYD* variants were found. MAFs of *DPYD* variants from these databases reflect those of the population due to the large group size in the databases. Since both ExAC and gnomAD do not contain individual matched or phased data, it is not possible to search for compound heterozygous patients in these databases.

Phasing in compound heterozygous DPYD carriers

Three databases were used to identify compound heterozygous *DPYD* variant allele carriers and determine the phasing, i.e. allelic location, of variants.

Genome of the Netherlands Datasets

The Genome of the Netherlands (GoNL) trio datasets contain information of related fathers, mothers, and children, and phasing information is therefore available. Datasets were previously processed and phased using trio-aware variant calling.[34] After the exclusion of children, phased variant call format (VCF) files for 496 subjects (fathers and mothers) were obtained from the GoNL repository. The toolset Bedtools (v2) was used to extract all variants found in the *DPYD* locus (chr1:97,543,300—98,386,615). Next, for all individuals, the carrier status of *DPYD**2A, *DPYD**13, c.1236G>A and c.2846A>T was examined. Individuals who carry at least one of the four actionable *DPYD* variants were identified and, using a custom Python script,[35] the phasing of variants was assessed for individuals with multiple variants.

1000 Genomes Database

The 1000 Genomes Project is the largest publicly available catalogue of human variation and genotyped phased data. It originally ran from 2008 until 2015, and thereafter it was maintained and expanded by the IGSR (International Genome Sample Resource).[36] On 27 October 2016, phased data of the *DPYD* gene (chr1: 97,543,300—98,386,615) was downloaded from the 1000 Genomes ftp server (phase 3; GRCh37; chr1: 97,543,300—98,386,615) using Tabix (v1.1).[37] The statistical program R (v3.2.5)[38] was used to select the genotypes at four *DPYD* risk alleles in unrelated individuals of Caucasian descent.

Exome Trios Leiden University Medical Centre Database

This diagnostic database of the clinical genetics department of LUMC contains 433 complete exome trios (father, mother, and child). The exome was enriched by the Agilent sureselect v5 kit and sequenced using various Illumina (San Diego, CA, USA) sequencers (Hiseq 2000, 2500, 4000, Nextseq). Carrier status of the abovementioned *DPYD* variants was established by querying the trio VCF files. We also investigated all samples with sufficient coverage of this region to obtain a reliable frequency estimate. In the case of trios, only parents were taken into account.

Results

Patient cases and clinical implications

Details of the demographics and clinical characteristics of the seven cases are described in the Supplementary Material (patient cases). All patients received treatment with fluoropyrimidines and were identified as compound heterozygous *DPYD* variant allele carriers, either prior to the start of treatment or retrospectively. Table 1 shows an overview of the cases. Table 2 shows all genotype and phenotype results. With additional genetic testing phasing could be determined in four out of seven patients. In three patients, the variants were located in *trans* and one patient carried the variants in *cis*. With the phasing information available, it is possible to calculate a dose recommendation using publicly

available pharmacogenetic dosing guidelines.[19,20] For example, patient 1 carried *DPYD**2A and c.1236G>A in *trans*. The gene activity values range from inactive (0) to fully active (1). *DPYD**2A and c.1236G>A/HapB3 have values of 0 and 0.5, respectively. As this patient carries the variants in *trans*, each allele contains one variant and no fully functional allele remains. Therefore, the cumulated gene activity score (GAS) is 0.5. The GAS can be used to determine dose recommendations according to the genotype, as was previously described.[12] The GAS ranges from 0 to 2, and a score of 0.5 corresponds to a dose recommendation of 25%. The DPD enzyme activity of patient 1 was 0.9 nmol/(mg*h). This was divided by the mean of the reference value (9.9), which results in a theoretical DPD activity of 9%. For each patient for whom phasing details were known, the GAS was determined and compared to the theoretical DPD activity. Dose recommendations according to the GAS (genotype) and theoretical DPD activity (phenotype) were divergent in almost all cases, as shown in Table 2.

Table 1. Characteristics of patient cases

Shown per patient are primary tumor, treatment, capecitabine dose, executed assays (genotype, dihydropyrimidine dehydrogenase (DPD) enzyme activity, and additional assays) information. Additional assays are droplet digital PCR, PacBio sequencing (Menlo Park, CA, USA), or an in-house developed technique. For the executed assays it is shown whether these were executed prior to treatment (P) or retrospectively (R).

Patient #	Primary Tumor	Treatment	Capecitabine dose	Executed assays
1	BC	CAP	1,000 mg/m^2/bid	Genotyping (R), DPD activity (R), in-house technique (R), droplet digital PCR (R)
2	BC	CAP	800 mg bid (50%)	Genotyping (P), DPD activity (R), in-house technique(R)
3	CRC	CAP+OX	900 mg bid (50%)[a]	Genotyping (P), DPD activity (P), PacBio (R)
4	BC	CAP	1,500 mg bid	Genotyping (R), DPD activity (R[b])
5	CRC	CAP+RT	800 mg bid (50%)	Genotyping (P+R[c]), DPD activity (R[d]), PacBio (R)
6	CRC	CAP+OX	1,000 mg/m^2/bid	Genotyping (R), DPD activity (R)
7	CRC	CAP+OX+ BEV	1,000 mg/m^2/bid	Genotyping (R), DPD activity (R)

[a] Increased to 70% in the second cycle;

[b] During hospital admission;

[c] *DPYD**2A was prospectively identified, c.2846A>T was retrospectively identified;

[d] During treatment.

Abbreviations: BC: breast cancer; CRC: colorectal cancer; CAP: capecitabine; RT: radiotherapy; OX: oxaliplatin; BEV: bevacizumab; bid: *bis in die*/twice a day.

Table 2. Dose advice for compound heterozygous *DPYD* variant allele carriers

Shown per patient are *DPYD* variants, phasing of the *DPYD* variants, GAS, retrospective DPWG dosing advice based on phasing, DPD enzyme activity, and percentage of DPD enzyme activity considered for dose advice. According to the DPWG guidelines [19] a gene activity score can be given to compound heterozygous patients when phasing is known. Fully functional/reduced functionality: gene activity score of 1.5; fully functional/inactive: gene activity score of 1; reduced functionality/reduced functionality: gene activity score of 1; reduced functionality/inactive: gene activity score of 0.5; inactive/inactive: gene activity score of 0.

Patient #	*DPYD* variants	Phasing	GAS[12]	DPWG dose advice (% of regular dose)	DPD activity (nmol/(mg*h))	Percentage of DPD activity[a]
1	*DPYD*2A + c.1236G>A	in *trans*	0.5	25%	0.9	9%
2	*DPYD*2A + c.2846A>T	in *trans*	0.5	25%	6.0	60%
3	c.1236G>A + c.2846A>T	in *trans*	1	50%	4.5	45%
4	*DPYD*2A + c.2846A>T	unknown	X	X	0.11	1%
5	*DPYD*2A + c.2846A>T	in *cis*	1	50%	7.2	72%
6	*DPYD*2A + c.1236G>A	unknown	X	X	3.8	38%
7	*DPYD*2A + c.1236G>A	unknown	X	X	1.6	16%

[a] The reference DPD activity ranges from 5.9-14 nmol/(mg*h) [28], and therefore the percentage of DPD activity can be calculated using the average of the reference (9.9 nmol/(mg*h)). This percentage could be used as a percentage of the regular dose.

Abbreviations: DPD: dihydropyrimidine dehydrogenase; GAS: gene activity score; DPWG: Dutch Pharmacogenetic Working Group; X: could not be determined.

Preventing toxicity

Three of the seven case patients were identified as carriers of one or more *DPYD* variants prior to the start of therapy. For one patient, the DPD enzyme activity was determined prior to the start of therapy. Based on their genotype or phenotype, these three patients received initially reduced fluoropyrimidine dosages of 50%. They experienced limited and reversible toxicity (CTC-AE grades 0—2). The dose of one patient was increased to 70% in the second treatment cycle, after which CTC-AE grade 3 toxicity occurred.

Four of the seven case patients received a full dose, since their genotype was unknown prior to the start of therapy. These patients all experienced severe toxicity (CTC-AE grades 3—5), and three of them were admitted to the hospital for seven to 14 days. An overview of cases, including the toxicity, is shown in Table 3.

Frequencies of compound heterozygous DPYD carriers without phasing information

The ExAC and gnomAD databases revealed an average MAF for *DPYD*2A, *DPYD*13, c.1236G>A, and c.2846A>T of 0.55%, 0.03%, 1.43%, and 0.27%, respectively. MAFs for ExAC and gnomAD separately are summarized in Table 4. The probability of identifying a compound heterozygous *DPYD* patient for two variants according to these databases was ≤0.008%,

as was calculated using frequencies of combinations of *DPYD* variants. Results for each combination of *DPYD* variants are shown in Table 5. With several million fluoropyrimidine users each year, thousands of patients worldwide are compound heterozygous for a subset of these four *DPYD* variants.

Table 3. Toxicity profiles of compound heterozygous *DPYD* variant allele carriers

Shown per patient are *DPYD* variants, fluoropyrimidine dose as a percentage of the regular dose, and experienced toxicity with this dose. All patients retrospectively identified as *DPYD* variants carrier received full doses and experienced severe (CTC-AE ≥3) toxicity. All patients prospectively identified as *DPYD* variant(s) carrier received dose reductions and experienced a maximum of CTC-AE grade 2 toxicity with the initial dose.

Patient #	*DPYD* variants	Dose (% of regular dose)	Toxicity (maximal CTC grade)
1	*DPYD**2A + c.1236G>A	100%	4
2	*DPYD**2A + c.2846A>T	50%	1—2
3	c.1236G>A + c.2846A>T	50% → 70%	0 (on 50% dose) → 3 (on 70% dose)
4	*DPYD**2A + c.2846A>T	100%	5
5	*DPYD**2A + c.2846A>T	50%	0
6	*DPYD**2A + c.1236G>A	100%	4
7	*DPYD**2A + c.1236G>A	100%	3

Abbreviations: CTC-AE: common terminology criteria for adverse events.

Frequencies of compound heterozygous DPYD carriers with phasing information

In the GoNL database, genetic data from 496 subjects (fathers and mothers only) was reviewed. One subject was found who carried two *DPYD* variants. This subject was a carrier of the *DPYD* c.1236G>A and *DPYD* c.2846A>T variants, both of which were located on a single allele (in *cis*). Based upon the data in GoNL, the probability of having compound heterozygosity of the four *DPYD* variants is <0.2%.

In the 1000 Genomes database, data of 2,513 individuals was available. After the selection of unique, unrelated individuals, 407 individuals remained. One subject was found who carried two *DPYD* variants. This subject was a carrier of *DPYD* c.1236G>A and *DPYD* c.2846A>T, both of which were located on different alleles (in *trans*). Based upon the data in 1000 Genomes, the probability of having compound heterozygosity of the four *DPYD* variants is <0.3%.

In the LUMC clinical genetics database (exome trios LUMC), the analysis was restricted to the children, since this would allow phasing. None of the 433 children carried more than one *DPYD* variant, thus compound heterozygosity in this database is <0.2%.

Despite the low frequency, compound heterozygous patients were identified in all databases except the LUMC clinical genetics database. However, the low frequency did not allow to determine the probability of in *cis* or in *trans* phasing of variants in a patient.

Table 4. MAF per database
Three databases (GoNL, 1000Genomes, and exome trios LUMC) containing phased data were checked for four *DPYD* variants. Two large online databases (ExAC and gnomAD) were checked to identify the MAFs of the individual *DPYD* variants. For each *DPYD* variant, the genotype distribution and MAF are shown.

variants / databases	*DPYD**2A (rs3918290)		*DPYD**13 (rs55886062)		c.1236G>A (rs56038477)		c.2846A>T (rs67376798)	
	HW/HE/HM	MAF	HW/HE/HM	MAF	HW/HE/HM	MAF	HW/HE/HM	MAF
GoNL	489/7/0	0.7%	494/2/0	0.2%	475/21/0	2.1%	490/6/0	0.6%
1000 Genomes	405/2/0	0.2%	406/1/0	0.1%	389/18/0	2.2%	403/4/0	0.5%
Exome Trios LUMC	946/15/0	0.8%	946/0/0	0.00%	946/46/0	2.3%	946/2/0	0.1%
ExAC	60,627/624/5	0.5%	60,320/42/0	0.03%	60,652/1,808/27	1.5%	60,687/317/1	0.3%
gnomAD	138,489/1,586/10	0.6%	138,166/83/0	0.03%	138,407/3,841/39	1.4%	138,478/792/1	0.3%

Abbreviations: MAF: minor allele frequency; HW: homozygous wild-type; HE: heterozygous carrier; HM: homozygous carrier; GoNL: Genome of the Netherlands; ExAC: Exome Aggregation Consortium; gnomAD: Genome Aggregation Database.

Table 5. Calculated frequency for compound heterozygous *DPYD* patients
Using the average MAFs of the ExAC and gnomAD databases (for *DPYD**2A, *DPYD**13, c.1236G>A, and c.2846A>T, these are 0.55%, 0.03%, 1.43%, and 0.27% respectively), possible combinations for two out of four currently genotyped *DPYD* variants are shown.

Combination of *DPYD* variants	Calculated frequency
*DPYD**2A + *DPYD**13	0.0002%
*DPYD**2A + c.1236G>A	0.008%
*DPYD**2A + c.2846A>T	0.001%
*DPYD**13 + c.1236G>A	0.0005%
*DPYD**13 + c.2846A>T	0.0001%
c.1236G>A + c.2846A>T	0.004%

Abbreviations: MAF: minor allele frequency; ExAC: Exome Aggregation Consortium; gnomAD: Genome Aggregation Database.

Discussion

Prospective genotyping of *DPYD* variants followed by individual dose adjustments is increasingly applied as the standard of care for patients starting fluoropyrimidine therapy. Standard dose reductions from CPIC and DPWG guidelines cannot be applied in patients who carry more than one *DPYD* variant, as the phasing of the variants is unknown. Despite the low population frequency of <0.2%, the absolute number of identified compound heterozygous patients will increase as the number of genotyped patients increases and the panel of tested variants is expanded. To the best of our knowledge, this is the first study that describes a case series of compound heterozygous *DPYD* variant allele carriers and investigates diagnostic and therapeutic strategies for these patients.

Our study shows the clinical need for further information on the genotype, as four patients were identified as compound heterozygous carriers retrospectively and all of them experienced severe toxicity. These compound heterozygous *DPYD* variant allele carriers have an increased risk of developing severe fluoropyrimidine-induced toxicity if dosages are not adequately adjusted. Previously, compound heterozygous patients have been described with severe or even lethal side effects after fluoropyrimidine treatment.[39,40] Three patients in this study were prospectively identified as compound heterozygous carriers, received initial dose reductions, and developed only mild toxicities.

Out of the four patients for whom we were able to retrieve phasing information, three were in *trans* and one was in *cis* orientation. Data from publicly available databases also showed that both in *cis* and in *trans* orientations exist. However, the recently updated CPIC guidelines on *DPYD* assumes in *trans* phasing for compound heterozygous patients.[20] The DPWG guidelines do not mention phasing; however, the dosing recommendations of the DPWG use the GAS, a score based on the activity of individual alleles.[19] This implies the need for phasing information. The assumption of in *trans* phasing could result in the underdosing of patients with variants phased in *cis*, and thus exemplifies the need for the determination of the phasing of variants.

In this study, we looked at different diagnostic strategies to determine the phasing of *DPYD* variants in compound heterozygous patients. In four patients, the phasing of *DPYD* variants could be determined using one of three different molecular methods. These methods are in the early phases of development, not routinely available, quite expensive, and not always conclusive. For two of these techniques, patient RNA is used, which degrades quickly after the blood draw unless specifically designed blood tubes are used. Compound heterozygous patients are rare, yet here we describe seven patients heterozygous for multiple *DPYD* variants. A limitation of our study is that most patients were identified retrospectively and in different institutions. Because of this, not enough of or not the right material was available for analysis, thus not all genotyping techniques could be executed in each patient. For two samples, tests failed or produced inconclusive results (data not shown). For this reason, a formal comparison of their suitability to identify phasing was not possible. However, of the three explored molecular methods, PacBio sequencing seems most promising. While phasing improved the prediction of DPD enzyme activity, patients with identical combinations of *DPYD* variants and identical phasing showed considerable differences in DPD enzyme activity, which could potentially limit the added value of the determination of

the phasing of *DPYD* variants. However, larger numbers of compound heterozygous *DPYD* variant allele carriers would be necessary to draw a firm conclusion.

The measurement of DPD enzyme activity in PBMCs was used as a reference to assess DPD activity. The method is well-established, commonly available, and shows limited intra- and interpatient variability.[27] However, recently, differences in intrapatient variability in DPD enzyme activity related to circadian rhythm were shown,[41] which can result in the under- or overestimation of DPD enzyme activity. In this study, we present one patient with extremely low DPD enzyme activity, which could possibly be influenced by the presence of severe neutropenia, as DPD activity is normally measured in mononuclear cells. Therefore, DPD enzyme activity can differ depending on the clinical condition of the patient, and should thus be measured prior to treatment.

A major question is whether genotyping or phenotyping is the best method to determine DPD activity to guide fluoropyrimidine dosing in patients carrying multiple *DPYD* variants. Despite the low population frequency, we present seven patients carrying multiple *DPYD* variants, of which three received initially reduced fluoropyrimidine dosages. However, based on these data, it is not possible to determine if a dose recommendation based on phased genetic information or DPD enzyme activity measured in PBMCs is safer. In three out of four cases, differences were observed between the theoretically calculated DPD activity using genotyping or phenotyping. These differences would result in different dosing recommendations. For example, there is a considerable interpatient variability in DPD enzyme activity in carriers of the *DPYD* variant c.1236G>A/HapB3.[12] Due to this variability, genetic dose recommendations are categorized (e.g. 25 or 50%) on the average of the phenotypes. This categorization could explain the observed dosing differences derived from genotyping and phenotyping. Other variants of *DPYD* currently not routinely tested for or variants in other genes, e.g. *MIR27A*,[42] might also be involved in reducing DPD activity or explaining fluoropyrimidine-induced toxicity. DPD enzyme activity measurements are well-established, and additional molecular methods to resolve phasing are still in early phases of development. Therefore, in our opinion, the current therapeutic strategy for compound heterozygous *DPYD* variant allele carriers should be to determine initial dose reductions based on a DPD phenotyping test, for example by measuring enzyme activity in PBMCs. Dosing could be adjusted by the treating physician in subsequent cycles based on observed severe toxicity (or lack thereof).

Conclusions

In conclusion, patients carrying multiple *DPYD* variants are at high risk of developing severe toxicity. Additional analyses are required to determine the correct dose of fluoropyrimidine treatment. In patients carrying multiple *DPYD* variants, we recommend that a DPD phenotyping assay be carried out to determine a safe starting dose. The dose could be titrated in subsequent cycles based on observed toxicity.

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SUPPLEMENT CHAPTER 11

Diagnostic and therapeutic strategies for fluoropyrimidine treatment of patients carrying multiple *DPYD* variants

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Patient cases

Patient 1

A 50 year old patient was diagnosed with breast cancer (cT3N3M0, triple negative), for which neo-adjuvant chemotherapy was started, followed by a breast saving surgery and axillary lymph node dissection (ypT1N1), and thereafter radiotherapy. Approximately a year later, lymph node metastases were found, for which capecitabine (1,000 mg/m^2 bid) was started. The patient experienced severe toxicity in the first cycle of capecitabine and was hospitalized for one week (allergic reaction —red swollen face—, complaints in the mouth, neutropenic fever, nausea, diarrhea, leukopenia CTC-AE grade 4). The patient was genotyped hereafter and turned out to be a carrier of *DPYD**2A and c.1236G>A. DPD enzyme activity was determined and was reported to be as low as 0.9 nmol/(mg*h) (normal range 5.9—14 nmol/(mg*h)). Internal evaluation using plasmids showed that these variants were both located at a different chromosome (in *trans*). Digital droplet PCR showed inconclusive results.

Patient 2

A 44 year old patient was diagnosed with breast cancer (cT1N1M0), for which the patient underwent surgery followed by radiotherapy and adjuvant chemotherapy (cyclophosphamide/methotrexate/5-FU dosed 600 mg/m^2 iv). Approximately seven years later, a relapse was discovered and one breast was removed followed by hormonal therapy (tamoxifen for three years, anastrozol for three years). Three years hereafter, palpable axillary lymph nodes were found, which turned out to be metastases of the tumor. TAC (docetaxel, doxorubicin, cyclophosphamide) cycles were started, however poorly tolerated and therefore cycles 2—6 were continued on 75% of the dose. Axillary radiotherapy was given hereafter. All was followed by letrozole. Then, metastases in the liver, bones and adrenal glands were found. Palliative chemotherapy with capecitabine (1,000 mg/m^2 bid) was started, for which *DPYD* genotyping was performed. She turned out to carry both *DPYD**2A and c.2846A>T. Taking this result into account and previous 5-FU combination therapy without any problems, capecitabine was started at 50% dose (800 mg twice daily). The therapy was discontinued due to side effects (hand-foot syndrome CTC-AE grade 1, diarrhea CTC-AE grade 1—2 and abdominal cramps CTC-AE grade 1—2). Internal evaluation using plasmids showed that the SNPs were both located at a different chromosome (in *trans*). For the purpose of this study, additional material was collected for DPD enzyme activity, which was low, but within normal range 6.0 nmol/(mg*h) (normal range 5.9—14 nmol/(mg*h).

Patient 3

A 61 year old patient was diagnosed with metastatic colorectal cancer (pT4N2M1). After a laparoscopic hemicolectomy was performed, chemotherapy with capecitabine and oxaliplatin was scheduled. The *DPYD* genotype was determined and two variants (c.2846A>T and c.1236G>A) were found. In addition to this, the DPD enzyme activity was measured, which was 4.5 nmol/(mg*h). The capecitabine dose was adjusted to ~50% (1,800 mg per day). Because of good tolerance, the capecitabine dose was increased to ~70% in the second

cycle. Upon this increased dose, the patient developed thrombocytopenia CTC-AE grade 3 and complaints of anorexia, nausea and fatigue CTC-AE grade 1—2. In the third cycle, 57% dose was applied, resulting in a new thrombocytopenia CTC-AE grade 2. After these three cycles disease progression was noted and treatment was discontinued. For the purpose of this study, additional material was collected for the PacBio analysis and this patient carried both *DPYD* variants on different alleles (in *trans*). (Also described by Henricks *et al.*).[1]

Patient 4

A 38 year old patient was diagnosed with breast cancer. After surgery (pT2N0) and hormonal therapy (tamoxifen for three years) disease progression was established, and other hormonal therapies (anastrozole for two years, exemestane for two years andfulvestrant) followed. Due to liver metastases treatment was continued with capecitabine (1,500 mg twice daily). Severe side effects (CTC-AE grade 3 oral mucositis) occurred after seven days of treatment for which chemotherapy was discontinued immediately and the patient was admitted to the hospital. During two weeks of hospital admission severe thrombocytopenia (CTC-AE grade 3), neutropenia and leukopenia (both CTC-AE grade 4) occurred. The patient deteriorated (respiratory problems, multiple organ failure), treatment against side effects was stopped and the patient died. During hospital admission, DPD enzyme activity was measured, which was extremely low (0.11 nmol/(mg*h)). Also, the *DPYD* genotype was determined and two variants (*DPYD**2A and c.2846A>T) were found. No additional analyses for this study were performed for this patient.

Patient 5

A 57 year old patient was diagnosed with colorectal adenocarcinoma (T4N0) for which a right hemicolectomy and trans anal endoscopic microsurgery of the rectum were performed. Approximately one year later, recurrence of rectal carcinoma was discovered (T4N2). The patient was also diagnosed with prostate cancer around the same time. Chemo radiotherapy was planned, consisting of radiotherapy on the rectum (25x2Gy) and prostate (total of 78Gy) combined with capecitabine. The patient participated in a clinical trial (NCT00838370) for which prospective genotyping (*DPYD**2A) was performed.[2] The patient tested positive and treatment was adjusted on the second day to 50% of capecitabine dose (800 mg twice daily). No severe side effects occurred. During treatment, DPD enzyme activity was measured, and was within normal range (7.2 nmol/(mg*h)). After treatment the patient was genotyped retrospectively for additional *DPYD* variants and was also a carrier of the c.2846A>T variant. For the purpose of this study, extra material was collected for the PacBio analysis and this patient carried both *DPYD* variants on a single allele (in *cis*).

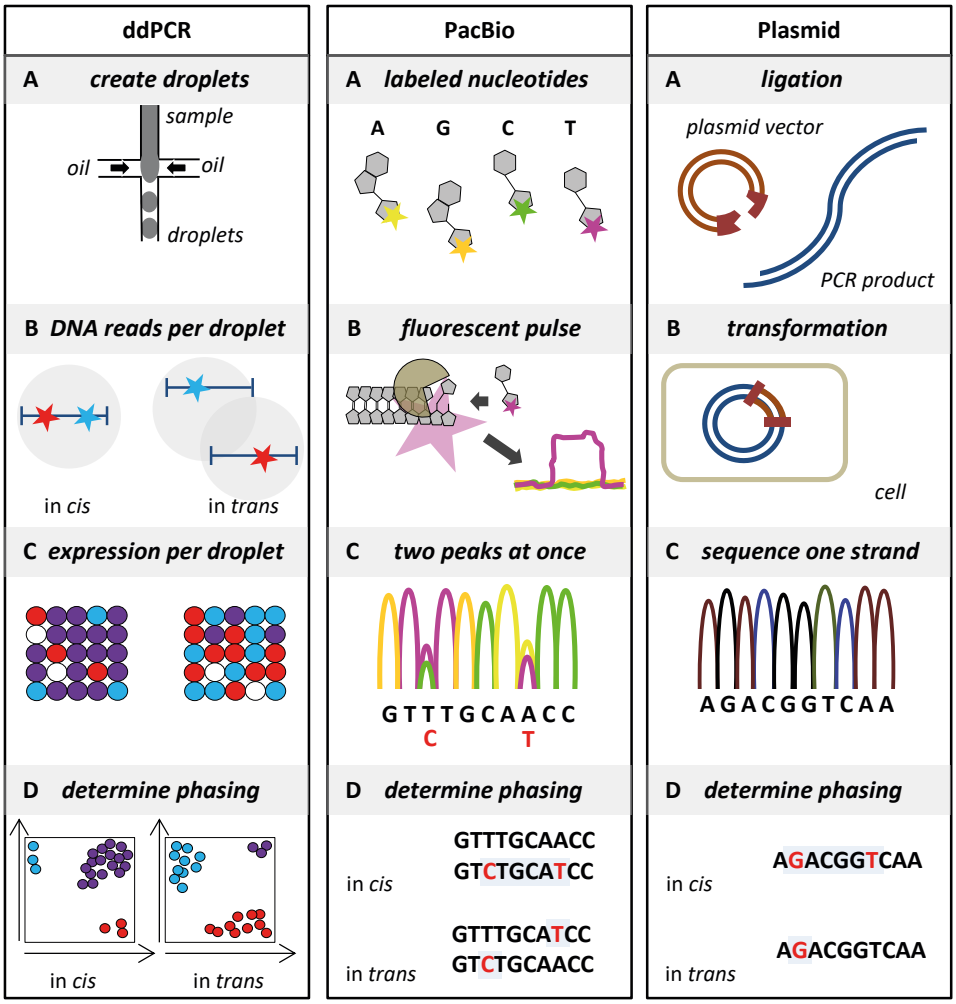
Patient 6

A 67 year old patient was diagnosed with metastasized colorectal adenocarcinoma and treated with capecitabine (1,000 mg/m^2 twice daily) and oxaliplatin. The patient experienced CTC-AE grade 4 neutropenia, thrombocytopenia and leukopenia, CTC-AE grade 3 nausea, vomiting, diarrhea, stomatitis and anorexia. Toxicity resolved after continuing with an unknown dose reduction. The DPD enzyme activity and *DPYD* genotype were determined

after toxicity was resolved. A reduced enzyme activity of 3.8 nmol/(mg*h) and two variants (*DPYD**2A and c.1236G>A/c.1129-5923C>G) were found. Approximately a year later, the patient was again treated with the unknown reduced capecitabine dose and oxaliplatin, which was stopped after six cycles due to toxicity. No additional analyses for this study were performed for this patient.

Patient 7

A 61 year old patient was diagnosed with disseminated colorectal cancer (pT4N1M1) who underwent surgical hemicolectomy. Palliative chemotherapy consisting of capecitabine (1,000 mg/m^2 twice daily, day 1—14), oxaliplatin (130 mg/m^2, day 1) and bevacizumab (7.5 mg/kg, day 1) was started. After eleven days of chemotherapy, the patient was admitted to the hospital with CTC-AE grade 3 diarrhea and nausea with vomiting, and CTC-AE grade 2 fever. Capecitabine was stopped immediately. Loperamide therapy was started but the diarrhea persisted. The patient was discharged from the hospital after 13 days. The measured DPD enzyme activity was low (1.6 nmol/(mg*h)), and *DPYD* genotype was *DPYD**2A and c.1236G>A/c.1129-5923C>G, both measured after therapy. No additional analyses for this study were performed for this patient.



Supplementary Figure 1. Illustration of molecular methods

ddPCR: Droplet Digital PCR (ddPCR)[3] is a method based on water-oil emulsion droplet technology. DNA isolation is performed using MagNA Pure Compact Nucleic Acid Isolation Kit I (Hoffmann-La Roche, Basel Switzerland). A DNA sample is fragmented into tens of thousands of droplets **(A)** and PCR amplification of DNA is present in each droplet **(B)**. PCR is based on standard TaqMan probe-based assays. In this study, we used FAM and HEX fluorescent labels, shown in red and blue (primers and probes used are; F-primer: CTGATCTTCATCTTCATTCC, R-primer: AGGTGGGAGAATTGTTGCTAT, probe: HEX-CCAGTTTCATCTTGTTCTGTCCGAACAA-BHQ, F-primer: TCACTGAACTAAAGGCTGA, R-primer: CAACTTATGCCAATTCTCTTG, probe: FAM-CTTTCCAGACAACATAAGTGTGATTTAAC-BHQ). In patients with two variants on a single allele (in *cis*) most droplets will emit both fluorescent labels, resulting in a combined color purple, where in patients with two variants on different alleles (in *trans*) most

droplets only emit one fluorescent label, red or blue, when the DNA strands are being amplified. All droplets are read out one by one **(C)** and in *cis* or in *trans* phasing can be determined **(D)**. ddPCR can be used for DNA samples and detect phasing of variants in up to 200 kb. For *DPYD,* combinations of *DPYD**2A+*DPYD**13 (66 kb distance) and *DPYD**2A+c.1236G>A (124 kb distance) can be determined using ddPCR.

PacBio: Pacific Biosciences RSII (PacBio)[4] starts with RNA isolation from PAXgene tubes using RNeasy® Mini Kit (Qiagen, Hilden Germany) according to manufacturer's protocol. Then, cDNA is synthesized using SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad CA USA) using oligo-dT or *DPYD* gene specific reverse primers (F2: GTTTGCCAGAACCCAATAAAGA, F3: CGTCATTGTACTTGGAGCTGG, Rev: CCACAAAACCTGTATTACTGAATAA, Rev-comp: TTATTCAGTAATCAGGTTTTGTGG). cDNA is amplified using KAPA HiFi HotStart ReadyMixPCR Kit (Kapabiosystems, Wilmington MS USA). Amplicon preparation is executed according to PacBio® Procedure and Checklist - Amplicon Template Preparation and Sequencing. PacBio is based on single molecule real-time (SMRT) sequencing. On each of the four nucleotides different fluorophore labels are attached **(A)**, which will emit when the nucleotide is build-in, which is shown as a fluorescence pulse or color peak **(B)**. When a patient carries multiple variants, multiple fluorescent labels will be emitted at the same time, resulting in two color peaks simultaneously **(C)**. Variants can either be located on the same strand (in *cis*) or on different strands (in *trans*), determined by reading the strands **(D)**. The advantage of SMRT-sequencing is that longer read lengths of DNA or RNA are possible, therefore phasing of variants in the large *DPYD* gene can be determined.

Plasmid: cloning as described previously.[5] First, patient RNA was isolated using RNeasy® Mini Kit (Qiagen, Hilden Germany). With 500 ng RNA, cDNA was synthesized using 10 mol/µl oligodT primer in a 10.25 µl volume which was incubated for 10 minutes at 70 degrees Celsius. After cooling, 2 µl 0.1M DTT, 2 µl dNTPs (5 mM), 4 µl first strand buffer, 0.5 µl reverse transcriptase (RT), and 0.25 µl RNasin® (Invitrogen, Bleiswijk the Netherlands) was added and incubated for 1 hour at 37 degrees Celsius. Thereafter, a PCR was performed using Qiagens universal mastermix and primers approximately 500 nucleotides up or downstream of the variants (*DPYD**2A: ACCACCTCTGGCCCCATG, c1236G>A: GGTGGGAGAATTGTTGCTATG and c.2846A>T: GTAGCCAGAATCATTACAGG). Plasmids were created by ligation of the specific PCR products into pGEM-T Easy vector **(A)** (Promega, Leiden Netherlands) as follows: 0.5 µl pGEM-T Easy, 0.5 µl Ligase, 3 µl PCR product and 4 µl buffer was incubated for 2 hours at room temperature. Ligation mixture was transformed to competent E coli cells (JM109) **(B)** and plated on IPTG/Xgal (Promega, Leiden Netherlands) containing LB-ampicillin agar plates (Acumedia Neogen, Ayrshire UK). Plates were incubated overnight at 37 degrees Celsius. Next day, cells with successful insertions (resulting in white colonies) are grafted in 2 ml LB-ampicillin and shaken overnight at 37 degrees Celsius. Plasmid DNA was isolated using Miniprep Kit (Qiagen, Hilden Germany) and restriction enzyme EcoR1 as used to check for insertion of PCR product (approximately 1000 bp insert). Thereafter, sequencing was performed by Macrogen (Amsterdam, the Netherlands) using primers T7 (GTAATACGACTCACTATAGGGC) and SP6 (ATTTAGGTGACACTATAGAA) located on both sides of A-T ligation side **(C)**. Plasmids contain only one allele of the PCR product, thus combined sequence result of T7 and SP6 primers determines the haplotype. Thus, when only one variant was found, the unidentified variant is located on the other allele, and therefore phasing results are in *trans*. When both variants, or no variants were found, phasing results are in *cis* **(D)**.

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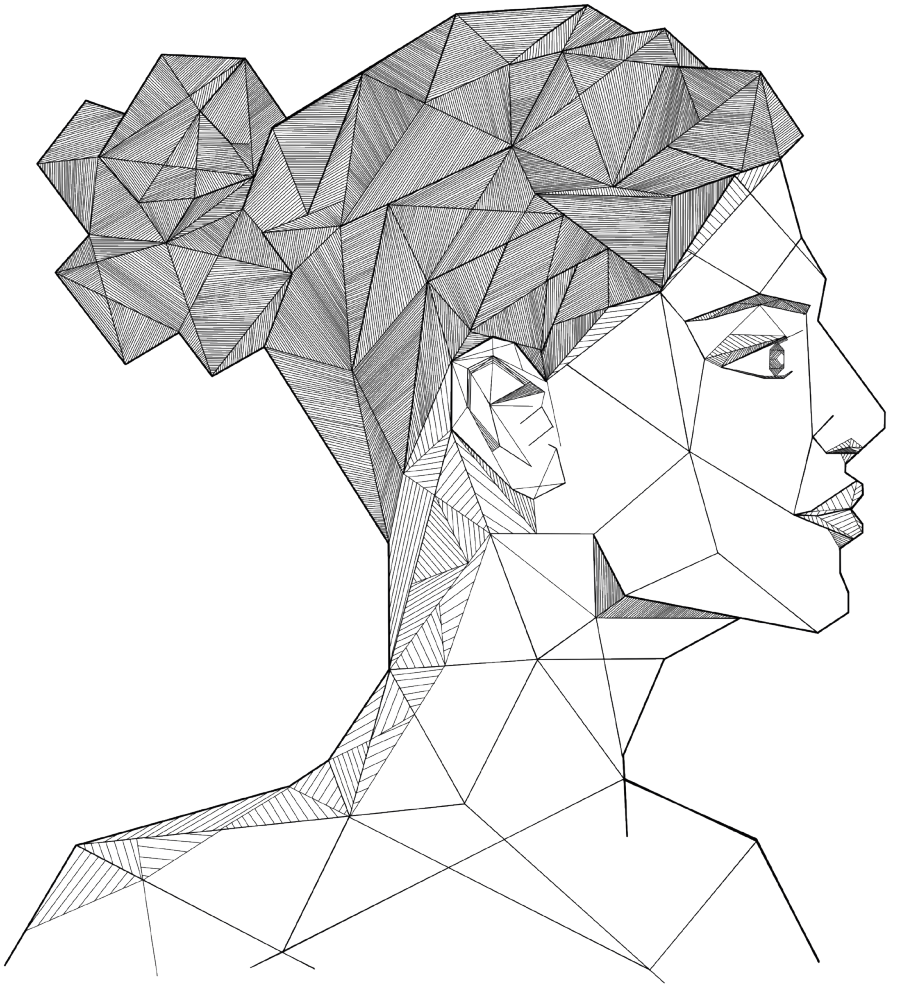

CHAPTER 12

Genome-wide association study to discover novel genetic variants related to the onset of severe toxicity following fluoropyrimidine use

Manuscript in preparation

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Abstract

Fluoropyrimidines are widely used anticancer drugs, but may lead to severe toxicity in up to 30% of patients. Prospective *DPYD* genotyping is increasingly used in clinical practice to predict and prevent severe toxicity, by means of initial dose reductions in *DPYD* variant allele carriers. While this strategy successfully reduces the incidence of severe toxicity, substantial toxicity remains that is not attributable to genetic variation in *DPYD*. A genome-wide association study (GWAS) was initiated to discover novel genetic variants associated with the onset of severe fluoropyrimidine-induced toxicity.

We conducted a GWAS in 1,146 patients treated with fluoropyrimidines who participated in the Alpe DPD study. Patients were genotyped using the Illumina Global Screening Array and data was imputed using the 1000 Genomes reference panel. The primary outcome was severe (grade ≥3) fluoropyrimidine-induced toxicity, compared to grade 0 or 1 fluoropyrimidine-induced toxicity. Variants were tested for association with severe fluoropyrimidine-induced toxicity using logistic, Cox, and ordinal regressions. A Polygenic Risk Score (PRS) was constructed by extracting all variants with $p<0.01$ in the association test.

1,101 patients passed the quality control (QC) analyses and 599 patients were included in the primary analysis. After imputation, 4,650,899 variants were included in the analysis. None of the genetic variants showed genome-wide significance ($p<5x10^{-8}$). Six variants were suggestive ($p<5x10^{-6}$) for the onset of severe fluoropyrimidine-induced toxicity. A PRS was constructed including 5,055 variants and predicted 62% of severe toxicity by non-genetic covariates alone and 96% by the combined analysis including covariates.

While no genome-wide significant variants could be identified, six variants were suggestive for the onset of severe toxicity in merely Caucasian patients. These variants are located outside of known fluoropyrimidine-pathway genes. Using a PRS consisting of 5,055 variants combined with clinical variables explained 96% of toxicity in this discovery cohort. This GWAS is one of the first attempts to identify variants predictive for fluoropyrimidine-induced toxicity and identified variants and the PRS require replication in an independent cohort.

Acknowledgements

The authors thank the Human Genotyping Facility of the Erasmus Medical Center for the execution of the genotyping and all participating centres of the NCT02324452 study for their help in including patients in the study.

Introduction

Fluoropyrimidines, including 5-fluorouracil (5-FU) and capecitabine, represent the backbone of chemotherapeutic regimens used to treat solid tumours, such as colorectal and breast cancer. Severe (grade ≥3) fluoropyrimidine-induced toxicity can occur in up to 30% of the patients, depending on the treatment regimen and may even be lethal in up to 1% of the patients experiencing toxicity.[1,2] Common fluoropyrimidine-induced adverse events are diarrhoea, mucositis, hand-foot syndrome and myelosuppression.[1,3] Dihydropyrimidine dehydrogenase (DPD) plays a key role in the degradation of 5-FU into inactive metabolites[4] and is encoded by the gene *DPYD.* Both DPD and genetic variants in *DPYD* have been widely investigated to explain severe fluoropyrimidine-induced toxicity. Recently, we have shown that prospective genotyping and dose reduction based on four variants in *DPYD* (*DPYD**2A, c.2846A>T, c.1679T>G and c.1236G>A) reduces severe fluoropyrimidine-induced toxicity in these *DPYD* variant allele carriers.[5] These four variants were selected based on previous studies and meta-analyses in which the association with fluoropyrimidine-induced toxicity was reported.[6-13] Nonetheless, severe toxicity did still occur in 23% of patients wild-type for these four variants, showing that other genetic variants or non-genetic factors may play a role in the onset of severe toxicity.[5]

Variants in genes other than *DPYD* could also play a role in the onset of severe fluoropyrimidine-induced toxicity. Previously, research to identify genetic variants has been conducted based on the pharmacological background of fluoropyrimidines, for example in pathway analyses or candidate gene studies. Several variants in *CDA* (cytidine deaminase), *CES1* (carboxylesterase 1), *TYMS* (thymidylate synthase), *MTHFR* (methylenetetrahydrofolate reductase), *ENOSF1* (enolase superfamily, member 1), *SLC22A7* (solute carrier family 22, member 7), *UMPS* (uridine monophosphate synthase) and *TYMP* (thymidine phosphorylase) genes were previously identified and associated with severe fluoropyrimidine-induced toxicity.[1,14-21] However, genome-wide association studies (GWAS) have the potential to identify novel variants without making assumptions based on a pharmacological background. Previously, O'Donnell *et al*. executed a GWAS on 503 cell lines to identify novel single nucleotide polymorphisms (SNPs) associated with capecitabine sensitivity.[22] Five variants showed genome-wide significance in this cell-line based GWAS, but replication in 268 patients only showed an association with sensitivity for capecitabine for *ADCY2* rs4702484.[23] Fernandez-Rodzilla *et al.* analysed data of 221 colorectal cancer patients treated with 5-FU or FOLFOX (folinic acid, 5-FU and oxaliplatin).[24] Seven SNPs were associated with adverse drug reactions, yet none reached the genome-wide significance level. Low *et al.* executed a GWAS on 13,220 patients in total, of which 1,460 patients received 5-FU, focussing on neutropenic and leukopenic toxicities.[25] For 5-fluorouracil, they identified four SNPs associated to neutropenia and leukopenia, yet none reached the genome-wide significance level. We conducted a GWAS to discover novel genetic variants associated with the onset of severe fluoropyrimidine-induced toxicity.

Materials and methods

Patients

Patients were recruited for the Alpe DPD study (clinicaltrial.gov identifier NCT02324452)[5] between April 30, 2015 and December 21, 2017, and were newly treated with fluoropyrimidines and genotyped prospectively for four *DPYD* variants (*DPYD**2A, rs3918290, c.1905+1G>A, IVS14+1G>A; c.1679T>G, *DPYD**13, rs55886062, I560S; c.1236G>A/HapB3, rs56038477, E412E; and c.2846A>T, rs67376798, D949V). Upon identification of one of these variants, heterozygous variant allele carriers received an initial dose reduction (25 or 50%) based on pharmacogenetic guidelines to prevent severe fluoropyrimidine-induced toxicity. Wild-type patients for these four *DPYD* variants received standard fluoropyrimidine dosages. After the second cycle the dose could be titrated upwards or downwards according to the occurrence of toxicity. The study was reviewed and approved by the medical ethical committee of the Netherlands Cancer Institute, Amsterdam, the Netherlands, and approval of the board of directors of each individual hospital was obtained for all participating centres. All patients signed informed consent prior to inclusion in the study, which included approval for the use of clinical data and remaining DNA to perform the current GWAS. All patients of whom sufficient DNA was available were genotyped. *DPYD* variant allele carriers (*N*=85) received dose reductions based on the four variants mentioned and were therefore excluded in the GWAS analyses.

Clinical data

Baseline characteristics, treatment type and toxicity data were collected for each patient. Ethnicity of the patients was self-reported, merely Caucasian patients participated in the Alpe DPD study. Toxicity was graded according to the National Cancer Institute common terminology criteria for adverse events (CTC-AE; version 4.03) and severe toxicity was defined as CTC-AE grade ≥3.[26] Relation to the study drugs 5-FU and capecitabine was recorded for each adverse event and only adverse events classified as possible, probable, or definite were taken into account.

Genotyping and quality control

Patient DNA remaining from the Alpe DPD study was collected. For each patient 200 ng of DNA was required and genotyping was executed at the Human Genotyping Facility of the Erasmus Medical Center, using the Illumina Global Screening Array (GSA).[27] The array contains 692,842 SNPs and includes rare variants with allele frequencies <1%. 1000 Genomes reference phase 3 GRCh37.p13 was used to impute the data. Quality control (QC) checks were performed using software R version 3.5.0[28] and PLINK software, version 1.07.[29,30] Patients were excluded from analyses based on an individual genotype call rate <97%, gender mismatch between reported and estimated sex based on genotypes of the X-chromosome (using PLINK), or excess of heterozygous genotypes as measured by the inbreeding coefficient. An inbreeding statistic of F>0.1 was judged to be outlying and patients were removed from the analysis. Genetic markers were excluded based on a SNP call rate <97% and a p-value $\leq 10^{-7}$ for the Hardy-Weinberg equilibrium (HWE) goodness-of-fit test. After exclusion of patients and markers in these marginal QCs, the remaining set was used

for integrative QC assessment. In order to evaluate the possibility of population stratification or outliers, multidimensional scaling (MDS) analysis was performed in PLINK. In addition, pairwise identity by state (IBS)/identity by descent (IBD) statistics was calculated to assess duplicates. MDS, IBS and IBD were computed using PLINK. Patients who were identified as outliers based on IBS clustering were excluded from the analysis. MDS coordinates were extracted and used as covariates in the association analysis. SNP imputation was performed using the programs *shapeit* and *impute2* with default parameters in which the reference panel 1000Genomes build version 3 was used with total, 'cosmopolitan', set of individuals.[31] An MDS plot was created to compare self-reported ethnicity of patients.

Statistical analyses

Genetic variants were tested for an association with the onset of severe fluoropyrimidine-induced toxicity. The primary outcome was severe (grade ≥3) fluoropyrimidine-induced toxicity, compared to grade 0 or 1 fluoropyrimidine-induced toxicity. Grade 2 toxicity was excluded from this analysis to maximize the contrast between toxicities. Gender, age, baseline BSA and treatment type (grouped as previously published)[5] were used as pre-specified covariates. Statistical analyses were performed in R statistics version 2.3.2. Base packages *stats, survival* and *MASS* were used to evaluate logistic, Cox, and ordinal regressions, respectively. Associations with a p-value $\leq 5x10^{-8}$ were considered statistically genome-wide significant. Associations with a p-value between $5x10^{-8}$ and $\leq 5x10^{-6}$ were considered suggestive. Post association QC was performed by visual inspection of Quantile-Quantile (QQ) plots of p-values of association tests and computation of the inflation factor given as: $\lambda=(\text{median}(T_1,\ldots,T_n)/0.675)^2$, where $T_1,\ldots,T_n$ are square roots of χ^2 quantiles.

A Polygenic Risk Score (PRS) was constructed by extracting all SNPs with a p-value <0.01 in the association test. To avoid problems due to collinearity, in the list sorted according to p-values, SNPs in a window of 100 kb were excluded after inclusion of a SNP. A penalized regression model was fitted using R-package *glmnet*. Included clinical parameters were gender, age, baseline BSA and treatment schedule.

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Results

Patients

Sufficient DNA was available for 1,146 out of 1,181 recruited patients (97%). These patients entered the QC procedure prior to association analyses. The flowchart on patient inclusion is shown in Figure 1. The observed individual genotype call rates varied between 97% and 100% and therefore meet the quality criteria. Based on subsequent QC steps, 45 patients (3.9%) were excluded from the analyses. Of these 45 patients, 30 patients (2.6%) were excluded due to missing genotypes, four (0.3%) patients were excluded due to a gender mismatch with the clinical data, six patients (0.5%) were excluded based on outlier removal of IBS plots. The inbreeding coefficient was 0.01 (-0.03─0.004), therefore, five (0.4%) patients were excluded. Of the 1,101 remaining patients, screen failures (*N*=55), patients with missing BSA at baseline (*N*=24) and *DPYD* variant allele carriers who received initially reduced dosages (*N*=80) were excluded (Figure 1). In addition, we chose to exclude patients who experienced grade 2 toxicity (*N*=343) from the primary analysis to maximize contrast between severe and non-severe toxicity.

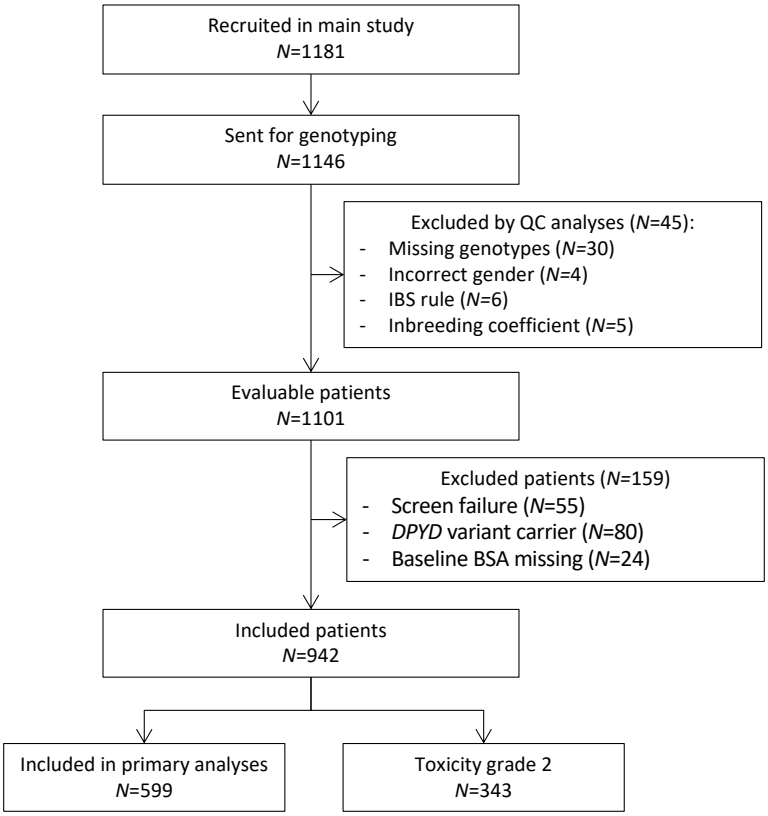

Figure 1. Flowchart of patients in the study

Patients who experienced toxicity grade 2 were excluded from the primary analyses to maximize contrast between severe and non-severe toxicity.

Abbreviations: QC: quality control; IBS rule: identity by state rule; *DPYD*: gene encoding dihydropyrimidine dehydrogenase; BSA: body surface area.

Association analysis

In the primary analysis, severe (grade ≥3) toxicity was compared to grade 0 and 1 toxicity in 599 patients. Patient characteristics of patients included in the primary analysis of this study and patients from the Alpe DPD study are shown in Table 1. There were no statistical differences between the cohorts, except for the number of treatment cycles.

The number of patients varied per SNP due to genotype missingness, which was limited to up to 3% as per QC. For the primary analysis, a MAF of 2% was used on imputed data to ensure stable numeric evaluation of all logistic regression models. This resulted in a total of 4,650,899 markers for which an association test was performed.

The primary analysis association test for severe fluoropyrimidine-induced toxicity (grades 3—5) includes covariates gender, age, baseline BSA and treatment type. The corresponding

Manhattan plot is shown in Figure 2. The corresponding QQ-plot of p-values is shown in Figure 3. The inflation factor is 1.04. Table 2 shows the list of the top 30 identified markers. No variants were identified to be statistically significant associated with severe fluoropyrimidine-induced toxicity at the genome-wide level. However, six SNPs were found to be suggestive. None of these SNPs have previously been reported in publications or in the ClinVar database of the National Center for Biotechnology Information (NCBI).[32] The variants are reported in the SNP database of the NCBI.[33] Three variants are stated on the website as having 'no gene consequence', two were listed as an intron variant in RNA gene LOC101927414 (rs114105116) and protein coding gene COL6A3 (rs12622722), and one was listed as an 2KB upstream variant in LOC107984256 (rs10786179).

Genotyping and quality control

A set of 692,367 markers was genotyped. After several QC steps, 186,920 markers were excluded. Of these, 18,114 markers (2.6%) were excluded based on a deviation from Hardy-Weinberg equilibrium (HWE). Filtering for allele frequencies (threshold 0.5%) resulted in the exclusion of 147,607 markers (21.3%). The missingness cut-off was set at 10%, 23,835 markers (3.4%) were excluded based on the missing data analysis. Of the abovementioned excluded markers, 2,636 had multiple QC failures. In total, 505,447 markers met the QC for statistical analyses. These markers were imputed using the 1000 Genomes dataset as a reference panel. In total, 4,650,899 variants were available for statistical analyses. In the integrative QC, individuals and markers from the marginal QC steps were excluded. An MDS was executed in order to detect population stratification. No individuals were excluded. IBD/IBS clustering was executed to assess duplicates. No individuals were excluded.

Polygenic risk score

To calculate the PRS all SNPs with a p-value <0.01 for their association with severe fluoropyrimidine-induced toxicity were selected. To reduce linkage disequilibrium, SNPs were pruned for a minimum distance of 10^5 bps. This resulted in a set of 5,055 SNPs. Finally, an elastic net regression (R package *glmnet*, α=0.5) was performed and evaluated by cross-validation. The receiver operating characteristic (ROC) curve is shown in Figure 4, where it is compared to the model containing only clinical covariates. The two corresponding areas under the curve (AUCs) were 96% and 62%, respectively.

Table 1. Patient characteristics

Patient characteristics of patients included in the primary analysis of this GWAS (*N*=599) and patients included in the Alpe DPD study (*N*=1,103). Data are *N*(%) or median(IQR). P-values comparing patients from the primary analysis to the Alpe DPD study patients. We used a nonparametric test for independent samples to compare medians of age, BSA, and number of treatment cycles; and a χ^2 test for gender, ethnic origin, tumour type, treatment regimen and WHO performance status.

Characteristic	**GWAS cohort (*N*=599)**	**Alpe DPD study (*N*=1,103)**	**P-value**
Gender			0.841
Male	319 (53.3%)	593 (53.8%)	
Female	280 (46.7%)	510 (46.2%)	
Age in years [IQR]	64 [57—71]	64 [56—71]	0.454
Ethnic origin			0.362
White	573 (95.7%)	1048 (95%)	
Black	14 (2.3%)	19 (1.7%)	
Asian	9 (1.5%)	24 (2.2%)	
Other[a]	3 (<1%)	12 (1.1%)	
Tumour type			0.991
Non-metastatic colorectal cancer	265 (44.2%)	472 (42.8%)	
Metastatic colorectal cancer	114 (19%)	232 (21%)	
Breast cancer	75 (12.5%)	141 (12.8%)	
Gastric cancer	32 (5.3%)	63 (5.7%)	
Other[b]	113 (18.9%)	195 (17.7%)	
Type of treatment regimen			0.234
Capecitabine monotherapy (±bevacizumab)	102 (17%)	205 (18.6%)	
Capecitabine + radiotherapy (±mitomycin)	172 (28.7%)	264 (23.9%)	
Capecitabine + oxaliplatin (±bevacizumab)	179 (29.9%)	374 (33.9%)	
Capecitabine + other anticancer drugs	41 (6.8%)	72 (6.5%)	
Fluorouracil monotherapy	-	2 (<1%)	
Fluorouracil + radiotherapy (±mitomycin)	43 (7.2%)	63 (5.7%)	
Fluorouracil + oxaliplatin + folinic acid (±bevacizumab)	18 (3%)	43 (3.9%)	
Fluorouracil + other anticancer drugs	44 (7.3%)	80 (7.3%)	
BSA [IQR]	1.9 [1.8—2.1]	1.9 [1.8—2.1]	0.503
WHO performance status			0.257
0	317 (52.9%)	554 (50.2%)	
1	241 (40.2%)	448 (40.6%)	
2	21 (3.5%)	42 (3.8%)	
Not specified[c]	-	59 (5.3%)	
Number of treatment cycles [IQR]	3 [1-7]	3 [1-8]	0.010
DPYD status			NA
Wild-type	599 (100%)	1018 (92.3%)	
c.1236G>A heterozygous		51 (4.6%)	
c.2846A>T heterozygous		17 (1.5%)	
*DPYD*2A heterozygous*		16 (1.5%)	
*DPYD*13 heterozygous*		1 (<1%)	

[a] Other ethnic origins included Hispanic descent, mixed racial parentage, and unknown ethnic origin;
[b] Other tumour types included anal cancer, oesophageal cancer, head and neck cancer, pancreatic cancer, bladder cancer, unknown primary tumour, vulva carcinoma, and several rare tumour types;
[c] WHO performance status was not specified for these patients, but was either 0, 1, or 2, as required by the study inclusion criteria.
Abbreviations: IQR: interquartile range; BSA: body surface area; DPD: dihydropyrimidine dehydrogenase; *DPYD*: gene encoding dihydropyrimidine dehydrogenase; WHO: world health organisation; NA: not applicable.

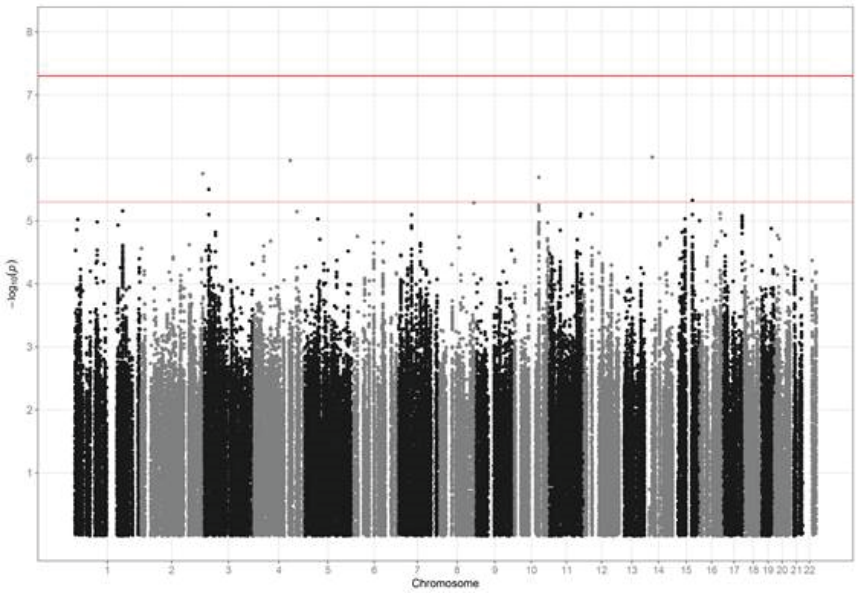

Figure 2. Manhattan plot
Manhattan plot for association with severe fluoropyrimidine-induced toxicity (grades 3—5), including de covariates gender, age, baseline BSA and treatment type. Genome-wide significance of the association with the onset of severe fluoropyrimidine-induced toxicity is indicated by the upper dark red line (≤p-value of $5x10^{-8}$). Suggestive association is indicated by the lower red line (p-value of ≤$5x10^{-6}$). No SNPs were found to be associated with severe fluoropyrimidine-induced toxicity. Six SNPs were found to be suggestive for association with severe fluoropyrimidine-induced toxicity, shown in Table 2.
Abbreviations: BSA: body surface area; SNPs: single nucleotide polymorphisms.

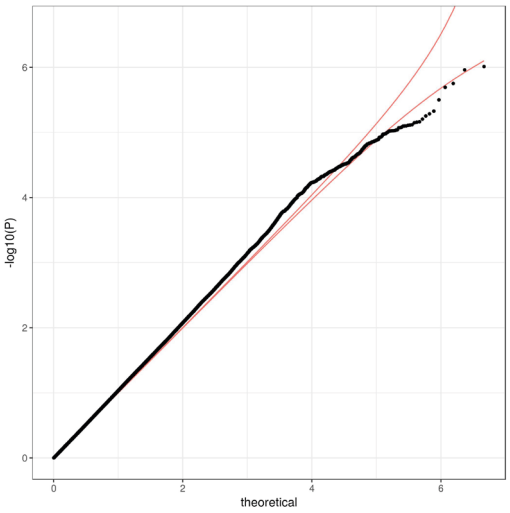

Figure 3. QQ-plot of p-values

The Quantile-Quantile (QQ)-plot shows the extent to which the observed distribution of the test statistic follows the theoretical null distribution. The inflation factor was λ=1.04.

Table 2. Thirty SNPs with lowest p-values

Variants are selected on allele frequency >0.01, β within -5 to 5, and are separated from another variant with more than 10 bps. Variants suggestive for the onset of severe toxicity are marked with an *.

Nr.	Marker	Chr	Position	A0	A1	AF	β	P-value
1	rs17114875 *	14	29999987	G	A	0,409	1,554562193	9,73E-07
2	rs114105116 *	4	138539880	T	A	0,02	1,213779485	1,1E-06
3	rs12622722 *	2	238269120	G	A	0,484	0,657789872	1,77E-06
4	rs10786179 *	10	96759531	T	G	0,889	-0,345506543	2,03E-06
5	rs367239 *	3	21421935	T	C	0,546	1,719787858	3,16E-06
6	rs11630087 *	15	75261673	G	T	0,456	0,757448146	4,71E-06
7	rs77579689	8	137130325	G	A	0,021	1,286849892	5,18E-06
8	rs11187969	10	96231169	G	A	0,129	1,381495967	5,59E-06
9	rs11187974	10	96239326	G	A	0,181	1,527446065	6,25E-06
10	rs12414693	10	97228795	C	T	0,259	0,713658584	6,87E-06
11	rs482061	1	182485749	T	C	0,847	-0,737925221	6,96E-06
12	chr4:164083322:D	4	164083322	TG	T	0,051	1,218271821	7,11E-06
13	chr16:78157332:I	16	78157332	G	GTT	0,065	1,023072586	7,63E-06
14	rs1838947	11	119691200	T	C	0,297	0,928423057	7,7E-06
15	rs495426	12	31021833	A	G	0,689	0,073481654	7,77E-06

table continues

Nr.	Marker	Chr	Position	A0	A1	AF	β	P-value
16	rs56338926	15	75259335	C	A	0,448	0,772401797	7,94E-06
17	rs449973	3	21425977	C	G	0,548	1,69493935	7,97E-06
18	rs1722291	7	56238936	G	A	0,198	1,553925389	8,02E-06
19	rs2344989	17	70924851	T	C	0,04	1,54932572	8,33E-06
20	rs2512155	11	117889448	C	T	0,179	1,449722565	8,45E-06
21	rs8076418	17	70921917	T	C	0,042	1,608309687	8,5E-06
22	rs2738545	16	78629320	G	A	0,673	0,420462271	9,13E-06
23	rs10851447	15	47411086	T	A	0,059	1,464774341	9,21E-06
24	rs722910	5	52781597	A	T	0,496	0,35577388	9,36E-06
25	rs8070810	17	70921851	G	A	0,042	1,61395951	9,42E-06
26	rs8067883	17	70921731	C	T	0,042	1,612564569	9,43E-06
27	rs6501582	17	70921801	T	C	0,042	1,613613576	9,44E-06
28	rs113309475	1	11430624	A	T	0,023	1,200617555	9,49E-06
29	rs9911437	17	70922305	T	C	0,042	1,614540275	9,49E-06
30	chr17:70923098:D	17	70923098	AC	A	0,042	1,614669511	9,65E-06

Abbreviations: Nr: number; Chr: chromosome; A0: nucleotide on allele 0; A1: nucleotide on allele 1; AF: allele frequency.

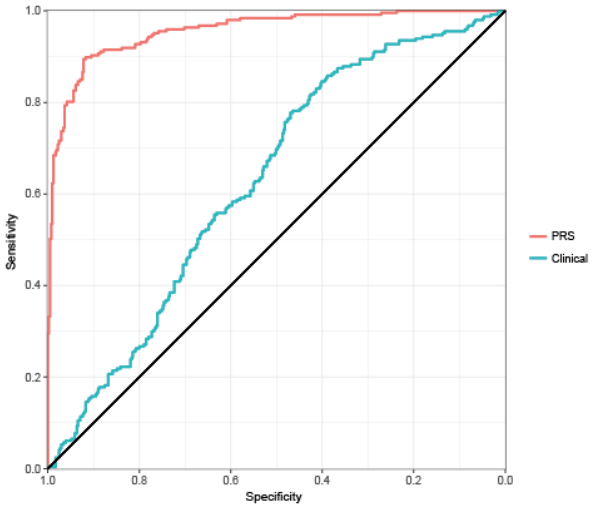

Figure 4. PRS-plot

The PRS-plot shows the predictive power of a covariate model (Clinical, blue line) and a covariate model plus 5,055 SNPs (PRS, red line). Included clinical parameters were gender, age, baseline BSA and treatment schedule. The AUC for the covariate model is 0.620 (95%CI: 0.577-0.663, $p=1.34^{-09}$), compared to the AUC for the combined model (prs) 0.956 (95%CI: 0.939-0.973, $p=3.98^{-140}$).

Abbreviations: PRS: polygenic risk score; AUC: area under the curve; 95%CI: 95% confidence interval.

Discussion

It is well recognized that *DPYD* genotyping is useful in preventing severe fluoropyrimidine-induced toxicity by applying initial dose reductions in patients who carry a specific *DPYD* variant.[5] However, not all toxicity can be predicted and prevented by the current four *DPYD* variants. Indeed, still ~20% of the patients experience toxicity, thus the search for genetic variants predictive for severe fluoropyrimidine-induced toxicity continues. We executed a GWAS in order to identify novel genetic variants possibly associated with the onset of severe fluoropyrimidine-induced toxicity.

To perform this GWAS on toxicity of fluoropyrimidines, over 1,100 patients were genotyped. Severe toxicity includes the National Cancer Institute CTC-AE grades 3—5. Scoring clinical toxicities can be difficult sometimes, as it can be open to interpretation. As severe toxicity has the most clinical impact, we chose to maximize contrast to the toxicity endpoint, and we excluded patients with grade 2 toxicity in the primary analysis. We were unable to identify genome-wide significant SNPs, yet we identified six SNPs suggestive (p-value of $5x10^{-6}$) of association with severe fluoropyrimidine-induced toxicity. Possibly the number of patients in our study is too small to reach genome-wide significance. However, we repeated the analysis including the patients with grade 2 toxicity, increasing the number of patients while reducing the contrast between toxicities. Yet, this did not result in a different outcome. The suggestive variants need to be re-tested in an independent cohort of patients who were treated with a fluoropyrimidine drug.

This GWAS was executed using DNA from patients participating in the Alpe DPD study. A formal comparison of GWAS analysed patients with the entire Alpe DPD cohort shows the cohorts were comparable. The range of number of treatment cycles was statistically different, with fewer cycles in the GWAS cohort. Possibly this is due to the exclusion of patients with grade 2 toxicity in the GWAS cohort, as grade 2 toxicity, if not developing into severe toxicity, may possibly arise from longer periods of fluoropyrimidine-treatment. We have no reason to believe that selection bias was introduced by leaving out patients with grade 2 toxicity. We believe the GWAS cohort is representative for patients in daily clinical care, as in the Alpe DPD study there were only limited restrictions on the inclusion criteria and the burden for patients to participate was very low.

With a large amount of genotyping data, we were able to compare ethnicity strings in the MDS plots to self-reported ethnicity from the Alpe DPD study. When adding two principal components, including ethnicity, to the statistical analysis, no differences were visible. Therefore, ethnicity was not of influence on the outcome of this GWAS and no patients were excluded based on self-reported ethnic origin.

Data on the functionality of the six SNPs suggestive of association with severe fluoropyrimidine-induced toxicity is limited. To the best of our knowledge, these six SNPs were not previously identified by other GWAS or other studies, or previously described in relation to the fluoropyrimidine pathway. Genome-wide significant SNPs (rs4702484, rs8101143, rs576523, rs361433) and suggestive SNPs (rs16857540, rs2465403, rs10876844, rs10784749, rs17626122, rs7325568, rs4243761, rs10488226, rs6740660, rs1567482 and rs6706693) identified in previously executed GWAS,[22,24,25] were not identified in this GWAS, possibly due to the differences in the design or endpoints of the study. For example, the

GWAS of Low *et al*. focused only on neutropenia and leukopenia as toxicity endpoint.[25] In the current GWAS we chose to include all types of fluoropyrimidine-induced toxicity, as we aim to improve fluoropyrimidine treatment by reducing all types of toxicity. Compared to the GWAS of O'Donnell *et al.* [22], we offer a cohort with clinical patient data representative of daily clinical care, in order to identify variants which could be clinically relevant. Compared to the GWAS of Fernandez-Rodzilla *et al.* our cohort is much larger.[24]

When applying prospective *DPYD* genotyping, still 23% of patients treated with fluoropyrimidines experience severe toxicity.[5] In order to further reduce this number, other genetic variants predictive for severe toxicity need to be identified. Options are to screen for rare variants in *DPYD*, investigate epigenetics, or look outside of the *DPYD* gene as was performed in this GWAS. The onset of severe toxicity might not only be linked to the start of fluoropyrimidines, but can be multifactorial and linked to patient characteristics or co-medication. In line of that thought, the onset of toxicity might be better predicted by multiple genetic variants. For this reason, we executed the PRS analysis. In addition, the future of genotyping is quickly evolving, with less single SNP-based assays and more assays with a panel of SNPs in genes or assays sequencing entire regions of genes, leading to future possibilities to apply a PRS in patients in clinical care. Our PRS analysis showed that a panel of 5,055 SNPs combined with clinical covariates outperforms clinical parameters alone, and can predict 96% of severe fluoropyrimidine-induced toxicity. Our PRS analysis possibly shows too optimistic results due to the pre-selection of significant SNPs into the score. Although we used cross-validation to verify the score, this step did not include the SNP selection as this would have been computationally prohibitive. We see this as an exploratory analysis that needs validation, but still suggests that low penetrance variants exist which are difficult to prove in a single-variant association test. The PRS analysis shows the possibility of future research with a multifactorial research approach. The panel of SNPs needs replication in a validation cohort and additional research is needed to be able to link the result to a dose adjustment advice in order to prevent toxicity.

In conclusion, while no genome-wide significant SNPs could be identified in our unique dataset of patients, six variants were suggestive for the onset of severe toxicity. These variants are located outside of known fluoropyrimidine-pathway genes. Using a PRS consisting of 5,055 SNPs combined with clinical variables explained 96% of toxicity in an optimistic analysis, suggesting highly polygenic nature of toxicity predisposition. This GWAS is one of the first attempts to identify variants predictive for fluoropyrimidine-induced toxicity. The identified variants and the PRS require replication in an independent cohort.

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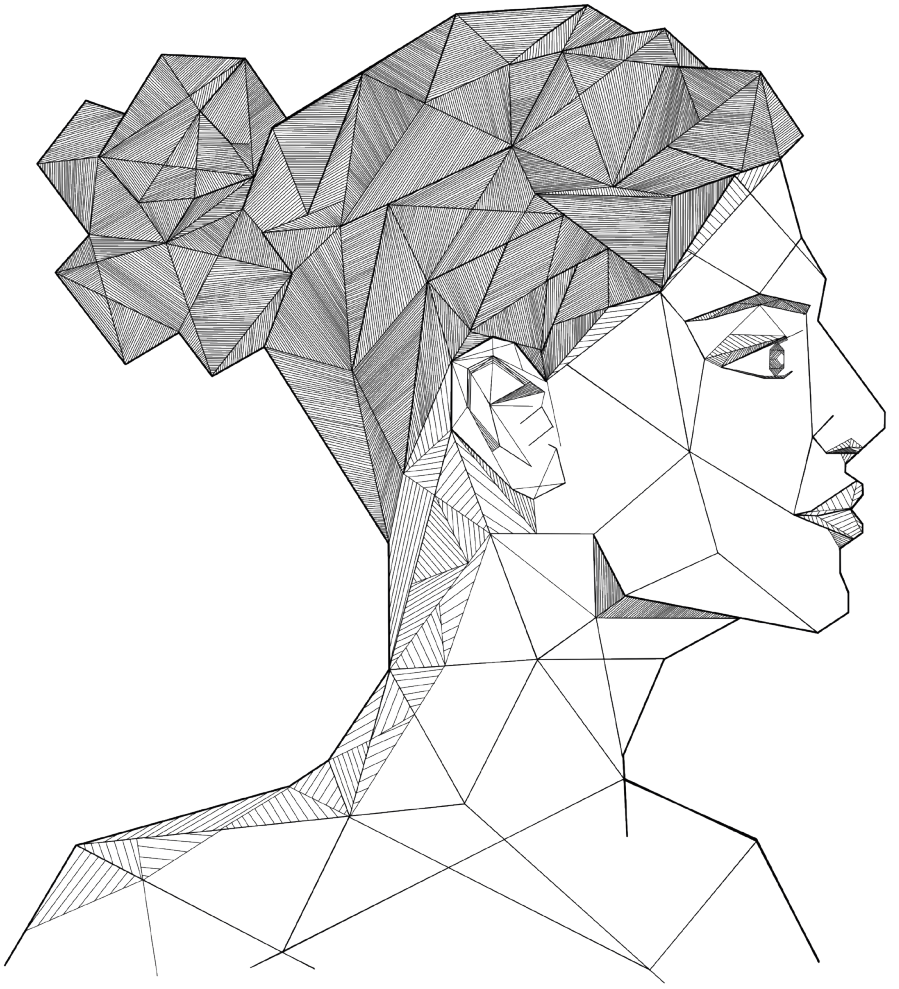
GENERAL DISCUSSION, SUMMARIES AND APPENDIX



CHAPTER 13

General discussion and future perspectives

Introduction

Severe (grade ≥3) toxicity remains a significant problem in treatment with fluoropyrimidines such as 5-fluorouracil (5-FU) and capecitabine. Personalised medicine, specifically *DPYD* genotyping, is a promising strategy to predict and prevent severe fluoropyrimidine-induced toxicity. This thesis focusses on reducing the risk of severe fluoropyrimidine-induced toxicity by optimizing *DPYD* genotyping and improving implementation of *DPYD* genotyping in daily clinical care. In addition, we investigate DPD phenotyping and innovative genotyping techniques beyond current *DPYD* pharmacogenetics (PGx) to prevent severe fluoropyrimidine-induced toxicity.

Personalised medicine: why choose pharmacogenetics (PGx)?

Up to 30% of patients treated with fluoropyrimidines experience severe treatment-related toxicity. Besides the direct consequences of severe fluoropyrimidine-induced toxicity, it additionally can affect patients' quality of life and efficacy of the therapy can be reduced when treatment cannot be resumed due to toxicity. A major contributor to the onset of severe fluoropyrimidine-induced toxicity is a reduced activity of the enzyme dihydropyrimidine dehydrogenase (DPD), as has been described since the eighties in several case reports.[1-3] Patients with a complete deficiency for DPD are rare (~0.1%) and have shown neurological disorders, such as convulsion, seizures and epileptic attacks.[4-7] Yet, there is great variation between patients. Also, patients who are partially DPD deficient generally do not show any phenotypic features. In order to predict and prevent severe fluoropyrimidine-induced toxicity, DPD deficient patients must be identified prospectively and treated individually (personalised medicine).

One way to identify DPD deficient patients, is to measure the DPD enzyme activity in peripheral blood mononuclear cells (PBMCs).[2,8,9] However, the method is not widely used since feasibility in clinical practice is difficult due to substantial costs, complex sample logistics and specific equipment required for the radio assay. In addition, there is substantial intra patient variability (up to 25%) in DPD enzyme activity, possibly caused by circadian rhythm.[10,11] An estimated 3—8% of the patients is DPD deficient. Therefore it is important to have inexpensive diagnostics for DPD deficiency, as all patients receiving fluoropyrimidines need to be tested while the majority of the tested patients does not require an adjusted dose or therapy. When a treatment plan has been decided, it is important to start the chemotherapy as soon as possible, thus short turn-around times of a test are essential as well.

Multiple genetic variants in *DPYD*, the gene encoding for DPD, lead to altered DPD enzyme activity.[12] Identifying such *DPYD* variants can indirectly identify DPD deficient patients. There are relatively quick, easy and inexpensive methods available to perform genotyping, therefore upfront *DPYD* genotyping can be used successfully to apply personalised medicine of fluoropyrimidines (pharmacogenetics, PGx).[13] This was shown in a prospective clinical trial by Deenen *et al.*[14] Prospective genotyping of the variant *DPYD**2A, followed by initial dose reductions in heterozygous carriers, reduced the risk of severe fluoropyrimidine-induced toxicity in these patients significantly. Also, this study showed that the genotyping approach did not increase costs, despite the fact that only 1.1% of tested patients was a carrier of the

*DPYD**2A variant. In chapter 5 and chapter 6, we have shown similar results, i.e. increasing patient safety without increasing treatment costs, for prospective genotyping of four *DPYD* variants (*DPYD**2A, rs3918290, c.1905+1G>A, IVS14+1G>A; c.1679T>G, *DPYD**13, rs55886062, I560S; c.1236G>A/HapB3, rs56038477, E412E; and c.2846A>T, rs67376798, D949V).[15,16]

Feasibility of *DPYD* genotyping in daily clinical care was shown in chapter 8 of this thesis.[17] *DPYD* genotyping at the Leiden University Medical Center (LUMC) was investigated, starting with the introduction as routine care in April 2013 until the end of the observation period in December 2014. This study showed that the implementation of *DPYD* genotyping was first characterised by a learning or acceptance curve, but was feasible thereafter in a real world clinical setting with 90-100% of the patients treated with fluoropyrimidines being genotyped. The dose adherence in this study was 90% instead of 100%, due to concerns of oncologists to reduce the dose in a *DPYD* variant allele carrier about to start chemoradiation therapy. The doubt was caused by the fact that fluoropyrimidine dosages in chemoradiation therapy are already lower compared to fluoropyrimidine dosages in other treatment regimens, and further reduction of the fluoropyrimidine dose could result in underdosing. To remove the uncertainty on fluoropyrimidine dose reductions in *DPYD* variant allele carriers who will receive chemoradiation therapy, we investigated this specific group in chapter 7.[18] *DPYD* variant allele carriers treated with regular fluoropyrimidine doses in chemoradiation therapy experienced more severe toxicity compared to *DPYD* variant allele carriers treated with reduced fluoropyrimidine doses in chemoradiation therapy, showing dose reductions are required as well in this treatment regimen.

The abovementioned studies show that *DPYD* genotyping to reduce severe fluoropyrimidine-induced toxicity is a useful strategy for all patients starting treatment with fluoropyrimidines. Both implementation of *DPYD* genotyping and adherence to a dose advice is feasible in a real world clinical setting.

Resistance and acceptance in implementation of *DPYD* genotyping

Despite substantial evidence on the association between *DPYD* variants and the onset of severe fluoropyrimidine-induced toxicity,[19-26] implementation of *DPYD* genotyping in clinical practice remained limited.[27,28] To improve uptake of genotyping an opinion review (chapter 2) was written, in which arguments for and against genotyping were discussed.[29] One of these arguments against genotyping was that a randomized clinical trial (RCT) is necessary to obtain the required evidence on *DPYD* genotyping prior to implementation. As described in chapter 2, there was one attempt to perform such an RCT. Dose adjustments were applied based on the prospectively determined *DPYD* genotype and DPD phenotype of patients in arm A, compared to patients in arm B who were retrospectively analysed and treated with full dose. This trial was stopped prematurely due to ethical reasons, and was later published in 2017.[30] Patients were in fact not randomized, as inclusion in either study arm was dependent on current practice of each participating institution and some patients were thus predestined to receive treatment in the control arm. However, this large trial of the group of Boisdron-Celle *et al.* was closest to the set-up of an RCT thus far performed and results were long awaited for. Unfortunately, significant differences in the frequency of DPD

deficient patients between study arms at baseline were detected, with more DPD deficient patients in the retrospectively screened study arm. This results in bias and could lead to the expectation of lower toxicity in the prospectively screened study arm, regardless of applying their multi-parametric approach.[31] Due to the available evidence on the increased risk of toxicity in DPD deficient patients or *DPYD* variant allele carriers, most researchers consider it unethical to perform an RCT and no further attempts are to be expected. Therefore, evidence from an RCT will never be gathered. In addition to this, it was debated that adequate (pharmacogenetic) evidence can also be provided by small-scale, innovative, prospective interventional studies,[32] and indeed, some other predictive biomarkers were previously implemented in clinical care without evidence from an RCT.[29] In the study of Deenen *et al*, a historic cohort of patients who appeared to be carrier of *DPYD**2A after treatment with fluoropyrimidines, was used to compare severe toxicity between groups.[14] The use of a historic cohort was applied as well in the clinical trial presented in this thesis (chapter 5).[15] Considering ethics, this study set-up is the best possible method to collect evidence in a prospective way, since an RCT is not possible.

Besides the lack of evidence from an RCT, there are other arguments against *DPYD* genotyping. The fear of underdosing patients is an often used argument not to implement *DPYD* genotyping. However, both the study of Deenen *et al.* and our study (chapter 5) show that *DPYD* variant allele carriers who received initial dose reductions have comparable 5-FU levels or 5-FU metabolite levels to *DPYD* wild-type patients treated with a standard dose,[14,15] therefore differences in efficacy are less likely. Secondly, treating physicians could increase fluoropyrimidine dosages in *DPYD* variant allele carriers during treatment based on the onset of severe toxicity (dose titration). In 55% of the *DPYD* variant allele carriers in whom the dose was increased during treatment, treatment had to be stopped or the dose had to be reduced again due to toxicity. Lastly, a recently published matched pair analysis by Henricks *et al.* showed no differences in efficacy, measured as overall survival and progression-free survival, between carriers of *DPYD**2A treated with a reduced dose and *DPYD**2A wild-type patients.[33] These results indicate that the fear of underdosing is unjustified.

Many of the arguments against *DPYD* genotyping can be refuted with the current evidence in favour of *DPYD* genotyping. Unfortunately, negative opinions on *DPYD* genotyping will always exist and maybe not everyone can be convinced. 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.[27] Yet, in order to align patient care, guidelines of health care authorities should be available.

Recommendations and guidelines

The Food and Drug Administration (FDA) state warnings or contraindications for the use of 5-FU or capecitabine in DPD deficient patients, however does not recommend to test for DPD deficiency.[34] No formal recommendations on DPD deficiency testing prior to treatment are given by health authorities, regulatory agencies or guideline committees from the National Comprehensive Cancer Network (NCCN) or American Society of Clinical Oncology (ASCO). In March 2018, the European Medicine Agency (EMA) has asked the involved pharmaceutical companies to update the Summary of Product Characteristics (SPC) of capecitabine by

including information on *DPYD* genotyping and the associated risk of severe fluoropyrimidine-induced toxicity.[35] An updated SPC, including a paragraph on *DPYD* genotyping, is attached to the European Public Assessment Report (EPAR) for capecitabine on the EMA website.[36] They state that genotyping of four variants is recommended, and variant carriers should be treated with extreme caution. Yet, it cannot be excluded that patients with a negative result can experience severe toxicity. The European Society for Medical Oncology (ESMO) explicitly states that they do not recommend upfront routine testing for DPD deficiency,[37] which was publicly questioned.[38,39] In October 2018, the results of chapter 5 were presented at the ESMO conference and the presenter suggested to ESMO to update their guidelines. In the Netherlands, updated guidelines (September 2017) for colorectal carcinoma from the Dutch Society of Medical Oncology clearly state that *DPYD* genotyping is recommended prior to treatment with fluoropyrimidines.[40] These updated guidelines were of assistance in the uptake of prospective *DPYD* genotyping in the Netherlands, which implies that the lack of official recommendations on pre-therapeutic genotyping is limiting the process of implementation of *DPYD* genotyping in other countries.

Dosing recommendations for *DPYD* genotyping

There are several pharmacogenetic dosing guidelines available for the use of fluoropyrimidines in *DPYD* variant allele carriers published by the Clinical Pharmacogenetics Implementation Consortium (CPIC), the Dutch Pharmacogenetics Working Group (DPWG) established by the Royal Dutch Pharmacists Association (KNMP), the French Network of Pharmacogenetics (RNPGx) and the Italian Association of Medical Oncology (AIOM-SIF, *unpublished guidelines, edited by the AIOM-SIF Working Group*).[41-43] In addition to dosing guidelines, the DPWG also describes an implication score in which *DPYD* genotyping is considered 'essential', directing *DPYD* genotyping prior to treatment with fluoropyrimidines (chapter 4). Both CPIC and DPWG guidelines recommend to treat carriers of the *DPYD**2A and *DPYD**13 variants with a 50% dose reduction. CPIC recommended to treat carriers of the c.2846A>T and c.1236G>A variants with a 25–50% dose reduction due to limited evidence for these variants, compared to the DPWG who recommended a 25% dose reduction. These dose reductions are based on the functional effect of a variant on the DPD enzyme activity and represent an expected remaining DPD enzyme activity, as described in chapter 3.[44] However, after publication of chapter 5, both groups discussed the results of this study and the possibility to adjust the recommendation from a 25% dose reduction to a 50% dose reduction for variants c.2846A>T and c.1236G>A/HapB3. This has resulted in an update from CPIC published online November 2018, in which dose reductions of 50% are recommended for all four *DPYD* variants.[45] An update from the DPWG is expected soon and will be implemented in the guideline.

In chapter 5 we indeed describe that a 25% dose reduction seems inadequate to reduce the risk of severe toxicity in carriers of c.2846A>T and c.1236G>A to the risk of severe toxicity for *DPYD* wild-type patients. We could not provide evidence that a 50% dose reduction is the best option for these patients. In fact, for carriers of c.2846A>T a 35% dose reduction seems more logical, which is based on the median DPD enzyme activity (67% of *DPYD* wild-type patients) and the additional dose reductions made by physicians in carriers of c.2846A>T (average dose titration from 73 to 64% during treatment) in our clinical trial (*unpublished*

data). On the other hand, a 35% dose reduction for carriers of c.2846A>T is not proven to be more adequate compared to a 50% dose reduction. In addition, a 50% dose reduction would be more feasible in clinical practice. The c.1236G>A variant has a large variation in DPD enzyme activity with a median of 74% activity of *DPYD* wild-type patients in our study. However, our study showed that a 25% dose reduction in carriers of c.1236G>A did not result in a reduction of the relative risk for these patients, as some patients require a larger dose reduction.[15] As was commented by Amstutz and Largiader, our study would support a 50% dose reduction in carriers of both c.2846A>T and c.1236G>A, provided that this should be used as a starting dose.[46] Further dose adaptations guided by the onset of toxicity (dose titration) are possible and should be applied slowly, as fluoropyrimidine-induced toxicity can occur with a certain delay.

Currently, there are no specific recommendations available on how to apply these additional dose adaptations. Recently, Kleinjan *et al.* retrospectively investigated dose escalations in *DPYD* variant allele carriers according to a local pre-specified protocol.[47] Eleven *DPYD* variant allele carriers were identified, of which six patients (55%) received a dose escalation of 15%. In two patients, the dose had to be reduced again due to toxicity, resulting in a median dose escalation of 9%. In two *DPYD* variant allele carriers (18%) the initially lower dose was further reduced. In the clinical trial (chapter 5) no pre-specified protocol was available for dose adjustments. We identified 85 *DPYD* variant allele carriers. In eleven patients (13%) the dose was increased by 21% on average, yet in five patients the dose had to be reduced again and one patient had to stop treatment, resulting in a mean dose escalation of 13%. In ten patients (12%) initially lower dosages were further reduced by 20% on average. Without a pre-defined protocol, the dose was increased in fewer patients, yet the dose adjustment steps were larger. The dose reductions applied after a dose escalation point out the importance of slowly applying dose escalations in relatively small steps. The additional dose reductions required after the low initial dose, again point out the variation in DPD enzyme activity in *DPYD* variant allele carriers, and could explain the higher overall severe toxicity rates in *DPYD* variant allele carriers of the clinical trial (39% versus 23% for wild-type patients).[15]

Dose adjustments after exposure to 5-FU or capecitabine

Therapeutic drug monitoring (TDM) is a useful method to guide dose adaptations after start of therapy. Unfortunately, the use of TDM for fluoropyrimidines in the Netherlands is limited as the wide majority of patients (approximately 90%) are prescribed capecitabine over 5-FU. For TDM of 5-FU defined target ranges and dosing algorithms are available.[48-50] Yet, the intracellular conversion of capecitabine into 5-FU and its metabolites result in low plasma concentrations of capecitabine and its metabolites, which makes it more difficult to develop TDM protocols for capecitabine.[51] Until such protocols have been established, TDM of fluoropyrimidines in the Netherlands will be used sparingly. Furthermore, TDM can be used to monitor drug levels after start of treatment, not to determine initial dose reductions in order to prevent quick-onset severe fluoropyrimidine-induced toxicity.

A method to determine if initial dose adaptations in patients are required, is to expose the patient prior to treatment to a 5-FU test dose of 250 mg/m^2.[52,53] After the test dose, 5-FU

and 5-fluoro-5,6-dihydrouracil (5-FDHU) plasma levels are used to calculate pharmacokinetic parameters. In a study setting, three patients had marked alterations in pharmacokinetic parameters and possibly severe toxicity was avoided by changing the 5-FU treatment into irinotecan treatment.[52] The 5-FU test dose did not result in side effects in any of the patients in this study, which questions the suitability of this test dose, as the metabolizing enzyme DPD has a certain overcapacity. As was stated by van Staveren *et al.,* a test dose of uracil of 500 mg/m^2 fully saturates the DPD enzyme.[54]

Implementation of *DPYD* genotyping in the Netherlands

Three Dutch hospitals participated in the study of Deenen *et al.*, applying *DPYD**2A genotyping in over 2,000 recruited study patients between May 2007 and October 2011. Thereafter, more studies on *DPYD* variants and their association with the onset of severe fluoropyrimidine-induced toxicity became available. Within this period, some hospitals in the Netherlands implemented routine *DPYD* genotyping of all patients starting fluoropyrimidines, e.g. the LUMC in April 2013 and the Maastricht University Medical Center in 2013 as well.[9,17] In April 2015 we started recruiting patients in our prospective study (chapter 5).[15] Seventeen hospitals in the Netherlands participated in this study and implemented or outsourced *DPYD* genotyping either for study patients only or for all patients starting fluoropyrimidine treatment. In 2016, a survey was published in the Dutch Medical Oncology Journal.[55] This survey was sent to oncologists in the Netherlands. Some remarkable results were found. First, 65% of the responders answered that DPD status was determined as standard for all patients starting treatment with fluoropyrimidines. Second, 80% of the oncologists used *DPYD* genotyping to determine DPD deficiency, compared to 15% of responders who used a DPD phenotyping test. Possibly these results were a little overestimated, as physicians who had experience with requesting these tests were more likely to reply to the survey compared to physicians who did not order DPD deficiency tests. Also, the results of the survey were not adjusted based on the number of respondents per hospital, which could give a misleading image on the status of *DPYD* genotyping in the Netherlands in 2016. Yet, it is clear that the use of *DPYD* genotyping in the Netherlands is ahead of the use in many other countries. Some research groups in France, the UK, Italy, Germany and the USA were able to implement *DPYD* genotyping, whether or not combined with DPD phenotyping, in their hospital or clinical institute and surrounding centres.

Other aspects of implementation

Treatment costs for patients did not increase when applying prospective genotyping of *DPYD**2A, or *DPYD**2A, *DPYD**13, c.2846A>T and c.1236G>A, as was shown by Deenen *et al*. and in chapter 6 of this thesis.[14,16] Expanding the genotyping panel from one variant to four variants did not increase the costs of genotyping much, while more patients at risk could be identified, and thus more (costs of) severe toxicity could be prevented. Currently, most hospitals can offer *DPYD* genotyping tests for approximately €100. Genotyping assays are becoming less expensive despite the addition of more variants to a genotyping panel, therefore it is expected that *DPYD* genotyping will probably remain cost-neutral. However, this holds to a current extend. If the panel of predictive variants becomes too large to be

genotyped with current genotyping techniques, and more expensive genotyping techniques need to be used, it is uncertain if *DPYD* genotyping remains cost-neutral. For example, at this moment sequencing the entire *DPYD* gene is too expensive to be used in a daily clinical care setting. Also, reimbursement for *DPYD* genotyping costs in the Netherlands is not (yet) covered by nationwide health care insurances. Therefore, hospitals in the Netherlands will cover costs in different ways, which leads to differences in health care between patients.

In chapter 9 we describe the dilemma of required confirmation practice as a quality control aspect of PGx testing.[56] Implementation of *DPYD* genotyping will benefit from the inexpensiveness of current genotyping arrays. Yet, as PGx tests are usually only executed once in a lifetime, it is of utmost importance to have a correct genotyping result. When applying the most adequate, but comprehensive, confirmation method, i.e. executing a second, independent genotyping assay, erroneous results can be discovered. In this study we discovered that, even after extensive validation, erroneous results can still occur due to misclassification of a genotype, e.g. caused by allele dropout. Despite the increase in costs and labour, a confirmation method is useful for genetic tests with a high clinical impact, such as *DPYD* testing. We also showed substantial variability between laboratories in the use of a second, independent technique for PGx testing. As is the case for applying *DPYD* genotyping in the first place, clear guidelines are required to align confirmatory laboratory practices for PGx as well.

Currently, mostly assays testing single variants are used to genotype *DPYD*. In case of a compound heterozygous *DPYD* variant carrier, a patient who carries multiple different *DPYD* variants, the genotyping result cannot be translated into a dose recommendation when phasing information (the allelic location of variants) is missing. Compound heterozygous *DPYD* variant allele carriers are at increased risk of severe fluoropyrimidine-induced toxicity when dose reductions cannot be applied. In chapter 11, we describe seven cases and examine diagnostic and therapeutic strategies for fluoropyrimidine treatment of patients carrying multiple *DPYD* variants.[57] The additional genotyping methods investigated in this study are still in early phases of development or currently too expensive to implement in clinical care, compared to a well-established DPD-phenotyping test. Therefore, we concluded to execute a phenotype test in these patients in order to determine a safe starting dose. When genotyping techniques which can determine the phasing of variants, such as long-read sequencing, will become less expensive in the future and are implemented in clinical care, phasing of variants of compound heterozygous *DPYD* variant allele carriers will be known directly and these patients can be treated according to dosing guidelines.

The probability of identifying a compound heterozygous *DPYD* variant carrier is low, yet while completing this chapter, five other patients were discovered in several genotyping facilities in the Netherlands, showing that this is a clinically relevant issue. Some of these patients were identified prospectively, after which the advice was given to determine the DPD enzyme activity. One patient was a carrier of three *DPYD* variants (*DPYD**2A, c.2846A>T and c.1236G>A) and was treated safely with a 40% dose based on the results of an executed DPD enzyme activity measurement. The other patients were carriers of two *DPYD* variants in different combinations (*DPYD**2A + c.2846A>T, c.2846A>T + c.1236G>A and *DPYD**13 + c.2846A>T).

Beyond current *DPYD* genotyping

It is known that *DPYD* variants are not the only risk factor for DPD deficiency, and DPD deficiency is not the only risk factor for severe fluoropyrimidine-induced toxicity. Approximately 17% of patients experiencing severe fluoropyrimidine-induced toxicity can be identified as carriers of one of the four currently genotyped *DPYD* variants. 39-61% of the patients who experienced severe toxicity were identified as DPD deficient patients, thus it was estimated that less than half of the DPD deficient patients could be identified by the four currently genotyped *DPYD* variants.[58] In order to increase the predictability of severe fluoropyrimidine-induced toxicity, we must better predict risk factors for DPD deficiency, and additionally look into factors outside of DPD. Recently, a study was published in which eight years of combining genotyping and phenotyping tests were described.[9] This study showed that only 25.3% of the DPD deficient patients was a carrier of one of the four currently genotyped *DPYD* variants. Patients with a DPD deficiency, but who did not carry the *DPYD**2A variant, were genotyped for the entire coding region of *DPYD*. DPD deficiency could be explained by *DPYD* variants in 23% of these patients. This results in an expected approximately 42% of DPD deficiency related to *DPYD* variants. Variants in other regions, which have not been sequenced before, could still contribute to DPD deficiency. Unfortunately, the abovementioned study had no toxicity data of the patients, thus the prediction of *DPYD* variants for DPD deficiency could be made, but not the prediction for severe toxicity.

It is clear that not only *DPYD* variants are involved in the onset of severe fluoropyrimidine-induced toxicity. Therefore the DNA of the patients participating in chapter 5 was analysed by genome-wide association study (GWAS), in order to discover novel variants related to the onset of severe fluoropyrimidine-induced toxicity. This study was described in chapter 12. Approximately 700,000 single nucleotide polymorphisms (SNPs) in different genes were genotyped, and imputed to over four million SNPs. While no genome-wide significant SNPs could be identified, six variants were suggestive for the onset of severe toxicity. These variants warrant replication in an independent cohort. After validation, variants can be added to the prospective genotyping panel. In addition to the variants in chapter 12, validation is required for all newly identified variants. For example, some newly identified variants were recently presented in a series of patients who experienced severe toxicity,[59] yet it is unclear if these variants could also be identified in patients who did not experience severe toxicity, and thus the clinical value of these variants needs to be determined. As described by Ciccolini *et al.* in 2010, both genetic and epigenetic factors, such as promotor hyper methylation or variations in transcriptional factor expression, play a role in *DPYD* dysregulations,[60] and should be a focus of future research in *DPYD* genotyping.

Phenotyping assays

DPD phenotyping could also be used to predict severe fluoropyrimidine-induced toxicity. As described before, the DPD enzyme activity measurement in PBMCs is a well-established method to determine DPD activity.[2,8,9] Additionally, DPD phenotyping assays were developed, such as the 2-^{13}C uracil breath test,[61-63] the uracil loading dose,[54,64] endogenous dihydrouracil/uracil (DHU/U) ratio and endogenous uracil concentrations.[65,66] The status of each DPD

phenotyping assay was summarized in two reviews.[58,67] Advantages and disadvantages per assay were discussed, such as the limited feasibility of an assay in clinical practice, lack of calculated test parameters (i.e. sensitivity, specificity), or lack of clear threshold values for patients who are prone to develop severe fluoropyrimidine-induced toxicity. In chapter 10, we executed a first-time head-to-head comparison of four DPD phenotyping assays in a patient cohort which was not selected based on –or enriched for– (severe) toxicity, but represents a daily clinical care patient cohort. We could not show associations with DPD deficiency or the onset of severe fluoropyrimidine-induced toxicity. The latter is possibly due to the fact that only ~30—50% of severe fluoropyrimidine-induced toxicity can initially be explained by DPD deficiency.[68] Previously it was described that clinical validity and utility were not yet determined for all phenotyping assays,[58] yet with this study we were unable to fully complement this lack of evidence. In order to determine the clinical value of DPD phenotyping assays additional research is required. DPD phenotyping assays, whether or not combined with *DPYD* genotyping, are already used in clinical care in some centres to predict and prevent toxicity. Yet, it is clear that additional research should be performed in order to determine and compare the clinical value of DPD phenotyping assays.

FUTURE PERSPECTIVES

Dosing algorithms

It is clear that toxicity is not caused by a single factor, but is due to a combination of multiple risk factors. In order to be able to predict and prevent severe fluoropyrimidine-induced toxicity in a larger number of patients, multiple risk factors should be taken into account. An algorithm in which multiple factors are included, can be used to calculate the total risk of severe toxicity and potentially required dose adjustments. This algorithm should include the abovementioned four *DPYD* variants, as they are proven to be associated to the onset of severe toxicity. However, the algorithm should be expanded by including other factors.

In an ongoing study, we investigate rare variants in *DPYD* by means of sequencing, as they might be predictive for the onset of severe fluoropyrimidine-induced toxicity. Besides the current four *DPYD* variants, identified rare *DPYD* variants, variants outside of the *DPYD* gene, or variants in modifier gene regions, could be added to the algorithm in the future when their association with toxicity has been validated. Possibly, a large panel of genetic variants could be used to calculate the 'genetic' risk, so-called polygenic risk score, which is increasingly being applied in research. Depending on which variants from the panel are identified in the patient, the patient has a different risk to develop severe toxicity.

The algorithm could also be supplemented by non-genetic factors, as they can play a role in the onset of (severe) toxicity. For example, results of phenotyping assays for DPD or other enzymes involved in the metabolism of 5-FU related to severe toxicity,[69] could be included in the algorithm. In addition, baseline characteristics of patients, such as age, gender, performance status or renal dysfunction, were described as risk factors for toxicity.[70-74] Also therapy-related factors, such as dosing schedule or co-medication, could influence the risk of toxicity.[75] Not all of the abovementioned risk factors have a similar effect on (severe) toxicity, therefore each risk factor included in the algorithm should have a corresponding

weighing factor, depending on the severity of the risk.

In addition to analysing *DPYD* genetics, baseline characteristics of patients and therapy-related factors to develop dosing algorithms, ethnicity should also be taken into account. The current four *DPYD* variants associated to the onset of severe fluoropyrimidine-induced toxicity are mainly identified in Caucasian patients. *DPYD**2A and c.2846A>T have been identified in ~0.1% in African-Americans, compared to a frequency of ~1% in Caucasians.[76-78] Novel deleterious *DPYD* variants can be identified in different ethnic populations, as was recently shown for an East African population.[79] Dosing algorithms might not predict DPD activity correctly in patients who are not Caucasian, depending on the variants included in the algorithm.

Current genotyping techniques are mostly single SNP-based assays or chip-based assays. In the near future extensive sequencing techniques will become less expensive and more available for daily practice in the laboratories of hospitals, or hospitals can outsource genotyping to special genotyping facilities. An increasing amount of genotyping data of patients will be known in a shorter period of time, and should be linked to clinical patient data in order to first translate the genotype into a prediction for toxicity, and second, the data can be used to complement and perfect the algorithm. The question that remains is, can we build an algorithm which can predict the majority of severe fluoropyrimidine-induced toxicity? When all previously reported risk factors for toxicity are validated and included, and when the complete genotype of patients is taken into account, what risk factors will remain to be discovered?

The future of fluoropyrimidines

5-FU has been used to treat cancer for decades and the first studies on DPD deficiency were published in the eighties.[1-3] Now, capecitabine is the preferred drug of use over 5-FU in various tumour types in several countries, including the Netherlands. To improve efficacy of cancer therapy, fluoropyrimidines are combined with several other anticancer drugs, yet they remain the backbone of therapy for a substantial number of tumour types.

To conclude with the following quote by Hamzic *et al*.: “While additional genetic factors or phenotyping approaches may complement pharmacogenetic testing in the future, *DPYD* genotyping provides an important tool that is available today to identify patients at increased risk of severe adverse effects from fluoropyrimidine-based therapies”.[80]

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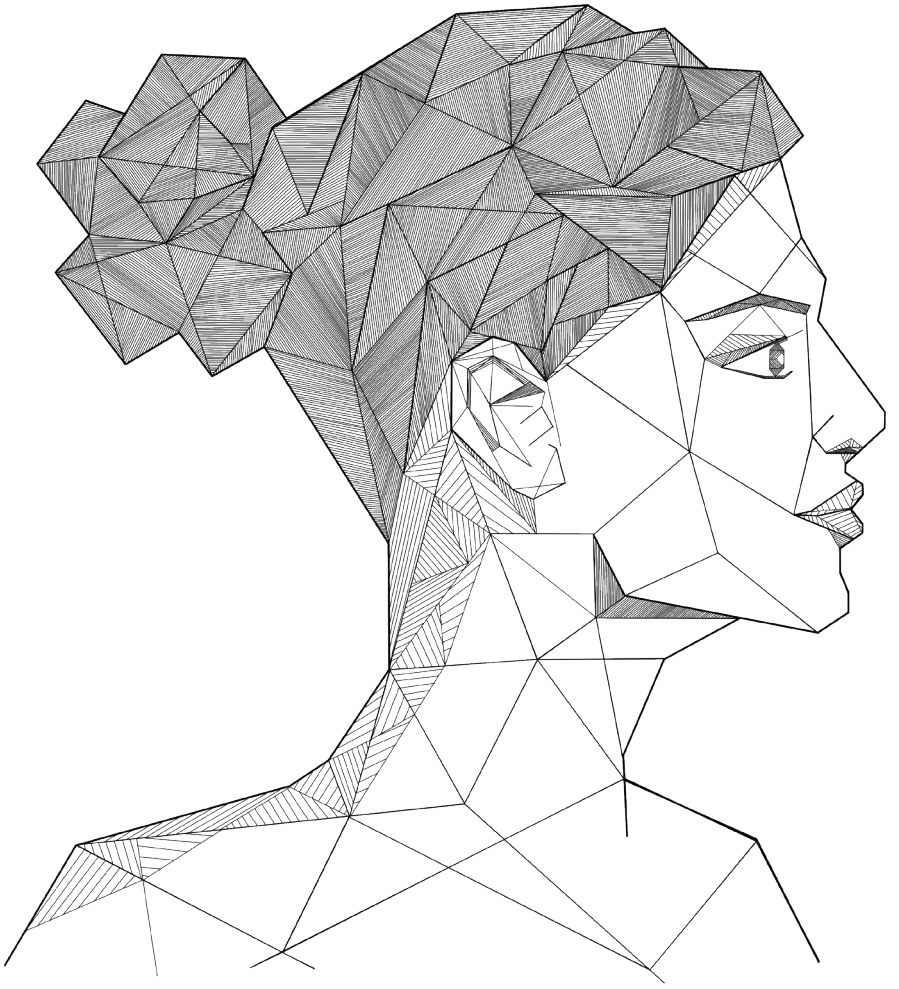


CHAPTER 14

Summary

Fluoropyrimidines, such as 5-fluorouracil (5-FU) and its oral pro-drug capecitabine, are widely used anti-cancer drugs in the treatment of several tumour types. Despite ample experience with these drugs, severe adverse drug reactions occur in up to 30% of patients treated with fluoropyrimidines. Over 80% of 5-FU is inactivated by the enzyme dihydropyrimidine dehydrogenase (DPD), which is encoded by the gene *DPYD*. Because of this, DPD plays an important role in the development of adverse drug reactions, mentioned here as toxicity. To prevent severe fluoropyrimidine-induced toxicity, it is important to identify patients who have an increased risk of toxicity and treat them in a personalised way. In other words, it is important to identify patients with a deficient DPD enzyme and treat them with reduced fluoropyrimidine dosages. Research has been executed on DPD deficiency, or variants in the *DPYD* gene, and the association with severe fluoropyrimidine-induced toxicity. This thesis focusses on reducing the risk of severe fluoropyrimidine-induced toxicity by optimising *DPYD* genotyping and improving implementation of *DPYD* genotyping in daily clinical care. In addition, we investigated DPD phenotyping and innovative genotyping techniques beyond current *DPYD* pharmacogenetics (PGx) to prevent severe fluoropyrimidine-induced toxicity.

***DPYD* genotyping: proof of principle and implementation in clinical practice**

Despite substantial evidence on the association between *DPYD* variants and the onset of severe fluoropyrimidine-induced toxicity, implementation of prospective *DPYD* genotyping in clinical practice remained limited. Therefore, an opinion review was written (chapter 2). In this review we summarize the available evidence on the association with severe fluoropyrimidine-induced toxicity for four variants in the *DPYD* gene. We discuss several advantages and disadvantages of *DPYD* genotyping. We substantiate why arguments against genotyping are unfounded and advocate implementation of prospective *DPYD* genotyping. In chapter 3 literature was extensively checked to discuss the functional effect of four *DPYD* variants on the DPD enzyme activity. This is converted into a gene activity score for each *DPYD* variant, which represents an expected remaining DPD enzyme activity, and which will be used in PGx guidelines to translate the *DPYD* genotype into a DPD phenotype. PGx guidelines by the Dutch Pharmacogenetics Working Group (DPWG) of the Royal Dutch Pharmacists Association (KNMP) were already present in the Netherlands for *DPYD* and fluoropyrimidines. This guideline is made available outside of the KNMP network in the Netherlands in chapter 4, and provides a dose reduction advice for heterozygous *DPYD* variant allele carriers of the following four *DPYD* variants: *DPYD**2A, rs3918290, c.1905+1G>A, IVS14+1G>A; *DPYD**13, c.1679T>G, rs55886062, I560S; c.1236G>A/HapB3, rs56038477, E412E; and c.2846A>T, rs67376798, D949V. In addition to dosing guidelines, the DPWG also described an implication score in which *DPYD* genotyping is considered 'essential', directing *DPYD* genotyping prior to treatment with fluoropyrimidines.

DPYD genotyping was applied prospectively in a nationwide clinical trial in chapter 5. Patients with an intention to treatment with fluoropyrimidines were genotyped for *DPYD**2A, *DPYD**13, c.2846A>T and c.1236G>A. Heterozygous carriers of a *DPYD* variant were treated with an initially reduced dose of fluoropyrimidine according to the DPWG PGx guidelines at the start of the study. This study showed that prospective *DPYD* genotyping followed by individualised dose adjustments improved patient safety by reducing the risk

of severe fluoropyrimidine-induced toxicity. No treatment-related deaths occurred in *DPYD* variant allele carriers who were treated with a reduced dose. Despite the low frequency of *DPYD* variant allele carriers, executing prospective *DPYD* genotyping did not increase costs, but reduced average costs slightly with €50 per patient, as was shown in the cost analysis of the trial (chapter 6).

Current PGx guidelines do not distinguish fluoropyrimidine dosing recommendations between treatment regimens. Fluoropyrimidine dosages in chemoradiation therapy are substantially lower compared to fluoropyrimidine dosages in other treatment regimens. Therefore, it was unclear if further fluoropyrimidine dose reductions could result in underdosing in *DPYD* variant allele carriers treated with chemoradiation therapy. In chapter 7 we compared severe toxicity between wild-type patients and *DPYD* variant allele carriers who received chemoradiation therapy, the latter group either treated with standard or reduced fluoropyrimidine dosages. *DPYD* variant allele carriers treated with regular fluoropyrimidine doses in chemoradiation therapy experienced severe toxicity more often compared to *DPYD* variant allele carriers treated with reduced fluoropyrimidine doses in chemoradiation therapy, showing dose reductions are required as well in this treatment regimen.

The feasibility of implementing prospective *DPYD* genotyping in daily clinical care was shown in chapter 8 of this thesis. The first 21 months of *DPYD* genotyping at the Leiden University Medical Center (LUMC) were investigated, starting with the introduction as routine care in April 2013 until the end of the observation period in December 2014. This study showed that the implementation of *DPYD* genotyping was first characterised by a learning or acceptance curve, but was feasible in a real world clinical setting with 90–100% of the patients treated with fluoropyrimidines being genotyped. This study also showed 90% dose adherence.

Another aspect of (*DPYD*) genotyping is the certainty of a test result, and the consequences of an erroneous result. In chapter 9 we describe the dilemma of confirmation practice as a quality control aspect of PGx testing. We discuss if it should be required to have two independent genotyping assays to correctly determine a genotype. In this study we discovered that, even after extensive validation, erroneous results can still occur due to misclassification of a genotype, e.g. caused by allele dropout. Despite the increase in costs and labour, a confirmation method is useful for genetic tests with high clinical impact, such as *DPYD* testing. Clear guidelines will help to align confirmatory laboratory practices for pharmacogenetics, which may need to be specified per gene and test.

Beyond current *DPYD* pharmacogenetics

In the first part of this thesis we describe how to reduce severe fluoropyrimidine-induced toxicity by *DPYD* genotyping of *DPYD**2A, *DPYD**13, c.2846A>T and c.1236G>A. Yet, it is known not all severe fluoropyrimidine-induced toxicity can be predicted by these four variants alone. Therefore, we investigated other options, beyond genotyping of the current four *DPYD* variants, to reduce severe fluoropyrimidine-induced toxicity. This is shown in the second part of this thesis, entitled “beyond current *DPYD* pharmacogenetics”.

In chapter 10, we present a first-time head-to-head comparison study of four DPD

phenotyping assays in a patient cohort which was not selected based on –or enriched for– (severe) toxicity. The goal was to determine the clinical value of each DPD phenotyping assay, by assessing clinical validity parameters (e.g. sensitivity and specificity) for DPD deficiency and the onset of severe fluoropyrimidine-induced toxicity. We could not show associations with DPD deficiency or the onset of severe fluoropyrimidine-induced toxicity. To determine the clinical value of DPD phenotyping assays additional research is required.

In chapter 11 we investigated a special subgroup of *DPYD* variant allele carriers, i.e. the compound heterozygous patients. These patients carry multiple *DPYD* variants and the effect of the *DPYD* variants on the DPD enzyme activity cannot be predicted using the gene activity score. Without dose reductions, these patients have an increased risk to develop severe toxicity. We describe seven cases and examine diagnostic and therapeutic strategies for fluoropyrimidine treatment of patients carrying multiple *DPYD* variants. The additional genotyping methods investigated in this study are still in early phases of development or currently too expensive to implement in clinical care, compared to a well-established DPD-phenotyping test. Therefore, we concluded to execute a phenotype test in these patients to determine a safe starting dose.

It is expected that other enzymes besides DPD, and thus other genes besides *DPYD,* are involved in the onset of severe fluoropyrimidine-induced toxicity. With the genome-wide approach in chapter 12 we aimed to discover other variants, mainly outside the *DPYD* gene, which are associated to the onset of severe fluoropyrimidine-induced toxicity. Approximately 700,000 single nucleotide polymorphisms (SNPs) in different genes were genotyped and imputed to over four million SNPs. We identified six variants suggestive of association to the onset of severe fluoropyrimidine-induced toxicity. In addition, we present an optimistic polygenic risk score analysis, suggesting highly polygenic nature of toxicity predisposition.

With the execution of the clinical trial described in chapter 5, an increasing number of hospitals in the Netherlands applied *DPYD* genotyping prior to start of therapy. An increased uptake in implementation of *DPYD* genotyping was thus visible, especially in the Netherlands. Outside of the Netherlands, great differences exist in the uptake of *DPYD* genotyping, whether or not including DPD phenotyping. In some countries initiatives to implement prospective testing for DPD deficiency are effective, where in other countries great differences in execution of tests exist between centres within that country. Uptake of *DPYD* genotyping will benefit from clear guidelines, i.e. recommendations whom and when to genotype, and dosing recommendations for *DPYD* variant allele carriers.

Currently four *DPYD* variants are included in the genotyping panel, yet it is known these four variants cannot predict all patients who will develop severe toxicity. It is likely other variants are associated to the onset of severe fluoropyrimidine-induced toxicity. To further improve the predictive power of the genotyping panel DPD phenotyping tests can be used, or novel variants can be added to the genotyping panel. Novel variants can be e.g. rare variants in the *DPYD* gene or variants in other genes.

The future of fluoropyrimidines

5-FU has been used to treat cancer for decades. Now, capecitabine is the preferred drug of use over 5-FU in various tumour types in several countries, including the Netherlands.

To improve efficacy of cancer therapy, fluoropyrimidines are combined with several other anticancer drugs, yet they remain the backbone of therapy for a substantial number of tumour types. Ample research on fluoropyrimidines and DPD (deficiency) has been executed. Right now, prospective *DPYD* genotyping should be executed for all patients starting treatment with fluoropyrimidines. Additional research will be executed to continue the search for other factors which could predict the onset of severe fluoropyrimidine-induced toxicity.

CHAPTER 14

Nederlandse samenvatting

Fluoropyrimidines, zoals 5-fluorouracil (5-FU) en haar orale prodrug capecitabine, zijn veel gebruikte kankergeneesmiddelen voor de behandeling van verschillende tumortypen. Ondanks vele ervaring met deze geneesmiddelen, treden ernstige bijwerkingen op bij 30% van de patiënten die met fluoropyrimidines worden behandeld. Meer dan 80% van 5-FU wordt geïnactiveerd door het enzym dihydropyrimidine dehydrogenase (DPD), dat wordt gecodeerd door het gen *DPYD*. Hierdoor speelt DPD een belangrijke rol bij de ontwikkeling van bijwerkingen, hier toxiciteit genoemd. Om ernstige fluoropyrimidine-geïnduceerde toxiciteit te voorkomen, is het belangrijk om patiënten met een verhoogd risico op toxiciteit te identificeren en hen op een gepersonaliseerde manier te behandelen (therapie op maat). Met andere woorden, het is belangrijk om patiënten met een deficiënt DPD-enzym te identificeren en ze te behandelen met gereduceerde fluoropyrimidine-doseringen. DPD-deficiëntie, of varianten in het *DPYD*-gen, en de associatie met ernstige fluoropyrimidine-geïnduceerde toxiciteit zijn reeds onderzocht. Dit proefschrift richt zich op het verminderen van het risico op ernstige fluoropyrimidine-geïnduceerde toxiciteit door *DPYD*-genotypering te optimaliseren en de implementatie van *DPYD*-genotypering in de dagelijkse klinische zorg te verbeteren. Om ernstige fluoropyrimidine-geïnduceerde toxiciteit te voorkomen hebben we daarnaast ook DPD-fenotypering onderzocht en meer innovatieve technieken voor genotypering vergeleken met de huidige farmacogenetica van *DPYD*.

***DPYD*-genotypering: bewijs van het principe en implementatie in de klinische praktijk**

Ondanks substantieel bewijs over de associatie tussen *DPYD*-varianten en het ontstaan van ernstige fluoropyrimidine-geïnduceerde toxiciteit, bleef de implementatie van prospectieve *DPYD*-genotypering in de klinische praktijk redelijk beperkt. Daarom werd een opiniërend review geschreven (hoofdstuk 2). In deze review vatten we het beschikbare bewijs over de associatie met ernstige fluoropyrimidine-geïnduceerde toxiciteit voor vier varianten in het *DPYD*-gen samen. We bespreken verschillende voordelen en nadelen van *DPYD*-genotypering. We onderbouwen waarom argumenten tegen genotypering ongegrond zijn en pleiten voor implementatie van prospectieve *DPYD*-genotypering. In hoofdstuk 3 wordt uitgebreide literatuur samengevat om het functionele effect van vier *DPYD*-varianten op de DPD-enzymactiviteit te bespreken. Dit wordt omgezet in een gen-activiteitscore voor elke *DPYD*-variant, die een verwachte resterende DPD-enzymactiviteit vertegenwoordigt. Deze zal worden gebruikt in farmacogenetica-richtlijnen om het *DPYD*-genotype in een DPD-fenotype te vertalen. Farmacogenetica-richtlijnen van de Nederlandse Farmacogenetica Werkgroep (DPWG) van de Koninklijke Nederlandse Maatschappij ter bevordering der Pharmacie (KNMP) waren reeds aanwezig in Nederland voor *DPYD* en fluoropyrimidines. Deze richtlijn wordt buiten het KNMP-netwerk in Nederland beschikbaar gesteld in hoofdstuk 4 en geeft een advies voor dosisreductie voor heterozygote dragers van *DPYD*-varianten van de volgende vier *DPYD*-varianten: *DPYD**2A, rs3918290, c.1905+1G>A, IVS14+1G>A; *DPYD**13, c.1679T>G, rs55886062, I560S; c.1236G>A/HapB3, rs56038477, E412E; en c.2846A>T, rs67376798, D949V. Naast doseringsrichtlijnen beschreef de DPWG ook een implicatiescore waarin *DPYD*-genotypering als 'essentieel' wordt beschouwd, waarbij *DPYD*-genotypering wordt geadviseerd voorafgaand aan de behandeling met fluoropyrimidines.

DPYD-genotypering werd prospectief toegepast in een landelijke klinische studie in

hoofdstuk 5. Patiënten met een intentie om te starten met fluoropyrimidines werden gegenotypeerd voor *DPYD**2A, *DPYD**13, c.2846A>T en c.1236G>A. Heterozygote dragers van een *DPYD* variant werden bij de start van de therapie behandeld met een initieel gereduceerde dosis fluoropyrimidines volgens de farmacogenetica-richtlijnen van de DPWG. Deze studie heeft aangetoond dat het prospectief genotyperen van *DPYD* gevolgd door geïndividualiseerde dosisaanpassingen de veiligheid van de patiënt verbeterde door het risico op ernstige fluoropyrimidine-geïnduceerde toxiciteit te verminderen. Er zijn geen *DPYD*-variant dragers overleden naar aanleiding van een behandeling met een gereduceerde dosis. Ondanks de lage frequentie van *DPYD*-variant dragers, heeft het uitvoeren van prospectieve *DPYD*-genotypering de kosten niet verhoogd, maar de gemiddelde kosten licht zelfs verlaagd met €50,- per patiënt, zoals werd aangetoond in de kostenanalyse van het onderzoek (hoofdstuk 6).

De huidige farmacogenetica-richtlijnen maken geen onderscheid in fluoropyrimidine-doseringsaanbevelingen tussen de verschillende type behandelingen. Fluoropyrimidine-doseringen in chemoradiotherapie zijn aanzienlijk lager in vergelijking met fluoropyrimidine-doseringen in andere type behandelingen. Daarom was het onduidelijk of verdere verlaging van de fluoropyrimidine-dosis zou kunnen leiden tot onderdosering bij *DPYD*-variant dragers die werden behandeld met chemoradiotherapie. In hoofdstuk 7 hebben we de ernstige toxiciteit tussen wildtype-patiënten en *DPYD*-variant dragers die chemoradiotherapie kregen vergeleken. De *DPYD*-variant dragers werden behandeld met standaard of gereduceerde fluoropyrimidine-doseringen. *DPYD*-variant dragers die met reguliere fluoropyrimidine-doseringen in chemoradiotherapie werden behandeld, hadden vaker ernstige toxiciteit vergeleken met *DPYD*-variant dragers die met verder gereduceerde fluoropyrimidine-doseringen in chemoradiotherapie werden behandeld. Dit toont aan dat dosisreducties ook in dit type behandeling nodig zijn.

De haalbaarheid van het implementeren van prospectieve *DPYD*-genotypering in de dagelijkse klinische zorg werd aangetoond in hoofdstuk 8 van dit proefschrift. De eerste 21 maanden *DPYD*-genotypering in het Leids Universitair Medisch Centrum (LUMC) werden onderzocht, te beginnen met de introductie als standaardzorg in april 2013 tot het einde van de observatieperiode in december 2014. Deze studie toonde aan dat de implementatie van *DPYD*-genotypering eerst gekenmerkt was door een leer- of acceptatiecurve, maar daarna haalbaar bleek te zijn in de dagelijkse klinische zorg, waarbij 90–100% van de patiënten die werden behandeld met fluoropyrimidines waren gegenotypeerd. Deze studie toonde ook aan dat 90% van de aanbevelingen voor een dosisreductie werden opgevolgd.

Een ander aspect van (*DPYD*)-genotypering is de betrouwbaarheid van een testresultaat en de gevolgen van een incorrect resultaat. In hoofdstuk 9 beschrijven we het dilemma om de resultaten van farmacogenetica testen te bevestigen als een aspect van de kwaliteitscontrole. We bespreken of het nodig zou moeten zijn om twee onafhankelijke genotyperingstesten te gebruiken om een genotype correct te bepalen. In deze studie ontdekten we dat, zelfs na uitgebreide validatie van de testen, incorrecte resultaten kunnen optreden als gevolg van misclassificatie van een genotype, bijvoorbeeld veroorzaakt door *dropout* van allelen. Ondanks de toename in kosten en werkzaamheden, kan het bevestigen van het resultaat van farmacogenetica testen nuttig zijn voor testen met een hoge klinische impact, zoals

DPYD-genotypering. Duidelijke richtlijnen zullen helpen om de praktijk in laboratoria gelijk te trekken wat betreft het bevestigen van resultaten van farmacogenetica testen. Deze richtlijnen zullen mogelijk per gen en test moeten worden gespecificeerd.

Buiten de huidige *DPYD*-farmacogenetica

In het eerste deel van dit proefschrift beschrijven we hoe ernstige fluoropyrimidine-geïnduceerde toxiciteit kan worden verminderd door middel van het uitvoeren van *DPYD*-genotypering voor *DPYD**2A, *DPYD**13, c.2846A>T en c.1236G>A. Het is bekend dat niet alle ernstige fluoropyrimidine-geïnduceerde toxiciteit wordt voorspeld door deze vier varianten alleen. Daarom onderzochten we andere opties, naast de genotypering van de huidige vier *DPYD*-varianten, om ernstige fluoropyrimidine-geïnduceerde toxiciteit te verminderen. Dit wordt toegelicht in het tweede deel van dit proefschrift, getiteld “buiten de huidige *DPYD*-farmacogenetica”.

In hoofdstuk 10 onderzochten we vier DPD-fenotyperingtesten voor het eerst in een vergelijking binnen dezelfde patiënten. Het cohort was niet geselecteerd, of verrijkt, met patiënten die (ernstige) toxiciteit hebben ervaren. Het doel van deze studie was om de klinische waarde van elke DPD-fenotyperingtest te bepalen door klinische validiteitsparameters (bijvoorbeeld sensitiviteit en specificiteit) te beoordelen voor DPD-deficiëntie en het ontstaan van ernstige fluoropyrimidine-geïnduceerde toxiciteit. We konden geen relatie aantonen tussen de DPD-fenotyperingtesten en DPD-deficiëntie of het ontstaan van ernstige fluoropyrimidine-geïnduceerde toxiciteit. Om de klinische waarde van DPD-fenotyperingtesten te bepalen is aanvullend onderzoek nodig.

In hoofdstuk 11 onderzochten we een speciale subgroep van *DPYD*-variant dragers, namelijk de samengestelde heterozygote patiënten. Deze patiënten dragen meerdere *DPYD*-varianten en het effect van de *DPYD*-varianten op de DPD-enzymactiviteit kan niet worden voorspeld met behulp van de gen-activiteitscore. Zonder een dosisreductie hebben deze patiënten een verhoogd risico om ernstige toxiciteit te ontwikkelen. We beschrijven zeven patiënten en onderzoeken diagnostische en therapeutische strategieën voor behandeling met fluoropyrimidines bij patiënten die meerdere *DPYD*-varianten dragen. De aanvullende onderzochte genotyperingmethoden uit deze studie bevinden zich nog in de ontwikkelingsfase of zijn momenteel te duur om in de klinische zorg te implementeren, in vergelijking met een reeds gevestigde DPD-fenotyperingtest. Daarom adviseren we om bij deze patiënten een fenotypetest uit te voeren om een veilige startdosis te bepalen.

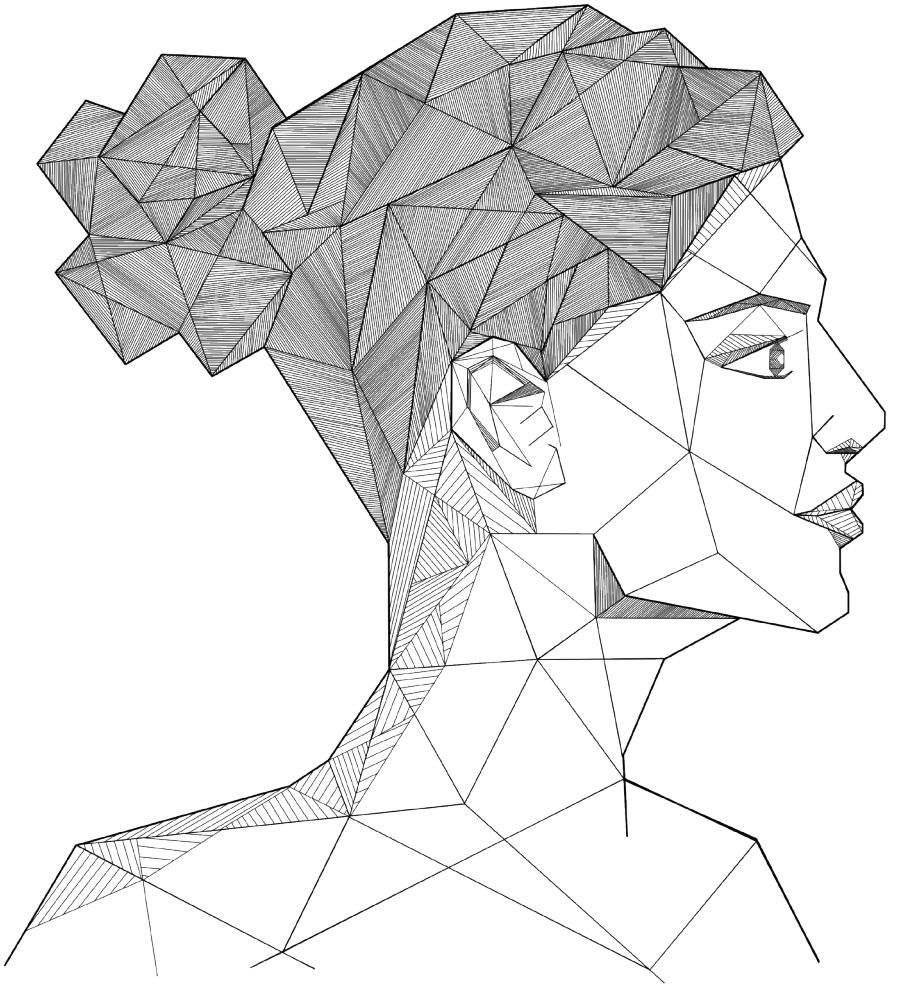
Verwacht wordt dat andere enzymen naast DPD, en dus andere genen naast *DPYD*, betrokken zijn bij het ontstaan van ernstige fluoropyrimidine-geïnduceerde toxiciteit. Met de genoomwijde associatie studie in hoofdstuk 12 hebben we geprobeerd andere varianten te ontdekken, voornamelijk buiten het *DPYD*-gen, die geassocieerd zijn met het ontstaan van ernstige fluoropyrimidine-geïnduceerde toxiciteit. Ongeveer 700,000 *single nucleotide polymorphisms* (SNP’s) in verschillende genen werden gegenotypeerd en geïmputeerd tot meer dan vier miljoen SNP’s. We hebben zes varianten gevonden die suggestief waren voor de associatie met ernstige fluoropyrimidine-geïnduceerde toxiciteit. Daarnaast laten we een optimistische analyse zien van een polygene risicoscore, wat duidt op een hoge polygene aard van het ontstaan van toxiciteit.

Vanwege de uitvoering van de klinische studie beschreven in hoofdstuk 5 pasten steeds meer ziekenhuizen in Nederland *DPYD*-genotypering toe voorafgaand aan het begin van de behandeling. Een toegenomen implementatie van *DPYD*-genotypering was dus zichtbaar, vooral in Nederland. Buiten Nederland bestaan er grote verschillen in de implementatie van *DPYD*-genotypering, al dan niet inclusief een DPD-fenotyperingtest. In sommige landen zijn initiatieven om prospectieve testen voor DPD-deficiëntie uit te voeren effectief, terwijl er in andere landen grote verschillen bestaan in de uitvoering van testen tussen verschillende centra in dat land. De implementatie van *DPYD*-genotypering zal profiteren van duidelijke richtlijnen, d.w.z. aanbevelingen wie en wanneer te genotyperen, en doseringsaanbevelingen voor *DPYD*-variant dragers.

Momenteel zijn vier *DPYD*-varianten opgenomen in het panel voor genotypering, maar het is bekend dat deze vier varianten niet alle patiënten kunnen voorspellen die ernstige toxiciteit zullen ontwikkelen. Het is waarschijnlijk dat andere varianten geassocieerd zullen zijn met het ontstaan van ernstige fluoropyrimidine-geïnduceerde toxiciteit. Om de voorspelling van het genotypepanel verder te verbeteren, kunnen DPD-fenotyperingtesten worden gebruikt, of kunnen nieuwe varianten aan het genotypepanel worden toegevoegd. Nieuwe varianten kunnen bijvoorbeeld zeldzame varianten zijn in het *DPYD*-gen of varianten in andere genen.

De toekomst van fluoropyrimidines

5-FU wordt al tientallen jaren gebruikt in de behandeling van kanker. Inmiddels is capecitabine het voorkeursgeneesmiddel boven 5-FU in de behandeling van verschillende tumortypes in verschillende landen, waaronder Nederland. Om de effectiviteit van kankertherapie te verbeteren, worden fluoropyrimidines gecombineerd met verschillende andere middelen tegen kanker, maar ze zullen een standaard onderdeel van de therapie blijven voor een aanzienlijk aantal tumortypen. Veel onderzoek naar fluoropyrimidines en DPD(-deficiëntie) is reeds uitgevoerd. Op dit moment dient prospectieve *DPYD*-genotypering te worden toegepast voor alle patiënten die een behandeling met fluoropyrimidines beginnen. Aanvullend onderzoek zal worden uitgevoerd waarin de zoektocht naar andere factoren die het ontstaan van ernstige fluoropyrimidine-geïnduceerde toxiciteit zouden kunnen voorspellen verder gaat.



APPENDIX

List of publications

Courses and training activities

Curriculum Vitae

Dankwoord

List of publications

Publications (this thesis)

Lunenburg CATC, Guchelaar HJ, van Schaik RHN, Neumaier M, Swen JJ. Confirmation practice in pharmacogenetic testing; how good is good enough? *Clin Chim Acta*. 2018;490:77-80

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In preparation

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Lunenburg CATC*, Henricks LM*, de Man FM*, Meulendijks D, van Kuilenburg ABP, Maring JG, van Staveren MC, de Vries N, Rosing H, Beijnen JH, Pluim D, Modak A, Imholz ALT, Schellens JHM, Swen JJ, Gelderblom H, Cats A, Mathijssen RHJ, Guchelaar HJ. Comparison of four phenotyping assays for predicting dihydropyrimidine dehydrogenase (DPD) deficiency and severe fluoropyrimidine-induced toxicity: a clinical study. *Manuscript in preparation.*

Courses and training activities

Courses at Leiden University Medical Center (LUMC)

2017	From database to dataset
2016	Statistical Aspects of Clinical Trials
2015	Introduction genetic epidemiology
2015	Basic methods and reasoning in biostatistics
2015	Basiscursus regelgeving en organisatie klinisch onderzoekers (BROK)

Courses at Leiden University

2018	Increasing your memory
2018	Time and self-management
2017	Motives and competences for PhD's

Other courses

2018	Summer course pharmacoepidemiology and drug safety, Utrecht University
2018	BioBusiness Summer School, Hyphen Projects
2015	PGx workshops, Nederlands Expertisecentrum Farmacogenetica

Symposia, meetings, congresses

2019	Oral presentation at the Dutch Internists Days in Maastricht
2018	Poster presentation at ESMO congress in Munich
2015-2018	Oral presentation at FOS course in Leiden
2015-2018	Oral presentation at annual meeting NNPM in Utrecht (2015), Leiden (2017) and Nijmegen (2018)
2017	Oral presentation at career evening MFLS in Leiden
2017	Oral presentation at lunch lecture LPSV Aesculapius in Leiden
2017	Oral presentation at annual meeting NVZA in Bunnik
2016	Poster presentation at annual meeting NFKFB in 's Hertogenbosch
2016	Oral and poster presentation at annual meeting NVT in Soesterberg

Awards and grants

2018	Award: LUMC best (clinical) poster prize, LUMC Research Conference 2018
2018	Grant co-applicant: Preventing fluoropyrimidine induced toxicity in patients of non-Caucasian descent, SFOH, 146.419 euro
2017	Grant co-applicant: Genome-wide association study to discover novel genetic variants related to the onset of severe toxicity following fluoropyrimidine use: beyond DPD, ZonMw GGG open ronde 6, 60.584,30 euro

Curriculum Vitae

Carin Lunenburg was born September 4th 1990 in Oss, the Netherlands. She grew up in Heesch as the youngest of three girls. After she completed her secondary school (VWO) at the Maasland College Oss in 2008, she started her study Biomedical Sciences at the Radboud University Nijmegen. During her studies, she participated in several committees and worked as a fulltime board member of the Medical Faculty Association Nijmegen (MFVN). Hereafter she started her masters Toxicology. She performed research internships under the supervision of dr. Marieke Coenen at the department of Human Genetics, Radboud University Medical Center Nijmegen, and under the supervision of dr. Björn Bauer at the College of Pharmacy, University of Minnesota, Duluth and University of Lexington in Kentucky, United States of America. She graduated in September 2014.

In December 2014, she started as a PhD candidate at the Leiden University Medical Center (LUMC) under the supervision of prof. dr. Hans Gelderblom (Medical Oncology), prof. dr. Henk-Jan Guchelaar and dr. Jesse Swen (Clinical Pharmacy and Toxicology). Her main project was the coordination of a large prospective clinical trial, which was executed in seventeen hospitals in the Netherlands. In 2017, she was co-applicant on a granted subsidy from ZonMw for conducting a genome-wide association study. In 2018, she was awarded the LUMC best (clinical) poster prize at the LUMC Research Conference.

At the end of her project, she worked part-time as an interim project-manager at the department of Clinical Pharmacy and Toxicology in collaboration with the Radiology department. As of March 2019, she works as a postdoctoral fellow at the Aarhus University Hospital, Denmark, in collaboration with dr. Christiane Gasse.

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