Treatment optimization of fluoropyrimidine-based chemotherapy

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Optimalisering van de behandeling met fluoropyrimidine-bevattende chemotherapie
(met een samenvatting in het Nederlands)

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Preface

Preface

Over the last decade cancer therapy has evolved from non-specific cytotoxic agents that kill cancer cells but also harm normal cells to specific agents targeting unique molecular features of tumor cells and immunotherapy that modulate the tumor immune response. However, despite this improved understanding of the molecular characterization of tumors non-targeted therapies such as chemotherapy are effective and still widely used and continue to be a mainstay in the systemic treatment of cancers.

Fluoropyrimidines

This thesis is focused on a group of anticancer agents called *fluoropyrimidines*. This group - which include 5-fluorouracil (5-FU), and its oral prodrugs capecitabine, and tegafur - has been and still is indispensable in the treatment of solid tumors, including colorectal, breast, gastric, and head- and neck cancer, since the introduction of 5-FU in the 1950s[1] and capecitabine in 2001.[2] Although fluoropyrimidines are reasonably well-tolerated by patients, ~20-30% experience severe toxicity which can even be fatal in up to 0.5-1% of patients.[3-5] The main severe fluoropyrimidine-related toxicities are diarrhea, nausea, vomiting, mucositis, neutropenia, and hand-foot syndrome; the latter especially in capecitabine. These drugs were used by an estimated amount of 2 million patients worldwide in 2002.[6] Due to the increase in the incidence of cancer it is expected that this number currently (2022) is even higher. Given this considerable number of patients treated with fluoropyrimidines, severe fluoropyrimidine-related toxicity is recognized as a major clinical problem.

DPD deficiency testing

Although fluoropyrimidines have been used as an anticancer treatment for over half a century, only recently it has come to the attention that the safety of fluoropyrimidine-based chemotherapy is greatly influenced by inter-individual variability in the activity of the DPD enzyme. Reduced activity of DPD is one of the main causes of fluoropyrimidine-related toxicity, due to the lower capacity to degrade 5-FU into inactive metabolites, resulting in higher exposure of 5-FU and cytotoxic metabolites and increased risk of developing severe toxicity.[8,9] This deficiency of the DPD enzyme is most often the result of deleterious polymorphisms in *DPYD*, the gene encoding for the DPD enzyme.[10] Throughout the last decade it has become clear that at least four *DPYD* variants (*DPYD**2A, c.1236G>A, c.2846A>T, and c.1679T>G) are clinically relevant and significantly increase the risk of developing severe fluoropyrimidine-related toxicity when treated with a standard dose of fluoropyrimidines.[11-13] Pre-therapeutic screening of these *DPYD* variants and subsequent dose-individualization has been shown to significantly decrease the incidence of severe fluoropyrimidine-related toxicity.[11,13] However, despite the reproducible link between the four *DPYD* variants and toxicity, ~23% of patients who do not carry any of these variants still experience severe fluoropyrimidine-related toxicity.[13] This is in line with previous findings in which was stated that approximately 17% of severe fluoropyrimidine-related toxicity can be traced back to the four previously mentioned *DPYD* variants and highlights the importance of identifying other *DPYD* variants and methods to identify patients at risk of severe fluoropyrimidine-related toxicity.[6]

A potential alternative to *DPYD*-genotyping with potential could be the use of pretreatment uracil concentrations as a DPD phenotyping method. Uracil is an endogenously present pyrimidine base used in RNA and is metabolized, similar to 5-FU, by DPD into dihydrouracil.[14,15] Therefore, it is hypothesized that the concentration of uracil could be used as a surrogate for the DPD activity with high levels of uracil being indicative of DPD deficiency and predictive of severe fluoropyrimidine-induced toxicity.[16] In line with this thought, it has been shown that elevated pretreatment uracil levels (>16 ng/mL) are associated with an increased risk of severe fluoropyrimidine-related toxicity.[15,17,18] The main advantage of the use of pretreatment uracil levels is that potentially all patients with a DPD deficiency can be identified with one measurement, including rare deleterious *DPYD* variant carriers which would not have been found using the conventional panel of *DPYD* variants. However, although evidence for *DPYD*-genotyping in preventing severe fluoropyrimidine-related toxicity is extensive and includes data from prospective clinical trials no such data to support to the use of pretreatment uracil levels is available.[11,13] In addition, large variability in uracil levels have been found within patients and between cohorts and hospitals, which have not yet been fully explained.[19,20]

Thesis outline

The general aim of this thesis it to study how treatment with fluoropyrimidine-based chemotherapy can be optimized and more specifically how severe fluoropyrimidine toxicity can be further reduced, in addition to *DPYD* genotype-guided dose-individualization.

In the first part of this thesis dose-individualizations strategies for fluoropyrimidine-based chemotherapy are discussed. In *Chapter 1* an overview of dose-individualization strategies and recommendations for fluoropyrimidines are discussed. In *Chapter 2* the combination of sequencing of the *DPYD* gene and a genome-wide association study to identify possible deleterious single nucleotide polymorphisms in- and outside of the *DPYD* gene are investigated as to explain remaining toxicity. *Chapter 3* focusses on the pitfalls and learned lessons from DPD phenotyping in a large prospective clinical study. In *Chapter 4* a large prospective clinical trial (Alpe2U-study) is described in which is studied whether dose-individualization of fluoropyrimidine-based chemotherapy based on pretreatment uracil levels can reduce the incidence of severe fluoropyrimidine-related toxicity. *Chapter 5* reports on the development of a model to predict severe fluoropyrimidine-related toxicity in *DPYD* wild type patients before start of treatment with fluoropyrimidines using easily measurable baseline variables.

The second part of this thesis focuses on clinical outcomes of fluoropyrimidine-based chemotherapy based. *Chapter 6* focusses on the impact of dose-individualization on treatment outcome in *DPYD* variant allele carriers compared to *DPYD* wild type patients treated with a standard dose. In *Chapter 7* a retrospective study investigating clinical parameters to predict the occurrence of fluoropyrimidine-related toxicity in elderly patients (≥ 65 years) was developed. In *Chapter 8* a prospective study investigating geriatric parameters related to the onset of severe fluoropyrimidine-related toxicity in elderly patients is described.

The third and last part of this thesis is focused on bioanalysis in support of fluoropyrimidine-based chemotherapy. *Chapter 9* provides an overview of bioanalytical challenges in the quantification of fluoropyrimidines and describes future wishes and perspectives that could serve as inspiration for future development of assays. In *Chapter 10* the development of a bioanalytical assay for the quantification of capecitabine and metabolites in a single assay is described. Lastly, in *Chapter 11* the quantification and stability of uracil in clinical practice is described.

Finally, the main conclusions of the research described in this thesis are summarized and are put in perspective in the *Conclusions and Perspectives*.

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Part I - Dose-individualization of fluoropyrimidine-based chemotherapy

Chapter 1

Individualized dosing of fluoropyrimidine-based chemotherapy to prevent severe fluoropyrimidine-related toxicity - What are the options?

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Abstract:

Fluoropyrimidines are widely used in the treatment of several types of solid tumors. Although most often well tolerated, severe toxicity is encountered in approximately 20-30% of the patients. Individualized dosing for these patients can reduce the incidence of severe fluoropyrimidine-related toxicity. However, no consensus has been achieved on which dosing strategy is preferred. The most established strategy for individualized dosing of fluoropyrimidines is upfront genotyping of the *DPYD* gene. Prospective research has shown that *DPYD*-guided dose-individualization significantly reduces the incidence of severe toxicity and can be easily applied in routine daily practice. Furthermore, the measurement of the DPD enzyme activity has shown to accurately detect patients with a DPD-deficiency. Yet, because this assay is time-consuming and expensive, it is not widely implemented in routine clinical care. Other methods include the measurement of pre-treatment endogenous serum uracil concentrations, the uracil/dihydrouracil-ratio and the 5-fluorouracil (5-FU) degradation rate. These methods have shown mixed results. Next to these methods to detect DPD-deficiency, pharmacokinetically-guided follow-up of 5-FU could potentially be used as an addition to dosing strategies to further improve the safety of fluoropyrimidines. Furthermore, baseline characteristics such as gender, age, body composition and renal function have shown to have a relationship with the development of severe toxicity. Therefore, these baseline characteristics should be considered as a dose-individualization strategy. We present an overview of the current dose-individualization strategies and provide perspectives for a future multiparametric approach.

Introduction

5-Fluorouracil (5-FU), and its oral prodrug capecitabine belong to the group of fluoropyrimidines and are the backbone of several treatment regimens in a wide range of cancer types including colorectal cancer (CRC), breast cancer, and head and neck cancer.[1] Although fluoropyrimidines are reasonably well tolerated by patients, approximately 20-30% experience severe (Common Terminology Criteria for Adverse Events (CTC-AE) grade 3-5) toxicity. The most common toxicities attributed to fluoropyrimidine-based chemotherapy are diarrhea, nausea, vomiting, mucositis, neutropenia and hand-foot syndrome; the latter especially with capecitabine.[2,3] Severe fluoropyrimidine-related toxicity can be fatal in up to 1% of patients.[4] Given the considerable number of patients (~2 million) treated with fluoropyrimidines worldwide every year, severe fluoropyrimidine-related toxicity is a well-recognized and significant clinical problem. Therefore, accurate biomarkers or methods that can predict and prevent severe fluoropyrimidine-related toxicity are of high interest. Over the years, several approaches for prediction of toxicity and guidance of dose-individualization of fluoropyrimidines have been studied. The probably most studied biomarker is the activity of the main catabolic enzyme dihydropyrimidine dehydrogenase (DPD), which is strongly correlated to the pharmacokinetics of 5-FU[5]. Despite extensive research identifying biomarkers predicting severe toxicity is challenging, and a consensus in approach for individualizing fluoropyrimidine dosing is lacking. In this review, we present an overview of the various possible strategies for dose-individualization of fluoropyrimidine-based chemotherapy (see Table 1). This review distinguishes itself from other reviews and guidelines by not only including strategies such as *DPYD*-genotyping and DPD-phenotyping, but also discuss less-known strategies such as patient characteristics and multiparametric approaches in detail. Additionally, we will evaluate the level of evidence, discuss the feasibility and provide recommendations regarding these dose-individualization strategies. This review only focusses on 5-FU and capecitabine as the vast majority of studies have been conducted in patients receiving 5-FU and capecitabine, excluding other fluoropyrimidines such as tegafur.

Metabolism of fluoropyrimidines

Capecitabine is metabolized into the active agent 5-FU through three steps (see Figure 1).[6] First, capecitabine is converted to 5'-deoxy-5-fluorocytidine (5'-dFCR) by carboxylesterase, which is an enzyme located mainly in the liver. Second, 5'-dFCR is converted to 5'-deoxy-5-fluorouridine (5'-dFUR) by cytidine deaminase which is mainly located in the liver and tumor tissue. Third, 5'-dFUR is converted to 5-FU by thymidine phosphorylase. This last conversion primarily takes place in tumor tissue, due to higher concentrations of thymidine phosphorylase compared to normal, healthy tissue6. Thereupon, 5-FU enters the cell through a facilitated transmembrane carrier. Subsequently, 5-FU is enzymatically converted to the active intracellular cytotoxic metabolites 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP), 5-fluorouridine 5'-triphosphate (FUTP), and 5-fluoro-2'-deoxyuridine 5'triphosphate (FdUTP).[7] Approximately 80-90% of 5-FU is catabolized by dihydropyrimidine dehydrogenase (DPD) into metabolite 5-dihydrofluorouracil (5-FUH2) which is neither cytotoxic to the tumor

cells nor toxic to normal cells. This conversion undergoes a circadian rhythm as DPD enzyme activity changes over time during the day.[8] Afterwards α-fluoro-β-ureidopropionic (FUPA) and α-fluoro-β-alanine (FBAL) are formed which are excreted through the urine with the remaining ~10% of 5-FU.[1,7]

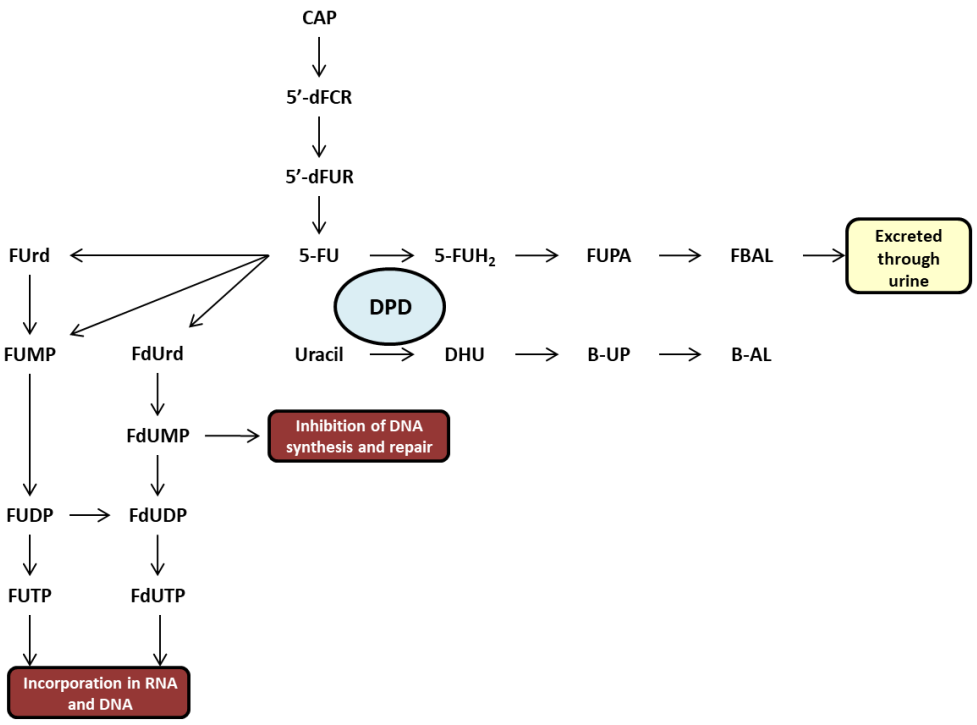

Figure 1: Metabolism of fluoropyrimidines. *Abbreviations*: 5'-dFCR = 5'-deoxy-5-fluorocytidine; 5'-dFUR = 5'-deoxy-5-fluorouridine; 5-FU = 5-fluorouracil; 5-FUH2 = 5,6-dihydro-5-fluorouracil, B-AL = β- alanine, B-UP = β-ureidopropionate; DHU = Dihydrouracil, FBAL = α-fluoro-β-alanine; FdUDP = 5-fluoro-2'-deoxyuridine 5'-diphosphate; FdUMP = 5-fluoro-2'-deoxyuridine 5'-monophosphate; FdUrd = 5-fluoro-2'-deoxyuridine; FdUTP = 5-fluoro-2'-deoxyuridine 5-'triphosphate; FUDP = 5-fluorouridine 5'-diphosphate; FUMP = 5-fluorouridine 5'-monophosphate; FUPA = α-fluoro-β-ureidopropionic acid; FUrd = 5-fluorouridine; FUTP = 5-fluorouridine 5'-triphosphate

Dosing

Historically, most chemotherapeutic drugs are dosed based upon the patient's body surface area (BSA). The same accounts for 5-FU and capecitabine. BSA-guided dosing intents to minimize inter-patient variability in exposure due to differences in body size, resulting in less toxicity.[9] However, no correlation was found between BSA and 5-FU plasma clearance by Gamelin *et al.*[10] Furthermore, Ratain addressed a few problems with dosing capecitabine such as a large interpatient variability (greater than 85%) in 5-FU concentration and Area-under-the-Curve (AUC) the limited tablet strengths available (150 mg and 500 mg) and the lack of evidence for suggested dose modifications.[11] The interpatient variability in 5-FU concentration and AUC are most likely caused by various enzymes involved in the conversion of capecitabine to 5-FU.[12] In the summary of product characteristics (SmPC) it is mentioned that a dose-reduction of 25% is recommended for patients with grade ≥ 3 toxicity treated with capecitabine, although limited prospective

research has been performed regarding dose modification in patients with grade ≥ 3 toxicity. It is questioned why a dose-reduction of 25% is recommended while the calculation for the starting dose is very precise. Yet, alternative dosing strategies have been studied scarcely.[13] Recently, de Man *et al.* have shown that the tolerance and effectiveness of fixed-dose capecitabine are comparable to BSA-guided dosing and therefore fixed-dosing could be an alternative for BSA-guided dosing. However, fixed-dosing of capecitabine did not lead to a decrease of severe toxicity.[14] Therefore, alternative strategies should be explored to optimize and individualize the treatment with fluoropyrimidines.

DPYD-guided dosing

Dihydropyrimidine dehydrogenase

One of the main factors influencing drug exposure in fluoropyrimidine-based chemotherapy is DPD enzyme activity. The *DPYD* gene encodes for the DPD enzyme. The availability of 5-FU for conversion into cytotoxic metabolites is primarily determined by the activity of the DPD enzyme.[12] Reduced activity of DPD is one of the main causes of fluoropyrimidine-related toxicity, due to the lower capacity to degrade 5-FU into the inactive metabolites, resulting in higher exposure of 5-FU and cytotoxic metabolites.[1,7] Most often a DPD-deficiency is the result of a deleterious single nucleotide polymorphism (SNP) in *DPYD*, altering the DPD enzyme activity.[15] A DPD-deficiency is classified as partial if there is remaining DPD activity (e.g., 25%-50% of normal) and as complete if no or almost no DPD enzyme activity (e.g., <5%) is detectable.

In the Caucasian population approximately 3-7% have a DPD-deficiency and 0.01-0.1% have a complete deficiency.[16] However, the frequency of DPD deficiencies can differ between ethnicities. For example, Mattison *et al.* found that approximately 8% of the African American population have a partial DPD-deficiency.[17]

DPYD variants

The first functionally relevant *DPYD* variant reported was the *DPYD**2A (c.1905+1G>A; IVS14+1G>A; rs3918290) variant.[18] The DPD enzyme activity in heterozygous carriers of *DPYD**2A is approximately 50% compared to wildtype. In addition to *DPYD**2A, several other SNPs in *DPYD* have been reported that are associated with a reduced DPD enzyme activity, including c.1236G>A (rs56038477; Haplotype B3), c.2846A>T (D949V, rs67376798) and c.1679T>G (*DPYD**13, I560S; rs55886062).[15] However, the decrease in DPD enzyme activity between these variants differ ranging from ~25% for c.1236G>A and c.2846a>T and 50% for c.1679T>G.[15,19] Furthermore, it is also possible that patients carry multiple *DPYD* variants simultaneously. Homozygous patients carry two identical *DPYD* variants which results in reduced or inactive alleles and therefore a reduced or absent DPD enzyme activity. Compound heterozygous patients carry two or more *DPYD* variants either on one allele (in cis) or on different alleles (in trans) leading to differences in DPD enzyme activity. When two or more *DPYD* variants are present on different alleles, both alleles are impacted and DPD enzyme activity is impacted more severely. For example, patients which are compound heterozygous carriers of a c.1236G>A and *DPYD**2A variants have a ~75% reduced DPD enzyme activity, theoretically. If these *DPYD* variants were present on the same allele the DPD enzyme activity would have been reduced by

Table 1: Overview of dose-individualization strategies, including their principles, advantages, and limitations

Strategy	Principle	Advantages	Limitations
***DPYD*-guided dose-individualization**			
	Dose-modifications based on single nucleotide polymorphisms in the *DPYD* gene	- Prospectively shown to significantly reduce incidence of severe toxicity - Simple and unequivocal results - Dosing guidelines available - Easily implemented in routine clinical care - Shown to be cost-saving	- Limited sensitivity - Current variants most likely only predictive for western population
DPD-phenotype guided dose-individualization			
Endogenous uracil and dihydrouracil	Measurement of plasma/serum uracil and dihydrouracil concentration as a surrogate marker for a DPD deficiency	- High sensitivity - Patient friendly	- Lacks prospective validation - Instability uracil/dihydrouracil *in vitro* - Influence food and circadian rhythm
Administration of uracil	Measurement of pharmacokinetic parameters or metabolites	- Accurate prediction of DPD deficiency	- Patient unfriendly - Demanding on clinical staff and resources - Lacks prospective validation
DPD enzyme activity	Measurement of the DPD enzyme activity in PBMCs	- Direct way of measuring DPD deficiency - High sensitivity	- Complex and laborious - Specific equipment necessary - Costly - Lacks prospective validation - No specific threshold established
5-FU degradation rate	Measurement of the degradation of 5-FU in PBMCs	- Takes complete degradation of 5-FU into account - Cheap	- No prospective validation available - Specific equipment necessary
Pharmacokinetically-guided dosing			
	Dosing strategy based on the concentration and pharmacokinetic characteristics of 5-FU	- Reduces toxicity while maintaining adequate exposure and efficacy	- Only applicable to 5-FU - Initially treated with full dose (possibility of severe toxicity) - Patient unfriendly - Lacks prospective validation
5-FU test dose			
	Administration of very low dose 5-FU, after which blood samples are taken to determine the exposure	- Direct way of assessing DPD deficiency - Small dose, so less risk of severe toxicity	- Lacks prospective validation - Currently only applicable to 5-FU - Possibility of rapid severe toxicity in patients with complete deficiency - No dosing guidelines
Patient characteristics at baseline			
Gender	Dose adjustment of baseline characteristics that are associated with an increased risk of developing severe toxicity	- Easily measured (gender, age and renal function) - Can easily be combined with other dose-individualization strategies	- Limited information available - No dose modifications based on baseline characteristics have been studied
Age			
Body composition			
Renal function			

only ~50%.[15,20] This can make compound heterozygous genotypes difficult to interpret.

The relation between these *DPYD* variants and severe fluoropyrimidine-related toxicity is widely accepted. Multiple meta-analyses have shown that these variants are associated with severe fluoropyrimidine-related toxicity.[2,21,22] Consequently, upfront genotyping for these variants and adjusting the dose according to the reduction in DPD enzyme activity was the next step.

DPYD-guided dosing

Deenen *et al.* were the first to prospectively evaluate the safety of *DPYD**2A-guided dose-individualization of fluoropyrimidines.[23] Before treatment with fluoropyrimidine-based chemotherapy patients (N=2039) were prospectively screened for *DPYD**2A and received a dose-reduction of 50% if carrying *DPYD**2A, followed by dose-titration if tolerated. Toxicity was compared to a historical cohort of patients carrying a *DPYD**2A variant treated with a standard dose and wildtypes (WTs) treated with a standard dose in this study. The risk of developing severe fluoropyrimidine-related toxicity was significantly reduced from 73% (95%-CI, 58%-85%) in the historical cohort (N=48) to 28% (95%-CI, 10%-53%) by *DPYD*-guided dosing (P<0.001). This was similar compared with WTs receiving the standard dose (23%; P=0.64). Pharmacokinetic (PK) analysis showed that patients carrying *DPYD**2A treated with a 50% dose-reduction achieved similar 5-FU exposure as WT patients treated with a standard dose suggesting that dose-reduction by 50% in *DPYD**2A carriers does not lead to undertreatment.[23]

Subsequently a similar prospective study was conducted in which c.1236G>A, c.2846A>T and c.1679T>G were added to the screening panel. Patients carrying a *DPYD* variant received a dose-reduction of either 50% (*DPYD**2A and c.1679T>G carriers) or 25% (c.1236G>A or c.2846A>T carriers) after which the dose could be escalated when treatment was well-tolerated. The incidence of toxicity was compared to a historical cohort similarly as described by Deenen *et al.*[16,23] A total of 1103 patients were included and deemed evaluable of which ~8% (N=85) were heterozygous carriers of one of the four *DPYD* variants. It was shown that the relative risk (RR) of developing severe fluoropyrimidine-related toxicity was reduced in *DPYD* *2A (1.31 (0.63-2.72) vs. 2.87 (2.14-3.86)) and c.2846A>T (2.00 (1.19-3.34) vs. 3.11 (2.25-4.28)) carriers compared to a historical cohort. Furthermore, the 25% dose-reduction for the c.1236G>A variant proved to be insufficient to reduce the RR (1.69 (1.18-2.42) vs. 1.72 (1.22-2.42). Only one patient was included carrying the c.1679T>G variant and was treated safely with a dose-reduction of 50%. PK-analysis showed that the mean exposure to 5-FU was similar between the group *DPYD* carriers treated with a reduced dose and WTs treated with a full dose.[16]

Additionally, both Deenen *et al.* and Henricks *et al.* showed that upfront genotyping of *DPYD* and subsequent dose-individualization is cost saving.[23,24] Although drug exposure is similar, uncertainty exists about the effectiveness of treatment with a reduced dose for variant carriers, as the often-mentioned fear is that this dose-reduction could result in underdosing. This was studied by Henricks *et al.* who compared *DPYD**2A carriers treated with a 50% dose-reduction with matched controls of WTs treated with a full dose (37 *DPYD**2A carriers and 37 controls). The applied dose-reduction did not negatively influence overall survival (OS) (median 27 months vs. 24 months, P=0.47) nor progression-free survival (median 14 months vs. 10 months, P=0.54). This suggests that a 50% dose-reduction in *DPYD**2A does not negatively impact effectiveness, while improving the patient safety.[25] However, this study only focused on *DPYD**2A and had a relatively small sample size. The impact of dose-reductions on the effectiveness of treatment remains to be studied for c.1236G>A, c.2846A>T and c.1679T>G carriers. These studies, among other published studies, have led to the update of the Clinical Pharmacogenetics Implementation Consortium (CPIC) and Dutch Pharmacogenetics Working Group (DPWG) guidelines for fluoropyrimidines and *DPYD*. These are evidence-based guidelines focusing on the drug-gene interaction of *DPYD* and fluoropyrimidines. The purpose of the CPIC guideline is to provide information for clinical interpretation of *DPYD*-genotype test results to guide the dosing of fluoropyrimidines.[26] The DPWG aims to expedite pharmacogenetics implementation by developing evidence-based guidelines to optimize pharmacotherapy.[27] Similar guidelines have been developed by the French Network of Pharmacogenetics (RNPGx) and the Italian Associazione Italiana di Oncologia Medica (AIOM) but are not available in English. A dose-reduction of 50% (instead of 25%) for c.1236G>A or c.2846A>T carriers is now recommended in both the CPIC as the DPWG guideline.[27,28] Furthermore, information about *DPYD*-genotyping has been added to the SmPC of capecitabine and the EMA has recently recommended that patients treated with fluoropyrimidines should be tested for the lack of DPD before start of treatment.[13,29] Similarly, the US FDA added statements to the label of 5-FU and capecitabine warning for the increased risk of severe toxicity in patients with a DPD-deficiency.[30]

DPYD-guided genotyping has shown to be an effective and cost-saving strategy for individualized dosing of fluoropyrimidine-based chemotherapy. Other advantages of *DPYD*-guided dosing are that genotyping of the *DPYD* gene is relatively simple and gives unequivocal results. In addition, dosing-guidelines based on *DPYD*-genotype are readily available and have been implemented in routine clinical car.[31] However, there are also a few drawbacks. The first and main drawback is that only a part of severe fluoropyrimidine-related toxicity can be traced back to genetic variants of the *DPYD* gene.[32] Meulendijks *et al.* reported that ~17% of the patients experiencing severe fluoropyrimidine-related toxicity are identified by genotyping for the four *DPYD* variants.[21] Furthermore, these *DPYD* variants are most likely only predictive of severe toxicity in the Western population. It has been shown by Elraiyah *et al.* that these variants were not present in patients of the East African descent. However, twelve non-synonymous *DPYD* variants were identified in this study of which seven variants showed a significantly decreased DPD enzyme activity *in vitro*.[33] In addition Offer *et al.* also showed that patients of African American descent carry unique variants such as *DPYD*-Y186C which was not present in patients of European American descent.[34] Furthermore, Hariprakash *et al.* studied *DPYD* variants associated with toxicity in south-Asian populations and showed that certain variants (e.g. rs1801160 and rs12022243) are observed in higher frequency in south-Asia compared to other populations.[35] This problem has been acknowledged and further research regarding *DPYD* variants in patients of non-Western descent is being conducted (NCT04300361). Lastly, another disadvantage of *DPYD*-guided dosing is the lack of options for patients with a homozygous or compound heterozygous *DPYD*-genotype. These patients are generally not treated with fluoropyrimidines.

Phenotype-guided dosing

Endogenous Uracil and Dihydrouracil

The variability in DPD enzyme activity can only partly be traced back to SNPs in the *DPYD* gene. Therefore, DPD-phenotyping could be useful to identify more patients with a DPD-deficiency. Several DPD-phenotyping methods have been described over the years and are mostly based on the conversion of the endogenous substrate of DPD, uracil (U), to dihydrouracil (DHU) (see Figure 1).It is thought that a DPD-deficiency decreases the conversion rate of U to DHU, resulting in higher U concentrations in DPD-deficient patients. Pre-treatment serum U concentrations have been measured in 550 patients and the predictive value of U for early severe fluoropyrimidine-related toxicity were compared. It was shown that a high pre-treatment serum U concentration (>16 ng/mL) was strongly associated with global severe toxicity (OR 5.3, P=0.009).[36] In addition to this, Etienne-Grimaldi *et al.* have shown that patients with a U concentration above 16 ng/mL were significantly prone to develop grade 4 toxicity compared with patients with a lower U concentration (RR 20.6, P=0.021).[37]

Moreover,a significant correlation was found by Boisdron-Celle *et al.* between U plasma concentrations and 5-FU toxicity with a threshold value of 15 ng/mL for toxicity.[38] Furthermore, an abstract of a prospective pilot study showed an association between U and DHU concentration and the development of severe fluoropyrimidine-related toxicity (median concentration 12.7 ng/ml (U) and 110 ng/ml (DHU) vs. 10.2 ng/ml (U) and 93 ng/mL (DHU) in patients with and without toxicity, P=0.014 (U) and P=0.011 (DHU)). ROC analysis showed that these differences were too small to use as predictor for toxicity.[39]

The endogenous U concentration is an interesting biomarker for the prediction of severe fluoropyrimidine-related toxicity, yet most phenotyping studies conducted have been aimed towards DHU/U-ratio rather than U concentration alone. Several studies have shown that there is an association between DHU/U-ratio and 5-FU plasma concentration and severe fluoropyrimidine-related toxicity.[10,40-43] On the contrary, no correlation was found between DHU/U-ratio and 5-FU clearance by Boisdron-Celle *et al.* while a significant correlation was found with severe toxicity (P<0.001) with a threshold of 6.[38]

In addition, Etienne-Grimaldi *et al.* could not establish correlation between DHU/U-ratio and toxicity (median 9.1 vs 9.6 in patients with and without toxicity, P=0.80).[39] The earlier mentioned retrospective study by Meulendijks *et al.* also showed that the DHU/U-ratio was a less accurate in predicting severe toxicity compared to the pre-treatment U concentration.[36] It has been shown that there is most likely an association between these phenotypes and severe toxicity. However, the major concern with the use these of phenotyping methods is the lack of prospective validation confirming that dose adjustments based upon U or DHU/U-ratio lead to a decreased incidence of severe toxicity. Despite the lack of prospective validation, the French National Authority for Health and French National Cancer Institute recently recommended testing for DPD-deficiency by determination of U concentration for patients treated with fluoropyrimidines in France.[44] Recently, a study in the Netherlands has started (NCT04194957, The Alpe2U-study) in which patients are prospectively screened for pre-treatment serum U concentration and a dose-reduction of 50% is applied to patients with a pre-treatment

serum U concentration above 16 ng/ml. Another important issue is the limited information concerning the sensitivity and specificity of U as a biomarker. It has been mentioned that the sensitivity of U is better compared to *DPYD*-genotyping by Captain *et al.*[45] However, this analysis was performed on selected patients with severe toxicity. This influences the results as no information is available on patients with no severe toxicity and high U concentrations (>16 ng/mL) and vice versa, which would reduce the sensitivity of U as a biomarker. Furthermore, U is measured in low concentrations which requires specific equipment. This equipment is not readily available at all hospitals, which complicates the implementation in the clinic. In addition, the limited stability of U and DHU has to be taken into account. It has been shown that the concentration of U and DHU increase over time at room temperature after samples have been taken.[46,47] This could significantly influence the possible dose-individualization based on these methods and indicates that samples need to be processed as soon as possible to minimize the increase of U and DHU concentration. This could be challenging in clinical practice where samples most often are not processed immediately. Lastly, the conditions under which blood samples are taken for determination of U and DHU should be chosen carefully as U is influenced by circadian rhythm and food.[8,48] It has been shown that U levels were higher in fasted state compared to fed state. It is recommended that sampling should be performed preferably between 8:00 and 9:00 AM after overnight fasting to avoid bias introduced by circadian rhythm and food effects.[48]

Administration of uracil

Other phenotypic methods based on the conversion of U to DHU are the U loading dose and the U breath test. The U loading dose consists out of oral administration of U and blood sampling at specific time points. After sampling, the concentration of U and DHU are measured. Staveren *et al.* have shown that PK-parameters such as the AUC and the maximum concentration (C_{max}) of U and DHU significantly differ between subjects with a DPD-deficiency and without.[49] Additional research was performed to assess the sensitivity and specificity of this test to identify patients with a DPD-deficiency. A sensitivity and specificity of 80% and 98%, respectively, was obtained for the U/DHU-ratio a t=120 min to discriminate between subjects with a normal DPD activity and DPD-deficient subjects. This shows that DPD-deficient patients can be accurately identified using this method.[50] An advantage of this strategy is that the DPD enzyme temporarily is saturated and therefore U is eliminated following zero-order kinetics. This is a better representation of the DPD enzyme activity than measuring endogenous U concentrations as under normal conditions the elimination of U follows first-order kinetics. This suggests that the rate of U elimination is more depended on the amount of U and not primarily on the amount of DPD enzyme activity.[51] However, the administration of U followed by a blood draw after 2 hours is relatively patient-unfriendly and demanding on the clinical staff and resources. More research is needed to further establish the correlation between the U loading dose and the prediction of severe toxicity. Furthermore, a prospective study in which dose-adaptions are applied based on this method needs to be conducted to see if the incidence of severe toxicity can be reduced.

Another phenotypic method in which U is administered orally is the U breath test. This method is based on the production $^{13}CO_2$ from 2-^{13}C-uracil by enzymes in the metabolism of U. First, baseline samples of patients are taken by collecting breath samples in bags.

Second, 2-^{13}C-uracil is ingested orally in an aqueous solution after which breath samples are taken. Thirdly, concentrations of $^{13}CO_2$ and $^{12}CO_2$ are measured by infrared spectrometry and expressed as a delta-over-baseline (DOB) ratio. This ratio represents a change in the ratio of $^{13}CO_2$ / $^{12}CO_2$ of the samples collected before and after administration of 2-^{13}C-uracil. [52,53] Mattison *et al.* have shown that the concentration of exhaled $^{13}CO_2$ is reduced in patients with a DPD-deficiency.[52] This was based on a single time-point determination at 50 min after administration.[52] In addition to this, it has also been shown that the U breath test correlates with DPD enzyme activity in peripheral blood mononuclear cells (PBMCs) (R=0.78) and plasma [2-^{13}C]-uracil AUC (R =-0.73).[54] In addition to this, Cunha-Junior *et al.* studied the ability of the U breath test to identify patients at risk of severe toxicity. Mean DOB_{50min} significantly differed between patients with grade 0-1 and grade 3-4 toxicity. A DOB_{50min} cutoff of ≤161.4 was found which could fairly accurate discriminate individuals who experienced severe toxicity from those who did not (sensitivity = 61%; specificity= 85%).[55] However, DPD is not the only enzyme involved in the conversion of U to $^{13}CO_2$. Several other enzymes are involved in the complete conversion and, therefore, could influence the outcome. Furthermore, due to the complex and laborious logistics, clinical implementation of the breath test could be hampered.

DPD enzyme activity in peripheral blood mononuclear cells

A more direct way of determining a DPD-deficiency is by measuring the DPD enzyme activity in PBMCs. DPD enzyme activity can be detected in multiple human tissues, with the highest activity found in the liver and lymphocytes.[56] A prospective study was conducted with 27 patients in which a significant linear correlation was found between DPD enzyme activity in the liver and in PBMCs (R=0.59, P=0.002). This indicates that DPD enzyme activity measured in PBMCs reflects DPD enzyme activity expressed in the liver.[57] Therefore, PBMCs are often used to measure the DPD enzyme activity and identify patients with a DPD-deficiency. Kuilenburg *et al.* demonstrated that in ~60% of the cases with severe toxicity a decreased DPD enzyme activity could be detected in PBMCs. In addition, 55% of patients with decreased DPD enzyme activity developed severe grade 4 neutropenia versus 13% in patients with a normal DPD enzyme activity (P=0.01). Moreover, the onset of toxicity was significantly faster in patients with a decreased DPD enzyme activity compared to patients with a normal DPD enzyme activity (10.0±7.6 vs. 19.1±15.3 days, P<0.05).[58] Over the years several assays have been developed for the determination of the DPD enzyme activity in PBMCs and this has led to different thresholds for DPD-deficiency. By our knowledge no consensus has been reached about a uniform threshold to determine DPD-deficiency based on DPD enzyme activity, making it hard to properly interpret and compare results. A pragmatic approach for determination of the threshold is described by Milano *et al.* who define a significant DPD-deficiency as the DPD enzyme activity in PBMCs less than 70% of the mean population value.[59] In addition, as earlier mentioned for U, DPD enzyme activity is influenced by a circadian rhythm which could influence the measured activity and therefore the subsequent dose-adaption.[8] Furthermore, the clinical implementation of the measurement of DPD enzyme activity in PBMCs is hampered by its complex and laborious sample processing, which makes it also time-consuming and expensive. In addition, not all laboratories (especially in smaller hospitals) have the specific equipment to perform this assay which also does not add to a widespread implementation. However, in the rare case of a homozygous or compound heterozygous *DPYD*-genotype the DPD enzyme activity test in PBMCs could still be extremely useful. Patients with these genotypes most likely have very low DPD enzyme activity (or a complete DPD-deficiency) and in general will not be treated with fluoropyrimidine-based chemotherapy, as these genotypes are difficult to interpret and the risk of severe toxicity is too high. For these rare cases the DPD enzyme activity could be determined and treatment could be tailored based on the remaining DPD enzyme activity compared to a normal DPD enzyme activity as described by Henricks *et al.*[60]

5-FU degradation rate

Another method to predict the risk of severe toxicity based on PBMCs is the determination of 5-FU degradation rate (5-FUDR). This assay measures the rate of 5-FU degradation in intact PBMCs. 5-FUDR distinguishes itself from DPD enzyme activity measured in PBMCs by incorporating the complete metabolism involved in drug catabolism instead of focusing on a specific enzyme.[61] This phenotypic method was tested and three metabolic classes were identified: poor metabolizers (PM, 5-FUDR≤0.85 ng/ml/10^6 cells/min), normal metabolizers (NM, 0.85 ng/ml/10^6 cells/min<5-FUDR≤2.20 ng/ml/10^6 cells/min) and ultra-rapid metabolizers (UM, 5-FUDR>2.20 ng/ml/10^6 cells/min). As expected, PMs showed an increased risk of developing severe toxicity compared to NMs. However, it was also seen that UMs were at increased risk of developing severe toxicity. It was hypothesized that this could be caused by an increased activity of the enzymes producing the active and cytotoxic metabolites.[62,63] Two retrospective studies also showed a similar association between low and high (OR 11.14, 95%CI:1.09-113.77 (low) and OR 9.63, 95%CI:1.70-54.55 (high), P=0.002) 5-FUDR and severe toxicity.[64,65] Furthermore, due to low-costs (mentioned to be only €10 per sample), non-invasive sampling and quick test results (within 1 working day) 5-FUDR seems suitable for clinical implementation.[65] Although promising, 5-FUDR has similar disadvantages as measurement of DPD enzyme activity in PBMCs as it requires specific equipment.[61] Furthermore, 5-FUDR lacks prospective validation which makes it difficult to assess clinical utility. More research is needed to assess the ability to predict severe toxicity and how fluoropyrimidine treatment should be individualized based on 5-FUDR.

Pharmacokinetically-guided dosing

In addition to *DPYD*-genotyping and DPD-phenotyping, PK-guided dosing of fluoropyrimidines has been studied extensively as a measure to individualize dosing. Use of a PK-based dosing approach could assist in dose-individualization of fluoropyrimidines and optimal systemic exposure, which would be ultimately more effective and less toxic for the patient. PK-guided dosing is better known as therapeutic drug monitoring (TDM). As mentioned earlier, no correlation has been found between BSA and the 5-FU clearance.[10] Therefore, an alternative could be to adjust the dose based on direct monitoring of the blood levels of 5-FU, as it has been shown that there is a relationship between 5-FU plasma concentration and biological effect, toxicity and efficacy.[66-68] It should be mentioned that limited data is available for TDM of capecitabine and therefore only 5-FU will be discussed in this subsection. Although capecitabine shares the same metabolic pathway, it is hypothesized that TDM is most likely not applicable for capecitabine in a clinical setting due to the complex pharmacokinetics.

Over the years several studies have been performed in which PK-guided dosing was

applied.[67,69-72] Fety *et al.* conducted a randomized clinical trial in which 122 head and neck cancer patients were treated with a continuous infusion of 5-FU (96 hours).[72] Patients received a standard dose (4 g/m^2) after which the dose was modified based on either toxicity (St-arm) or PK-parameters (PK-arm). In the PK-arm (N=49), the AUC and 5-FU doses were significantly reduced during cycle 2 and 3 compared with the St-arm (P<0.001), while maintaining a comparable response rate. In addition, grade 3-4 neutropenia and thrombopenia were significantly more frequent in the St-arm compared to the PK-arm (17.5% vs. 7.6%, P=0.013).[72] In another study by Gamelin *et al.*, a PK-guided dosing approach in 280 patients with metastatic CRC was studied.[67] Patients were randomly assigned to either arm A (BSA-guided dosing of 5-FU) or arm B (PK-guided dosing of 5-FU). The initial dose was 1500 mg/m^2 5-FU plus 200 mg/m^2 folinic acid during a continuous 8-hour infusion. In arm B 5-FU doses were adjusted weekly based on single point measurements of 5-FU plasma concentrations at steady state until the therapeutic range of 2.5-3.0 mg/L (AUC range of 20-24 mg*h/L) was reached.[67] This range was established by Gamelin *et al.* in previous studies.[73,74] It was shown that patients in arm A received a mean 5-FU dose of 1500 mg/m^2 throughout treatment compared with 1790 mg/m^2 in arm B, while significantly more patients experienced severe toxicity in arm A (P=0.003). Furthermore, a trend towards a better median OS was seen in arm B compared to arm A (22 months vs. 16 months, P=0.08). Showing that arm B was treated with a higher dose-intensity without experiencing more toxicity and most likely improved OS.[67] Dosing based on the proposed range by Gamelin *et al.* of 2.5-3.0 mg/L has shown to reduce toxicity without the loss of efficacy.[67] However, this range is rather small, especially knowing that there is a large intra-patient variability in PK of 5-FU. This could lead to unnecessary or incorrect dose-adjustments. Therefore, Kaldate *et al.* proposed a wider $AUC_{0-\geq 18h}$ range of 20 - 30 mg*h/L.[70]

Furthermore, a dosing algorithm was proposed for $AUC_{0-\geq 18h}$ values of 8 mg*h/L to values higher than 40 mg*h/L, with corresponding dose adjustments.[70] This algorithm was prospectively validated by Wilhelm *et al.* in 75 patients with metastatic CRC.[71] After the fourth cycle 54% of patients had an AUC within the target range and the incidence of severe fluoropyrimidine-related toxicity was significantly reduced compared to historical data, despite 55% of patients receiving an increased dose.[71] Also, Goldstein *et al.* have shown that PK-guided dose-individualization is a cost-effective strategy compared to conventional BSA-guided dosing.[75]

These studies show that PK-guided dosing of 5-FU is a viable strategy to individualize dosing of 5-FU which can reduce toxicity while maintaining adequate exposure to 5-FU and efficacy. However, patients are still initially treated with a full dose. Severe fluoropyrimidine-related toxicity can occur rapidly (especially in DPD-deficient patients) and PK-guided dose-individualization does not prevent that. Furthermore, additional blood samples need to be taken which is relatively patient unfriendly and could require an additional visit to the hospital, depending on the 5-FU scheme. In addition, PK-guided dosing only applies to treatment with 5-FU which limits the application of this method. Nevertheless, PK-guided follow-up of patients in combination with another dosing strategy could improve the safety and efficacy. An initial dose-reduction could be applied based on, for example, the *DPYD*-genotype, after which the AUC could be evaluated every cycle and dose adjustments can be made to achieve maximal safe exposure.

5-FU test dose

A more direct way to identify patients at risk of toxicity is by administrating a very low dose of 5-FU or capecitabine followed by blood sampling to assess the exposure to treatment with the fluoropyrimidine drug. This was first tested by Bocci *et al.* in 20 CRC patients who were given two dose-levels of 5-FU, 250 and 370 mg/m^2 administered by i.v. bolus. Afterwards, 5-FU and 5-FUH2 were determined in plasma samples obtained at baseline and several time points between 5 min and 4 hours after i.v. bolus. Significant differences in the plasma PK-parameters (AUC, C_{max} and total body clearance) of 5-FU and 5-FUH2 were found between the test-dose and the treatment dose. This is expected as these parameters are influenced by the administered dose. In contrary, no correlations were found between 5-FU or 5-FUH2 at the two dose-levels and the DPD enzyme activity in PBMCs.[76] This was further studied by Bocci *et al.* in 188 patients with gastrointestinal cancer who were treated with 5-FU. Patients were given a 5-FU test-dose of 250 mg/m^2 two weeks before starting initial treatment with 370 mg/m^2. The 5-FU test dose was well-tolerated in all patients. In 3 out of 188 patients marked reduced drug clearance was seen in the presence of a normal DPD enzyme activity. Therefore, these patients were treated with irinotecan instead of 5-FU, which was well tolerated. An association was found between 5-FUH_{2tmax} values higher than 30 min and the risk of moderate to severe neutropenia and diarrhea (P=0.0323 and P=0.0138). This suggests that a 5-FU test dose might be useful for the identification of patients at risk of severe fluoropyrimidine-related toxicity.[77] However, very limited data is available and more research is needed. In addition, to our knowledge no studies have been conducted in which a test dose of capecitabine has been studied. This could limit the use of a test dose as in certain countries capecitabine is used more frequently than 5-FU. Furthermore, it is not certain that both 5-FU or capecitabine will behave similarly when given at such low dose-levels compared to normal dose-levels. Lastly, administration of a test dose of 5-FU to patients with a complete DPD-deficiency could lead to possibly life-threatening toxicity. Therefore, the 5-FU test dose should be combined with at least one other method which can detect a DPD-deficiency upfront before administration.

Patient characteristics at baseline

Gender

While numerous studies have explored the use of the above-mentioned methods to predict severe fluoropyrimidine-related toxicity, few have studied the use of patient characteristics at baseline. Gender-dependent differences in response rates and the probability of toxicity in patients treated with chemotherapy have been seen. It has been suggested that these differences are explained by variation in expression levels of metabolic enzymes and differences in body composition leading to different pharmacokinetics. It has often been seen that the half-life of drug therapy for oncologic diseases are longer in women compared to men, which is associated with improved survival, however, also with increased toxicity.[78] In the SmPC of capecitabine it has been stated the AUC and C_{max} of FBAL are approximately 10% and 20%, respectively, higher in women compared to me.[13] This suggests that capecitabine is catabolized slower in women compared to men. Yet, gender did not have any clinical significant effect on the pharmacokinetics of the main metabolites of capecitabine (5'-dFUR, 5-FU, FBAL).[13]

The pharmacokinetics of fluoropyrimidines have been studied by several researchers and showed different results. Milano *et al.* determined the 5-FU clearance for 380 patients (301 men and 79 women) treated for head and neck cancer with a 5-day continuous intravenous infusion.[79] The 5-FU clearance levels showed a large variation in both men and women, but was significantly lower in women (median 155 L/h/m^2 vs. 179 L/h/m^2, P=0.0005). When adjusted for age and dose, the influence of gender remained significant (P=0.013).[79] This indicates that women have less capacity to clear 5-FU compared to men, and are more likely to develop severe fluoropyrimidine-related toxicity.[79] These differences in 5-FU clearance were later also shown by Mueller *et al.*[80] PK-sampling was performed at baseline for 32 patients receiving a 46h continuous infusion of 5-FU and showed that men had a higher elimination of both 5-FU and 5-FUH2 (26% and 18% higher, respectively). In addition, a significant lower AUC was found in men (18 vs. 22 mg*h/L, P=0.04), independent of weight or BSA, indicating that exposure to fluoropyrimidines is higher in women compared to men.[80] Another study by Stein *et al.* in which the toxicity of 331 patients was analyzed showed that gender is an independent risk predictor, which strengthen the findings of Milano *et al.*[79,81] In addition, two meta-analyses of North Central Cancer Treatment Group trials have been undertaken.[82,83] The first meta-analysis included data from 731 patients (402 men and 329 women) and focused on the incidence of 5-FU-induced stomatitis. Stomatitis was more frequently reported for women and with greater severity compared to men. The incidence of severe or very severe stomatitis for women and men was 22% and 16% (P=0.0006), respectively. Additionally, women were also more likely to experience grade ≥ 3 leukopenia (18% vs. 11%, P=0.004).[82] The second meta-analysis included data from 2.348 patients (1.093 men and 1.093 women) and focused on the incidence of stomatitis, leukopenia, alopecia, diarrhea, nausea and vomiting. Significant differences were found between incidence of severe toxicity between women and men (51% vs. 38%, P<0.0001) across cycles 1 to 3 adjusted for study, dose BMI, and age.[83] Several other studies have also reported the association between gender and severe fluoropyrimidine-related toxicity.[80,84-86]

These studies indicate that women have a decreased 5-FU clearance leading to an increased exposure to fluoropyrimidines and an increased risk of developing severe fluoropyrimidine-related toxicity. Therefore, gender-based dose-individualization should be considered. To our knowledge, this has not been studied yet. In future studies women could be treated with an initially reduced dose after which according to toxicity or PK the dose could be increased. A major advantage of this is that no additional tests or blood sampling are initially required. However, as not all studies have adjusted the results for body size it cannot be stated that the increased risk of developing severe fluoropyrimidine-related toxicity is caused by a decreased 5-FU clearance. Furthermore, prospective studies are needed to confirm the clinical significance of gender-based dosing.

Age

Age has also been studied as a risk factor of developing severe fluoropyrimidine-related toxicity. The decision to treat elderly patients with a reduced dose due to being more fragile and therefore more prone to develop severe fluoropyrimidine-related toxicity has been frequently discussed. Milano *et al.* and Stein *et al.* both studied the influence of age on severe toxicity.[79,81] Interestingly, mixed results were found. Milano *et al.* did not find an association between age and risk of developing severe toxicity, whereas Stein *et al.* found that age was a significant risk factor for severe toxicity (P<0.0001).[79,81] Furthermore, Meulendijks *et al.* retrospectively studied the relationship between age and the risk of developing severe toxicity in 1463 patients of which 231 (16%) experienced early severe toxicity and 132 (9%) were hospitalized.[87] They found that age was a predictor of early severe toxicity, yet not statistically significant (OR 1.14 per 10 years, P=0.0891). However, age was significantly associated with fatal treatment-related toxicity (OR 5.75, P= 0.0008).[87] Recently, a large retrospective study was published by in which the impact of age on toxicity and efficacy of 5-FU-based combination chemotherapy was studied.[85] A total of 3.223 patients were included of which 2.488 patients were <70 years and 735 were ≥75 years. Older age was associated with a higher probability of serious adverse events (OR 0.649; 95%CI 0.545-0.772; P<0.001) and separate toxicities such as all-grade diarrhea, high-grade diarrhea, high-grade stomatitis, high-grade thrombocytopenia, all-grade neutropenia, and high-grade neutropenia.[88] Another study showed that older age was associated with a higher risk of hospitalization. A total of 2.533 patients were included of which 1.010 experienced at least one serious adverse event. In total 945 (39.9%) patients were hospitalized one or more times and 148 (5.8%) patients suffered from fatal events. It was shown that older age was predictive of hospitalization (P<0.001). Older age might be associated with a higher risk of developing severe fluoropyrimidine-related toxicity, however limited information is available. More research is necessary to properly establish the relationship between age and severe toxicity.

Body composition

Another patient characteristic that has been associated with an increased risk of severe toxicity is body composition. Gusella *et al.* have studied the relationship between body composition parameters including body cell mass (BCM), total body water (TBW) and lean body mass (LBM) and 5-FU pharmacokinetics.[89] This relationship was studied in 34 CRC patients (13 women and 21 men) treated with intravenous 5-FU. This study showed that the clearance of 5-FU better correlated with the LBM than the standard measures such as body weight and BSA.[89] This was further studied by Prado *et al.* who used data from a prospective study to determine if the highest doses of 5-FU per kilogram LBM would be associated with dose-limiting toxicity in colon cancer patients treated with 5-FU and leucovorin.[90] A cut-off point of 20 mg 5-FU/kg LBM was found as threshold for developing severe toxicity (P=0.005). This was only found in women (OR 16.73, P=0.021), which had a relatively low proportion LBM compared to their body weight.[90] This could explain the difference in relationship between men and women and severe toxicity found in other studies. Other body composition parameters such as (skeletal) muscle mass have also been studied as predictors of severe toxicity. Williams *et al.* examined the association of low skeletal muscle (sarcopenia) on PK-parameters of 5-FU.[91] No significant differences in AUC were found between sarcopenic and non-sarcopenic patients. However, LBM was also studied and a significant association was found between 5-FU per kg LBM and hematological toxicities (110 vs. 94 mg/kg, P=0.002). Yet, no correlation between the dose/LBM and 5-FU AUC was found.[91] Another study examined the association of sarcopenia and dose-limiting toxicity (DLT) during treatment with capecitabine combination therapy in patients with metastatic CRC. In contrary to Williams *et al.* sarcopenia and/or muscle loss was associated with increased risk of DLTs.[92] Furthermore, Jung *et al.* reviewed the data of 229 patients with colon cancer treated with 5-FU, oxaliplatin, and leucovorin and studied the association of muscle mass and toxicity.[93] It

was shown that a decreased muscle mass was associated with an increased risk of grade 3-4 toxicity and poor prognosis.[93] These studies suggest that body composition parameters such as LBM and muscle mass could be an interesting marker to predict severe toxicity. However, more research is needed to confirm these associations and to determine the corresponding dose-modifications.

Renal function

5-FU is predominantly metabolized in the liver and tumor tissues.[6] Therefore, at first it is not expected that renal impairment would influence the exposure to 5-FU. However, pooled data from phase I studies showed that creatinine clearance has a significant influence on the AUC of 5-FU. On the contrary, a population PK-analysis of phase III trials, did not reveal a significant effect of the creatinine clearance on the pharmacokinetics of 5-FU and 5-FUH2. A significant effect was observed for FBAL, and a positive relationship was seen between AUC of FBAL and treatment-related grade 3-4 diarrhea and C_{max} of FBAL and treatment related grade 3-4 AE's. However, this does not necessarily mean that FBAL causes these AE's. FBAL might be a marker of the amount of 5-FU that is formed in tissues. Meaning that patients with high FBAL concentrations might be patients with a high exposure to 5-FU. Renal impairment leads to a major increase in the systemic exposure to FBAL, but did not significantly impact the pharmacokinetics of capecitabine and 5-FUH_2.[6] Another study by Cassidy *et al.* showed that creatinine clearance is inversely correlated to risk of toxicity and recommended a dose-reduction of 25% for patients with moderate renal impairment (calculated creatinine clearance 30-50 ml/min) and contra-indicate capecitabine for patients with a severe renal impairment (<30 ml/min).[94] This recommendation was followed-up and taken up in the SmPC in 2005.[13] Furthermore, Meulendijks *et al.* also found that renal function is a clinically relevant predictor of severe fluoropyrimidine-related toxicity in a dataset of 1.463 patients treated with capecitabine or 5-FU.[21] However, the precise mechanism by which renal impairment increases risk of severe fluoropyrimidine-related toxicity is unclear.

Multiparametric approaches

Information about patient characteristics such as gender, age and renal function are easily obtained or measured and have shown to most likely have a relationship with the development of severe fluoropyrimidine-related toxicity. Therefore, the logical next step would be to combine these patient characteristics with the more established strategies such as *DPYD*-genotyping and DPD-phenotyping to develop a dosing algorithm.

In 2007 a decision-tree was described by Boisdron-Celle *et al.* in which *DPYD*-genotyping was combined with the measurement of endogenous U concentration, DHU/U-ratio and individual PK follow-up.[38] This algorithm was further developed and a multicenter prospective cohort study was performed to assess the clinical benefit of this new multiparametric approach. In this study two parallel cohorts were treated with 5-FU-based chemotherapy. In arm A, patients were screened upfront for DPD-deficiency with the multiparametric approach, whereas no screening for DPD-deficiency was performed in arm B. In total 1,142 patients were included of which 718 in arm A and 398 in arm B. The percentage of patients experiencing grade 4-5 toxicity in Arm A was 1.2% vs. 3.0% in arm B (P=0.0406) and 10.9% vs. 17.6% (P=0.497) for grade 3-5 toxicity, respectively. It was concluded that this multiparametric approach significantly reduced the risk of developing severe fluoropyrimidine-related toxicity.[95] Although promising, some serious questions are raised regarding the methodology of this study as mentioned by Etienne-Grimaldi *et al.* in a letter to the editor.[96] It was noted that the prevalence of DPD-deficiency based on the multiparametric approach and DHU/U-ratio in arm A was 2.5-fold (P=0.00017) and 4-fold fewer (P=0.00007) compared to arm B, respectively. This means that the two arms were incomparable at baseline resulting in less toxicity in arm A.[96] The most important factor which makes it difficult to properly interpret these results is the fact that this multiparametric approach is protected by a patent, therefore it is unknown what this approach consists out of and could be seen as a so called 'blackbox'. It is mentioned that *DPYD*-genotyping is combined with DPD-phenotyping (DHU/U-ratio) and that demographic parameters are used, but how this is converted into a dose-recommendation is not described.

Similarly, Botticelli *et al.* aimed to develop a nomogram which could accurately predict toxicity.[97] This nomogram consisted of metabolic parameters and clinical patient characteristics. Fluoropyrimidine-related toxicity was correlated with patient specific and treatment-related factors. Univariate logistic regression analyses were performed to identify predictive variables. Variables with a P-value less than 0.10 in the univariate model were entered into a multivariate model. Multivariate logistic regression showed that age, *DPYD* status, the number of drugs administered, and 5-FUDR value were associated with severe fluoropyrimidine-related toxicity (P-values below 0.05). Based on these findings a nomogram was structured to assess a score to predict the probability of developing severe fluoropyrimidine-related toxicity before starting treatment. However, no corresponding dose-modification is mentioned. Therefore, it is unclear how much the dose should be reduced if a patient has a certain probability of developing severe fluoropyrimidine-related toxicity. Furthermore, it is unclear why the chosen variables were selected to include in the univariate analysis. In addition, this nomogram has not been validated either internally or externally, therefore it is difficult to assess how accurate this nomogram can predict the probability of developing severe fluoropyrimidine-related toxicity.[97]

Recently, Etienne-Grimaldi *et al.* presented the results of the FUSAFE meta-analysis in which the performance of *DPYD*-genotyping to predict fluoropyrimidine-related toxicity was studie.[98] A clinical model was developed to assess the prognostic value of consensual deleterious *DPYD* variants on grade 4-5 toxicity. This model was based on data of 6,403 Caucasian patients from 7 studies and included age, sex, BMI, fluoropyrimidine administration mode and associated anti-cancer drugs as predictors of grade 4-5 toxicity. The presence of *DPYD**2A, c.2846A>T and c.1679T>G improved the model and showed to be relevant in predicting grade 4-5 toxicity. Despite its association with toxicity, c.1236G>A did not improve the ability of the model to identify patients at risk of grade 4-5 toxicity.[98]

Conclusion and future perspectives

Numerous strategies for dose-individualization have been discussed in this review. However, the level of evidence and feasibility differs a lot between these strategies. Currently, the most established and evidence-based strategy for dose-individualization

of fluoropyrimidine-based chemotherapy is *DPYD*-guided dosing. It has been shown that this strategy significantly reduces the incidence of severe fluoropyrimidine-related toxicity, does not negatively impact efficacy and is cost-effective.[16,23-25] Therefore, we think that *DPYD*-guided dosing should be the cornerstone in dose-individualization of fluoropyrimidines and recommend that this strategy is implemented in routine clinical care. However, only a limited number of patients experiencing severe toxicity can be identified with the four current variants and these variants are most likely only predictive for severe toxicity in patients of western descent. Therefore, additional screening methods are needed and more research should be conducted in ethnicities which are underrepresented in genetic studies.

The major issue with these additional screening methods is the lack of prospective validation. Multiple screening methods (e.g., DPD-phenotyping) have shown to be promising, but due to the lack of prospective studies are scarcely being implemented. Measuring the DPD enzyme activity in PBMCs would probably be the choice for which most evidence is available, yet due to the complicated and laborious method is not recommended for application in clinical routine care. Measurement of U or the DHU/U-ratio could be a good alternative. Previous studies have shown that U could be an accurate predictor of severe fluoropyrimidine-related toxicity. Therefore, results of the recently started prospective clinical trial which combines *DPYD*-genotyping and U measurements (NCT04194957) are awaited. In addition to these methods, PK-guided follow-up of patients could further improve the safety of treatment with fluoropyrimidine-based chemotherapy, especially for 5-FU treated patients.

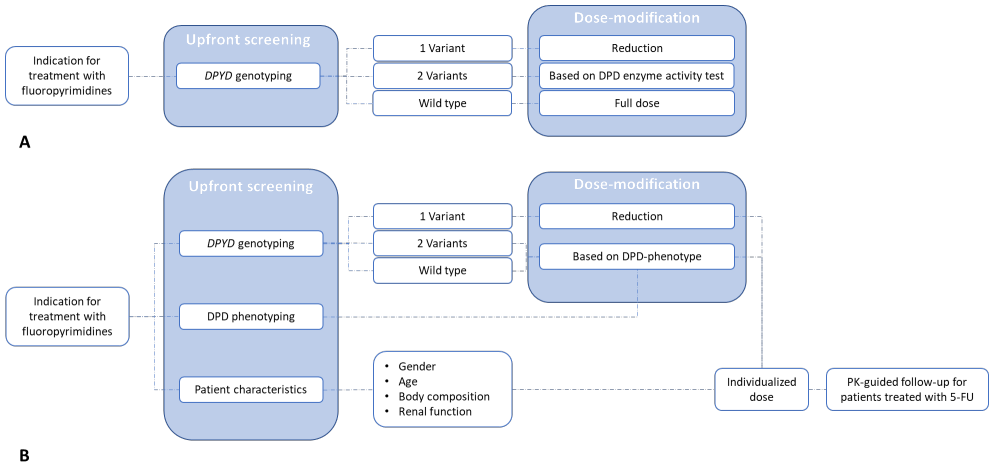

Figure 2: (A) Current dosing strategy. (B) Potential future dosing strategy in which upfront screening is performed which includes *DPYD*-genotyping, DPD-phenotyping and screening of baseline characteristics and PK-guided follow-up. *Abbreviations*: 5-FU = 5-fluorouracil; DPD = dihydropyrimidine dehydrogenase; PK = pharmacokinetic.

Set dose adjustments based on *DPYD*-genotype or DPD-phenotype can reduce the incidence of severe toxicity but are not suited for all patients in a similar manner. With PK-guided follow-up patients could be monitored and treatment could be altered if

concentrations are outside of the therapeutic range. However, this is only possible for patients treated with 5-FU due to the complex metabolism of capecitabine. An interesting addition to these dosing strategies could be the use of patient characteristics at baseline. Patient characteristics such as age, gender and renal function are easily obtained or measured and have shown to most likely have a relationship with the development of severe fluoropyrimidine-based toxicity. However, only limited information is available. Studies in which the dose of fluoropyrimidines are individualized based on these characteristics are needed. All the strategies described in this review have shown to have potential, however the limitations of these strategies need to be overcome by conducting additional research before combining of strategies is possible.

In an ideal world all the proposed strategies could be combined into an algorithm or model which could accurately predict the probability of developing severe fluoropyrimidine-related toxicity and translate this probability into a dose recommendation (Figure 2). By combining all these strategies all known factors that have been associated with severe fluoropyrimidine-related toxicity are covered which could significantly improve the safety of fluoropyrimidine-based chemotherapy.

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Chapter 2

Discovering novel germline genetic variants outside and inside *DPYD* associated with the onset of severe fluoropyrimidine-related toxicity

Submitted

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Abstract

Background: The Alpe-DPD study (NCT02324452) demonstrated that prospective genotyping and dose adjustment using four alleles in *DPYD* (*DPYD**2A/rs3918290, c.1236G>A/rs75017182, c.2846A>T/rs67376798 and c.1679T>G/rs56038477) can mitigate the risk of severe fluoropyrimidine toxicity. However, dose reductions for carriers of these variants could not prevent all toxicities. The goal of this study was to identify additional genetic variants, both inside and outside *DPYD*, that may contribute to fluoropyrimidine toxicity.

Methods: Bio specimens and data from the Alpe-DPD study (clinicaltrial.gov identifier NCT02324452) were used. Exon sequencing was performed to identify risk variants inside *DPYD*. In silico and *in vitro* analyses were used to classify *DPYD* variants. A genome-wide association study (GWAS) with severe fluoropyrimidine-related toxicity (Common toxicity criteria grade ≥3) was performed to identify variants outside *DPYD*. Association with severe toxicity was assessed using matched-pair analyses for the exon sequencing and logistic, Cox, and ordinal regression analyses for GWAS.

Findings: Twenty-four non-synonymous, frameshift, and splice site *DPYD* variants were detected in ten of 1,103 patients. Seven of these variants (c.1670C>T, c.1913T>C, c.1925T>C, c.506delC, c.731A>C, c.1740+1G>T, c.763-2A>G) were predicted to be deleterious. The carriers of either of these variants showed a trend towards a 2.14-fold (95% CI, 0.41-11.3, P=0.388) increased risk of severe toxicity compared to matched controls (N=30). After GWAS of 942 patients, no individual single nucleotide polymorphisms achieved genome-wide significance ($P \leq 5x10^{-8}$), however, five variants were suggestive of association ($P < 5x10^{-6}$) with severe toxicity.

Interpretation: Our results from *DPYD* exon sequencing and GWAS analysis suggest that at a population level, testing for single markers in addition to the four established *DPYD* variants, currently has limited value in improving fluoropyrimidine toxicity prediction.

Introduction

Fluoropyrimidines, including 5-fluorouracil (5-FU) and capecitabine represent the backbone of chemotherapeutic regimens used in the treatment of solid tumours, such as gastroesophageal, colorectal, and breast cancer. Depending on the treatment regimen administered, severe fluoropyrimidine-induced toxicity affects approximately 35% of recipients and can be lethal in up to 1% of the patients.[1] Common fluoropyrimidine-induced adverse events include diarrhoea, mucositis, hand-foot syndrome, and myelosuppression. An increased risk for the development of fluoropyrimidine-induced toxicity exists in patients with a deficiency of dihydropyrimidine dehydrogenase (DPD), an enzyme that is encoded by the *DPYD* gene and responsible for catalysing 5-FU degradation into inactive metabolites.[2] Both DPD activity and genetic variants in *DPYD* have been widely investigated and partially explain severe fluoropyrimidine-induced toxicity. Previous studies and meta-analyses have shown a strong association between four *DPYD* variants (c.1905+1G>A/rs3918290, c.1236G>A/ rs56038477, c.2846A>T/ rs67376798, and c.1679T>G/rs55886062) and severe fluoropyrimidine-induced toxicity.[3,4] Recently, we showed that patients' safety indeed improved following fluoropyrimidine dose individualization based on *DPYD*-genotyping of the four *DPYD* variants mentioned above.[5] Consistent with these findings, the European Medicine Agency (EMA) recently recommended that all patients scheduled for fluoropyrimidine treatment should be tested for DPD deficiency before the start of treatment.[6]

Despite the recognition of the importance of the abovementioned four variants in reducing toxicity, approximately 23% of *DPYD* wild-type patients still experienced severe fluoropyrimidine-induced toxicity.[5] This suggests that additional factors, including other *DPYD* genetic variants and/or variants affecting other genes involved in fluoropyrimidine metabolism, may contribute to toxicity. Indeed, low frequency germline variants (minor allele frequencies (MAFs) <1%) may explain approximately 30-40% of inter-individual functional variability in pharmacogenes.[7] However, the effect of these low-frequency variants in *DPYD* has not been assessed comprehensively in fluoropyrimidine-treated patient populations.

In the present study, we sought to identify potential biomarkers of severe fluoropyrimidine toxicity risk in a patient population that did not carry any of the four well-characterized risk alleles in *DPYD*. To accomplish this goal, we used complementary approaches for genotyping that included both targeted sequencing of the exon-coding region for *DPYD* and genome-wide association study (GWAS) in cancer patients treated with fluoropyrimidines.

Methods

Patients

Clinical data including baseline characteristics and toxicity data were derived from patients included in the Alpe-DPD study (clinicaltrial.gov identifier NCT02324452).[5] All patients (N=1,181) signed informed consent before inclusion in the study, which included approval for the use of clinical data and remaining DNA to perform *DPYD* sequencing and GWAS. Eighty-five patients carrying one of the 4 *DPYD* variants *DPYD**2A, c.1236G>A/

HapB3, c.2846A>T, or c.1679T>G received a dose reduction to prevent severe toxicity and were therefore excluded from the analyses performed in this 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.[8] Only the highest graded adverse events classified as possible, probable, or definite related to fluoropyrimidines were included in the analyses.[5]

DPYD sequencing

Genotyping

Targeted DNA sequencing was performed for specimens with adequate DNA (N=1,103). Sequencing libraries were generated using Access Array chemistry (Fluidigm, South San Francisco, CA) as previously described, with modifications.[9] Custom primer panels were designed to cover all 23 exons of the *DPYD* gene and the intronic region containing rs75017182, the causal single nucleotide polymorphisms (SNP) in perfect linkage disequilibrium with c.1236G>A/HapB3. Target amplification and sample indexing were performed using Juno Targeted Sequencing LP 192.24 Integrated Fluidic Circuits (IFCs) on a Juno instrument (Fluidigm). Indexed sequencing libraries from 2 IFCs were pooled, and paired-end sequencing was performed using an Illumina HiSeq 4000 in the Mayo Clinic Cancer Center Genome Analysis Core. Patient sequence data were demultiplexed using barcode sequences added during library preparation. Adapter and region-specific primer sequences were pruned, and reads were aligned to targeted regions of the hg38 human reference genome using BWA-MEM. Variants were identified using GATK HaplotypeCaller. A QUAL score of ≥500 across the population of samples tested was used as a threshold for variant inclusion in subsequent analyses. The presence of toxicity-associated variants (*DPYD**2A, c.1236G>A, c.2846A>T, or c.1679T>G) was confirmed using previous genotyping data.[5] The genotypes for additional rare variants with allele frequencies less than 1% in the study population were confirmed in carriers by Sanger sequencing of the relevant exon at the Mayo Clinic Cancer Center Genomics Analysis Core using methods that have been previously described.[9]

Variant classification

In this study, different *in vitro* and in silico approaches were used to assess the potential effect of identified *DPYD* variants (Figure 1). Missense variants were evaluated using a previously published *in vitro* expression system in HEK293T/c17 cells. If available, results for variants where reused, otherwise, novel variants were expressed in the *in vitro* system.[10,11] Detailed primer sequences used to generate the expression plasmids for selected variants are reported in the supplementary (Table 1). 2) the *DPYD*-Varifier, a *DPYD*-specific in silico prediction tool applied for eligible variants.[12] Frameshift variants were considered deleterious based on previous findings.[11] The potential impact of splice variants was predicted using MMsplice, a modelling-based tool to predict genetic variation effects on splicing.[13]

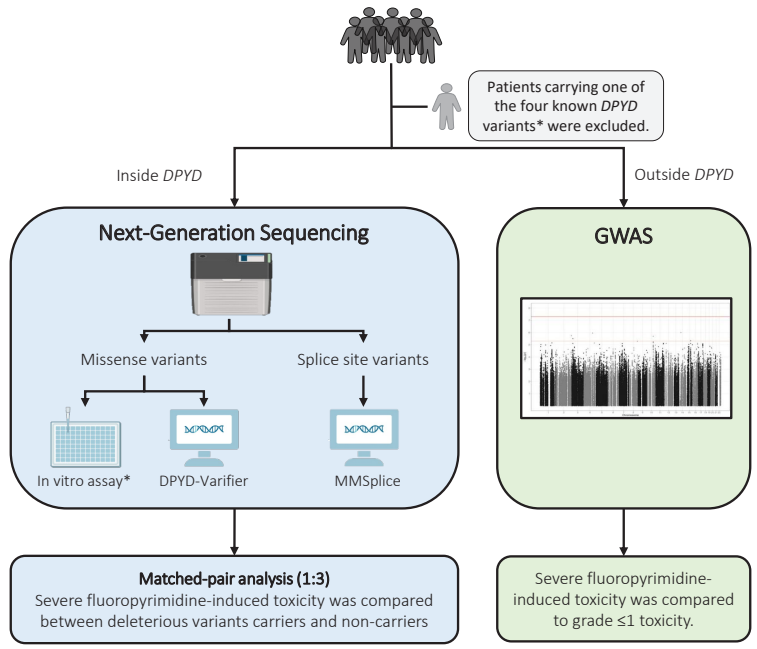

Figure 1: Study design. Severe fluoropyrimidine-related toxicity was defined as CTC-AE grade ≥3. *The four known *DPYD* variants are *DPYD**2A, c.1236G>A, c.2846A>T, and c.1679T>G.

Statistical analysis

After *DPYD* sequencing, all patients who carried at least one predicted deleterious variant were matched with wild-type controls identified from the Alpe-DPD study participants to compare toxicity. Automatic matching (in a 1:3 ratio) was performed based on three parameters that are most strongly associated with toxicity: treatment regimen, tumour type, and disease stage. If more than three eligible wild-type controls that fulfilled all matching criteria were available, the paired matches were selected at random from these eligible controls. Fisher's exact test was conducted to compare the incidence of severe toxicity between deleterious variants carriers and their matched controls. Statistical analyses were conducted using SPSS version 25.0.

Genome-Wide Association Study

Genotyping was performed at the Human Genotyping Facility of the Erasmus University Medical Center, using the Infinium Global Screening Array (GSA) v1.0.[14] The array contains 692,842 SNPs and includes rare variants with allele frequencies <1%. A minor allele frequency (MAF) of 0.5% was used for the primary analysis. 1,000 Genomes reference phase 3 GRCh37.p13 was used to impute the data.[15,16] Quality control (QC) checks can be found in the supplementary information. 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 ≤1 fluoropyrimidine-induced toxicity. Grade 2 toxicity was excluded from the primary analysis to maximize the contrast between toxicities (Figure 1). Additionally, as a sensitivity analysis, severe fluoropyrimidine-induced toxicity was also compared to grade ≤2

fluoropyrimidine-induced toxicity. Gender, age, baseline body surface area (BSA), and treatment regimen (grouped as previously published)[5] were used as pre-specified covariates. Statistical analyses were performed in R statistics version 2.3.2.[17] Base packages stats, survival, and MASS were used to evaluate logistic, Cox, and ordinal regression analyses, respectively. A p value threshold of $\leq 5x10^{-8}$ was used for determining significance at the genome-wide level. 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. Online databases (Linkage-Disequilibrium tools, hapreg, and genome browser) were used to explore possible biological mechanisms of genome-wide associated or suggestive novel SNPs.[18-20]

Results:

Cohort

Patient characteristics are shown in Table 1. In total, 1,181 were included in the Alpe-DPD study, of which 1,103 were evaluable (Figure 2). Of these, 85 *DPYD* variant carriers (*DPYD**2A, c.1236G>A/HapB3, c.2846A>T, or c.1679T>G) were treated with a reduced dose and consequently excluded from analyses, resulting in 1,018 patients being evaluable for *DPYD* sequencing and GWAS analysis. Whole exon sequencing failures and GWAS quality control checks led to the exclusion of 32 and 74 patients, resulting in 986 and 942 patients being included in the *DPYD* sequencing analysis and GWAS, respectively. As stated in the methods section, patients with grade 2 toxicity were disregarded in the GWAS analysis, leading to 599 patients in the GWAS cohort.

DPYD sequencing and variant function prediction

A total of 24 non-synonymous, frameshift, and splice site variants (in addition to the four variants being tested for in the original Alpe-DPD study) were detected in 986 individuals. Of these variants, 20 were in exons (Table 2) and four were in introns (Table 3). The frequencies and results of the functional assessment with the three prediction tools are described in Table 2 and Table 3, respectively. In total, seven rare deleterious variants were identified, which were carried by 10 individuals. Five variants in the coding region (c.1670C>T, c.1913T>C, c.1925T>C, c.506delC, and c.731A>C) and two variants in the flanking splice region (c.1740+1G>T and c.763-2A>G) were predicted to be deleterious. Of these seven variants, only c.1670C>T and c.763-2A>G have been reported previously. The remaining seventeen non-synonymous variants were predicted benign, of which 3 have not yet been previously noted in dbSNP. A comparison of the three prediction tools can be found in supplementary Table 2.

Out of the patients who carried predicted deleterious variants, 3 of 10 (30%) patients developed severe toxicity. No statistically significant difference in severe toxicity was found between patients carrying a predicted deleterious variant and 30 matched non-carriers (OR 1.47, 95% CI, 0.38 to 5.74, P<0.703). In matched control patients who did not carry any deleterious *DPYD* variant, 16.7% (5 out of 30 patients) experienced severe toxicities (OR 2.143, P<0.388; Supplementary Table 3). The patient characteristics of ten carriers and their matched control are shown in the supplementary Table 4.

Table 1: Patient characteristics. Patient characteristics of evaluable patients (N=1,103) and the patients included in the primary analysis of the GWAS (N=599). Data are n (%) or median (IQR).

Characteristic	Evaluable patients (N=1,103)	GWAS cohort (N=599)
Gender		
Male	593 (54%)	319 (53%)
Female	510 (46%)	280 (47%)
Age in years, median, (IQR)	64 (56-71)	64 (57-71)
Ancestry		
White	1048 (95%)	573 (96%)
Black	19 (2%)	14 (2%)
Asian	24 (2%)	9 (2%)
Other[a]	12 (1%)	3 (<1%)
Tumor type		
Non-metastatic colorectal cancer	472 (43%)	265 (44%)
Metastatic colorectal cancer	232 (21%)	114 (19%)
Breast cancer	141 (13%)	75 (13%)
Gastric cancer	63 (6%)	32 (5%)
Other[b]	195 (18%)	113 (19%)
Type of treatment regimen		
Capecitabine monotherapy (±bevacizumab)	205 (19%)	102 (17%)
Capecitabine + radiotherapy (±mitomycin)	264 (24%)	172 (29%)
Capecitabine + oxaliplatin (±bevacizumab)	374 (34%)	179 (30%)
Capecitabine + other anticancer drugs	72 (7%)	41 (7%)
Fluorouracil monotherapy	2 (<1%)	-
Fluorouracil + radiotherapy (±mitomycin)	63 (6%)	43 (7%)
Fluorouracil + oxaliplatin + folinic acid (±bevacizumab)	43 (4%)	18 (3%)
Fluorouracil + other anticancer drugs	80 (7%)	44 (7%)
BSA, median, (IQR)	1.9 (1.8-2.1)	1.9 (1.8-2.1)
WHO performance status		
0	554 (50%)	317 (53%)
1	448 (41%)	241 (40%)
2	42 (4%)	21 (4%)
Not specified[c]	59 (5%)	20 (4%)
Number of treatment cycles, median, (IQR)	3 (1-8)	3 (1-7)

[a] Other ethnic origins included Hispanic descent, mixed racial parentage, and unknown ethnic origin;
[b] Other tumor types included anal cancer, oesophageal cancer, head and neck cancer, pancreatic cancer, bladder cancer, vulvar cancer, unknown primary tumors, and rare tumor 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 organization

Genome-wide association analysis

GWAS was assessed for severe (grade ≥3) toxicity and was compared to grade 0 or 1 toxicity in 599 patients (excluding 343 patients with grade 2 toxicity, Figure 2). The number of patients varied per SNP due to genotype missingness, which was limited to up to 3% as per QC. An association test for severe fluoropyrimidine-induced toxicity (CTC-AE grades 3-5) was performed for a total of 4,650,899 markers. Gender, age, baseline BSA, and treatment type were included as covariates. The corresponding Manhattan and QQ plots are shown in the supplementary (Figure 1 and 2). The inflation factor is 1.04. While none of the individual SNPs achieved genome-wide significance as per the pre-specified definition ($p \leq 5x10^{-8}$), five variants (rs17114875, rs367239, rs77579689, rs114105116, and

Table 2: Frequencies and results of functional assessment of all variants in exons.

Non-synonymous and frameshift variants							
Transcript change	**dbSNP**	**Amino acid change**	**Carriers**	**Prediction tool**		**Predicted activity[†]**	**Observed severe toxicity**
				***In vitro* assay**	***DPYD*-Varifier**		
c.1218G>A	rs61622928	M406I	5 (0.45%)	Neutral[#]	Neutral	Neutral	0 (0)
c.1601G>A	rs1801158	S534N	47 (4.26%)	Neutral[#]	Neutral	Neutral	14 (29.8%)
c.1627A>G	rs1801159	I543V	366 (33.2%)	Neutral[#]	Neutral	Neutral	91 (24.9%)
c.1670C>T	rs754125729	T557I	2 (0.18%)	Deleterious	Deleterious	Deleterious	0 (0)
c.1796T>C	rs147601618	M599T	1 (0.09%)	Neutral[#]	Neutral	Neutral	0 (0)
c.1913T>C	NA	I638T	2 (0.18%)	Deleterious	Deleterious	Deleterious	0 (0)
c.1925T>C	NA	M642Ts	2 (0.18%)	Deleterious	Deleterious	Deleterious	1 (50%)
c.2087G>A	rs778298325	R696H	2 (0.18%)	Neutral	Neutral	Neutral	1 (50%)
c.2194G>A	rs1801160	V732I	103 (9.33%)	Neutral[#]	Neutral	Neutral	26 (25.2%)
c.2194G>T	NA	V732F	1 (0.09%)	Neutral	Deleterious	Neutral	1 (100%)
c.2621A>G	rs1164428597	K874R	1 (0.09%)	Neutral	Neutral	Neutral	1 (100%)
c.2806G>T	rs137878450	G936C	1 (0.09%)	Neutral[#]	Neutral	Neutral	1 (100%)
c.3067C>A	rs114096998	P1023T	2 (0.18%)	Neutral[#]	NA	Neutral	2 (100%)
c.482A>G	NA	E161G	1 (0.09%)	Neutral	Neutral	Neutral	0 (0)
c.496A>G	rs2297595	M166V	176 (15.96%)	Neutral[#]	Neutral	Neutral	53 (30.1%)
c.506delC	NA	c.506delC	1 (0.09%)	Deleterious[#]	NT[*]	Deleterious	1 (100%)
c.731A>C	NA	E244A	1 (0.09%)	Deleterious	Deleterious	Deleterious	0 (0)
c.768T>G	rs556933127	I256M	3 (0.27%)	Neutral	Neutral	Neutral	0 (0)
c.775A>G	rs45589337	K259E	13 (1.18%)	Neutral[#]	Neutral	Neutral	2 (15.4%)
c.85T>C	rs1801265	C29R	411 (37.26%)	Neutral[#]	NA	Neutral	95 (23.1%)

[*]Outside of structurally defined regions of human DPD protein and therefore cannot be classified using *DPYD*-Varifier. NT, Not testable. [†]An identified variant was regarded as deleterious if at least one of these two tools predicted the variant to be deleterious. Notably, if there is a contradictory result from *in vitro* assay and *DPYD*-Varifier, the effect identified by *in vitro* assessment is preferred. [#]The *in vitro* assessment results of these variants have been published previously.[10-12] The primer sequences used to perform the novel site directed mutagenesis on the expression plasmids are included in the supplementary files. NA, Not assigned.

rs12622722) showed a suggestive association with severe toxicity, with p values between $5x10^{-8}$ and $5x10^{-6}$.

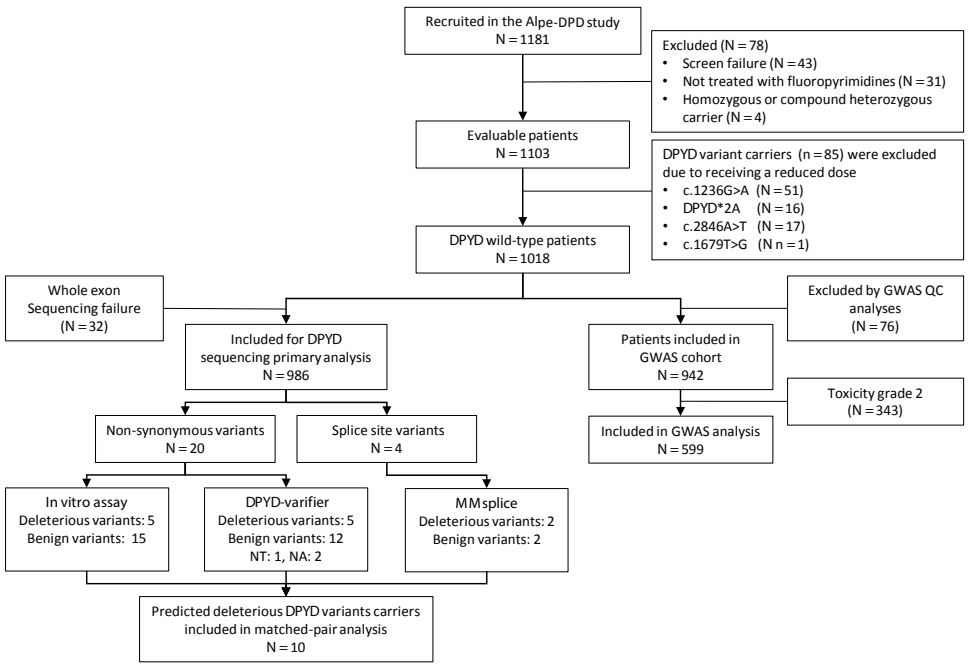

Figure 2: Flowchart of patient inclusion. Patients who experienced grade 2 toxicity were excluded from the GWAS analyses to maximize the contrast between severe and non-severe toxicity. *Abbreviations*: QC: quality control; *DPYD*: gene encoding dihydropyrimidine dehydrogenase; NT: Not tested, NA: Prediction not available

The closest annotated genes to rs171114875 are PRKD1 and MIR548AI. The closest annotated gene to rs77579689 is KHDRBS3. The closest annotated genes to rs367239 are VENTXP7, and ZNF385D. Additionally, rs367239 is in linkage disequilibrium with rs1396004 and rs341838 which are both SNPs located in VENTXP7. The other two suggestive variants are listed as intronic variants of the non-coding RNA gene LOC101927414 (rs114105116) and protein-coding gene COL6A3 (rs12622722). The 30 most significantly associated markers are shown in Table 4. None of these SNPs have previously been reported in publications or the ClinVar database of the National Center for Biotechnology Information (NCBI).[15]

Genome-wide association analysis

GWAS was assessed for severe (grade ≥3) toxicity and was compared to grade 0 or 1 toxicity in 599 patients (excluding 343 patients with grade 2 toxicity, Figure 2). The number of patients varied per SNP due to genotype missingness, which was limited to up to 3% as per QC. An association test for severe fluoropyrimidine-induced toxicity (CTC-AE grades 3-5) was performed for a total of 4,650,899 markers. Gender, age, baseline BSA, and treatment type were included as covariates. The corresponding Manhattan and QQ plots are shown in the supplementary (Figure 1 and 2). The inflation factor is 1.04. While

Table 3: Frequencies and results of functional assessment of all variants in splice region.

Splice site variants				
c	**dbSNP**	**Carriers**	**MMsplice**	**Observed Severe toxicity, No. (%)**
c.1129-3delT	NA	1 (0.09%)	Neutral	0 (0)
c.1740+1G>T	NA	1 (0.09%)	Deleterious	1 (100%)
c.1905C>T	rs3918289	2 (0.18%)	Neutral	0 (0)
c.763-2A>G	rs1300669537	1 (0.09%)	Deleterious	0 (0)

Abbreviations: NA, Not applicable; No, Number.

none of the individual SNPs achieved genome-wide significance as per the pre-specified definition ($p \leq 5x10^{-8}$), five variants (rs17114875, rs367239, rs77579689, rs114105116, and rs12622722) showed a suggestive association with severe toxicity, with p values between $5x10^{-8}$ and $5x10^{-6}$. The closest annotated genes to rs171114875 are PRKD1 and MIR548AI. The closest annotated gene to rs77579689 is KHDRBS3. The closest annotated genes to rs367239 are VENTXP7, and ZNF385D. Additionally, rs367239 is in linkage disequilibrium with rs1396004 and rs341838 which are both SNPs located in VENTXP7. The other two suggestive variants are listed as intronic variants of the non-coding RNA gene LOC101927414 (rs114105116) and protein-coding gene COL6A3 (rs12622722). The 30 most significantly associated markers are shown in Table 4. None of these SNPs have previously been reported in publications or the ClinVar database of the National Center for Biotechnology Information (NCBI).[15]

Discussion

While applying prospective *DPYD* genotyping to clinical practice has successfully reduced the incidence of severe toxicity, a substantial number of patients treated with fluoropyrimidines still experience severe treatment-related toxicity.[5] We hypothesized that in addition to the four established *DPYD* variants, other genetic variations in- and outside *DPYD* might be associated with the onset of severe fluoropyrimidine-related toxicity. Therefore, we performed comprehensive genetic analyses including whole exon sequencing of *DPYD* and a GWAS analysis in a large well-characterized cohort derived from a prospective clinical study consisting of 1,103 mostly Caucasian patients (95%) treated with fluoropyrimidine-based chemotherapy.[5] Within *DPYD*, we detected 24 non-synonymous and splice site variants, of which 7 allele variants that were carried in 10 patients were predicted to be deleterious. In the matched-pair analysis, the carriers of these deleterious variants showed a statistically non-significant 2-fold higher risk of severe toxicity. These findings imply that patients with rare deleterious variants may be at increased risk of severe fluoropyrimidine-related toxicity.

Out of the 24 detected variants, 5 deleterious variants are novel and would have been missed with a pre-designed panel test, highlighting the potential of the combination of next generation sequencing (NGS) with available functionality assessment tools in detecting deleterious variants and preventing life-threatening toxicity. Yet, despite analysis of a large cohort of over 1,000 patients, the number of novel deleterious *DPYD*

variants remains low. Moreover, it is challenging to connect these unique variants to clinical decisions or upfront dose reductions because of the risk of undertreatment, limiting clinical application. Therefore, additional studies on implementing these approaches are needed, especially in understudied populations, which are more likely to carry other deleterious *DPYD* variants in addition to the four commonly tested ones.[9] However, even after accounting for the additional deleterious variants in *DPYD*, unexplained severe fluoropyrimidine-induced toxicity remained. Potentially, this remaining toxicity is the result of genetic variation outside *DPYD*. Several GWAS studies have been performed in patients and cell lines in attempts to identify novel risk variants.[21-23] These previous studies failed to identify associations that reached genome-wide significance, possibly due to limitations including small sample size and focus on specific toxicities such as neutropenia or leucopenia.[21-23] Similarly, no variants in our GWAS reached genome-wide significance despite the comparatively large sample size and broader definition of fluoropyrimidine-associated toxicity, suggesting that non-genetic variables and/or more complex interactions between genetic components, with each exerting a small effect size, contribute to the occurrence of severe fluoropyrimidine induced toxicity. Polygenic risk models are an attractive approach to address this issue; however, such analyses require far more patients than available in our study.

Although no genome-wide significant SNPs were identified, we did identify five variants suggestive of association with severe fluoropyrimidine-induced toxicity that might provide insight into possible alternative mechanisms that contribute to fluoropyrimidine toxicity. To the best of our knowledge, these SNPs have not been previously described in relation to fluoropyrimidines. Three variants (rs17114875, rs367239, rs77579689) are stated as having 'no gene consequence' in the digital ClinVar database of the NCBI. PRKD1 and MIR548AI are the closest annotated genes to rs171114875. PRKD1 encodes for protein kinase D1 (PKD1), which is an important kinase and contributes to several cancer-related signaling pathways including the NFκB and JNK pathways. Up and downregulation of PKD1 have been associated with cancer development depending on the tissue type.[24] Therefore it is possible that the regulation of PKD1 levels or activity through genetic variation could influence treatment with fluoropyrimidines through a currently unrecognized regulatory pathway. Furthermore, both PKRD1 and MIR548AI play a role in cardiac function and could conceivably contribute to a higher risk of cardiac toxicity.[25,26]

However, it is noted that cardiac toxicity was rare (<1%) in the Alpe-DPD study, so this mechanism likely does not explain the association noted in the GWAS. KHDRBS3 is the closest annotated gene to rs77579689, which has been associated with significantly worse progression-free survival and drug resistance to 5-FU in gastrointestinal cancer patients.[27] Drug resistance to 5-FU might lead to faster deterioration of patients' health and might therefore indirectly increase the likelihood of developing severe-fluoropyrimidine toxicity. The closest annotated genes to rs367239 are VENTXP7, and ZNF385D. Very little is known of VENTXP7 and ZNF385D and it is unclear how both genes could be involved in the development of severe toxicity. The other two suggestive variants are listed as intronic variants of the non-coding RNA gene LOC101927414 (rs114105116) and protein-coding gene COL6A3 (rs12622722). The COL6A3 gene encodes for the α-3-chain of type VI collagen, which is primarily associated with the extracellular matrix of skeletal muscle, skin, tendons, and vessels.[28] It has been shown that COL6A3 has been

Table 4: Thirty genetic variants with the 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 of the onset of severe toxicity are marked with an *. *Abbreviations*: Nr: number; Chr: chromosome; A0: nucleotide on allele 0; A1: nucleotide on allele 1; AF: allele frequency.

Nr.	Marker	Chr	Position	A0	A1	AF	β	*P* value
1	rs17114875*	14	29999987	G	A	0.409	-0.65087	5.14×10^{-07}
2	rs114105116*	4	138539880	T	A	0.020	3.124216	1.13×10^{-06}
3	rs367239*	3	21421935	T	C	0.546	-0.59915	2.45×10^{-06}
4	rs12622722*	2	238269120	G	A	0.484	0.627528	4.55×10^{-06}
5	rs77579689*	8	137130325	G	A	0.021	-3.90131	4.64×10^{-06}
6	rs74910762	8	81109425	C	A	0.044	1.419904	5.29×10^{-06}
7	chr16:78157332:I	16	78157332	G	GTT	0.065	1.20317	5.55×10^{-06}
8	rs12414693	10	97228795	C	T	0.259	0.672817	5.73×10^{-06}
9	rs449973	3	21425977	C	G	0.548	-0.56792	6.23×10^{-06}
10	rs495426	12	31021833	A	G	0.689	0.661952	6.63×10^{-06}
11	chr4:164083322:D	4	164083322	TG	T	0.051	1.624069	7.09×10^{-06}
12	rs1722291	7	56238936	G	A	0.198	-0.75534	7.81×10^{-06}
13	rs147501714	15	102309786	G	A	0.041	2.183951	8.07×10^{-06}
14	rs76146060	8	81120217	A	T	0.044	1.404553	8.12×10^{-06}
15	rs12415681	10	97233085	T	C	0.258	0.657462	8.66×10^{-06}
16	rs11595114	10	97231520	G	T	0.258	0.658815	8.67×10^{-06}
17	rs12415079	10	97229543	G	C	0.257	0.660208	8.70×10^{-06}
18	rs2344989	17	70924851	T	C	0.040	-1.72137	8.96×10^{-06}
19	rs8076418	17	70921917	T	C	0.042	-1.70739	9.53×10^{-06}
20	rs184137490	4	64175576	A	T	0.028	-2.76836	9.54×10^{-06}
21	rs2085003	2	5323126	A	T	0.930	1.524253	9.99×10^{-06}
22	rs10742634	11	42623993	G	A	0.458	0.550361	1.01×10^{-05}
23	rs77635577	16	61042468	A	T	0.108	-0.96048	1.05×10^{-05}
24	rs8067883	17	70921731	C	T	0.042	-1.70883	1.06×10^{-05}
25	rs4304264	7	89699913	A	T	0.191	-0.76196	1.06×10^{-05}
26	rs6501582	17	70921801	T	C	0.042	-1.70574	1.06×10^{-05}
27	rs8070810	17	70921851	G	A	0.042	-1.70479	1.06×10^{-05}
28	rs10895872	11	105604719	A	G	0.553	-0.58102	1.06×10^{-05}
29	rs9911437	17	70922305	T	C	0.042	-1.70383	1.07×10^{-05}
30	chr17:70923098:D	17	70923098	AC	A	0.042	-1.7027	1.09×10^{-05}

associated with the development of colorectal cancer possibly through regulation of the PI3K-AKT signalling pathway, which in turn has been associated with increased 5-FU sensitivity.[29]

One trade-off of not considering patients who experienced grade 2 toxicity is that not all patients of the cohort are included in the association analysis. Therefore, we conducted a sensitivity analysis by including the patients with grade 2 fluoropyrimidine-related toxicity

(grade 0-2 vs. grade 3-5), thereby increasing the number of patients while reducing the contrast between toxicities. Yet, this did not result in a different outcome (Supplementary Figure 3 and Table 5). Furthermore, as toxicities can differ between capecitabine and 5-FU, we repeated the GWAS with patients receiving capecitabine as this was the majority of patients (494 (82%) of 599). This analysis did not result in a different outcome. Our results indicate that *DPYD* exonic variants, especially predicted deleterious variants, as well as the five GWAS variants that were found to be suggestive of association with severe fluoropyrimidine-induced toxicity, are candidate SNPs that are valuable for further study. However, a substantial part of the observed fluoropyrimidine-related toxicity remains unexplained and other explanations such as the contribution of variants in non-coding regions should also be considered. Furthermore, while exon sequencing had a limited contribution to explaining the remaining severe fluoropyrimidine-toxicity in our patient population consisting of mostly Caucasians (95%), other genetic variants may be present in more ethnically diverse study populations as it is well-known that these facilitate the identification of genetic risk factors.[30] Additional studies in populations with greater ancestral diversity are therefore needed.

In conclusion, our results from *DPYD* exon sequencing and GWAS analysis suggest that at the population level it is not likely that besides the four established *DPYD* variants, genetic variants either inside or outside *DPYD* have a clinically relevant contribution to severe fluoropyrimidine-induced toxicity in patients treated with fluoropyrimidines.

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Supplemental - Chapter 2

Supplementary methods

Supplementary table 1: *In vitro* assay - The primer sequences used to perform site-directed mutagenesis on the expression plasmids

Variants	Primer
T557I	5′ TATCAATGATTCGAAGAGCTTTTGAAGCTGG 5′ TGCTGGTGGCTGGAGTTGC
I638T	5′ CTGCTAGCATTATGTGCAGTTACAATAAAAATGAC 5′ TCACAATGTTGTCTGGAAAGTCAGCC
M642T	5′ CGTGCAGTTACAATAAAAATGACTGGACGG 5′ TAATGCTAGCAATCACAATGTTGTCTGGA
R696H	5′ ACTGGGTTAGGCAAGCTGTTCAG 5′ GGCAGATGTTCCGCACCAGC
V732F	5′ TTTACAGCCACCAACACTGTCTCAG 5′ GCCATTGGCACCACCTTCCT
K874R	5′ GGAAACTGCCAAGTTTTGGACCTTATCT 5′ TGTCCATGAGTTCAGCTATACGTGG
E161G	5′ GGGTATTCAAAGCAATGAGTATCCCACA 5′ CAGTAGCAAATTGCTGCAATCCACC
E244A	5′ CGATTGAGCTAATGAAGGACCTTGGT 5′ CAAAATTCACTACATCATACGGCAGCC
I256M	5′ GTGCGGTAAAAGCCTTTCAGTGAATG 5′ ATTATCTTTACACCAAGGTCCTTCATTAGCTC

GWAS - Quality control

Quality control (QC) checks were performed using software R version 3.5.0[1] and PLINK software, version 1.07.[2,3] 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. Patients were removed from the analysis if the inbreeding statistic F>0·1, which was judged to be outlying. Genetic markers were excluded based on a SNP call rate <97% and a p-value ≤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. 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 were 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*[4,5] with default parameters in which the reference panel 1000Genomes build version 3 was used with a total, 'cosmopolitan', set of individuals.[6] An MDS plot was created to compare the self-reported ethnicity of patients.

Supplementary results

DPYD-sequencing - Comparison between three variants of function predictive tools

In this study, three tools were used to assess the effect of *DPYD*-variants, including *in vitro* assay, *DPYD*-Varifier, and MMsplice. Out of 24 variants, five variants were predicted to be deleterious by the *in vitro* assay. In contrast, *DPYD*-Varifier has a similar capability for novel variants prediction, but two variants failed to be assigned (Supplementary Table 2). Carriers of deleterious variants identified by *in vitro* assays had a 25% severe toxicity equivalent to that of the *DPYD*-Varifier. Notably, most of the variants identification results via those two systems are consistent except for one common missense variant c.2194G>T (*DPYD**6), which is neutral based on *in vitro* assay but deleterious via the *DPYD*-varifier (Table 2). Although some *DPYD* variants may be predicted by only one of these tools, such as c.506delC, a deleterious variant identified only by *in vitro* assays, most deleterious variants in the coding region can eventually be identified by the combination of two prediction tools. Besides, MMsplice also recognized two more deleterious variants in flanking regions, and those deleterious variants carriers had a 50% possibility to experience severe toxicity (Supplementary Table 2).

Supplementary Table 2: Comparison of deleterious variants identified by different predictive tools

	Variants tested	Prediction results	Predicted deleterious	Carriers	Carriers with overall toxicity	Carriers with severe toxicity
In vitro assay	20	20	5	8	8	2 (25%)
DPYD-varifier	19	17	5[a]	8	8	2 (25%)
MMsplice	4	4	2	2	2	1 (50%)

[a] Data in n (%). A c.2194G>T was deleterious based on *DPYD*-varifier, but due to the *in vitro* assay, it was considered a neutral variant in the final decision.

Matched-pair analysis

Supplementary Table 3: Matched pair analysis of novel deleterious variants

	Predicted deleterious variants carriers (N=10)	Matched patients without deleterious variants (N=30)	*P* value	Odds ratio (95% CI)	Positive predictive value	Negative predictive value
≥ grade 1 toxicity,	9 (90%)	24 (80%)	0.656	2.250 (0.237-21.367)	27.2	85.7
Severe toxicity (grade≥ 3)	3 (30%)	5 (16.7%)	0.388	2.143 (0.408-11.255)	37.5	78.1

Abbreviations: CI, Confidence interval.

Supplementary Table 4: The characteristics of patients included in the matched-pair analysis

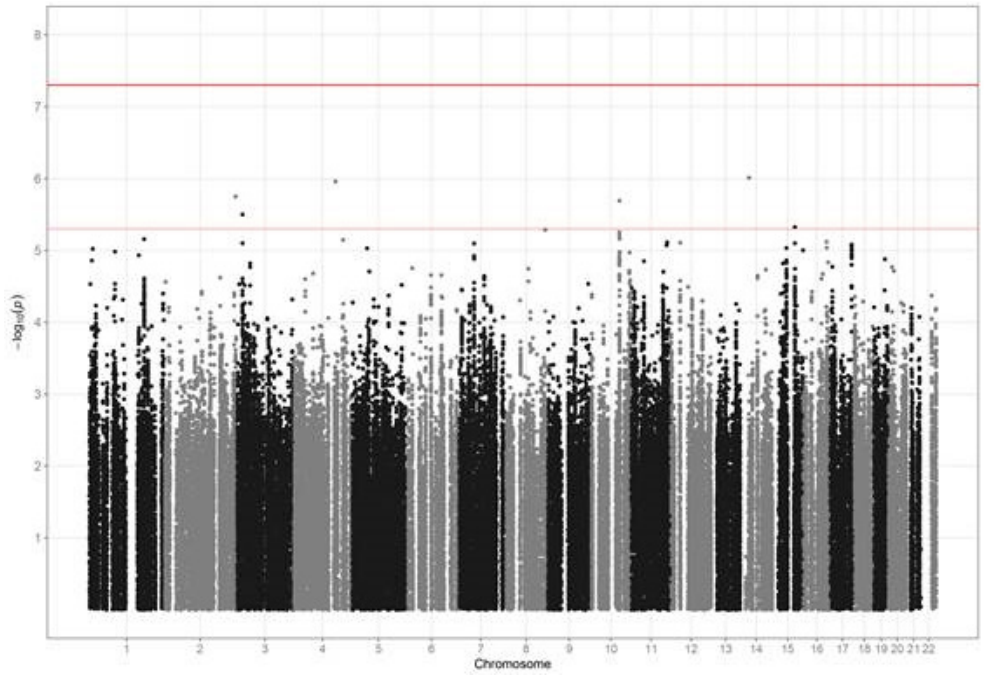
	Predicted deleterious variants carriers (N=10)	Matched patients without deleterious variants (N=30)
Gender		
Male	7 (70%)	15 (50%)
Female	3 (30%)	15 (50%)
Age in years	63 (55-69)	63 (55-70)
Ancestry		
White	10 (100%)	30 (100%)
BSA	1.9 (1.8-2.1)	2.0 (1.8-2.1)
WHO performance status		
0	6 (60%)	11 (37%)
1	4 (40%)	15 (50%)
2	0	2 (7%)
Not specified	0	2 (7%)
Number of treatment cycles	3 (2-6)	3 (2-6)

Data in n (%) or median (IQR). *Abbreviations*: BSA-Body surface area. WHO-World Health Organization.

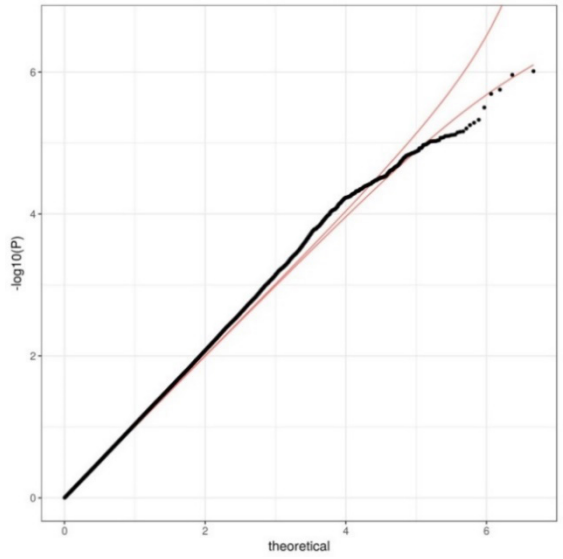
Genome-wide association analysis

Genotyping and quality control

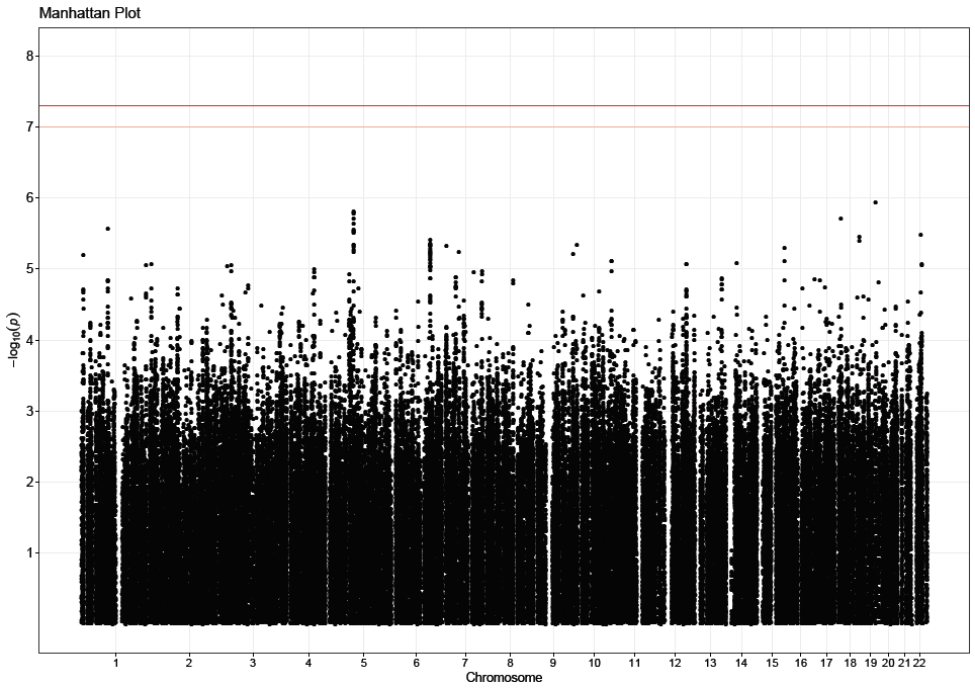
A set of 692,367 markers was genotyped. After several QC steps, 186,920 markers were excluded. Of these, 18,114 markers (2·6%) were excluded based on a deviation from Hardy-Weinberg equilibrium (HWE). Filtering for allele frequencies (threshold 0·5%) resulted in the exclusion of 147,607 markers (21·3%). In total, 23,835 markers (3·4%) were excluded based on the missing data analysis (missingness cut-off at 10%). Of the abovementioned excluded markers, 2,636 had multiple QC failures. In total, 505,447 markers met the QC for statistical analyses. After imputation with using the 1000 Genomes dataset as a reference panel 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 analysis was executed to detect population stratification. IBD/IBS clustering was executed to assess duplicates. No individuals were excluded based on this analysis.



Supplementary Figure 1: Manhattan plot for association with severe fluoropyrimidine-induced toxicity. Manhattan plot for association with severe fluoropyrimidine-induced toxicity (grade ≥3), 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 significantly associated with severe fluoropyrimidine-induced toxicity. Five 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.



Supplementary Figure 2: 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.



Supplementary Figure 3: Manhattan plot for association with severe fluoropyrimidine-induced toxicity. Manhattan plot for association with fluoropyrimidine-induced toxicity (grade ≥2), including de covariates gender, age, baseline BSA, and treatment type. *Abbreviations*: BSA: body surface area; SNPs: single nucleotide polymorphisms.

Supplementary Table 5: Variants are selected on allele frequency >0·01, β within -5 to 5, and are separated from another variant with more than 10 bps. Abbreviations: Nr: number; Chr: chromosome; A0: nucleotide on allele 0; A1: nucleotide on allele 1; AF: allele frequency.

Nr.	Marker	Chr	Position	A0	A1	AF	β	*P* value
1	rs2627645	19	45705323	C	A	0·932	-1·61014	$1·18 \times 10^{-06}$
2	rs2897989	5	56313932	A	C	0·248	-1·17743	$1·59 \times 10^{-06}$
3	rs115429202	5	56312774	T	G	0·247	-1·18033	$1·60 \times 10^{-06}$
4	rs57877390	5	56312654	T	A	0·247	-1·17787	$1·65 \times 10^{-06}$
5	rs518004	18	7485914	C	A	0·139	-1·65712	$1·98 \times 10^{-06}$
6	rs62356628	5	56315591	A	G	0·248	-1·17094	$1·98 \times 10^{-06}$
7	rs62356625	5	56314320	T	C	0·254	-1·14236	$2·35 \times 10^{-06}$
8	rs75025959	1	96902149	C	T	0·828	-1·06894	$2·77 \times 10^{-06}$
9	rs62356624	5	56314210	A	G	0·251	-1·13938	$2·83 \times 10^{-06}$
10	rs62356627	5	56315157	C	T	0·251	-1·13858	$2·95 \times 10^{-06}$
11	rs62356626	5	56314910	G	T	0·251	-1·13827	$3·01 \times 10^{-06}$
12	rs17732457	5	56301089	T	C	0·262	-1·09054	$3·08 \times 10^{-06}$
13	rs72396379	22	28868536	GTGTA	G	0·413	0·831405	$3·38 \times 10^{-06}$
14	chr18:68930999:D	18	68930999	CTAT	C	0·026	2·225657	$3·59 \times 10^{-06}$
15	rs142815469	6	130656827	A	G	0·026	1·835159	$3·96 \times 10^{-06}$
16	rs117010467	18	68949442	A	G	0·03	2·161431	$4·12 \times 10^{-06}$
17	rs74537901	6	130646607	G	A	0·027	1·767477	$4·49 \times 10^{-06}$
18	rs117198473	6	130651408	G	A	0·027	1·767392	$4·50 \times 10^{-06}$
19	chr6:130656050:D	6	130656050	CCAGCAGA	C	0·027	1·767073	$4·52 \times 10^{-06}$
20	rs75556955	6	130643789	C	A	0·027	1·767295	$4·53 \times 10^{-06}$
21	rs117001051	6	130660192	G	A	0·027	1·766165	$4·57 \times 10^{-06}$
22	chr5:56308030:D	5	56308030	CAT	C	0·275	-1·04733	$4·65 \times 10^{-06}$
23	rs12188896	5	56298297	G	A	0·259	-1·07756	$4·68 \times 10^{-06}$
24	rs145061688	10	8347578	A	G	0·031	-151·26	$4·68 \times 10^{-06}$
25	rs117376718	7	13342578	C	T	0·027	1·889196	$4·70 \times 10^{-06}$
26	rs75361266	6	130664619	C	T	0·027	1·763235	$4·73 \times 10^{-06}$
27	rs116940099	6	130665596	G	A	0·027	1·761975	$4·80 \times 10^{-06}$
28	chr5:56297015:D	5	56297015	AG	A	0·259	-1·07594	$4·92 \times 10^{-06}$
29	rs61201669	5	56295995	T	C	0·259	-1·07489	$5·07 \times 10^{-06}$
30	rs117481946	6	130668660	G	C	0·027	1·755796	$5·12 \times 10^{-06}$

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Chapter 3

Dihydropyrimidine dehydrogenase phenotyping using pretreatment uracil: a note of caution based on a large prospective clinical study

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Abstract

In clinical practice, 25-30% of the patients treated with fluoropyrimidines experience severe fluoropyrimidine-related toxicity. Extensively clinically validated *DPYD* genotyping tests are available to identify patients at risk of severe toxicity due to decreased activity of dihydropyrimidine dehydrogenase (DPD), the rate limiting enzyme in fluoropyrimidine metabolism.

In April 2020, the European Medicines Agency recommended that, as an alternative for *DPYD* genotype-based testing for DPD deficiency, also phenotype testing based on pretreatment plasma uracil levels is a suitable method to identify patients with DPD deficiency. While the evidence for genotype-directed dosing of fluoropyrimidines is substantial, the level of evidence supporting plasma uracil levels to predict DPD activity in clinical practice is limited. Notwithstanding this, uracil-based phenotyping is now used in clinical practice in various countries in Europe.

We aimed to determine the value of pretreatment uracil levels in predicting DPD deficiency and severe treatment-related toxicity. To this end, we determined pretreatment uracil levels in 955 cancer patients, and assessed the correlation with DPD activity in PBMCs and fluoropyrimidine-related severe toxicity. We identified substantial issues concerning the use of pretreatment uracil in clinical practice, including large between study center differences in measured pretreatment uracil levels, most likely as a result of pre-analytical factors. Importantly, we were not able to correlate pretreatment uracil levels with DPD activity nor were uracil levels predictive of severe treatment-related toxicity. We urge that robust clinical validation should first be performed before pretreatment plasma uracil levels are used in clinical practice as part of a dosing strategy for fluoropyrimidines.

Introduction

Fluoropyrimidines, including 5-fluorouracil (5-FU) and its oral prodrug capecitabine, are indispensable drugs in the treatment of different solid tumors. A consistent concern in clinical practice however, is that 25-30% of patients treated with a standard dose experience severe toxicity, which can result in early treatment discontinuation, hospital admission, and even death.[1-6] Deficiency of the main enzyme metabolizing 5-FU, dihydropyrimidine dehydrogenase (DPD), strongly increases a patient's risk of experiencing severe fluoropyrimidine-related toxicity.[1,7] Both genotype- and phenotype-based methods to test for DPD deficiency have been developed, which allow identification of patients at risk of severe toxicity and reduction of their starting dose.[8,9] The clinical validity of genotyping-based tests, which typically test for four *DPYD* genotypes (*DPYD**2A, c.1679T>G, c.2846A>T and c.1236G>A/HapB) has been established in multiple meta-analyses and two large prospective studies.[1,7,10-12] These studies have shown that genotype-based DPD testing in routine clinical practice leads to improvement of patient safety and is cost-effective.[13] As a result, *DPYD* genotyping is now widely recommended in clinical practice guidelines, in predominantly Caucasian patient populations where these four *DPYD* deficient alleles occur at a consistent frequency, and used in different countries in Europe (Supplementary Table S1). Recently, the European Medicines Agency (EMA) has concluded that product labels needed to be updated.[14,15] Since April 2020, based on EMA's conclusions, product labels of fluoropyrimidines recommend that:

- Patients treated with fluoropyrimidines (fluorouracil, capecitabine, tegafur) should be tested for DPD deficiency before starting treatment;
- Patients with partial DPD deficiency should be treated with an adjusted starting dose;
- Genotyping and phenotyping based on plasma uracil levels are currently the most suitable methods to identify patients with DPD deficiency.[14,15]

The recommendation on DPD phenotyping, specifically on pretreatment uracil levels, is of note considering the absence of both a prospective validation on the uracil threshold as a marker for fluoropyrimidine-related severe toxicity and evidence that uracil testing improves patient safety when used to individualize dose. Since endogenous plasma uracil is converted into dihydrouracil (DHU) by DPD, the concentration of uracil in plasma is thought to be a proxy for DPD activity, with (exceptionally) elevated levels of endogenous plasma uracil being reflective of a (complete) DPD deficiency and therefore predictive of increased risk for severe toxicity. Consistent with this rationale, it has previously been shown that pretreatment plasma uracil concentrations higher than 15 or 16 ng/ml, depending on the study, were associated with increased risk of severe fluoropyrimidine-related toxicity.[16-18] However, while the evidence for *DPYD* genotyping in preventing severe fluoropyrimidine-related toxicity is extensive and includes data from well-designed prospective clinical studies data showing that testing leads to improved patient safety, there are no such data to support the use of pretreatment uracil levels.[7,9] Moreover, uracil cut-off levels that predict toxicity have not been validated. In addition to this, prior studies have highlighted extensive variability in uracil measurements when different cohorts were compared, which to date remains insufficiently explained.[19] Therefore, the evidence available thus far regarding validation of pretreatment uracil and other DPD phenotyping methods appears insufficient to warrant routine use in clinical practice.

In the study reported here, we determined the value of pretreatment uracil levels in predicting fluoropyrimidine-related severe toxicity and assessed the correlation between pretreatment uracil levels and DPD activity in PBMCs - which is considered the reference assay/gold standard for measuring *in vivo* DPD activity.[9]

Patients and methods

This study was part of the previously reported large prospective multi-center study in 1103 patients (clinicaltrials.gov identifier NCT02324452).[12] Patient recruitment for this study was open from 30 April 2015 until 21 December 2017. Eligibility criteria have been reported previously[12]; key criteria were: eligible to start with fluoropyrimidine-based therapy, age ≥18 years, performance status ≤2, adequate bone marrow, renal and liver function, and no prior treatment with fluoropyrimidines. Ethical approval was granted by the medical ethical committee of The Netherlands Cancer Institute (NCI), Amsterdam, the Netherlands. All patients provided written informed consent before enrolment. Pretreatment uracil (U) levels and pretreatment DHU/U ratio were measured in the main study cohort of patients recruited in 17 Dutch hospitals. Protocols for sample collection, handling, and processing for DPD phenotyping were available prior to study start.

The DPD enzyme activity in PBMCs was measured in all *DPYD* variant allele carriers and in a subgroup of wild type patients. To assess pretreatment DPD enzyme activity and uracil levels, a blood sample was drawn before the start of fluoropyrimidine treatment. The blood samples for pretreatment uracil levels were stored on ice directly and centrifuged within 30 minutes and the plasma stored at -80°C. Uracil levels were measured centrally in the NCI in Amsterdam using a validated bioanalytical method.[20,21] Samples for DPD enzyme activity in PBMCs were shipped to the Academic Medical Center (AMC) in Amsterdam for further processing, or processed at the hospital of blood drawn as described previously.[22] After processing, isolated PBMCs were stored at -80°C before measurement of DPD activity at the AMC in Amsterdam with a validated bioanalytical assay.[22] Patients who received at least one fluoropyrimidine administration were followed for toxicity during the entire treatment period. Association between DPD activity in PBMCs, pretreatment uracil levels and fluoropyrimidine-related toxicity was assessed in patients wild type for *DPYD* variants, as patients who were identified as *DPYD* variant allele carriers (either *DPYD*2A*, *DPYD*13*, c.2846A>T or c.1236G>A) underwent a per protocol dose adjustment at start of therapy.[12] Toxicity was graded according to the National Cancer Institute common terminology criteria for adverse events (CTC-AE; v4.03) and severe toxicity was defined as CTC-AE grade ≥3.[23] Toxicities defined by the treating physician as possibly, probably, or definitely related to fluoropyrimidine treatment were taken into account. Uracil concentrations were compared between hospitals using Kruskal-Wallis test. The median uracil concentrations in the hospitals were also individually compared with the reference hospital (NCI). The correlation between uracil levels an DPD enzyme activity was assessed by calculating the R^2. Furthermore, the uracil level was compared between patients who developed severe toxicity and patients who did not, using a Wilcoxon signed-rank test. Lastly, the uracil levels and DPD enzyme activity were compared between *DPYD* genotypes using Kruskal-Wallis test. The threshold for significance was $P<0.05$. All statistical analyses were performed using R v3.6.3.

Results

In total, 1,037 patients participated in this study. Of these, 82 patients were identified as being *DPYD* variant allele carriers and 955 patients were *DPYD* wild type. DPD enzyme activity in PBMC's was determined in 138 patients. (Supplementary Figure S1) Pretreatment plasma uracil levels were determined in all patients and were analyzed in relation to DPD activity, *DPYD* genotype and fluoropyrimidine-related severe toxicity. Median pretreatment DPD enzyme activity, uracil levels and DHU/U ratios are summarized in Supplementary Table S2. The results from subsequent analyses showed unexpected findings of potential clinical importance. First, there were unexpectedly large between-center differences in measured pretreatment uracil levels (Kruskal-Wallis test, $P<0.001$; Figure 1). The median uracil concentration of *DPYD* wild type patients was 9.63 ng/mL (range: 3.76-188 ng/mL) in the reference hospital (NCI) compared to a range of 7.59 - 16.30 ng/mL in the other hospitals, with significant differences between hospitals and the reference hospital in 8 cases (Figure 1). In addition to these between-center differences there appeared to be an effect of sex on uracil concentrations but this effect was smaller than compared to the effect of the study center (Supplementary Table S3). Age and body surface area (BSA) were not associated with uracil levels (Supplementary Table S3).

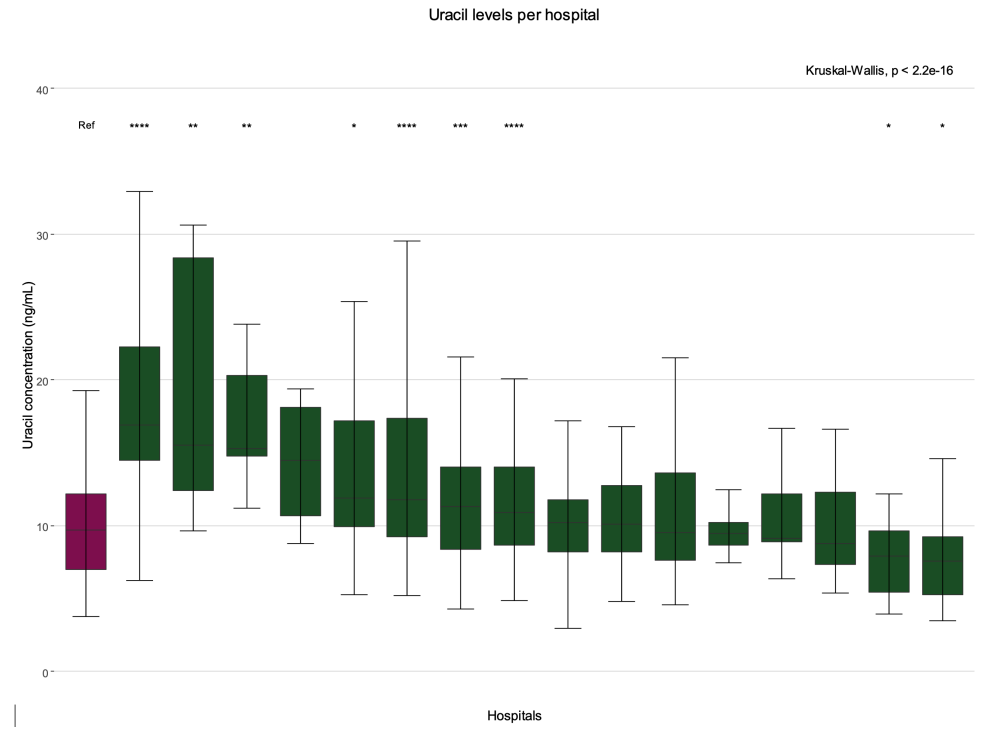

Figure 1: Differences in measured pretreatment uracil levels between hospitals. Differences in uracil concentrations (ng/mL) between the participating hospitals in an explorative substudy of a prospective multicenter study in 955 patients (clinicaltrials.gov identifier NCT02324452). All the samples were measured centrally therefore, the central hospital was chosen to be the reference hospital (indicated in red). Differences between medians were determined using one-way analysis of variance (Kruskal-Wallis). *P ≤ 0.05; ***P ≤ 0.001; ****P ≤ 0.0001.

Secondly, there was no correlation between pretreatment uracil concentrations and the reference assay (DPD activity in PBMCs; R^2<0.01, P=0.391; Figure 2A). However, when performing the analyses without the outlier with an uracil concentration of 188.0 ng/mL a significant correlation was found (R^2<0.04, P=0.022). Importantly, there was no association between uracil and severe fluoropyrimidine-related toxicity, as the median pretreatment uracil level was 10.10 ng/mL in patients without severe toxicity compared to 10.35 ng/mL in the patients with severe toxicity (P=0.73; Figure 2B). Multivariable analysis to adjust for other potential risk factors (body surface area, age, sex, treatment regimen and cancer stage) did not result in a different association between pretreatment uracil levels (both as continuous variable or as dichotomous variable with a cut-off of 16 ng/ml) and severe toxicity (OR 0.997, 95% CI 0.97-1.01, P=0.71). While ECX (epirubicin, cisplatin and capecitabine)/EOX (epirubicin, oxaliplatin and capecitabine) treatment regimen, concomitant radiotherapy and sex are associated with severe toxicity (P=0.03, P=0.04 and P=0.04, respectively). There was no association found between pretreatment DHU/U ratio and severe toxicity (Supplementary Figure S2). Lastly, and of note, pretreatment uracil levels did differ as expected between *DPYD* wild types, and *DPYD* variants c.1236G>A/HapB, c.2846A>T, *DPYD**2A, and c.1679T>G with median uracil levels of 10.10, 12.20, 14.60, 16.80, and 40.10 ng/mL, respectively (Figure 2C). Also, DPD activity in PBMCs correlated with *DPYD* genotypes as expected and as previously reported (Figure 2D).[24]

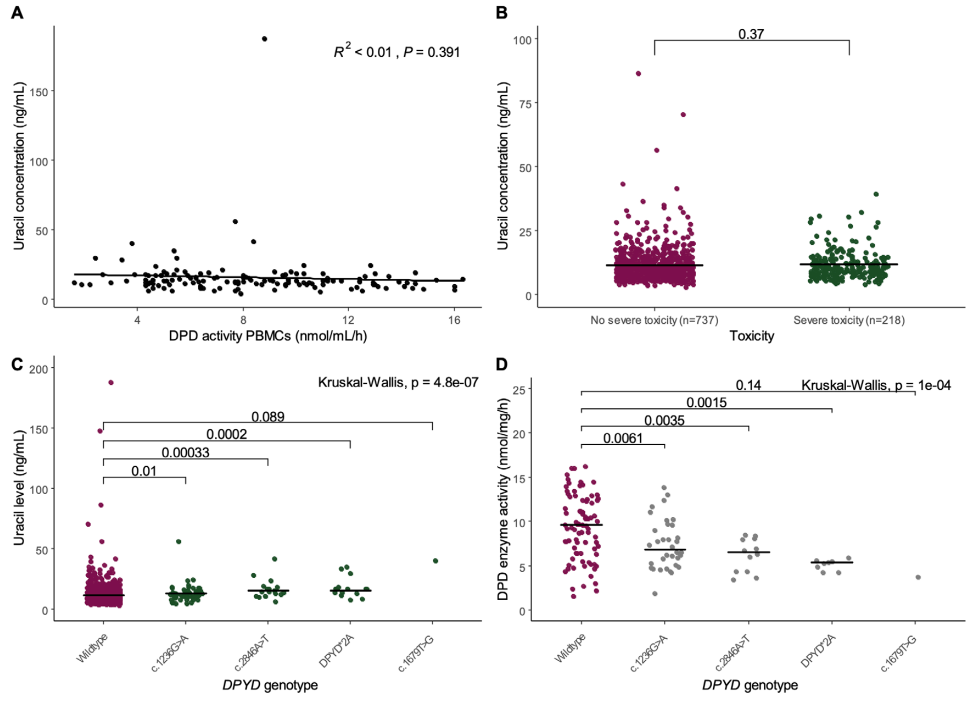

Figure 2: Correlations of endogenous uracil levels, DPD enzyme activity in PBMCs, toxicity, and *DPYD* genotype. Dots represent individual results. Black lines represent the median of the data. (a) Shows the correlation between endogenous uracil levels and DPD activity. (b) Shows the endogenous uracil concentration in patients with and without severe toxicity. *DPYD* variants were excluded from the analysis as they received initial dose reductions based on their genotype results. (c) Shows the endogenous uracil levels in patients by *DPYD*- genotype. (d) Shows the DPD enzyme activity measured in PBMCs of 138 patients (both *DPYD* variant carriers and wild type patients). *Abbreviations*: DPD, dihydropyrimidine dehydrogenase; *DPYD*, gene encoding dihydropyrimidine dehydrogenase; NS, not significant; PBMCs, peripheral blood mononuclear cells; P value; vs, versus.

Discussion

In this study we were not able to confirm that pretreatment uracil levels can predict DPD deficiency and severe fluoropyrimidine-related toxicity. The results showed no association between pretreatment uracil levels and both DPD activity in PBMCs and occurrence of severe fluoropyrimidine-related toxicity. More importantly, very large between-center differences in the uracil measurements were observed. These results are in contrast with the prior single center study that showed a clear correlation between high endogenous uracil levels (>16 ng/mL) and early severe toxicity[17] and which has been the basis for some of the recommendations in current clinical practice guidelines.

We identified potential pitfalls in the clinical use of pretreatment uracil levels to test for DPD deficiency. As uracil concentration in whole blood samples is stable for at least 4 hours when stored at 2-8˚C, and in heparin plasma for at least 5 days when stored at 2-8˚C,[21,25] the observed large variability between study centers could therefore probably be explained by differences in the duration of pre-analytical sample handling at room temperature and processing among the 17 hospitals that participated in the prospective study.[21,25] The current study and the prior retrospective study[17] that was performed at one of the participating centers used the same validated bioanalytical assay, which was performed centrally, and it is therefore unlikely that the results are explained by the bioanalytical method.[21] Our hypothesis therefore is that between-center differences in pre-analytical sample processing are the main cause for the observed unexpected results. Second, the influence of circadian rhythm and food intake cannot be excluded.[25,26] In this study, both the time of sampling and the time of last meal before blood drawl was not standardized in all patients, which has been shown to affect DPD enzyme activity.[26]

Hence, we feel that differences in pre-analytical sample handling and processing are the main causes of the variability seen in pretreatment uracil concentration between hospitals. Previous data have also raised potential concerns regarding between-center variability in observed measurements for pretreatment uracil/dihydrouracil ratio.[19] This should therefore be regarded as a note of caution for institutes that are currently using these DPD phenotyping tests.

The measurement of uracil levels prior to fluoropyrimidine-based treatment is now advised by health authorities, reimbursed and used in at least two countries in Europe (Supplementary Table S1).[27,28] EMA's recommendations will possibly further increase the uptake of pretreatment uracil tests. The concerns raised in this study and the fact that previous studies also raised concerns about between-center variability in observed measurements add to the uncertainty around the test.[19] Considering the above, prospective validation of DPD phenotyping tests, including implementation of robust sample handling procedures and a personalized dosing advice in patients with high uracil concentration, is urgently needed. In addition, bioanalytical cross validation of the uracil

test should be conducted. A large prospective study investigating the effect of phenotype-guided dosing based on pretreatment uracil levels is currently being conducted (clinicaltrials.gov identifier NCT04194957). In this study, the time between sampling, processing, storage, and transportation is standardized to avoid pre-analytical errors as much as possible. In addition, blood samples are taken between 8 and 10 in the morning and patients are required to be fasted to minimize the influence of circadian rhythm on the DPD enzyme and food intake on the uracil levels, respectively. This study may provide further insight into the validity of pretreatment uracil levels as way to prevent severe fluoropyrimidine-related toxicity. Besides pretreatment uracil, various other DPD phenotyping tests have been explored to identify patients with DPD deficiency, which include measurement of DPD enzyme activity in PBMCs, a 2-^{13}C uracil-based breath test, and a uracil test dose.[29] The level of clinical validation in the predicting of severe toxicity of these tests varies, but is currently considerably lower compared to pretreatment uracil levels. Therefore, further research will be needed to understand the clinical utility of these tests.

Conclusion

In conclusion, the most important learning from this study is that measurement of pretreatment uracil concentration as a DPD-phenotyping method to predict severe toxicity, is prone to pre-analytical error. This is in contrast to genotyping methods which have shown to yield consistent results across centers and regions using available standardized protocols. Misclassification of patients in terms of DPD deficiency will have potentially relevant impact on patients' safety and treatment outcome. We therefore urge that before using pretreatment uracil levels as part of routine clinical practice to adjust starting doses of fluoropyrimidines, robust clinical validation is performed, standardized protocols for sample handling and processing are developed, and bioanalytical cross validation is conducted.

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Supplemental - Chapter 3

Table S1: Most recent guidelines regarding DPD testing

Guideline:	Date	Screening test	Translation	Dosing advise of fluoropyrimidines	
Clinical Pharmacogenetics Implementation Consortium (CPIC)[1]	Nov 2017	Genotyping[A]	*DPYD* genotype is translated into DPD phenotype (activity score)	*DPYD* normal metabolizer	Standard dose
				Gene activity score:	Dose-modifications
				- 0 - 0.5	Contraindicated
				- 1	50% reduction
				- 1.5	25-50% reduction
L'Institut national de cancer (INCa) and la Haute Autorité de Santé (HAS)[2]	Dec 2018	Phenotyping: *measurement of plasma uracil concentration*[B,C]		*Uracil concentration*	Dose modification
				- 16 - 150 ng/ml	Dose reduction
				- > 150 ng/ml	Contraindicated
Associazione Italiana di Oncologia Medica (AIOM)	Oct 2019	Genotyping[A,D]	*DPYD* genotype is translated into wild type, heterozygous variant or homozygous variant	*Wild type*	Standard dose
				Heterozygous	
				- c.1236G>A	25% reduction
				- c.1679T>G / c.1905+1 / c.2846A>T	50% reduction
				Homozygous	
				- c.1236G>A	25% reduction
				- c.1679T>G / c.1905+1 / c.2846A>T	50% reduction
Dutch Pharmacogenetics Working Group (DPWG)[3]	Nov 2019	Genotyping[A] *Phenotyping: measurement of DPD enzyme activity for some phenotypes*	*DPYD* genotype is translated into DPD phenotype (gene activity score)	*Gene activity score*	*Dose modification*
				- 2	Standard
				- 1 - 1.5	50% dose reduction
				- PHENO	Dose based on DPD enzyme activity
				- 0	Contraindicated
European Medicines Agency (EMA)[4]	July 2020	Genotyping[A] and/or Phenotyping: *measurement of pretreatment endogenous uracil concentration*	Genotype and/or phenotype is translated into partial DPD deficiency or complete DPD deficiency	*Partial DPD deficiency*	Dose reduction
				Complete DPD deficiency	Contraindicated
Joint Belgian recommendation[5]	Jan 2021	Phenotyping: *measurement of plasma uracil and dihydrouracil concentration*[C] Genotyping[A]: *when uracil > 14 ng/mL*	*DPYD* genotype is translated into genotype score	*Uracil concentration*	*Dose-modification*
				- ≥ 100 ng/mL	Contraindicated
				- > 14 ng/mL and <100 ng/mL	50% reduction
				- ≤ 14 ng/mL	Standard dose
				Genotype score	*Dose-modification*
				- 2	Standard dose
				- 1 - 1.5	50% reduction
				- 0 - 0.5	Contraindicated

The current guidelines regarding DPD testing are listed in chronological order. [A]Genotyping of the following *DPYD* variants: *DPYD**2A (c.1905+1G>A, IVS14+1G>A), *DPYD**13 (c.1679T>G), c.2846A>T, and c.1236G>A (in linkage disequilibrium with c.1129-5923C>G). [B]Use of tubes without separating gel. Use of tubes with anticoagulant. [C]Max time between sampling and centrifugation: at room temperature: 1 hour 30 min, at 4°C: 4 hours. Centrifugation at 4°C. After centrifugation immediately freezing of the plasma obtained. [D]In case of toxicity, genotyping of c.2194G>A additionally. *Abbreviations:* DPD = dihydropyrimidine dehydrogenase; *DPYD* = gene encoding dihydropyrimidine dehydrogenase

Table S2: Overview of DPD phenotyping measurements by *DPYD* genotype status

DPD enzyme activity assay and two phenotyping assays are shown, including the number of patients included per assay and how many were *DPYD* wild type or variant allele carrier for the *DPYD**2A, *DPYD**13, c.2846A>T or c.1236G>A variants. The calculated medians and the interquartile range are shown. The DPD enzyme activity was only measured in a subset of patients (138) of the original NCT02324452 trial which included 1103 patients in total.

Phenotyping assay	All patients		*DPYD* wild type patients		*DPYD* variant allele carriers	
	Median [IQR]	N of patients	Median [IQR]	N of patients	Median [IQR]	N of patients
DPD enzyme activity, nmol/(mg*h)	8.0 [5.4-10.9]	138	9.6 [6.4-12.4]	83	6.2 [4.8-8.1])	55[A]
Endogenous uracil levels, ng/mL	10.4 [8.0-13.8]	1037	10.1 [7.9-13.4]	955	13.4 [10.7-16.9]	82
Endogenous DHU/U ratio	8.7 [6.6-11.6]	1037	9.0 [6.8-11.6]	955	7.2 [5.4-9.1]	82

[A]Of these patients, 34 were c.1236G>A variant carriers, twelve were c.2846A>T, eight were *DPYD**2A variant carriers and one was a c.1679T>G carrier. *Abbreviations*: DHU=dihydrouracil; DPD=dihydropyrimidine dehydrogenase; IQR=interquartile range; U=uracil.

Table S3: Results of factors influencing phenotyping measurements

A

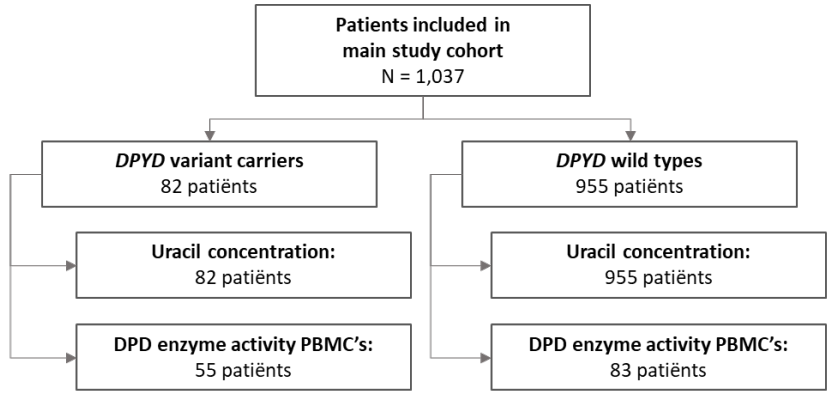
DPD phenotyping method	N	Sex[a]		*P* value
		Men (N=81), Median [IQR]	Women (N=57), Median [IQR]	
DPD enzyme activity, nmol/mg/h	138	8.1 [5.7-10.7]	7.4 [5.1-11.0]	0.466
Endogenous uracil levels, ng/mL	955	12.6 [10.90-16.90]	12.70 [10.0-16.80]	0.689
Endogenous DHU/U ratio	955	7.51 [5.67-10.63]	7.81 [5.79-11.08]	0.905

B

Factors influencing phenotyping results	DPD enzyme activity (N=138)			Endogenous uracil levels (N=955)			Endogenous DHU/U ratio (N=955)		
	Estimate	95%CI	*P*-value	Estimate	95%CI	*P*-value	Estimate	95%CI	*P*-value
Age[B]	0.003	-0.048 - 0.054	0.903	0.011	-0.045 - 0.066	0.7	0.037	0.014 - 0.060	0.013
BSA[C]	0.61	-1.92 - 3.14	0.633	0.4	-2.46 - 3.26	0.784	0.348	-0.77 - 1.47	0.544

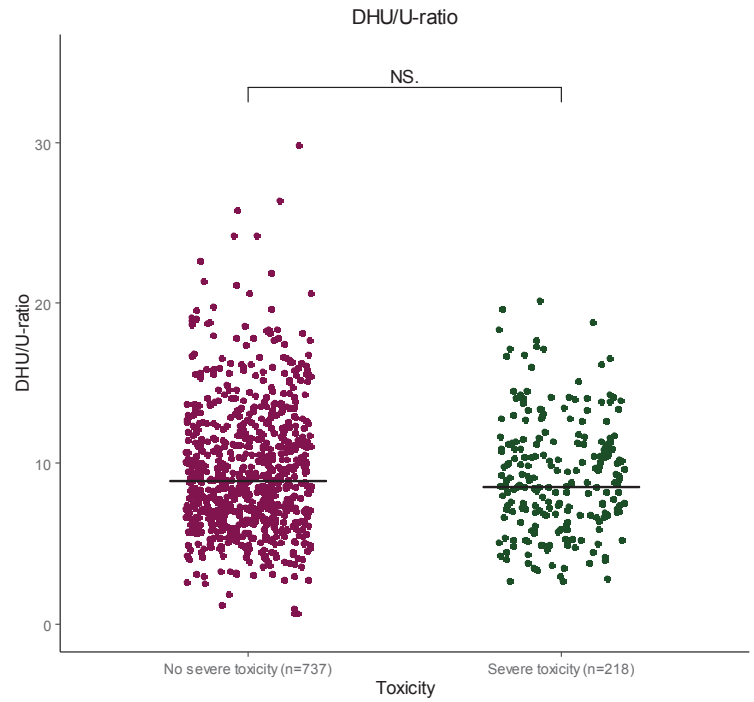
[A]In case of sex, men were compared to women using the Wilcoxon rank sum test. [B]Age was considered as continuous variable by year increase, and tested using a linear regression. [C]BSA was considered as continuous variable by 0.1 BSA increase, and tested using a linear regression. *Abbreviations:* BSA=body surface area; CI=confidence interval; DHU=dihydrouracil; U=uracil.

Figure S1: Flowchart of patient inclusion



Flowchart of patient inclusion and the number of *DPYD* variant and *DPYD* wild type patients of whom is measured the pretreatment uracil concentration and the DPD enzyme activity in PBMC's. *Abbreviations*: DPD = dihydropyrimidine dehydrogenase; PBMC's = peripheral blood mononuclear cells.

Figure S2: DHU/U-ratio versus severe fluoropyrimidine-associated toxicity



The DHU/U-ratio in patients with and without severe toxicity. *DPYD* variants were excluded from the analysis as they received initial dose reductions based on their genotype results. Groups were not significantly different (medians 8.9 and 9.015). *Abbreviations*: DHU=dihydrouracil; NS.=not significant; U=uracil.

Chapter 4

Dose-individualisation of fluoropyrimidines based on pre-treatment serum uracil levels: A safety and pharmacokinetic analysis from the Alpe2U study

Interim analysis

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Abstract

Background: *DPYD*-guided dosing significantly improves the safety of fluoropyrimidine-based chemotherapy. However, severe toxicity remains in ~23% of patients treated with fluoropyrimidines, which is not explained by the four common *DPYD* variant alleles. Elevated pre-treatment uracil levels have shown to be associated with severe fluoropyrimidine-related toxicity. In this prospective clinical trial, we investigated whether uracil-based dose-individualisation can further reduce severe fluoropyrimidine-induced toxicity in *DPYD* wild-type patients.

Methods: Uracil levels were measured prior to fluoropyrimidine-based treatment. *DPYD* wild-type patients with uracil levels > 16 ng/mL ($DPYD_{wt}/U_{high}$) received an initial dose reduction of 50%. The incidence of severe (CTCAE grade ≥ 3) fluoropyrimidine-related toxicity in the $DPYD_{wt}/U_{high}$ patients was compared to: $DPYD_{wt}$ patients with U ≤ 16 ng/mL from this study and a historical cohort of $DPYD_{wt}/U_{high}$ patients, both treated with full fluoropyrimidine dose. Pharmacokinetic data were compared to $DPYD_{wt}$ patients form another historical cohort.

Findings: 612 evaluable patients were enrolled, of whom 22 (3.6%) were $DPYD_{wt}/U_{high}$. The incidence of severe toxicity in the $DPYD_{wt}/U_{high}$ patients treated with a reduced dose was significantly lower compared to the historical cohort (20% vs. 43%, P=0.029, respectively), however the incidence during the first 2 treatment cycles was comparable to *DPYD*wt patients with U ≤16 ng/mL from this study (10% *vs.* 11%, P=1.00, respectively). Pharmacokinetic analysis of $DPYD_{wt}/U_{high}$ patients (N=19) treated with a 50% dose showed that 5-fluorouracil exposure was substantially lower compared to the exposure from the historical cohort (179 vs. 381 ng*h/mL, respectively). No correlation (R^2=0.014, P=0.64) between uracil levels and DPD enzyme activity was found.

Interpretation: Pre-treatment uracil levels as an indicator for DPD enzymatic activity, accompanied by a 50% dose reduction in $DPYD_{wt}/U_{high}$ patients results in a reduction of fluoropyrimidine-associated toxicity, but also leads to inadequate 5-FU exposure in our study population. Therefore, this strategy should not be recommended for dose-individualization of fluoropyrimidine-based chemotherapy.

Introduction

Fluoropyrimidines, including 5-fluorouracil (5-FU) and its oral prodrug capecitabine, play a key role in the treatment of multiple solid tumour types. Despite the large amount of experience and research regarding the safety of fluoropyrimidine treatment, approximately 20-30% of patients experience severe toxicity.[1-4] Moreover, severe fluoropyrimidine-related toxicity can result in hospitalisation, early treatment discontinuation, and can even be lethal.[1,5] Risk of developing severe fluoropyrimidine-related toxicity strongly increases in patients with a deficiency of the main catabolizing enzyme of 5-FU, dihydropyrimidine dehydrogenase (DPD), which is encoded by the *DPYD* gene.[6,7] *DPYD*-guided dosing of fluoropyrimidines, which typically tests for *DPYD**2A (rs3918290, c.1905+1G>A, IVS14+1G>A), c.1236G>A (rs56038477, E412E, in haplotype B3), c.2846A>T (rs67376798, D949V), and c.1679T>G (rs55886062, *DPYD**13, I560S) has shown that pre-therapeutic screening of *DPYD* and subsequent dose-individualization in routine clinical practice is feasible, improves safety, and is cost-effective.[4,8] Hence, the European Medicines Agency (EMA) recommended in 2020 that all patients scheduled to be treated with fluoropyrimidines should be tested for DPD deficiency before treatment with fluoropyrimidines.[9-11] However, despite *DPYD* genotype-guided dosing, severe fluoropyrimidine-related toxicity remains present in approximately 23% of patients, indicating that severe toxicity can only partially be attributed to these four variants.[4] This shortcoming of *DPYD* genotype-guided dosing might potentially be overcome by using a phenotype-directed approach for identifying DPD deficiency. The most direct way of identifying DPD deficient patients is by measuring the DPD enzyme activity in peripheral blood mononuclear cells (PBMCs). However, this test is expensive, has a high turn-around time (>1 week), and requires specific equipment that is not readily available in all hospitals.[12]

A promising alternative is the measurement of pre-treatment serum uracil concentrations. Uracil is an endogenous pyrimidine base and is, similar to 5-FU, metabolized by DPD to dihydrouracil.[13-15] Therefore, uracil has been considered to act as a surrogate marker for DPD enzyme activity, with elevated levels of uracil corresponding with a DPD deficiency, being predictive of severe fluoropyrimidine-induced toxicity.[15] In line with this, it has previously been shown in retrospective studies that pre-treatment uracil concentrations above 16 ng/mL are associated with a significantly increased risk of severe fluoropyrimidine-induced toxicity.[14,16] This suggests that patient safety could be further improved by dose-individualization based on pre-treatment uracil levels in addition to *DPYD* genotype-guided dosing. However, the use of pre-treatment uracil levels for fluoropyrimidines dose-individualization has yet not been studied prospectively. Moreover, a cut-off value for pre-treatment uracil levels that predict severe fluoropyrimidine-related toxicity has not been validated yet. Therefore, we assessed the effect of prospective DPD phenotype-guided dosing on patient safety by pre-treatment serum uracil concentration measurements and subsequent fluoropyrimidine dose-individualization in daily clinical care.

Patients and methods

Study design and participants

This prospective multicentre clinical trial was performed in 15 participating hospitals in the Netherlands (NCT04194957). This study was approved by the institutional review board of the Netherlands Cancer Institute (NKI), Amsterdam, the Netherlands, and approval from the board of directors of each hospital was obtained for all participating centres. All patients provided written informed consent before enrolment in the study. Patients intended to start with fluoropyrimidine-based chemotherapy, both monotherapy and combination therapy were included. Combination therapy including irinotecan was excluded. Prior chemotherapy was allowed, except for prior treatment with fluoropyrimidines.

According to the standard of care in the Netherlands, all patients were genotyped for *DPYD**2A, c.1236G>A, c.2846A>T, and c.1679T>G variant alleles. Genotyping was performed in the clinical chemistry laboratory of the local hospital or in one of the other participating centres. Patients not carrying any of the mentioned four *DPYD* variant alleles were considered *DPYD* wild-type. Additionally, the pre-treatment serum uracil concentration was measured in all patients. The DPD enzyme activity in PBMCs was measured before treatment in all *DPYD* wild-type patients with uracil levels above 16 ng/mL ($DPYD_{wt}/U_{high}$) and in patients carrying a homozygous or compound heterozygous *DPYD* genotype. $DPYD_{wt}/U_{high}$ and all *DPYD* variant allele carriers received an initial dose reduction of 50%. The dose of fluoropyrimidine treatment in *DPYD* homozygous or compound heterozygous patients was based on the measured DPD enzyme activity in PBMCs. *DPYD* wild-type patients with normal uracil levels ($DPYD_{wt}/U_{normal}$) were treated according to the current standard of care. To ensure safe and adequate exposure after a dose reduction, dose escalation was recommended with small increments (10-20%) after the first two cycles (or two weeks in case of chemoradiation therapy) and onwards when treatment was well-tolerated and was left to the discretion of the treating physician. Complete inclusion and exclusion criteria can be found in the Supplementary Methods.

Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 5.0.[17] Severe toxicity was defined as grade three or higher. $DPYD_{wt}/U_{high}$ patients, c.1236G>A and c.2846A>T variant allele carriers, and *DPYD* homozygous or compound heterozygous variant allele carriers were followed for toxicity during the entire treatment period. All other patients were followed for toxicity during the first two treatment cycles. Only toxicities scored for causality as possibly, probable, or definitely related to fluoropyrimidines-therapy were taken into account for fluoropyrimidine-related toxicity, see also Supplementary Methods. Toxicity was compared to a subset of a historical cohort of patients which were included in a large prospective trial in which patients were genotyped for *DPYD**2A and of which pre-treatment serum uracil levels were measured, as previously described.[8,14] The group of $DPYD_{wt}/U_{high}$ patients included 3 *DPYD* variant allele carriers (two c.1236G>A and one c.2846A>T) which were excluded from analysis, resulting in 14 $DPYD_{wt}/U_{high}$ patients of which 6 experienced severe fluoropyrimidine-related toxicity.[14] Patients were considered evaluable if they received at least one fluoropyrimidine administration.

Procedures

DPD phenotyping

DPD phenotyping was performed by measuring the pre-treatment serum uracil concentration before the start of treatment using a validated method UPLC-MS/MS method as described previously.[18] Uracil concentrations were measured centrally once a week at the department of pharmacy of the NKI. Blood samples for measurement of uracil concentration were taken between 8:00 - 10:00 a.m. in a fasted state to limit the influence of circadian rhythm and food effect on the uracil concentration.[19,20] Subsequently, blood samples were centrifuged at 4°C and 3300 rpm within 1 hour at room temperature after sampling and serum was directly frozen (-20°C) to ensure the stability of uracil. Samples drawn outside of NKI were transported on dry ice to ensure stability or uracil. Additional DPD phenotyping was performed by measuring the DPD enzyme activity in PBMCs in $DPYD_{wt}/U_{high}$ patients and patients carrying a homozygous or compound heterozygous *DPYD* genotype. DPD enzyme activity in PBMCs was measured centrally at the laboratory of Maastricht University Medical Center using a validated assay, as described previously.[21]

Pharmacokinetics

$DPYD_{wt}/U_{high}$ patients underwent pharmacokinetic sampling in which plasma levels of capecitabine, and metabolites (5'-deoxy-5-fluorocytidine (5'-dFCR), 5'-deoxy-5-fluorouridine (5'-dFUR), 5-FU, and fluoro-β-alanine (FBAL)) were determined at the first day of the first cycle of fluoropyrimidine treatment to assess the pharmacokinetic (PK) profile. A validated UPLC-MS/MS method was used for the quantification of capecitabine and metabolites (details in Supplementary Methods). Results of PK parameters, such as dose-normalized area-under-the-curve ($AUC_{0\text{-}8h}$), and half-life ($t_{1/2}$) were calculated using a non-compartmental analysis, and compared to reference values derived from both $DPYD_{wt}/U_{high}$ patients treated with full dose from this study and wild-type patients with advanced cancer of the stomach or the gastroesophageal junction with unknown uracil levels treated with a full dose (850 mg/m^2, twice daily on days 1-14 of 3 week cycles) from literature.[22]

Outcomes

The primary endpoint of our study was the frequency of severe fluoropyrimidine-related toxicity (CTCAE grade 3 to 5). The incidence of severe toxicity in $DPYD_{wt}/U_{high}$ patients treated with a reduced fluoropyrimidine-dose of 50% was compared with the incidence in both $DPYD_{wt}/U_{normal}$ from this study and a historical cohort of $DPYD_{wt}/U_{high}$ patients, both treated with a full dose.[14] *DPYD* wild-type patients from the historical cohort, described by Meulendijks *et al.*, were not identified prior to start of treatment of fluoropyrimidines and were therefore treated with a full dose.[14] Secondary endpoints included assessment of pharmacokinetic parameters and DPD enzyme activity in patients given a reduced dose of fluoropyrimidines.

Sample size calculation and statistical analysis

The sample size was calculated under the assumption that severe toxicity could be reduced from 43% ($DPYD_{wt}/U_{high}$ receiving standard dose from a historical cohort)[14] to 23% by applying a 50% dose-reduction in this group of patients. A total of 36 $DPYD_{wt}/U_{high}$ patients were required based to detect this difference of 20% with a binomial test for one

proportion, assuming a one-sided type I error equal to 5%. It was expected that 2.5 to 3.0% of all wild-type patients would have a uracil concentration above 16 ng/mL[14] and 93% of all patients was expected to be *DPYD* wild-type.

A pre-planned interim-analysis was performed when 50% (N=18) of the 36 required $DPYD_{wt}/U_{high}$ patients were included. Pharmacokinetic data (dose-normalized $AUC_{0\text{-}8h}$) and DPD enzyme activity in PBMCs were measured and compared to reference values from a historic cohort[22] for assessment of adequacy of the pre-specified fluoropyrimidine dose reduction in the study protocol. In the interim-analysis it was pre-specified that in case of inadequate 5-FU exposure the fluoropyrimidine dose would be optimized.

To test whether the incidence of severe toxicity in wild-type patients was lowered after a 50% reduced dose based on high uracil pre-treatment concentrations (>16 ng/mL) compared to the historical incidence of 43%, the binomial test for one proportion was used.[14] Descriptive statistics were employed to evaluate toxicity in $DPYD_{wt}/U_{high}$ and $DPYD_{wt}/U_{normal}$ patients, and to compare toxicity outcomes by use of adaptive dosing based on DPD phenotype. Descriptive tables summarising the number and percentage of patients experiencing adverse events as categorized in the NCI-CTCAE version 5.0 were generated. Baseline characteristics between $DPYD_{wt}/U_{high}$ and $DPYD_{wt}/U_{normal}$ patients were compared with Fisher's exact test for categorical variables or the Mann-Whitney-U test for continuous variables. A p value <0.05 was considered to indicate a statistically significant difference. Analyses were performed using R statistical software (version 4.2.1).

Results:

Patients and treatment characteristics

Between January 13, 2020, and July 1st, 2022, 677 patients who intended to start fluoropyrimidine-based chemotherapy were prospectively phenotyped by pre-treatment uracil level measurement and genotyped for *DPYD* variant alleles before start of treatment. As a result of the interim-analysis (2nd trimester of 2022) the study protocol was adapted and the dose reduction for $DPYD_{wt}/U_{high}$ patients was halted. Patient inclusion continued, however, all $DPYD_{wt}/U_{high}$ patients were treated with a full dose of fluoropyrimidines to be able to compare exposure to 5-FU and other metabolites with the $DPYD_{wt}/U_{high}$ patients previously treated with a reduced dose as an internal control. Sixty-five patients were considered unevaluable (Figure 1), due to missing inclusion criteria, ultimately not being treated with fluoropyrimidines, or missing phenotyping and/or genotyping results. This resulted in 612 evaluable patients of whom 46 were *DPYD* variant allele carriers, 566 (93%) were *DPYD* wild-type patients of whom 22 had elevated uracil levels. Two of the 22 wild-type patients with elevated uracil levels received a full fluoropyrimidine-dose at start of treatment and were therefore excluded from analysis. Baseline characteristics of the *DPYD* wild-type patients are shown in Table 1 and the baseline characteristics of *DPYD* variant allele carriers are shown in Supplementary Table S1. The most prevalent tumour type was colorectal cancer (61%) and 556 of 612 (91%) patients were treated with a capecitabine-based treatment regimen.

$DPYD_{wt}/U_{high}$ and $DPYD_{wt}/U_{normal}$ patients had median pre-treatment uracil levels of 18.4 ng/mL and 9.2 ng/mL, respectively. Overall, uracil levels ranged from 3.1 to 29.3 ng/mL. Heterozygous carriers of *DPYD**2A, c.2846A>T, and c.1236G>A had a median uracil concentration of 16.8, 13.0, and 10.9 ng/mL (Supplementary Figure S1), respectively. In addition, one homozygous c.1236G>A variant carrier was included, who had a pre-treatment uracil level of 13.4 ng/mL.

The mean fluoropyrimidine dose-intensity during the first treatment cycle for $DPYD_{wt}/U_{high}$ patients was 48.4% (range 35.8% to 51.3%) of the standard indicated dose. Initial dose reductions were therefore in line with the pre-specified dose reductions. Additionally, mean dose intensity for *DPYD* variant allele carriers was close to 50% (52%, range: 25-100) which is in line with pre-specified dose reductions.

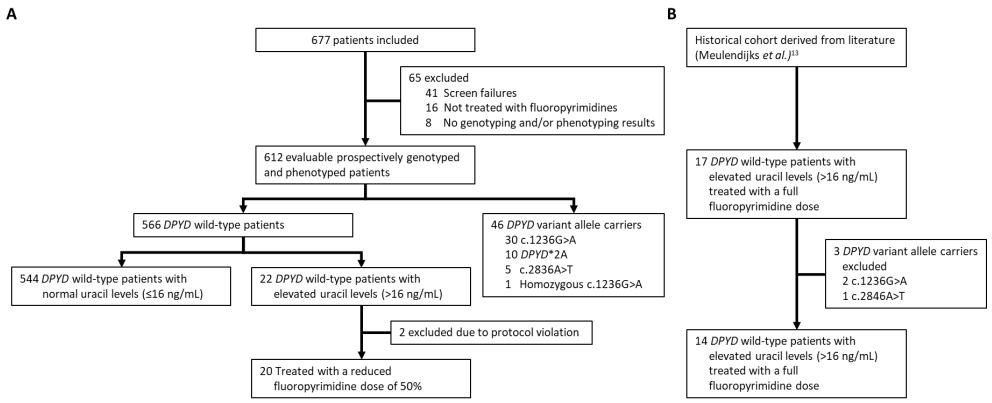

Figure 1: Flow diagram. A) Present study. B) Previous study by Meulendijks et al.[14]

Toxicity in $DPYD_{wt}/U_{high}$ patients treated with a reduced dose versus $DPYD_{wt}/U_{high}$ patients treated with a full dose

Uracil-guided dosing resulted in 10 (50%), six (30%), and four (20%) of the 20 $DPYD_{wt}/U_{high}$ patients experiencing overall grade 0, 1 to 2, and ≥3 toxicity when treated with a reduced dose of 50%, respectively. Toxicity during the whole treatment period in $DPYD_{wt}/U_{high}$ patients treated with a reduced dose was compared with the same patients treated with a full dose, from a historical cohort.[14] This analysis showed that phenotype-guided dosing based on pre-treatment uracil levels reduces the risk of developing severe fluoropyrimidine-related toxicity (20% vs 43%, P=0.029) in $DPYD_{wt}/U_{high}$ patients when treated with a reduced fluoropyrimidine-dose of 50%.

Table 1: Baseline characteristics of *DPYD* wild-type patients.

	DPYD wild-type with uracil levels > 16 ng/mL (N=20)	*DPYD* wild-type with uracil levels ≤ 16 ng/mL (N=544)	Total (N=564)	*P* value*
Sex				0.822
Male	10 (50%)	290 (53%)	300 (53%)	
Female	10 (50%)	254 (46%)	264 (46%)	
Age, years	66 (57-75.0)	65 (56-73)	65 (56-73)	0.584
Primary tumor type				0.503
Colorectal	10 (50%)	336 (62%)	346 (61%)	
Breast	3 (15%)	73 (13%)	76 (14%)	
Eosophagogastric	6 (30%)	90 (16%)	96 (17%)	
Head and neck	0 (0%)	4 (0%)	4 (1%)	
Other[†]	1 (5%)	41 (8%)	42 (7%)	
Type of treatment				0.319
Capecitabine monotherapy (with or without monoclonal antibodies)	3 (15%)	127 (23%)	130 (23%)	
Capecitabine combined with radiotherapy (with or without mitomycin)	3 (15%)	109 (20%)	112 (20%)	
Capecitabine combined with oxaliplatin (with or without monoclonal antibodies)	11 (55%)	248 (46%)	259 (46%)	
Capecitabine combined with other anticancer drugs[ε]	2 (10%)	15 (3%)	17 (3%)	
Fluorouracil combined with radiotherapy (with or without mitomycin)	0	17 (3%)	17 (3%)	
Fluorouracil combined with oxaliplatin and folinic acid (with or without monoclonal antibodies)	1 (5%)	10 (2%)	11 (2%)	
Fluorouracil combined with other anticancer drugs[∞]	0	18 (3%)	18 (3%)	
Body surface area	1.9 (1.8-2.0)	2.0 (1.8-2.1)	2.0 (1.8-2.1)	0.652
WHO Performance status				0.084
0	6 (30%)	286 (53%)	292 (52%)	
1	12 (60%)	209 (39%)	221 (40%)	
2	2 (10%)	37 (7%)	39 (7%)	
Not specified[‡]	0	12 (2%)	12 (2%)	
Stage of cancer				0.139
Local	1 (5%)	136 (25%)	137 (24%)	
Locally advanced	5 (25%)	175 (32%)	180 (32%)	
Metastatic	14 (70%)	229 (42%)	243 (43%)	
Other	0	4 (<1%)	4 (<1%)	
Uracil concentration (ng/mL)	18.4 (17.3-22.3)	9.2 (7.5-11.1)	9.4 (7.5-11.3)	< 0.001[*]
Dihydrouracil concentration (ng/mL)	149.8 (125.4-163.1)	117.0 (100.5-135.4)	117.0 (101.0-136.4)	< 0.001[*]
DHU/U-ratio	7.5 (6.5-8.0)	12.7 (10.5-15.5)	12.6 (10.3-15.4)	< 0.001[*]

Data are n (%) or median (IQR). *DPYD*-gene encoding dihydropyrimidine dehydrogrenase. NA-not applicable. [*]p value comparing *DPYD* wild-type patients with a uracil concentration > 16 ng/mL to *DPYD* wild-type patients with a uracil concentration ≤ 16 ng/mL. We used a Mann-Whitney-U test for age, body surface area, uracil concentration, dihydrouracil concentration; a Fisher's exact test for WHO performance status; and a Fisher's exact test for sex, tumour type, and treatment regimen.

Other tumor types included anal cancer, bladder cancer, pancreatic cancer, unknown primary tumour type, vulvar carcinoma, urothelial cancer and several rare tumour types. [ε]Other anticancer drugs include anastrozol, carboplatin, cyclophosphamide, docetaxel, gemcitabine, leuprorelin, temozolomide, tucatinib, and vinorelbine (with or without monoclonal antibodies). [∞]Other anticancer drugs include carboplatin, cyclophosphamide, docetaxel, and epirubicin (with or without monoclonal antibodies). [‡]WHO performance status was not specified for these patients, but was ≤2, as required by study inclusion criteria.

Toxicity in DPYD$_{wt}$/U$_{high}$ patients treated with a reduced dose versus DPYD$_{wt}$/U$_{normal}$ patients treated with full dose

When comparing the incidence of overall grade ≥3 toxicity during the first two treatment cycles with the *DPYD*$_{wt}$/U$_{normal}$ patients treated with full dose, no significant difference could be found (2 [10%] of 20 vs 58 [11%] of 544, P=1.00, Table 2). In this study, grade 4 toxicity was not present in *DPYD*$_{wt}$/U$_{high}$ patients, but was present in four *DPYD*$_{wt}$/U$_{normal}$ patients. One *DPYD*$_{wt}$/U$_{high}$ patient died due to pneumonia during treatment with fluoropyrimidines. Frequencies and percentages of severe fluoropyrimidine-related toxicity of *DPYD*$_{wt}$/U$_{high}$ patients treated with a reduced fluoropyrimidine-dose of 50% and DPY$_{wt}$/U$_{normal}$ patients treated with a full dose during the first two treatment cycles are depicted in Table 2.

Table 2: Treatment outcomes for *DPYD* wild-type patients (during first 2 treatment cycles) included in this study

	***DPYD* wild-type with uracil levels > 16 ng/mL (N=20)**	***DPYD* wild-type with uracil levels ≤ 16 ng/mL (N=544)**	***P* value**
Relative dose intensity first cycle*	49% (36-51) [4]	96% (37-107) [10]	NA
Relative dose intensity after 2 cycles*	50% (31-91) [13]	94% (37-107) [11]	NA
Overall grade ≥3 toxicity	2 (10%)	58 (10.7%)	1.00
Grade ≥3 Gastrointestinal toxicity	1 (5%)	33 (5.1%)	1.00
Grade ≥3 Cardiac toxicity	0	4 (<1%)	1.00
Grade ≥3 Haematological toxicity	0	7 (1.3%)	1.00
Grade ≥3 Hand-foot syndrome	0	2 (<1%)	1.00
Grade ≥3 Other treatment-related toxicity	1 (5%)	18 (3.3%)	0.50
Overall grade ≥4 toxicity	0	4 (<1%)	1.00
Grade ≥4 Gastrointestinal toxicity	0	1 (<1%)	1.00
Grade ≥4 Cardiac toxicity	0	1 (<1%)	1.00
Grade ≥4 Haematological toxicity	0	1 (<1%)	1.00
Grade ≥4 Hand-foot syndrome	0	0	
Grade ≥4 Other treatment-related toxicity	0	1 (<1%)	1.00
Stop of fluoropyrimidines because of adverse events?	3 (15%)	30 (5.5%)	0.11

Data are mean (range)[SD] or n (%). *DPYD*-gene encoding dihydropyrimidine dehydrogenase. NA-not applicable. *Relative dose intensity was calculated as the given dose divided by the standard dose in mg/m^2 given for the indication and treatment regimen that was applicable for the patient.

Pharmacokinetics and DPD enzyme activity in DPYD$_{wt}$/U$_{high}$ patients

Pharmacokinetic analysis was performed during the interim-analysis and data was available of 19 *DPYD*$_{wt}$/U$_{high}$ patients treated with a 50% reduced dose of fluoropyrimidines. Mean AUC$_{0\text{-}8h}$ values of the *DPYD*$_{wt}$/U$_{high}$ patients treated with a fluoropyrimidine dose of 50%, 100%, and the reference cohort treated with 100% are depicted in Figure 2 (detailed information in Supplementary Table S2). Mean exposure to 5-FU showed to be 179 ng*h/mL compared to the reference AUC$_{0\text{-}8h}$ of 381 ng*h/mL. Mean exposure to capecitabine and other metabolites was also substantially lower in these patients compared to control patients receiving a full dose of fluoropyrimidines[22] (Supplementary Table S2).

Pharmacokinetic analysis of the two *DPYD*$_{wt}$/U$_{high}$ patients treated with a full dose showed that the mean exposure to 5-FU was comparable with the reference AUC$_{0\text{-}8h}$ (341.2 ng*h/mL, Figure 2).

For DPD enzyme activity in PBMCs of the *DPYD*$_{wt}$/U$_{high}$ patients treated with a 50% fluoropyrimidine dose, no correlation was found with pre-treatment uracil levels (R^2=0.014, P=0.64, Figure 3). In addition, no correlation was found between DPD enzyme activity and the ratio of dihydrouracil to uracil and the AUC$_{0\text{-}8h}$ of 5-FU (Supplementary Figure S2).

Discussion:

The results of this study suggest that uracil-guided dosing potentially poses a risk for underexposure to 5-FU in patients with elevated uracil levels when treated with a reduced fluoropyrimidine dose of 50%. Pharmacokinetic analysis during the interim-analysis showed that exposure to 5-FU was 53% lower in *DPYD*$_{wt}$/U$_{high}$ patients treated with a reduced dose of 50% compared to a historic cohort of 20 *DPYD* wild-type patients treated with a full dose of fluoropyrimidines.[22] In addition, no correlation between uracil levels and DPD enzyme activity was found. This suggests that uracil-guided dosing on average resulted in undertreatment and may be unsuitable for phenotype-guided dosing of fluoropyrimidines, which is not in line with latest EMA recommendations regarding DPD deficiency testing.[11]

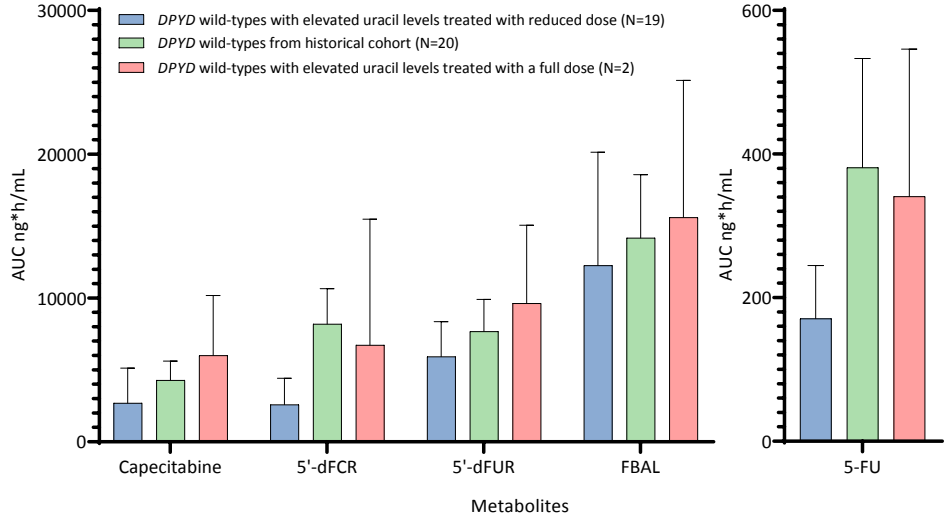

Figure 2: Pharmacokinetics of uracil-guided capecitabine dosing. Dose-normalized mean AUC$_{0\text{-}8h}$ of capecitabine and metabolites for *DPYD* wild-type patients with elevated uracil levels (>16 ng/mL) treated with reduced dose of 50% (blue), *DPYD* wild-type control patients from a previously published study (green)[22], and *DPYD* wild-type patients with elevated uracil levels treated with a full fluoropyrimidine-dose (red). All AUCs were dose-normalized to 850 mg/m^2 because patients were treated at various dosages. 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. FBAL = α-fluoro-β-alanine.

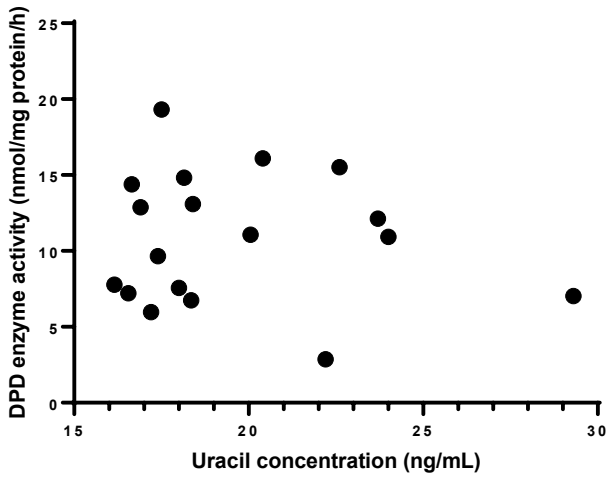

Figure 3: Scatter plot of DPD enzyme activity versus uracil concentration in *DPYD* wild-type patients with uracil levels above 16 ng/mL (Pearson correlation, R2=0.014, 95% CI -0.55 - 0.37, P=0.64). CI-Confidence interval. DPD-Dihydropyrimidine dehydrogenase.

As expected, in the current study, the incidence of severe fluoropyrimidine-related toxicity in wild-type patients with U_{high} could be decreased from 43% to 20% by a 50% dose reduction in those patients. Thereby the incidence was comparable with wild-type patients with uracil levels ≤ 16 ng/mL within our study as with data of wild-type patients derived from literature.[4] Previous research also showed an association between increased pre-treatment uracil levels and increased risk of fluoropyrimidine-related toxicity.[14,16,23] Whereas other studies could not find this association, neither an association between pre-treatment uracil levels and DPD enzyme activity.[24] The latter was also lacking in our study. Additionally, a large retrospective clinical trial (N=4215) could not find a correlation between *DPYD* genotype and uracil levels. Therefore, they concluded that the evidence supporting the use of the current cut-off is currently inadequate.[25] Furthermore, it was shown in a cohort of 573 patients that DPD phenotype was discordant in 17% of the patients when comparing the uracil levels between two samples taken at the same time. This suggests that approximately 1 out of 5 patients are at risk of being falsely classified as being DPD deficient.[26] Based on above, it is questionable whether the increased uracil value is actually a predictor for fluoropyrimidine-related toxicity, especially since with a substantial dose reduction there is less chance of toxicity anyway. Interestingly, the studies in which a correlation was described have also been the basis for the recommendation of the EMA to include DPD phenotyping based on pre-treatment uracil levels to identify patients with a DPD deficiency.[11]

In our previous study, we found large differences in uracil levels between hospitals, in addition to a lack of correlation between pre-treatment uracil levels and severe fluoropyrimidine-related toxicity.[24] It was hypothesised that these large between-centre differences and this lack of correlation possibly were caused by pre-analytical factors as uracil is highly instable at room temperature and is affected by circadian rhythm and food intake.[18-20] Hence, in this study, blood sampling for the measurement of uracil levels was standardized and blood samples were taken between 08:00 and 10:00 AM in a fasted

state and processed within one hour at room temperature after sampling to minimize the influence of these factors. Subsequently, all samples were transported on dry ice and measured centrally. Considering these factors were taken into account, it can be questioned whether pre-treatment uracil levels are predictive of DPD deficiency and subsequent severe fluoropyrimidine-related toxicity. Furthermore, recently a case was described in which it was suggested that uracil levels are possibly influenced by renal function resulting in false-positive results.[27] Based on the above, the influence of pre-analytical factors may be even greater than expected.

Based on the results of the interim-analysis it was concluded that the dose reduction of 50% in $DPYD_{wt}/U_{high}$ patients was not justified and therefore halted. The AUC0-8h of 5-FU in $DPYD_{wt}/U_{high}$ patients treated with a full dose was comparable to the reference value derived from Deenen *et al.*,[22] further adding to the assumption that elevated uracil levels may not be associated with DPD deficiency, increased exposure to 5-FU, and a higher risk of developing severe fluoropyrimidine-related toxicity. Moreover, these results are in line with a recent study in which was described that patients with elevated uracil levels (≥ 16 ng/mL) receiving a reduced 5-FU dose were at risk of under-exposure and 45% of these patients benefitted from a dose increase between the first two cycles of treatment because of a low exposure.[28] However, the small number of $DPYD_{wt}/U_{high}$ patients treated in our study with a full dose makes interpretation complex. Based on the results of our current study the EMA recommendation is worrying as a substantial number of patients with elevated uracil levels are potentially being treated with inadequate doses of fluoropyrimidines, possibly negatively impacting treatment outcome.

In *DPYD* variant allele carriers, the uracil levels were not as high as expected upfront with only the median uracil level of *DPYD**2A carriers exceeding the proposed threshold of 16.0 ng/mL for DPD deficiency. Of note, the one homozygous c.1236G>A variant carrier had a pre-treatment uracil level of 13.4 ng/mL, suggesting that no DPD deficiency was present in this patient. DPD enzyme activity in this patient was found to be below the threshold, although marginally. This indicates that *DPYD* genotyping should still be preferred over DPD phenotyping based on uracil.

A limitation of this study was the use of a historical cohort of $DPYD_{wt}/U_{high}$ patients treated with a full dose of fluoropyrimidines. In this historical cohort, uracil levels were retrospectively measured of patients from only one Dutch center of which serum was available without regard of sampling conditions.[14] Therefore, differences in study population could have influenced the observed safety outcomes. However, a randomized controlled clinical trial would have been unethical as patients with elevated uracil levels were expected to be at increased risk of developing severe fluoropyrimidine-related toxicity when treated with a full dose.[14] Moreover, this historical cohort only consisted of 14 $DPYD_{wt}/U_{high}$ patients making it difficult to compare groups.

To summarise, we showed that the incidence of severe fluoropyrimidine-related toxicity was lowered by dose-individualization on the basis of pre-treatment uracil levels in *DPYD* wild-type patients. However, pharmacokinetic analysis showed a substantial decrease in exposure to 5-FU in patients with elevated uracil levels when treated with a reduced dose of 50%. This indicates that these patients are treated sub optimally and that pre-treatment

uracil levels are not predictive of DPD deficiency. Therefore, pre-treatment uracil levels are, in our opinion, unfit for dose-individualization of fluoropyrimidine-based treatment regimens.

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Supplemental - Chapter 4

Supplementary methods

Inclusion and exclusion criteria

Patient with a pathologically confirmed malignancy for which treatment with fluoropyrimidine-based chemotherapy regimens were considered to be in the patient's best interest could be included in this study. Eligible patients were 18 years or older, of western-descent and able and willing to give written informed consent. Patients were required to have a WHO performance status of <2 and able and willing to undergo extra blood sampling for study related analysis. Patients were also required to have adequate baseline characteristics (complete blood count, hepatic function which involves serum bilirubin, AST, ALT, and renal function) left to the discretion of the treating physician.

Exclusion criteria were prior treatment with fluoropyrimidines, known substance abuse, psychotic disorders, and/or diseases expected to interfere with study or patient's safety in the opinion of the treating physician, and patients treated with a combination of fluoropyrimidines and irinotecan.

Toxicity assessments:

The following definitions were used for causality assessment of toxicity:

- **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.

Pharmacokinetic analyses

For the pharmacokinetic analysis blood was collected on the first day of treatment with fluoropyrimidines. Blood was collected in lithium heparin tubes of 4 ml at 9 different time points up to 8 hours after capecitabine intake (pre-dose, 0.25, 0.50, 1, 2, 3, 4, 6, and 8 hours). Samples were immediately centrifuged after blood sampling at 3300 rpm at 4°C and plasma was stored at -80°C until analysis. Capecitabine and the metabolites5'-deoxy-5-fluorocytidine (5'-dFCR), 5'-deoxy-5-fluorouridine (5'-dFUR), 5-FU, and fluoro-β-alanine (FBAL) were quantified in plasma samples using a validated ultra-performance liquid chromatography tandem mass-spectrometry (UPLC-MS/MS) method. The assay was validated over the range of 25.0 to 2500 ng/mL for capecitabine, 10.0 to 1000 ng/mL for 5'-dFCR, 5'-dFUR, and 5-FU and 50 to 5000 ng/mL for FBAL in human plasma. Stable isotopically labelled internal standards were used for all analytes. Sample preparation was performed by taking a sample aliquot of 300 µL and adding 20 µL internal standard working solution. Subsequently, proteins were precipitated with 900 µL of methanol:acetonitrile 1:1 (v/v), followed by short vortex mixing, 10 min of automatic shaking at 1250 rpm and centrifuging at 14000 rpm for 10 min at room temperature. The supernatant was evaporated under a gentle stream of nitrogen and thereupon dissolved in 100 µL of 0.1% formic acid in water followed by short vortex mixing and centrifuging at 14000 rpm at 4°C. Analytes were separated using an Acquity UPLC HSS T3 column (150 mm x 2.1 mm ID, particle size 1.8 µm). Chromatic separation was achieved by using a gradient consisting of mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) at a constant flow rate of 300 µL/min. The gradient started for the first 2.50 min with 100% mobile phase A after which the proportion of mobile phase B increased linearly to 90% until 7.50 min. At t=7.5 min, the column was brought back to its original state of 100% mobile phase A. Detection of the analytes was performed on a quadruple trap mass spectrometer with a Turbo Ion Spray Interface (Q-trap 5500 triple quadrupole, Sciex, Framingham, M, USA). 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, measured in the same laboratory as the current study.[1]

Supplementary results:

Detailed information on dose modification in DPYD wild-type patients with elevated uracil levels

Fluoropyrimidine doses were escalated during treatment in 9 out of 20 (45%) *DPYD* wild-type patients with elevated uracil levels treated with a reduced starting dose of 50%. In two patients this higher dose was not tolerated. In one of these patients the capecitabine dose was increased at the start of the 4th cycle from 50% to 57% resulting in grade 3 pain and a subsequent dose reduction to the previously administered dose of 50% at the start of the 5th cycle. However, toxicity did not improve and the dose was further reduced (44%) at the start of the 7th cycle after which treatment was prematurely stopped due to patient refusal. In the other patient, the dose was increased from 50% to 100% after six treatment cycles, which was tolerated for three cycles more, after which the patient developed grade 3 hand-foot syndrome. Thereupon the dose was decreased to 70% which was given for the remaining 12 treatment cycles (21 cycles in total). The other 7 patients were able to continue treatment with the escalated dose or were subsequently escalated to higher doses, although none were escalated to a full dose of fluoropyrimidines. In two patients the initial starting dose of 50% was not tolerated and further reduced which was re-escalated (58%) after one cycle in one patient who remained on this dose until the end of treatment. The other patient could tolerate the reduced dose of 18% and treatment with fluoropyrimidine was discontinued. The one homozygous c.1236G>A carrier had a DPD enzyme activity of 6.99 nmol/mg protein/h which corresponds to 44.5% remaining DPD enzyme activity (median reference value: 15.7 nmol/mg protein/h)[27] and thus was treated with this percentage of the full dose. No severe fluoropyrimidine-related toxicity was observed, although a dose reduction to 35.5% was applied after cycle 3 due to grade 2 nausea, after which treatment was completed one this dose level according to the treatment protocol.

Pharmacokinetic analysis

A total of 22 *DPYD* wild-type patients with elevated uracil levels (>16 ng/mL) were included and 20 were treated with an initially reduced fluoropyrimidine-dose of 50% and were included in the pharmacokinetic analysis during the interim-analysis. Pharmacokinetic results are shown in Figure 1 and supplementary Table S2. In 19 patients pharmacokinetic sampling was performed at day 1 of cycle 1, in one patient this was done at day 1 of the second cycle, after a week without capecitabine intake. One patient was treated with 5-FU of which blood samples for pharmacokinetic sampling were accidently only taken after ending of infusion and therefore unable to be used in the pharmacokinetic analysis.

References:

1 Deenen MJ, Meulendijks D, Boot H, *et al.* Phase 1a/1b and pharmacogenetic study of docetaxel, oxaliplatin and capecitabine in patients with advanced cancer of the stomach or the gastroesophageal junction. *Cancer Chemother Pharmacol* 2015; **76**: 1285-95.

Table S1: Baseline characteristics of *DPYD* variant allele carriers

	DPYD variant allele carriers (N=46)	*DPYD**2A (N=10)	c.2846A>T (N=5)	c.1236G>A (N=30)	Homozygous c.1236G>A (N=1)
Sex					
Male	29 (63%)	9 (90%)	5 (100%)	15 (50%)	0
Female	17 (37%)	1 (10%)	0	15 (50%)	1 (100%)
Age, years	63 (59-71)	63 (61-70)	74 (62-78)	62 (58-69)	61
Primary tumor type					
Colorectal	28 (61%)	7 (70%)	3 (60%)	17 (57%)	0
Breast	5 (11%)	1 (10%)	0	4 (13%)	1 (100%)
Eosophagogastric	8 (18%)	1 (10%)	1 (20%)	6 (20%)	0
Head and neck	0	0	0	0	0
Other[†]	5 (11%)	1 (10%)	1 (20%)	3 (10%)	0
Type of treatment					
Capecitabine monotherapy (with or without monoclonal antibodies)	6 (13%)	1 (10%)	2 (40%)	3 (10%)	0
Capecitabine combined with radiotherapy (with or without mitomycin)	6 (13%)	3 (30%)	0	3 (10%)	0
Capecitabine combined with oxaliplatin (with or without monoclonal antibodies)	25 (54%)	5 (50%)	1 (20%)	18 (60%)	1 (100%)
Capecitabine combined with other anticancer drugs[ε]	3 (7%)	0	0	3 (10%)	0
Fluorouracil combined with radiotherapy (with or without mitomycin)	2 (4%)	1 (%)	1 (20%)	0	0
Fluorouracil combined with oxaliplatin and folinic acid (with or without monoclonal antibodies)	2 (4%)	0	1 (20%)	1 (3%)	0
Fluorouracil combined with other anticancer drugs[∞]	2 (4%)	0	0	2 (7%)	0
Body surface area	2.0 (1.8-2.1)	2.1 (2.0-2.2)	2.0 (2.0-2.1)	1.9 (1.8-2.1)	1.7 (1.7-1.7)
WHO Performance status					
0	23 (50%)	6 (60%)	3 (60%)	14 (47%)	0
1	20 (44%)	3 (30%)	2 (40%)	14 (47%)	1 (100%)
2	3 (7%)	1 (10%)	0	2 (7%)	0
Stage of cancer					
Local	11 (24%)	4 (40%)	1 (20%)	6 (20%)	0
Locally advanced	10 (22%)	3 (30%)	0	6 (20%)	1 (100%)
Metastatic	25 (54%)	3 (30%)	4 (80%)	18 (60%)	0
***DPYD* status**					
*DPYD**2A, heterozygous	10 (22%)	10 (100%)	0	0	0
c.2846A>T, heterozygous	5 (11%)	0	5 (100%)	0	0
c.1236G>A, heterozygous	30 (65%)	0	0	30 (10%)	0
Homozygous c.1236G>A	1 (2%)	0	0	0	1 (100%)
Uracil concentration (ng/mL)	12.3 (9.8-14.3)	16.8 (14.6-19.1)	13.0 (12.8-14.0)	10.9 (9.2-12.3)	13.4 (13.4-13.4)
Dihydrouracil concentration (ng/mL)	114.0 (104.1-132.9)	113.5 (109.5-126.2)	107.0 (104.0-109.0)	117.2 (101.6-141.6)	115.0 (115.0-115.0)
DHU/U-ratio	9.4 (8.1-11.5)	7.7 (6.5-8.5)	8.2 (7.0-8.4)	11.2 (9.5-12.9)	8.6 (8.6-8.6)

Data are n (%) or median (IQR). *DPYD*-gene encoding dihydropyrimidine dehydrogrenase. NA-not applicable. U-Uracil. DHU-Dihydrouracil. [†]Other tumor types included pancreatic cancer, urothelial cancer and several rare tumour type, [ε]Other anticancer drugs include anastrozol and temozolomide, [∞ε]Other anticancer drugs includes docetaxel.

Table S2: Pharmacokinetic parameters of capecitabine and metabolites in *DPYD* wild-type patients treated with elevated uracil levels treated with a reduced dose, wild-type controls, and with elevated uracil levels treated with a full dose.

Metabolite	Mean dose-normalized AUC_{0-8} (ng*h/mL) [CV%][a]			Mean $T_{1/2}$ (h) [CV%]		
	***DPYD* wild-type patients with elevated uracil levels treated with 50% (*N*=19)**	***DPYD* wild-type patients (reference cohort)[b] (N=20)**	***DPYD* wild-type patients with elevated uracil levels treated with 100% (N=2)**	***DPYD* wild-type patients with elevated uracil levels treated with 50% (N=19)**	***DPYD* wild-type patients (reference cohort) [b] (N=20)**	***DPYD* wild-type patients with elevated uracil levels treated with 100% (N=2)**
Capecitabine	2789 [69%]	4281 [31%]	6002 [70%]	0·72 [131%]	0·76 [55%]	1·6 [74%]
5′-dFCR	2664 [59%]	8192 [30%]	6730 [130%]	0·79 [36%]	1·0 [45%]	1·4 [62%]
5′-dFUR	5955 [36%]	7673 [29%]	9634 [56%]	0·77 [82%]	0·9 [34%]	1·9 [84%]
5-FU	179 [46%]	381 [40%]	341 [60%]	0·67 [42%]	1·0 [57%]	1·3 [83%]
FBAL	10137 [45%]	14177 [31%]	15604 [61%]	2·7 [53%]	2·6 [33%]	4·3 [36%]

[a] Mean AUC_{0-8h} have been dose-normalized because patients were treated at various dose dosages, all AUCs were dose-normalized to 850 mg/m^2. [b]Control values are derived from Deenen *et al.* 1 for patients with advanced cancer of the stomach or gastroesophageal junction after administration of capecitabine 850 mg/m^2. *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: α-fluoro-β-alanine; $T_{1/2}$: half-life.

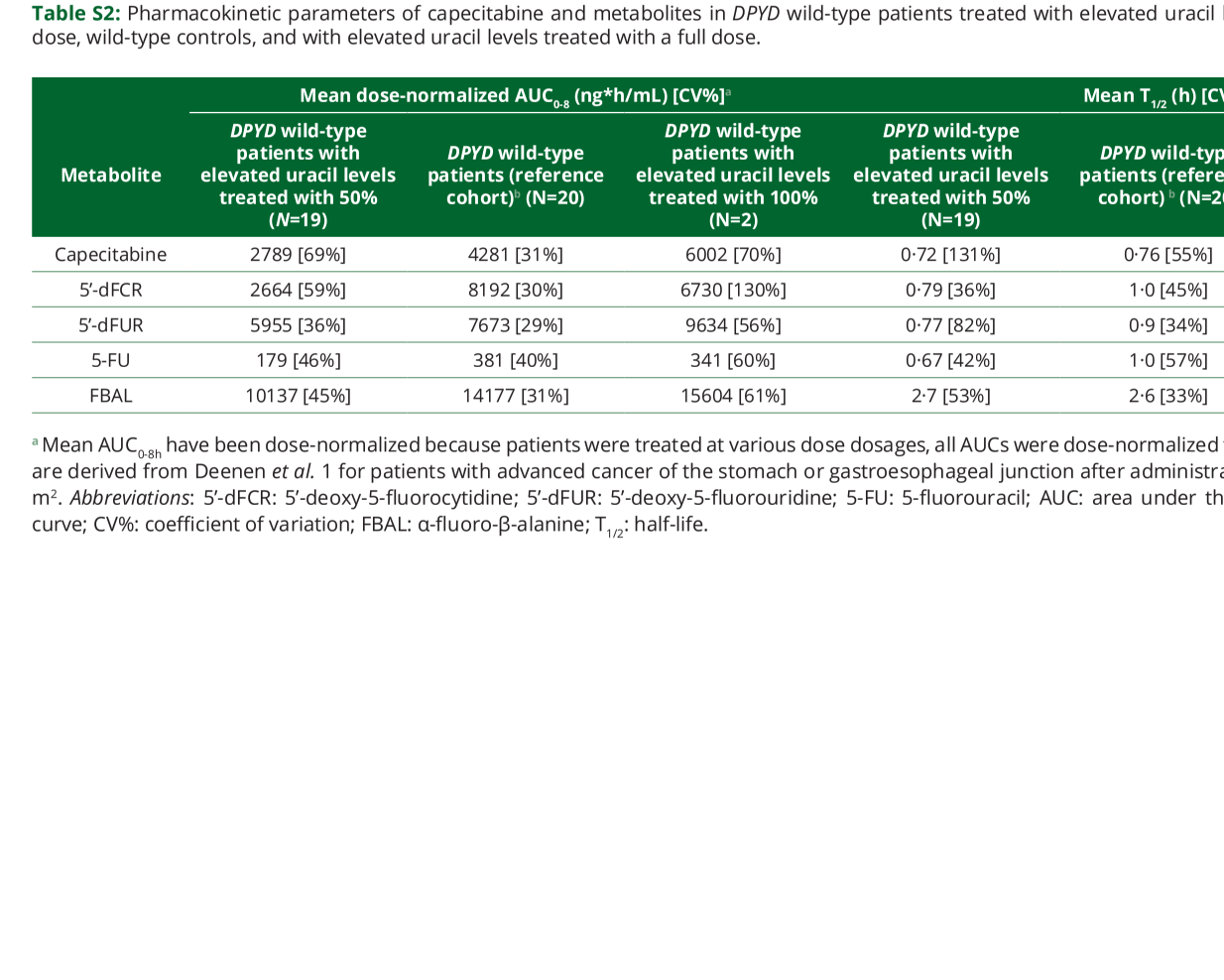

Figure S1: Uracil level per *DPYD* genotype. Red dotted line indicates threshold for uracil and DPD deficiency of 16 ng/mL.

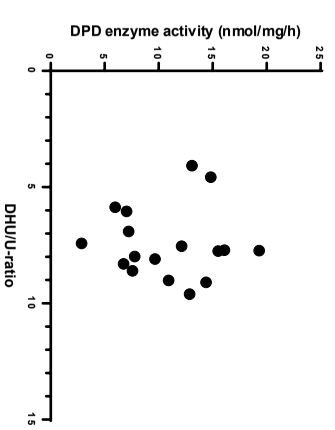

Figure S2: Scatter plot of DPD enzyme activity vs. (A) DHU/U-ratio (Pearson correlation, R^2<0.01, P=0.94) and (B) dose-normalized AUC_{0-8h} (ng*h/mL) in *DPYD* wild-type patients with uracil levels above 16 ng/mL (Pearson correlation, R^2<0.01, P=0.84). *Abbreviations*: AUC-Area-under-the-curve. DPD-Dihydropyrimidine dehydrogenase. DHU-Dihydrouracil. U-Uracil.

Chapter 5

A nomogram to predict severe toxicity in *DPYD* wild-type patients treated with capecitabine-based anticancer regimens

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Abstract

DPYD-guided dosing has improved the safety of fluoropyrimidine-based chemotherapy in recent years. However, severe toxicity remains in ~23% of patients not carrying *DPYD* variant alleles treated with capecitabine. Therefore, we developed a predictive model based on patient-related and treatment-related factors aimed at estimating the risk of developing severe capecitabine-related toxicity.

The nomogram was developed using data from two large clinical trials (NCT00838370 and NCT02324452). Cancer patients carrying a *DPYD* variant allele (*DPYD**2A, c.1236G>a, c.2846A>T, and c.1679T>G) were excluded. Univariable and multivariable logistic regression using predetermined predictors based on previous findings including age, sex, body surface area, type of treatment regimen, and creatinine levels were used to develop the nomogram. The developed model was internally validated using bootstrap resampling and cross-validation.

A total of 2147 *DPYD* wild-type patients with cancer treated with capecitabine-based chemotherapy regimens were included of which complete data of 1745 patients was available and used for the development of the nomogram. Univariable and multivariable logistic regression showed that age, sex, and type of treatment regimen were strong predictors of severe capecitabine-related toxicity in *DPYD* wild-type patients. Internal validation demonstrated a concordance index of 0.68 which indicates a good discriminative ability for prediction of severe capecitabine-related toxicity.

The developed nomogram includes readily available parameters and may be a helpful tool for clinicians to assess the risk of developing severe capecitabine-related toxicity in patients without known risk *DPYD* variant alleles treated with capecitabine-based anticancer regimens.

Introduction

Capecitabine is an anti-cancer agent belonging to the group of fluoropyrimidines and is a pro-drug of 5-fluorouracil (5-FU) and is widely used in the treatment of various cancers.[1-3] Despite being used for over two decades, the efficacy of capecitabine is often negatively impacted by severe fluoropyrimidine-related toxicity, resulting in dose reductions, delays, treatment discontinuation, loss of quality of life, and in some cases even death.[4-6] Approximately 10-30% of patients treated with capecitabine experience severe toxicity, which includes nausea, diarrhoea, vomiting, mucositis, neutropenia, and hand-foot syndrome.[4,5] One of the main causes of these toxicities during treatment with fluoropyrimidines is a deficiency of the main catabolic enzyme dihydropyrimidine dehydrogenase (DPD). Genetic polymorphisms in the *DPYD* gene, which encode for the DPD enzyme, can reduce the metabolism of 5-FU into inactive metabolites, thereby affecting the risk of severe fluoropyrimidine-induced toxicity.[7,8] Hence, pre-therapeutic screening for *DPYD* variant alleles (*DPYD**2A, c.1236G>A, c.2846A>T, and c.1679T>G) and subsequent dose-individualization was studied and proved to reduce severe fluoropyrimidine-related toxicity in *DPYD* variant allele carriers.[8,9] As a result, *DPYD* genotyping is now widely recommended by several clinical guidelines and the European Medicines Agency (EMA) and used in several European countries.[10-12]

Although *DPYD* genotype-guided dosing reduces the incidence of toxicity, nearly a quarter of the *DPYD* wild-type patients still experience severe fluoropyrimidine-related toxicity.[8,9] Besides *DPYD* genotyping, DPD phenotyping methods have been explored to further reduce the incidence of severe fluoropyrimidine-related toxicity.[13] However, these methods are also aimed towards detecting DPD deficiency and rarely take other factors into account which could influence the risk of developing severe toxicity emphasising the need for dose-individualisation strategies for cancer patients without DPD deficiency. Previously, other factors besides DPD such as sex, body composition, age, body surface area (BSA), type of capecitabine-based treatment regimen and renal function have been associated with the early onset of fluoropyrimidine-related toxicity.[14-16] It has been suggested that women have decreased 5-FU clearance, increased 5-FU exposure and therefore are at increased risk of developing severe fluoropyrimidine-related toxicity.[13] Moreover, the possible relation between body composition and severe toxicity could possibly be explained by the relatively low proportion of lean body mass or muscle mass in women compared to men. Furthermore, a higher clearance of 5-FU and subsequently a lower risk of severe toxicity has been found in patients with higher BSA.[14] Interestingly, the association between renal function and severe fluoropyrimidine-related toxicity was unexpected as 5-FU is predominantly metabolised in the liver and tumour tissue.[17] However, pooled data from phase I studies showed that creatinine clearance significantly influences exposure to 5-FU. Indicating that renal function needs to be considered when dosing fluoropyrimidines, even though the exact mechanism by which renal function increases risk of severe fluoropyrimidine-related toxicity is unclear.[13,17]

These patient- and treatment-related factors could potentially be used as a dose-individualisation strategy for *DPYD* wild-type patients treated with fluoropyrimidines to reduce the remaining risk for severe toxicity. Therefore, we aimed to develop a prediction tool based on patient-related and treatment-related factors that could accurately predict

severe toxicity in patients without known risk *DPYD* variant alleles treated with capecitabine.

Methods:

Patient population

Patients from two large multicenter clinical trials (Deenen *et al.*[8] (NCT00838370) and Henricks *et al.*[9] (NCT02324452)) including 1463 and 913 cancer patients respectively. Only patients treated with capecitabine-based treatment regimens, were included due to small number of patients treated with 5-FU in both trials.[8,9] The design and study population of both studies have previously been published.[8,9] Briefly, in Deenen *et al.*[8] patients were prospectively screened for *DPYD**2A, and heterozygous *DPYD**2A variant carriers received a 50% fluoropyrimidine dose reduction. In addition, patients were also retrospectively screened for c.1236G>A, c.2846A>T, c.1679T>G and c.1601G>A. In Henricks *et al.*[9] upfront genotyping of four *DPYD* variant alleles was performed. *DPYD**2A and c.1679T>G variant allele carriers received a 50% fluoropyrimidine dose reduction, and c.1236G>A and c.2846A>T variant allele carriers a 25% fluoropyrimidine dose reduction. Patients carrying a *DPYD* variant allele (*DPYD**2A, c.1236G>a, c.2846A>T, c.1679T>G, and c.1601G>A) were excluded from the analysis, resulting in 1302 and 845 patients, respectively (Figure 1). All toxicities were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTC-AE) version 3.0 or 4.0 and from day 1 of treatment until end of treatment with CTC-AE grade ≥ 3 being considered as severe toxicity. Only toxicities scored for causality as possibly, probable, or definitely related to fluoropyrimidines were taken into account for fluoropyrimidine-related toxicity.

Prediction model construction and nomogram

The outcome of interest in this study was severe (grade ≥ 3) capecitabine-related toxicity during treatment with capecitabine-based regimens. Age, sex, BSA, treatment regimen (supplementary table 1), and renal function were previously shown to be associated with capecitabine-related toxicity, and therefore included as covariates in the multivariable logistic regression model, regardless of their significance in the univariable logistic regression analysis.[13-15] However, due to the correlation between renal function (glomular filtration rate (GFR)) and age, sex and BSA (dependent on formula used to calculate GFR) serum creatinine levels was used as a marker for renal function instead. A nomogram was constructed from this model to facilitate its interpretation in a visual way, by computing predicted capecitabine-related severe toxicity probabilities and mapping them into points on a scale from 0 to 100. For this purpose, the estimates of effect of the different covariates in the multivariable model were ranked, regardless of their statistical significance, by absolute value. The biggest effect was assigned 100 points on the scale, while the rest of covariates in the multivariable model were assigned a number of points proportional to their effect size.

Statistical analysis

Patient characteristics for continuous variables were summarised as mean (±standard deviation) or median (interquartile range [IQR]), depending on their distribution. For categorical variables frequency and percentage were presented. Categorical variables were compared using Pearson's chi-square test (Fisher's exact test in case of sparse data) and the Mann-Whitney U-test was used to test differences in continuous variables.

Univariable and multivariable logistic regression models were used in the development of the prediction model for the nomogram. Correlations between variables were assessed using Pearson's and Spearman's correlation coefficients. The inclusion of interaction terms was explored by estimating pairwise interactions using a p-value of 0.01 as cut-off for inclusion in the model, and restricted cubic splines were used to assess nonlinear relationships with the regression outcome. The discriminative power of the model was evaluated by calculating the area under the receiving operating characteristics curve (AUC), which corresponds with the concordance index, and its corresponding 95% confidence interval (CI). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and prevalence were also calculated. Confidence intervals for predictive values were calculated according to Mercaldo *et al.*[18] Accuracy was evaluated with locally estimated scatterplot smoothing (loess)-based calibration curves and confidence bands (smoothing parameter 0.75) and the mean absolute error, which was calculated from the difference between the actual (observed) probability and the predicted probability of toxicity grade ≥3 with smoothing using the loess algorithm. The nomogram was internally validated using bootstrap resampling and leave-one-out cross-validation (LOOCV) to provide an unbiased estimate of the model performance with the concordance index. The clinical utility of the prediction model in the nomogram was estimated by decision analysis curves[19], based on the threshold probability (that is, the probability at which the harm of falsely declaring toxicity equals the harm of falsely declaring non-toxicity). Statistical analyses were performed using R statistical software (version 4.2.1).

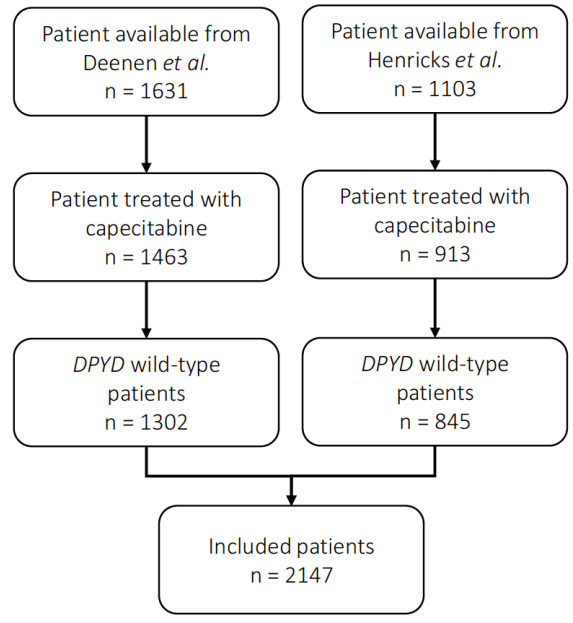

Figure 1: Flow diagram of patient inclusion.

Table 1: Patient characteristics per study

	Original dataset			Subset of patients used in prediction model		
	Deenen *et al.*[8] (N=1302)	**Henricks *et al.*[9] (N=845)**	**Total (N=2147)**	**Deenen *et al.*[8] (N=977)**	**Henricks *et al.*[9] (N=768)**	**Total (N=1745)**
Age (years)						
N	1302	845	2147	977	768	1745
Median (range)	61 (21-89)	64 (56-71)	62.0 (54-69)	60.5 (21-89)	64.0 (19-89)	62.0 (19-89)
Sex						
Female	741 (56.9%)	390 (46.2%)	1131 (52.7%)	578 (59.2%)	350 (45.6%)	928 (53.2%)
Male	561 (43.1%)	455 (53.8%)	1016 (47.3%)	399 (40.8%)	418 (54.4%)	817 (46.8%)
BSA						
N	1302	775	2077	977	768	1745
Missing	0	70	70	0	0	0
Median (range)	1.9 (1.1-2.7)	1.9 (1.3-2.7)	1.9 (1.1-2.7)	1.9 (1.1-2.7)	1.9 (1.3-2.7)	1.9 (1.1-2.7)
Primary tumour						
Breast cancer	318 (24.4%)	100 (11.8%)	418 (19.5%)	282 (28.9%)	87 (11.3%)	369 (21.1%)
Colorectal cancer	712 (54.7%)	601 (71.1%)	1313 (61.2%)	484 (49.5%)	547 (71.2%)	1031 (59.1%)
Gastric cancer	163 (12.5%)	50 (5.9%)	213 (9.9%)	127 (13.0%)	49 (6.4%)	176 (10.1%)
Other*	109 (8.4%)	94 (11.1%)	203 (9.5%)	84 (8.6%)	85 (11.1%)	169 (9.7%)
Type of regimen†						
Missing	1	0	1	0	0	0
Capecitabine - monotherapy	382 (29.4%)	171 (20.2%)	553 (25.8%)	300 (30.7%)	149 (19.4%)	449 (25.7%)
Capecitabine - platinum	345 (26.5%)	345 (40.8%)	690 (32.2%)	183 (18.7%)	312 (40.6%)	495 (28.4%)
Capecitabine - taxane	57 (4.4%)	1 (0.1%)	58 (2.7%)	57 (5.8%)	1 (0.1%)	58 (3.3%)
Capecitabine - triplet	105 (8.1%)	47 (5.6%)	152 (7.1%)	82 (8.4%)	45 (5.9%)	127 (7.3%)
Capecitabine - other	15 (1.2%)	35 (4.1%)	50 (2.3%)	12 (1.2%)	31 (4.0%)	43 (2.5%)
Capecitabine - radiotherapy	397 (30.5%)	246 (29.1%)	643 (30.0%)	343 (35.1%)	230 (29.9%)	573 (32.8%)
Creatinine (µmol/L)						
N	978	836	1814	977	768	1745
Missing	324	9	333	0	0	0
Median (range)	71 (35-354)	73 (34-213)	72 (34-354)	71.0 (35-354)	74.0 (34-213)	71.0 (34-354)

Abbreviations: BSA, Body surface area; CAP, Capecitabine; IQR, Interquartile range. *Other tumor types included: Head and neck cancer, anal cancer, vulvar cancer, urethral cancer, oesophagogastric cancer, and several rare tumor types. †Capecitabine - platinum includes combinations of capecitabine and cisplatin or oxaliplatin and monoclonal antibodies (Bevacizumab, trastuzumab or panitumumab); Capecitabine - taxane includes combinations of capecitabine and docetaxel or paclitaxel. Capecitabine - triplet includes combinations of docetaxel and oxaliplatin, cisplatin and epirubicin, oxaliplatin and epirubicin, and doxorubucin and cyclophosphamide. Capecitabine - other includes combination with irinotecan, monoclonal antibodies (bevacizumab, trastuzumab or panitumumab), temozolomide, and vinorelbine; Capecitabine-radiotherapy includes combinations of capecitabine, radiotherapy, and mitomycin C.

Results

Nomogram construction

A total of 2147 wild-type patients (Figure 1) were included. An overview of patient characteristics of included patients from both studies is shown in Table 1. For 1745 patients, all predefined predictors to be used in the nomogram were available for a complete case analysis. The prevalence of toxicity grade ≥ 3 among these patients was 20% (19% in Deenen *et al.* study and 21% in Henricks *et al.*). Univariable and multivariable logistic regression results are displayed in Table 2. Age, sex, and type of treatment regimen were strong predictors of toxicity with increasing risk of severe toxicity with age (per 10 years an increase in OR of 1.17, 95% CI 1.04-1.32, P=0.01) and male sex having a decreased risk of developing severe toxicity (OR 0.68, 95% CI 0.49-0.95, P=0.02). Pairwise interactions between all predictors in the model where explored, but for all of them global p-values were above 0.10 and thus not included.

Figure 2 shows the developed nomogram that can be used to predict the likelihood for a patient to develop severe capecitabine-related toxicity. For example, a female patient (17 points), aged 45 years (19 points), with BSA 2.7 (0 points), serum creatinine level of 100 µmol/L (15 points) and receiving capecitabine in combination with a platinum compound (34 points) would have a total of 85 points, which corresponds to a probability of severe toxicity of 20%. In order to obtain this, a vertical line can be drawn on Figure 2 intersecting sex equal to female, to then obtain at which number of points (first segment in Figure 2) the vertical line intersects. After performing these steps for each of the patient characteristics, the cumulative number of points is calculated and marked on the "Total Points" segment in Figure 2. From there, a vertical line crossing this number of total points can be drawn to obtain where it crosses the "Probability of Toxicity" segment right below. This will yield the probability of severe toxicity for this patient. As a second example, a male patient (0 points), aged 65 years (33 points), with BSA 1.7 (6 points), having a serum creatinine level of 135 µmol/L (22 points) and receiving capecitabine in combination with 2 other anticancer agents (capecitabine - triplet, 85 points) would have a total of 146 points, which corresponds to a probability of severe toxicity of 51%.

To accompany Figure 2, a dynamic nomogram was created using the shiny package in R software (URL: https://biometricsdept.shinyapps.io/dynamic_nomogram). It must be noted that the ranges of predictor values used in the nomogram displayed in Figure 2, as well as in the dynamic nomogram, correspond to ranges in the data used for building the prediction model (except for age which, for display purposes, has been represented ranging from 18 to 90 years). Applying a prediction model to patients with characteristics outside these ranges may compromise model performance, since this involves extrapolation of data.

Nomogram performance

The model's discriminative ability, as measured by the concordance index, was 0.68 (95% CI 0.64-0.71). See Figure 3 for the corresponding ROC curve. This indicates that our model can discern a patient with severe toxicity from a patient without severe toxicity 68% of the time. To correct for overfitting, the bias-corrected concordance index was obtained to be 0.67 using bootstrapping with 1000 repetitions, and 0.67 with 10-fold cross-validation.

The model's predictive accuracy can be observed in the calibration curve (supplementary Figure 1). This figure displays the predicted probabilities for the nomogram versus the actual probabilities, which would fall in a 45-degree line if the prediction model were perfectly accurate. Judging from this figure, the calibration curve stays close to the reference line, with slight under-prediction or over-prediction along the range of predicted values, and poorer precision with increasing predicted values as well as values close to zero. The mean absolute error was 0.006 and can thus be considered small (the smaller this value, the better the calibration, with a value of zero indicating perfect calibration). For obtaining measures of diagnostic accuracy, we contemplated different choices for a probability threshold. The prevalence in the data used to build the nomogram was 20% (351/1745), which led to sensitivity 0.54, specificity 0.71, PPV 0.32 and NPV 0.85. However, this threshold did not necessarily minimize misclassification of patients, and we aimed at maximizing the PPV and, in a lesser degree, the NPV. As the dose of capecitabine can be rapidly escalated in patients misclassified as high risk, those experiencing severe toxicity may need to interrupt treatment or, in severe cases, require hospitalization. We therefore chose a threshold of 0.4 and we obtained obtain a PPV of 0.49 (95% CI 0.41-0.56) and NPV 0.83 (95% CI 0.81-0.85). The relatively low value of the PPV is not surprising given that in our model prevalence is low (toxicity grade ≥3 occurs in 20% of patients), and it can be derived that the rarer the outcome, the higher the NPV and the lower the PPV.[20,21]

Table 2: Univariable and multivariable logistic regression results for probability of severe capecitabine-related toxicity

			Univariable logistic regression			Multivariable logistic regression		
Variable	**Events**	**N**	**OR**	**95% CI**	***P* value**	**OR**	**95% CI**	***P* value**
Sex								
Female	208	928	1 (ref.)			1 (ref.)		
Male	143	817	0.73	0.58 - 0.93	0.01	0.68	0.49 - 0.95	0.02
Age, per 10 years	351	1745	1.05	0.94 - 1.17	0.36	1.17	1.04 - 1.32	0.01
BSA	351	1745	0.68	0.4 - 1.17	0.17	0.87	0.44 - 1.72	0.69
Creatinine, per 10 µmol/L	351	1745	1.00	0.95 - 1.06	0.87	1.05	0.99 - 1.12	0.13
Type of regimen								
Capecitabine - monotherapy	83	449	1 (ref.)			1 (ref.)		
Capecitabine - platinum	101	495	1.13	0.82 - 1.56	0.46	1.32	0.94 - 1.86	0.11
Capecitabine - taxane	31	58	5.06	2.87 - 8.94	<0.001	5.95	3.29 - 10.75	<0.001
Capecitabine - triplet	57	127	3.59	2.35 - 5.48	<0.001	4.27	2.74 - 6.66	<0.001
Capecitabine - other	15	43	2.36	1.21 - 4.62	0.01	2.47	1.25 - 4.89	0.01
Capecitabine - radiotherapy	64	573	0.55	0.39 - 0.79	<0.001	0.61	0.42 - 0.88	0.01

Abbreviations: BSA, Body surface area; CAP, Capecitabine; CI, Confidence interval; OR, Odds ratio

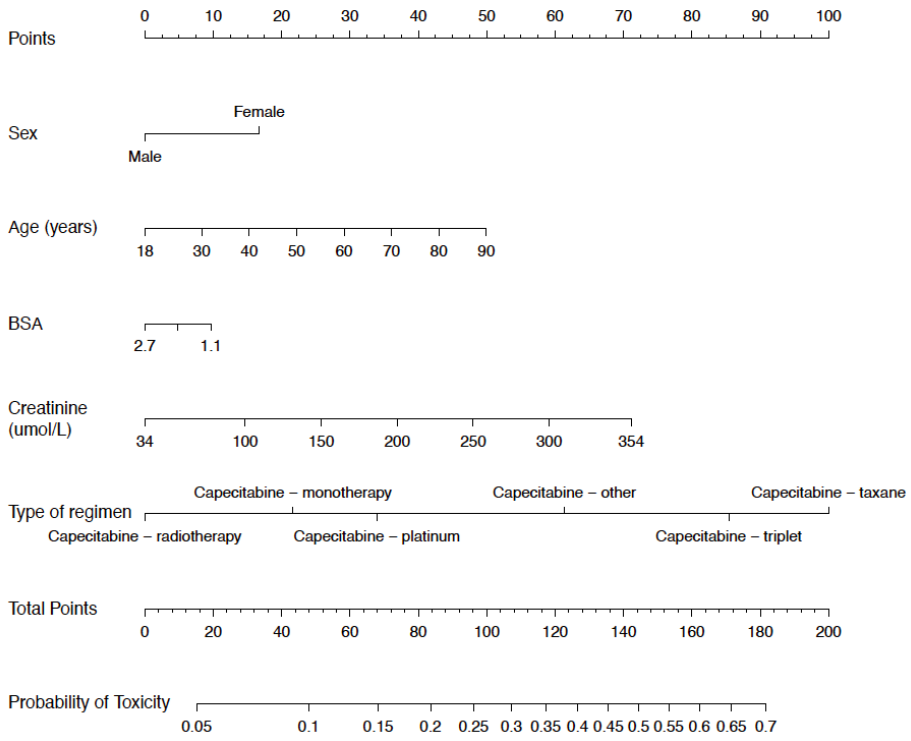

Figure 2: Nomogram to predict severe capecitabine-related toxicity using predetermined clinical predictors
Abbreviations: BSA, Body surface area; CAP, Capecitabine.

We also attempted to evaluate the clinical utility of our model. The net benefit is calculated in true-positive units, as the proportion of true positives in the sample (benefit of adjusting the treatment due to predicted toxicity) minus the proportion of false positives in the sample (harm of adjusting treatment due to predicted toxicity) weighted by the odds of the threshold. The net benefit is calculated across all possible thresholds from 0 to 1 and is depicted for our prediction model as well as for default decisions of not adjusting treatment for anyone (net benefit zero) and adjusting treatment for all. Concerning our model, if the probability of severe toxicity is deemed high for a particular patient according to our chosen threshold of 0.4, a dose reduction might be proposed, which in turn might lead to reduced efficacy. From Figure 4 it can be derived that with threshold probabilities between 20% and 50% the net benefit of classifying patients at high risk of severe capecitabine-related toxicity would be higher than the default situations of assuming toxicity, and thus adjusting treatment, for all or none of the patients.

Additional analyses were performed to study the robustness of these results. A multivariable logistic model adjusted for study next to the predetermined predictors was also run to examine possible differences in severe toxicity between studies. No significant difference in severe fluoropyrimidine-related toxicity was found (HR=1.22, 95% CI 0.94-1.69, P=0.14; results not shown) for Henricks *et al.*[9] versus Deenen *et al.*[8] Also, the nomogram displayed in Figure 2 was based on a complete-case analysis that omitted patients with missing creatinine and BSA.

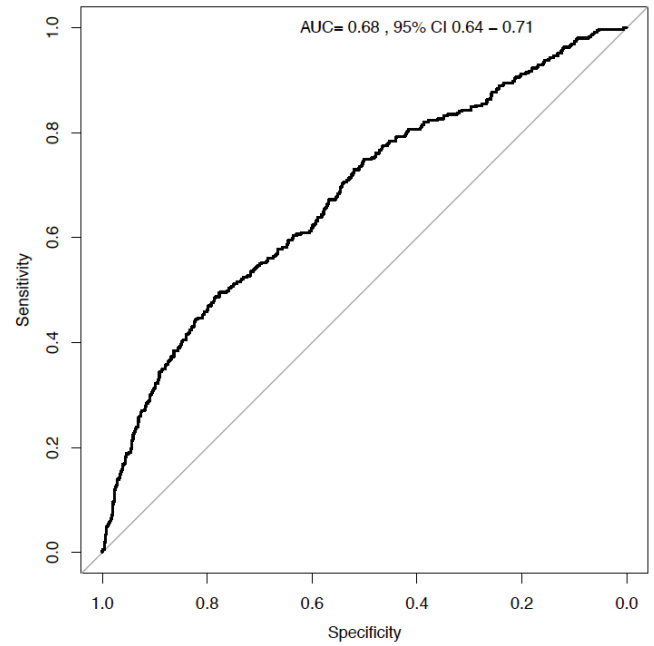

Figure 3: ROC curve for probability of severe capecitabine-related toxicity. *Abbreviations*: AUC, Area-under-the-curve; CI, Confidence interval

An analysis based on multiply imputed data was taken into consideration for tackling missing data. However, there was limited data available in both datasets that were correlated to the variables of interest in the model, or that helped to maintain the randomness of the missing process. For this reason, no appropriate auxiliary variables could be found for the imputation procedure and only a complete-case analysis was performed.

Discussion

Over the last decade safety of fluoropyrimidine-based treatment was greatly improved by *DPYD* genotype-guided dosing, significantly reducing the incidence of severe fluoropyrimidine-related toxicity.[8,9] However, despite the success of *DPYD*-guided dose-individualisation severe toxicity remains in approximately 23% of patients without one of the four known risk *DPYD* variant alleles treated with fluoropyrimidines.[9] Our study aimed to develop a tool that could accurately predict severe fluoropyrimidine-related toxicity in wild-type patients treated with capecitabine-based chemotherapy regimens. This resulted in a nomogram including creatinine concentration, sex, age, type of treatment regimen, and BSA which predicts the probability of developing severe capecitabine-related toxicity in patients treated with capecitabine-based treatment regimens. Our nomogram has a concordance-index of 0.67 after bias correction, which indicates a good discriminative ability of the model to predict severe capecitabine-related toxicity. This suggests that our model can relatively accurately predict the probability of severe fluoropyrimidine-related toxicity in wild-type patients treated with capecitabine-based

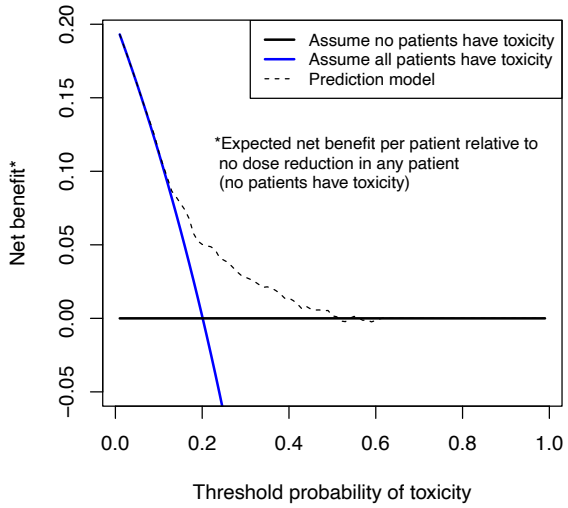

Figure 4: Decision curve for prediction model in nomogram

treatment regimens and can also be easily used by clinicians in daily clinical practice since all required model parameters are readily available.

The clinical validity of our model to predict severe toxicity was assessed by sensitivity, specificity, PPV and NPV. The main aim of our model was to accurately predict severe capecitabine-related toxicity, therefore a high PPV is desired. However, possible misclassification of patients being at high risk of severe fluoropyrimidine-related toxicity is also not desirable, and therefore NPV values should not be too low either. A PPV of 49% and NPV of 83% were found in our study. This PPV could be interpreted as low. However, both PPV and NPV are relative to frequency of patients with severe toxicity. PPV can remain limited even though there is a high risk of severe fluoropyrimidine-related toxicity, if adverse events are rare. This was also the case in our study, with 20% of patients experiencing severe fluoropyrimidine-related toxicity. We therefore regarded our PPV and NPV as acceptable. PPV and NPV of *DPYD* testing ranges from 23.5% to 100% and 50.5% to 91.5%, respectively. [22-24] These results indicate that patients who carry a *DPYD* variant allele have high risk of developing severe toxicity (high specificity). However, conversely non carriers still develop severe toxicity which can't be predicted by *DPYD* genetic testing. Additionally, due to the high prevalence of severe toxicity in *DPYD* variant carriers it was expected that the PPV would be relatively high. In our cohort the relative prevalence of toxicity is significantly lower compared to the prevalence of toxicity in *DPYD* variant carriers and therefore a lower PPV was expected when using our model. These results indicate that our model could be complementary to *DPYD* genotyping and could further reduce the risk of severe toxicity in patients treated with fluoropyrimidines without a large risk of sub optimal treatment.

Ideally, this model would be used in a multi-parametric approach as shown in Figure 5. Such a two-step decision tool could be used in patients who are first screened for *DPYD* variant alleles associated with severe fluoropyrimidine-related toxicity. Subsequently, if

none of the four *DPYD* variants are present our nomogram could be used to predict the probability of developing severe fluoropyrimidine-related toxicity. If, for example, the probability of severe toxicity exceeds 40% a dose reduction could be applied. As the optimal threshold has not been determined yet, up titration of individualised doses based on toxicity is recommended to ensure an adequate and safe dose for all patients.

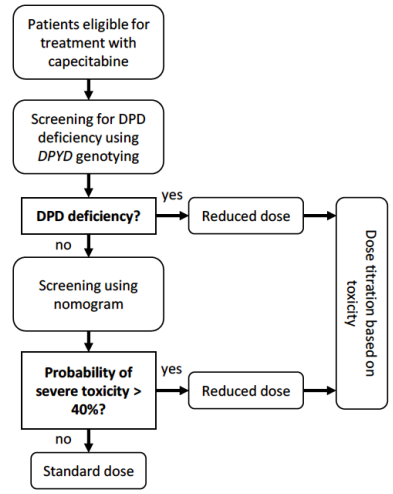

Figure 5: Example of possible approaches for dose-individualisation in patients treated with capecitabine-based treatment regimens using a two-step dosing strategy including *DPYD*-guided dosing and our multi-parametric nomogram.

A possible useful additional variable could be pre-treatment uracil levels as it has been shown to be associated with an increased risk of severe fluoropyrimidine-related toxicity.[25] However, due to critical pre-analytical factors, it is currently not yet deemed suitable to include uracil in the nomogram. It is therefore possible that our predictive accuracy may increase when using uracil levels as a predictor in our model. However, uracil is a complex biomarker influenced by multiple factors including food intake, circadian rhythm, and instability at room temperature after blood sampling.[26-28] By including uracil, our model would become more complex and difficult to use in clinical practice.

One of the main limitations of our nomogram is that it is only applicable to patients treated with capecitabine-based treatment regimens, as creatinine levels were missing for all patients treated with 5-FU in Deenen *et al.*[8] Moreover, even within the subgroup receiving capecitabine-based treatment regimens, serum creatinine levels were only available for patients from two participating hospitals in Deenen *et al.*[8] Lack of auxiliary data hampered the use of multiple imputation techniques to deal with missing creatinine for the remaining patients.[8] Due to exclusion of these patients for the complete-case analysis, selection bias may have been introduced, though there were no indications in our dataset that missing creatinine data was related to patient condition or particular patient characteristics. Furthermore, it could be questioned whether this model is best suited for specifically predicting capecitabine-related toxicity as multi-drug regimens are included in the model. A model specifically aimed towards capecitabine could be

considered to isolate the toxicity as being described to capecitabine. When applying the model only on capecitabine monotherapy patients (N=449) of whom 83 experienced severe toxicity the concordance-index was 0.58 (95% CI 0.51 - 0.65). Alternatively, a novel algorithm based on capecitabine patients only could be developed. However, in clinical practice patients are often treated with multi-drug regimens and therefore such a model may be of limited value. The multiple combination regimens, as shown in our study and collected from real world underscore this heterogeneity.

Conclusion

We developed a simple nomogram using easily measured or obtainable variables that can predict severe toxicity and may be useful in improving the safety of capecitabine-based treatment regimens in patients without the four known *DPYD* risk variant alleles. This nomogram requires further validation through external and prospective validation to ensure adequate prediction of toxicity. Nonetheless, our nomogram is a simple and easy tool for physicians to estimate the risk of severe capecitabine-related toxicity and to further personalise capecitabine treatment to reduce severe toxicity.

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5 Genetic markers of toxicity from capecitabine and other 5-fluorouracil- based regimens : investigation in the QUASAR2 study,systematic review and meta-analysis Rosmarin,*et al* METHODS Supplementary methods Item 1. FU metabolism pathways Item 2. Colle..

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Supplemental - Chapter 5

Supplementary Results:

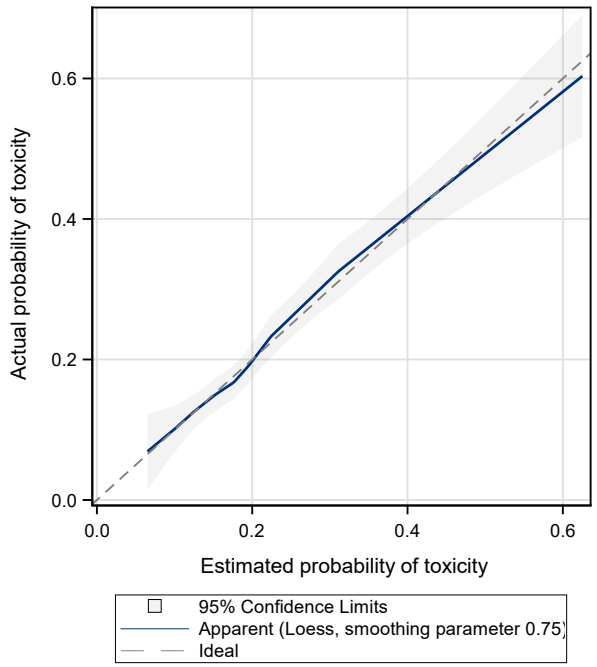

Figure 1: Calibration plot for probability of severe capecitabine-related toxicity. *Abbreviation*: Loess = locally estimated scatterplot smoothing

Table 1: Treatment regimens

Treatment regimen	Included regimens
Cap + Monotherapy	Capecitabine
Cap + Platinum	Capecitabine + Cisplatin Capecitabine + Cisplatin + Trastuzumab Capecitabine + Oxaliplatin Capecitabine + Oxaliplatin + Monoclonal Antibody (Bevacizumab, Trastuzumab or Panitimumab)
Cap + Radiotherapy	Capecitabine + Radiotherapy Capecitabine + Radiotherapy + Mitomycin
Cap + Taxane	Capecitabine + Docetaxel Capecitabine + Paclitaxel
Cap + Triplet	Capecitabine + Docetaxel + Oxaliplatin Capecitabine + Cisplatin + Epirubicin Capecitabine + Oxaliplatin + Epirubicin Capecitabine + Doxorubicin + Cyclophosphamide
Cap + Other	Capecitabine + Irinotecan Capecitabine + Monoclonal antibodies (Bevacizumab, Trastuzumab or Panintimumab) Capecitabine + Temozolomide Capecitabine + Vinorelbin

Construction of the nomogram:

Effect sizes (ln(OR)) for the different covariates in the model are ranked (in absolute value) for building of the nomogram. For sex, the absolute value is ln(1)-ln(0.68)=0.38, while for type of regimen this is ln(5.95)-ln(0.61)=2.28. For continuous predictors such as creatinine level, the range of available values (34 to 354 µmol/L) is taken into account, to obtain an absolute value of (354-34)*ln(1.05)/10=1.56 (dividing ln(1.05) by 10, since in Table 2 creatinine is input per 10 µmol/L for ease of interpretation). The highest effect size of all corresponds to treatment regimen capecitabine - taxane, which is converted into 100 points. For capecitabine - platinum, the number of points is computed as ln(1.32)-ln(0.61)=0.77 and divided by the highest effect size 2.28 described above, resulting in 0.34, and thus 34 points. For the remaining variables we proceed similarly.

Predicted probabilities can be calculated from the coefficients (ln(OR)) in the model following:

$$\frac{\exp\left(-2.4994-0.3808*X1+0.0158*X2-0.1377*X3+0.0051*X4+1.4515*X5+0.9047*X6+0.2795*X7-0.4934*X8+1.7840*X9\right)}{1+\exp\left(-2.4994-0.3808*X1+0.0158*X2-0.1377*X3+0.0051*X4+1.4515*X5+0.9047*X6+0.2795*X7-0.4934*X8+1.7840*X9\right)},$$

where:

- X1= sex (=1 if male, =0 if female),
- X2= age in years
- X3= BSA
- X4= creatinine (µmol/L)
- X5= treatment capecitabine - triplet (=1 if capecitabine - triplet, =0 otherwise)
- X6= treatment capecitabine - other (=1 if capecitabine - other, =0 otherwise)
- X7= treatment capecitabine - platinum (=1 if capecitabine - platinum, =0 otherwise)
- X8= treatment capecitabine - radiotherapy (=1 if capecitabine - radiotherapy, =0 otherwise)
- X9= treatment capecitabine - taxane (=1 if capecitabine - taxane, =0 otherwise)

Part II – Clinical outcomes of fluoropyrimidine-based chemotherapy

Chapter 6

Survival of cancer patients with *DPYD* variant alleles and dose-individualized fluoropyrimidine therapy - A matched-pair analysis

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Abstract

Purpose: *DPYD*-guided fluoropyrimidine dosing improves patient safety in carriers of *DPYD* variant alleles. However, the impact on treatment outcome in these patients is largely unknown. Therefore, progression-free survival (PFS) and overall survival (OS) were compared between *DPYD* variant carriers treated with a reduced dose and *DPYD* wild-type controls receiving a full fluoropyrimidine dose in a retrospective matched-pair survival analysis.

Methods: Data from a prospective multicenter study (NCT02324452) in which *DPYD* variant carriers received a 25% (c.1236G>A and c.2846A>T) or 50% (*DPYD**2A and c.1679T>G) reduced dose and data from *DPYD* variant carriers treated with a similarly reduced dose of fluoropyrimidines identified during routine clinical care, was obtained. Each *DPYD* variant carrier was matched to three *DPYD* wild-type controls treated with a standard dose. Survival analyses were performed using Kaplan-Meier estimates and Cox regression.

Results: In total, 156 *DPYD* variant carriers and 775 *DPYD* wild-type controls were available for analysis. Sixty-one c.1236G>A, 25 *DPYD**2A, 13 c.2846A>T and -when pooled- 93 *DPYD* variant carriers could each be matched to three unique *DPYD* wild-type controls. For pooled *DPYD* variant carriers PFS (HR, 1.23; 95% CI 1.00-1.51, P=.053) and OS (HR, 0.95; 95% CI 0.75-1.51, P=.698) were not negatively impacted by *DPYD*-guided dose-individualization. In the subgroup analyses, a shorter PFS (HR, 1.43; 95% CI 1.10 to 1.86, P=.007) was found in c.1236G>A variant carriers, whereas no differences were found for *DPYD**2A and c.2846A>T carriers.

Conclusion: In this exploratory analysis *DPYD*-guided fluoropyrimidine dosing does not negatively impact PFS and OS in pooled *DPYD* variant carriers. Close monitoring with early dose modifications based on toxicity is recommended, especially for c.1236G>A carriers receiving a reduced starting dose.

Introduction

Since the introduction of 5-fluorouracil (5-FU) and more recently capecitabine (oral prodrug of 5-FU), fluoropyrimidine-based chemotherapy has become a cornerstone in the treatment of many solid tumors.[1] Dihydropyrimidine dehydrogenase (DPD) plays a key role in fluoropyrimidine-related toxicity.[2,3] DPD deficiency leads to decreased catabolism of 5-FU, and consequently, to a shift towards its active metabolites.[4,5] Single nucleotide variants in the *DPYD* gene (*DPYD**2A, c.2846A>T, c.1236G>A, and c.1679T>G), encoding for DPD, are a dominant cause of decreased DPD enzyme activity, thereby increasing the exposure to fluoropyrimidines and the risk of developing severe fluoropyrimidine-related toxicity including diarrhea, mucositis, nausea, vomiting, and hand-foot syndrome.[6-10] In a large prospective clinical trial (the Alpe-DPD study, NCT02324452) we showed that by reducing the starting fluoropyrimidine dose to 50% for heterozygous *DPYD**2A carriers, these patients could be safely treated.[10] However, the application of a 25% dose reduction in heterozygous c.2846A>T and c.1236G>A carriers, was not accompanied by a significant decrease in severe toxicity. Consequently, the need for a larger dose reduction with toxicity-guided dose titration in heterozygous carriers of c.2846A>T and c.1236G>A was considered and is currently recommended by the Clinical Pharmacogenetics Implementation Consortium (CPIC).[11,12] In another study, we showed that progression-free survival (PFS) and overall survival (OS) were not negatively impacted by an initial dose reduction of 50% in 37 *DPYD**2A carriers.[13] Nonetheless, the impact of a reduced fluoropyrimidine dose on the survival in a larger cohort of *DPYD* variant carriers, consisting of c.2846A>T, c.1236G>A, and c.1679T>G carriers as well, has yet not been reported. A traditional approach using a randomized clinical trial comparing survival in *DPYD* variant carriers treated with a full dose would be unethical and unfeasible due to the known increased risk of severe fluoropyrimidine-related toxicity, as was recently also addressed by both Hertz and Baker *et al.*[6,14,15] Therefore, we compared the effectiveness of fluoropyrimidine treatment after dose reduction in patients carrying a *DPYD**2A, c.2846A>T, c.1236G>A or c.1679T>G variant to *DPYD* wild-type patients treated with a full dose using a matched-pair analysis.

Patients and methods

Study design and participants

The design, study population, and results of the Alpe-DPD (NCT02324452) study have previously been published.[10] Briefly, patients treated with fluoropyrimidines were included. Heterozygous *DPYD* variant carriers received an initial dose reduction of 25% (c.2846A>T and c.1236G>A) or 50% (*DPYD**2A and c.1679T>G). *DPYD* wild-type controls were treated with the full dose. A total of 1,103 evaluable patients were enrolled between April 2015 and December 2017, of whom 85 were heterozygous carriers of one of the abovementioned four *DPYD* variants. The Alpe-DPD study was approved by the medical ethical committees of each participating hospital and all patients provided informed consent before enrolment in the study. This included use of collected data for future studies. The present retrospective analysis investigates the effect of the reduced fluoropyrimidine dose on treatment efficacy in *DPYD* variant carriers (N=82) treated in 14 of the 17 hospitals (N=1,019) from the Alpe-DPD study, enriched with all *DPYD* variant

carriers (N=143) who were treated according a similar protocol with the same dose reductions as part of routine clinical care between February 2013 and March 2020 in the Netherlands Cancer Institute (Amsterdam, The Netherlands). Data regarding disease progression, survival, and treatment (follow-up until February 2021) were either used from the Alpe-DPD study or collected from electronic medical records. Data regarding toxicity was only available from patients included in the Alpe-DPD study.[10] This study was approved by the institutional review board or ethics committee at each hospital and was conducted following the principles of the Declaration of Helsinki and Good Clinical Practice. No additional informed consent was needed as patients from the Alpe-DPD study had already consented to use of their data for future studies and data from the patients from routine clinical care was anonymized.

Matching

Due to the large degree of heterogeneity among carriers, specifically in terms of treatment received and primary tumor type, a matched-pair analysis was chosen over a multivariable regression model accounting for these and other characteristics. In each matched group (pooled *DPYD* variant carriers and the individual *DPYD**2A, c.2846A>T and c.1236G>A carriers) each *DPYD* variant carrier was matched to three unique *DPYD* wild-type controls from the Alpe-DPD study.[10] Patients were matched based on available characteristics that we considered relevant for treatment outcome, i.e.: gender, age (±10 years), primary tumor type (colorectal, breast, gastric, other), stage of cancer (local, locally advanced, or metastatic), and treatment regimen (Supplementary Table 1). Patients with missing data regarding disease progression or death were excluded before matching. Exact matching without replacement was performed per matching group, using R-package "MatchIt" version 4.3.0.[16] Therefore, wild-type controls were only used once per matched group.

Statistical analysis

PFS was defined as the time between initiation of treatment and first signs of disease progression by either clinical signs or radiological imaging, or death from any cause, whichever came first. OS was defined as the time between initiation of treatment and death from any cause. Patients not experiencing disease progression or death before the end of follow-up were censored at the last date known to be alive. Standardized differences were used to examine the balance in baseline covariates between carriers and wild-type controls in the matched and unmatched samples. PFS and OS curves were generated using the Kaplan-Meier method. A (stratified) log-rank test was used to compare survival between *DPYD* variant carriers and wild-type controls. Univariable Cox regression analyses were performed to test the association between *DPYD* status and PFS and OS. Hazard ratios (HR) and their corresponding 95% confidence intervals (CI) were obtained. To account for the matched nature of the data Cox regression with standard errors calculated using the jackknife sandwich estimator (JSE) was used as the primary method and Cox regression stratified for matched groups as the secondary method [17-19]. The first approach results in an estimated HR equivalent to the one obtained by a conventional Cox regression, but with a robust variance estimator accounting for clustering within matched groups. The stratified Cox regression is an approach that conditions the matched groups and assumes a common HR, but different baseline hazards, which might not be realistic in many situations and therefore used as the secondary approach. Both approaches could be seen as complementary alternatives.[19]

Exploratory analysis was performed for all variants pooled and for the individual genetic subgroups *DPYD**2A, c.2846A>T, and c.1236G>A. In addition, multivariable Cox regression analyses were performed for all available *DPYD* variant carriers and wild-type controls (before matching), adjusted for matching variables. Schoenfeld residuals were used to verify the proportional hazards assumption. Median follow-up was calculated using the reverse Kaplan-Meier method. Given the small numbers of the variant carrier subgroups and the exploratory nature of these analyses, no multiplicity adjustments were performed. All statistical analyses were performed using R v3.6.3[20], and P values < .05 were considered statistically significant.

Results

In total, 1,162 patients were available for inclusion. Due to missing data regarding disease progression or death, 231 patients were excluded, resulting in a total number of 931 patients. Survival data from 72 *DPYD* variant carriers (13 *DPYD**2A, 14 c.2846A>T, and 45 c.1236G>A) included from the Alpe-DPD study[10] and 84 *DPYD* variant carriers (31 *DPYD**2A, 7 c.2846A>T, and 46 c.1236G>A) identified during routine clinical care in the Netherlands Cancer Institute and treated with a reduced dose of fluoropyrimidines were available for analysis. No survival data was available of the single c.1679T>G variant allele carrier, inhibiting further analysis of this variant. Survival data from 775 *DPYD* wild-type controls treated with a full dose identified during the Alpe-DPD study were available for matching. The characteristics of the matched groups are listed in Table 1.

In total, 156 *DPYD* variant carriers were available for matching. When pooled, 93 *DPYD* variant carriers (25 *DPYD**2A, 13 c.2846A>T, and 55 c.1236G>A) could be matched to three unique *DPYD* wild-type controls. These 93 *DPYD* variant carriers consisted of 52 carriers from the Alpe-DPD study and 41 from routine clinical care. When matched according to *DPYD* variant allele 25 *DPYD**2A, 13 c.2846A>T, and 61 c.1236G>A, carriers could be matched to three unique *DPYD* wild-type controls (Figure 1). Standardized mean differences were all within 0.1 for the matching variables between carriers and non-carriers, indicating a good balance in baseline covariates. Median follow-up time, and outcomes of PFS and OS are shown in Table 2. The Kaplan-Meier-estimated PFS and OS distributions for the matched groups are shown in Figure 2

Cox regression analysis using the JSE showed no statistically significant difference in PFS (Table 3) for the 93 pooled *DPYD* variant carriers compared to their matched wild-type controls (HR, 1.23; 95% CI 1.00 - 1.51, P=.053), but significantly shorter PFS in the subgroup of 61 c.1236G>A variant carriers (HR, 1.43; 95% CI 1.10 - 1.86, P=.007). No statistically significant difference in PFS was found between 25 *DPYD**2A and 13 c.2846A>T variant carriers and matched wild-type controls (Table 3). Alternatively, Cox regression analyses stratified for matched groups were also performed, finding significantly shorter PFS among the pooled *DPYD* group and the c.1236G>A variant carriers For the subgroup of c.2846A>T carriers a non-significant shorter PFS with HR of 2.48 was found (Supplementary Table 2). Though not significant, the multivariable Cox regression indicated a slightly attenuated difference for PFS for the pooled *DPYD* variants (HR, 1.18; 95% CI 0.95 - 1.46, P=.135), more pronounced for the c.1236G>A variant and no shorter PFS in the subgroup of c.2846A>T carriers. (Supplementary Table 3).

Table 1: Patient characteristics of matched groups

	DPYD Wild-type (N=279)	*DPYD* variant (N=93)	*DPYD* Wild-type (N=75)	*DPYD**2A (N=25)	*DPYD* Wild-type (N=39)	c.2846A>T (N=13)	*DPYD* Wild-type (N=183)	c.1236G>A (N=61)
Sex, No. (%)								
Male	144 (51.6)	48 (51.6)	36 (48.0)	12 (48.0)	24 (61.5)	8 (61.5)	96 (52.5)	32 (52.5)
Female	135 (48.4)	45 (48.4)	39 (52.0)	13 (52.0)	15 (38.5)	5 (38.5)	87 (47.5)	29 (47.5)
Age, Median, (IQR)	65 (57-72)	63 (55-71)	65 (58-71)	64 (57-69)	69 (54-71)	69 (53-72)	63 (54-71)	63 (53-71)
Stage of cancer, No. (%)								
Local	33 (11.8)	11 (11.8)	6 (8.0)	2 (8.0)	6 (15.4)	2 (15.4)	27 (14.8)	9 (14.8)
Locally advanced	102 (36.6)	34 (36.6)	30 (40.0)	10 (40.0)	15 (38.5)	5 (38.5)	57 (31.1)	19 (31.1)
Metastatic	144 (51.6)	48 (51.6)	39 (52.0)	13 (52.0)	18 (46.2)	6 (46.2)	99 (54.1)	33 (54.1)
Primary tumor type, No. (%)								
Colorectal	186 (66.6)	62 (66.6)	45 (60.0)	15 (60.0)	24 (61.5)	8 (61.5)	117 (63.9)	39 (63.9)
Breast	60 (21.5)	20 (21.5)	21 (28.0)	7 (28.0)	9 (23.1)	3 (23.1)	36 (19.7)	12 (19.7)
Gastric	3 (1.1)	1 (1.1)	3 (4.0)	1 (4.0)				
Other*	30 (10.8)	10 (10.8)	6 (8.0)	2 (8.0)	6 (15.4)	2 (15.4)	30 (16.4)	10 (16.4)
Treatment regimen†, No. (%)								
CAP / 5-FU monotherapy	72 (25.8)	24 (25.8)	21 (28.0)	7 (28.0)	12 (30.8)	4 (30.8)	45 (24.6)	15 (24.6)
CAP / 5-FU other	9 (3.2)	3 (3.2)	3 (4.0)	1 (4.0)	-	-	6 (3.3)	2 (3.3)
CAPOX regimens	108 (38.7)	36 (38.7)	27 (36.0)	9 (36.0)	15 (38.5)	5 (38.5)	72 (39.3)	24 (39.3)
Chemoradiotherapy	90 (32.3)	30 (32.3)	24 (32.0)	8 (32.0)	12 (30.8)	4 (30.8)	60 (32.8)	20 (32.8)
Number of treatment cycles, No. (%)								
Median (IQR)	3 (1 - 8)	3 (1 - 8)	3 (1 - 8)	4 (1- 8)	4 (1 - 8)	2 (1 - 5)	3 (1 - 8)	3 (1 - 8)
1	91 (32.6)	29 (25.8)	25 (33.3)	7 (28.0)	13 (33.3)	5 (38.5)	60 (32.8)	19 (31.1)
2	25 (9.0)	12 (12.9)	7 (9.3)	4 (16.0)	3 (7.7)	2 (15.4)	17 (9.3)	6 (9.8)
3	29 (10.4)	9 (9.7)	8 (10.7)	1 (4.0)	3 (7.7)	1 (7.7)	17 (9.3)	8 (13.1)
4	20 (7.2)	6 (6.5)	3 (4.0)	2 (8.0)	5 (12.8)	1 (7.7)	14 (7.7)	4 (6.6)
≥5	114 (40.9)	37 (39.8)	32 (42.7)	11 (44.0)	15 (38.5)	4 (30.7)	75 (41.0)	24 (39.3)
Dose-intensity (%), Median, (IQR)								
First cycle	100 (95.8-100.3)	73.4 (55.9-75.0)	100 (94.5-100.0)	49.4 (47.2-55.9)	100 (94.4-100.0)	72.5 (70.6-77.0)	100 (95.3-101.3)	75.0 (72.5-75.8)
All cycles	98.8 (88.4-100.0)	71.4 (56.5-75.0)	97.1 (87.9-100.0)	50.7 (47.2-56.5)	98.5 (86.8-100.0)	72.5 (62.9-75.0)	98.8 (89.1-100.0)	73.9 (68.4-75.6)
Dose-modification after initial dose, No. (%)								
No modification	201 (72.0)	58 (62.4)	56 (74.7)	15 (60.0)	30 (76.9)	6 (46.2)	135 (73.8)	41 (67.2)
Escalation	11 (3.9)	16 (17.2)	4 (5.3)	6 (24.0)	0	3 (23.1)	5 (2.7)	7 (11.5)
Reduction	67 (24.1)	19 (20.4)	15 (20.0)	4 (16.0)	9 (23.1)	4 (30.8)	43 (23.5)	13 (21.3)

Abbreviations: CAP / 5-FU - monotherapy, capecitabine or 5-fluorouracil monotherapy; CAP / 5-FU + other, capecitabine or 5-FU in combination with other anticancer drugs; CAPOX regimens, capecitabine and oxaliplatin-based regimens; IQR, Interquartile range. *Other tumor types included head and neck cancer, oesophagogastric cancer, anal cancer, vulva carcinoma, urethral cancer, and several rare tumor types. †CAP / 5-FU other includes combinations of capecitabine or 5-FU with cisplatin, carboplatin, docetaxel, irinotecan, vinorelbine, temozolomizde, streptozocin, or monoclonal antibodies (bevacizumab, panitimumab, and trastuzumab); Capecitabine and oxaliplatin-based regimens in combination with bevacizumab, panitimumab, or trastuzumab; Chemoradiotherapy regimens in combination with mitomycin, cisplatin, or oxaliplatin.

Table 2: Progression-free survival and overall survival in pooled and subgroup *DPYD* variant carriers matched to *DPYD* wild-type patients.

Matched groups	N	Median follow-up time	95% CI	PFS events No. (%)	Median PFS	95% CI	5-year PFS	95% CI	Deceased No. (%)	Median OS	95% CI	5-year OS	95% CI
		Months			Months		%			Months		%	
DPYD variant carriers	93	56.9	50.6 - 61.3	66 (71.0)	12.4	9.1 - 18.7	28.7	20.6 - 39.9	50 (53.8)	38.6	26.0 - NE	40.3	30.6 - 53.0
Matched wild-type	279	60.7	59.7 - 62.6	177 (63.4)	15.9	12.7 - 24.9	37.1	31.8 - 43.2	164 (58.8)	35.5	26.7 - 53.1	40.9	35.4 - 47.3
*DPYD**2A	25	54.9	29.7 - 66.0	16 (64.0)	17.3	10.2 - NE	37.7	22.2 - 63.9	9 (36.0)	NE	24.2 - NE	59.5	41.9 - 84.5
Matched wild-type	75	63.2	61.2 - 66.1	47 (62.7)	17.5	10.0 - NE	37.3	27.8 - 50.0	43 (57.3)	38.4	20.6 - NE	43.4	33.4 - 56.4
c.2846A>T	13	58.1	39.7 - NE	8 (61.5)	21.5	2.5 - NE	38.5	19.3 - 76.5	6 (46.2)	NE	12.6 - NE	52.7	31.2 - 89.2
Matched wild-type	39	61.7	59.1 - 65.4	21 (53.8)	25.6	13.2 - NE	48.7	35.3 - 67.2	19 (48.7)	65.2	20.5 - NE	53.9	40.3 - 72.0
c.1236G>A	61	56.9	49.0 - 63.1	48 (78.7)	9.1	7.2 - 17.1	20.2	12.0 - 33.8	40 (65.6)	27.0	22.2 - 48.0	26.9	16.8 - 43.1
Matched wild-type	183	60.7	59.4 - 63.7	123 (67.2)	13.8	10.9 - 21.6	33.5	27.2 - 41.2	114 (62.3)	30.3	21.4 - 48.0	38.0	31.3 - 46.1

Abbreviations: CI, Confidence interval; NE, Not estimable; No., Number; OS, Overall survival; PFS, progression-free survival.

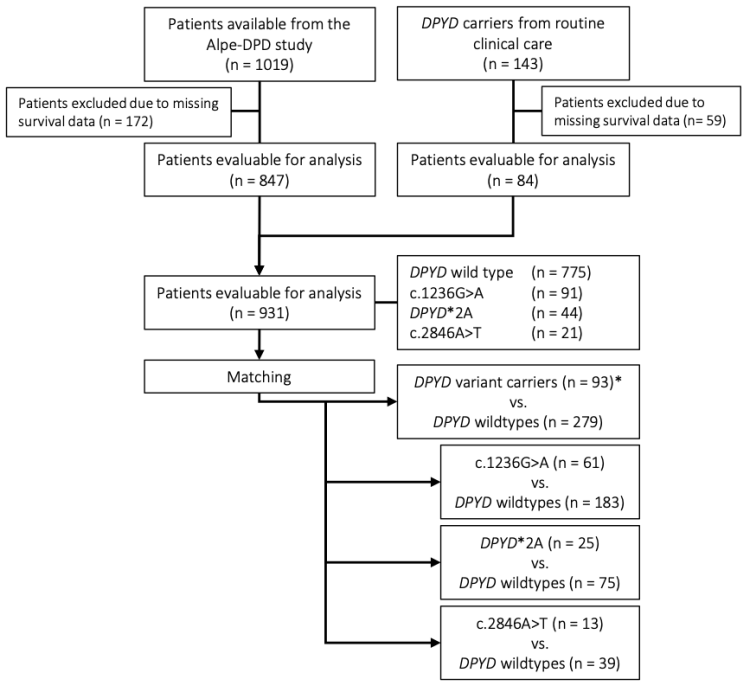


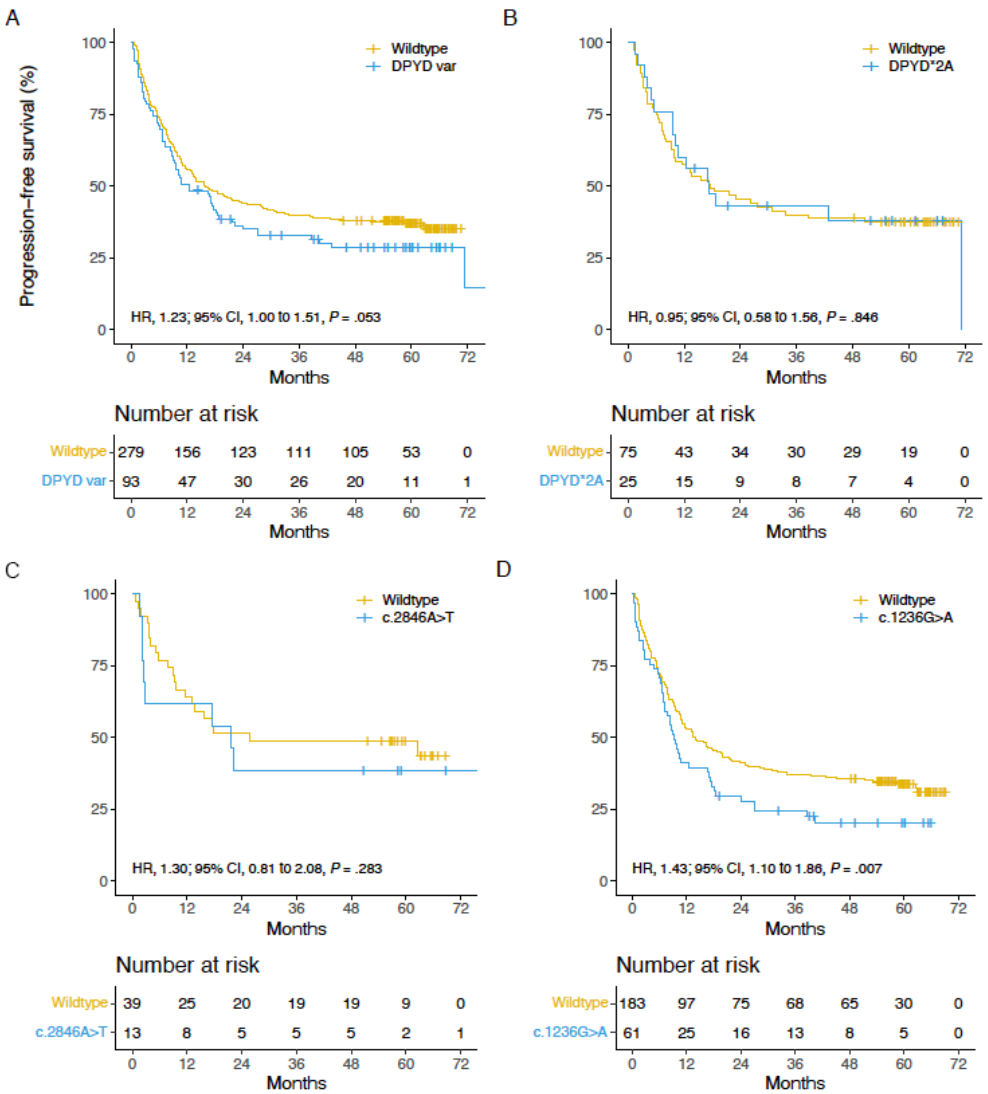
Figure 1: Flow diagram of patient inclusion and matching. *Unique controls were used per matched group, therefore, not all *DPYD* variant carriers could be matched and the total of included *DPYD* variants included in the pooled group (c.1236G>A (N=55), *DPYD**2A (N=25), and c.2846A>T (N=13)) is not the sum of individually matched *DPYD* variant carriers.

Table 3: Hazard ratios (HR) for progression-free survival calculated by matched-pair Cox regression analysis

DPYD variant allele	N	Cox regression			Cox regression with robust standard errors		
		HR	95% CI	*P* value	HR	95% CI	*P* value
DPYD variant carrier	93	1.23	0.92 - 1.63	.159	1.23	1.00 - 1.51	.053
*DPYD**2A	25	0.95	0.53 - 1.70	.869	0.95	0.58 - 1.56	.846
c.2846A>T	13	1.30	0.57 - 2.93	.535	1.30	0.81 - 2.08	.283
c.1236G>A	61	1.43	1.02 - 2.00	.036*	1.43	1.10 - 1.86	.007*

Robust standard errors (95% CI) were obtained using the jackknife sandwich estimator. Results of stratified Cox regression can be found in supplementary Table 2. (*) Indicates a significant difference with a P value of < .05. *Abbreviations*: CI, Confidence interval; HR, Hazard ratio.

Cox regression analysis did not show significant differences in OS (Table 4) for the pooled *DPYD*, c.2846A>T and c.1236G>A variant carriers compared to matched wild-type controls, and the corresponding HRs were close to the value of 1. In contrast, 25 *DPYD**2A carriers were found to have longer OS than wild-types (HR, 0.61; 95% CI 0.38 - 0.98, P=.042). Cox regression analysis stratified for matched groups did not show any differences in OS between all matched groups (Supplementary Table 2). The results of the multivariable Cox regression analyses were consistent with those of the primary matched-pair analysis (Supplementary Table 4). All Cox regression analyses did not violate proportional hazard assumptions.



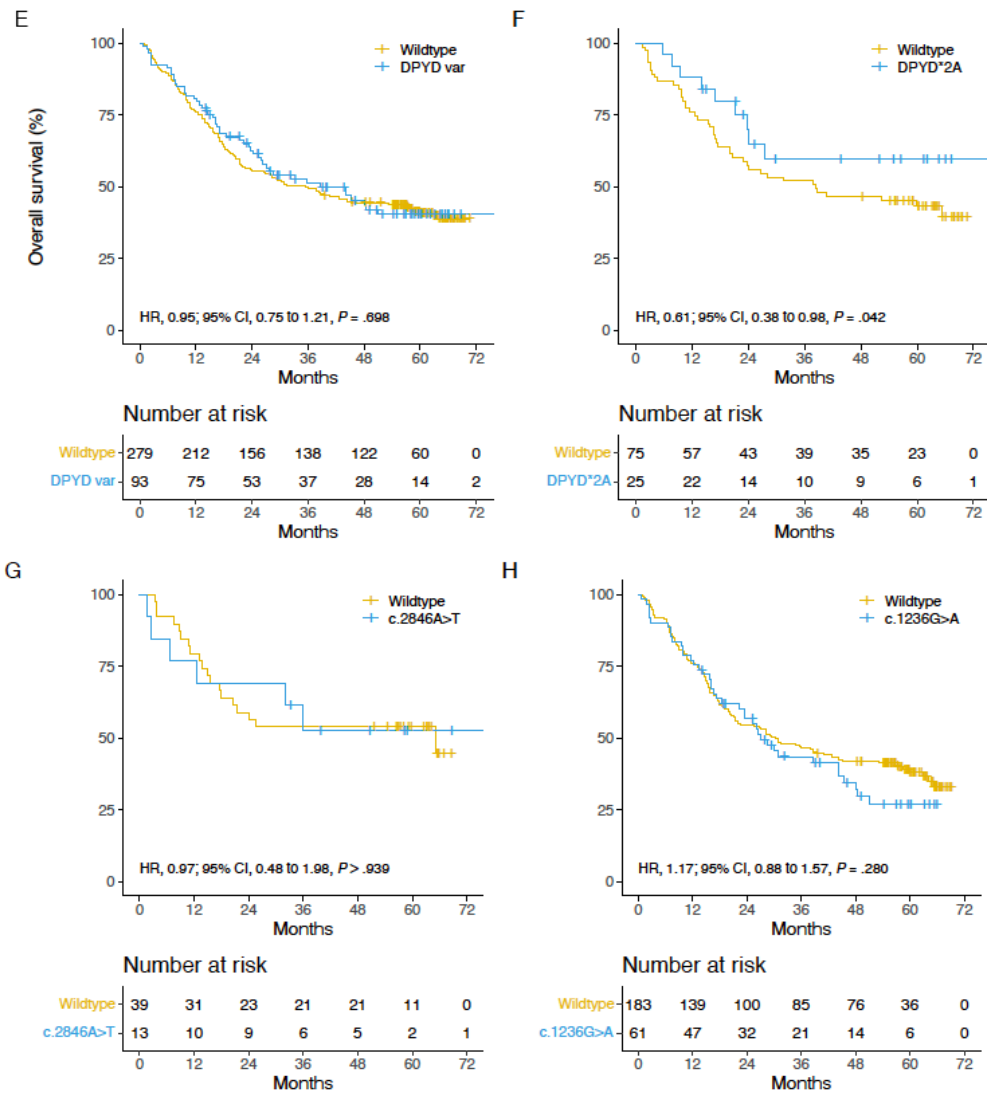


Figure 2: Kaplan-Meier plots and Hazard ratios for progression-free survival (PFS) and overall survival (OS) of pooled *DPYD* variant carriers (A and E), *DPYD**2A (B and F), c.2846A>T (C and G), and c.1236G>A (D and H) carriers. All P values indicated on the Kaplan-Meier curves were adjusted P values corresponding to the Cox regression analysis using the jackknife sandwich estimator. Censoring is indicated by tick marks. Abbreviations: CI, Confidence interval; HR, Hazard ratio

Table 4: Hazard ratios (HR) for overall survival calculated by matched-pair Cox regression analysis

DPYD variant allele	N	Cox regression			Cox regression with robust standard errors		
		HR	95% CI	*P* value	HR	95% CI	*P* value
DPYD variant carrier	93	0.95	0.69 - 1.31	.770	0.95	0.75 - 1.21	.698
*DPYD**2A	25	0.61	0.30 - 1.26	.184	0.61	0.38 - 0.98	.042*
c.2846A>T	13	0.97	0.39 - 2.44	.953	0.97	0.48 - 1.98	.939
c.1236G>A	61	1.17	0.82 - 1.69	.385	1.17	0.88 - 1.57	.280

Robust standard errors (95% CI) were obtained using the jackknife sandwich estimator. Results of stratified Cox regression can be found in supplementary Table 2. (*) Indicates a significant difference with a P value of < .05. *Abbreviations*: CI, Confidence interval; HR, Hazard ratio.

To explore the robustness of the results, sensitivity analyses were performed (Supplementary Tables 5-7). Alternative matching strategies were considered including one or two matched controls when less than three matches could be found and 1:2 matching. Adding the date (year) of start of treatment to the matching variables, allowing for a maximum difference of 2 years between matched patients, was also performed to account for possible secular trends. All alternative matching strategies resulted in similar results as compared to the primary analysis. Toxicity data was only available from patients included in the Alpe-DPD study and showed that severe fluoropyrimidine toxicity was substantially more present in *DPYD* variant carriers, despite dose reductions, compared to the matched *DPYD* wild-types (Supplementary Table 8).

Discussion

The results of our study showed no significant differences in PFS and OS between the pooled *DPYD* variant carriers and matched *DPYD* wild-type patients, suggesting that *DPYD*-guided dose-individualization can likely be performed safely without compromising effectiveness. For PFS, the primary matched-pair analysis using the JSE method was not significantly different, although this was borderline. While the stratified Cox regression indicated that PFS might be negatively impacted by a reduced fluoropyrimidine dose with HRs up to 1.76, the multivariable Cox regression analysis did not show a significant difference in PFS and a lower HR of 1.18. Furthermore, both PFS and OS were not negatively impacted in *DPYD**2A variant allele carriers treated with a reduced dose of 50% compared to matched wild-type patients, in line with previous findings. 13,21 Carriers of c.2846A>T were found to trend towards shorter PFS, although not significant, and results were hampered by low power due to the small sample size. Subgroup analysis revealed a consistently shorter PFS for c.1236G>A carriers. The trend towards shorter PFS accompanied by a borderline *P* value in the pooled *DPYD* variant carriers was therefore probably largely driven by the survival outcomes of c.1236G>A, which made up the majority of pooled *DPYD* variant carriers. For OS, no significant difference was found in c.1236G>A carriers. This discrepancy may be caused by differences in administration of other systemic treatment lines or other treatment modalities following fluoropyrimidine-based treatment between all studied patient

groups. Unfortunately, this information was not available.

Sensitivity analyses performed to explore the robustness of the results confirmed the abovementioned findings. Nonetheless, the incidence of severe fluoropyrimidine-related toxicity was higher in the c.1236G>A carriers (27.3%) compared to matched *DPYD* wild-types (17.2%) in this study (Supplementary Table 8), and are in line with results from the Alpe-DPD study.[10] Notably, no dose modifications after initial dose reduction were applied in 75.8% of the c.1236G>A carriers (Supplementary Table 8), who remained on the 75% starting dose throughout all treatment cycles, whereas in only 6.1% of c.1236G>A carriers dose-escalation was performed. These results suggest that a substantial number of c.1236G>A carriers may benefit from an upward dose titration when treatment is well tolerated, which was recommended in the Alpe-DPD study protocol,[10] but seemingly was applied to a limited extent. It also suggests that DPD enzyme activity is not impacted similarly across all c.1236G>A carriers. This is underscored by a large variation in DPD enzyme activity and exposure to 5-FU in c.1236G>A carriers.[10] Depending on the magnitude of impact on the DPD enzyme activity, this could result in both under- and overexposure. Moreover, previous research has shown that wild-type mRNA for DPD is still detectable in homozygous c.1236G>A carriers, indicating that at least some normal functional DPD can still be formed in these patients.[22,23] These findings, combined with the presented data regarding the treatment outcome, suggest that a dose reduction of 25% may not be beneficial for all c.1236G>A carriers.

A possible strategy to prevent both severe toxicity and potential sub-therapeutic dosing of fluoropyrimidines in c.1236G>A carriers would be individualized early dose-escalation after a reduced dose in the absence of severe toxicity.[3,15,24] Of note, a larger dose-reduction of 50% is currently recommended by the CPIC. This deserves further attention as this could negatively impact PFS in case dose-escalation is not applied when treatment is otherwise tolerated well.[12]

A limitation of this study is the use of matching, irrespective of the method used for adjustment. When carriers are left unmatched, like in our study, the estimation of the effect of mutation status is possibly biased and it is unclear to which population of carriers the results apply. Using less restrictive matching criteria as an alternative, leads to matching of less similar patients and introduces residual confounding. Ideally, matching could be avoided altogether performing instead a multivariable Cox regression analysis on all data. However, as in the current study, when there is a high degree of heterogeneity in variables like tumor type and treatment regimen, the estimation of this model and its interpretation is not straightforward. Nonetheless, multivariable Cox regression was performed and showed similar results (Supplementary Tables 3 and 4). Due to the retrospective design of our study, patient data regarding matching variables were not always complete, which hampered the matching and reduced the number of matches. Ideally, matching would be performed using additional variables that influence treatment outcome such as molecular tumor subtypes. However, this data was not available. Furthermore, due to the choice of 1:3 matching without replacement of patients, it was not possible to match each of the *DPYD* variant carriers to 3 *DPYD* wild-type controls. Hence, additional analyses using alternative matching strategies were also performed to include more *DPYD* variant carriers. Similar results were found using other matching strategies for c.1236G>A (Supplementary Tables

5-7), which further strengthens the assumption that a dose reduction of 25% in c.1236G>A may negatively impact disease progression if dose-titration is not applied when patients experience no or minimal toxicity. Another limitation was the use of an additional category "other" defined for primary tumor type including less prevalent tumor types that otherwise had to be left out of the main analyses due to not finding matches. Similarly, less frequent treatment regimens were also combined into one category to increase the number of matches. Although this could potentially introduce biases, it can reduce incomplete matching. Furthermore, patients with local or locally advanced disease were grouped for the endpoint PFS while disease-free survival (DFS) may be better suited, as the treatment goal in these patients is cure. However, data regarding DFS was not available. A post-hoc power analysis showed that our study was underpowered to completely exclude false negative findings. To detect the currently observed difference in PFS (HR=1.23) between *DPYD* variant carriers and *DPYD* wild-type controls with 80% power, with a 1:3 ratio, nearly twice as many PFS events would have been needed. All limitations must be taken into account, and the findings presented in this study must therefore be interpreted with appropriate caution.

Most limitations could be overcome by performing an adequately powered prospective study using novel trial designs as recently described[14,15] which include similar approaches using *DPYD* wild-type patients treated with a full dose as a comparator and the use of real-world evidence.[14,15] In addition, pharmacokinetic analyses of *DPYD* variant carriers may help to establish the relationship between 5-FU exposure and survival.

In summary, the results of this retrospective exploratory analysis suggest that PFS and OS are not negatively impacted by *DPYD*-guided dose-individualization in the pooled *DPYD* variant carriers and likely do not hamper the effectiveness of fluoropyrimidines as was also previously shown for *DPYD**2A.[13] However, a shorter PFS in c.1236G>A carriers receiving *DPYD*-guided fluoropyrimidine dosing cannot be excluded. Notwithstanding this, it should be considered that 75.8% of c.1236G>A carriers remained on the same dose throughout all treatment cycles, suggesting that a substantial number of c.1236G>A variant carriers could have benefited from upward dose titration when treatment was well-tolerated, as was dictated in the study protocol. Evidence on the impact on PFS for c.2846A>T was highly dependent on matching strategy and was hampered by the small numbers of carriers available and requires further research with a larger sample size. Notably, both c.1236G>A and c.2846A>T carriers still experienced significantly more severe fluoropyrimidine-induced toxicity after a 25% dose reduction compared to wild-type controls treated with a full dose.[11] Apparently, the 25% dose reduction was not sufficient to protect all variant carriers from developing severe toxicity and therefore more research is needed to explain the impact of heterogeneity in DPD enzyme activity in *DPYD* variant allele carriers. Meanwhile, close monitoring with early dose modifications, escalation when possible and reduction when necessary, based on toxicity is recommended when treating c.1236G>A variant carriers with a reduced fluoropyrimidine starting dose.

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Supplemental - Chapter 6

Supplementary tables

Supplementary Table 1: Treatment regimens included in each treatment group.

Group	Included treatment regimens
Cap / 5-FU - monotherapy	Capecitabine monotherapy
	5-Fluorouracil monotherapy
Cap / 5-FU + other	Capecitabine + Docetaxel Capecitabine + Monoclonal antibodies (Trastuzumab, Bevacizumab or Panitimumab) Capecitabine + Carboplatin or Cisplatin Capecitabine + Vinorelbine Capecitabine + Temozolomide
	5-Fluorouracil + Bevacizumab 5-Fluorouracil + Irinotecan 5-Fluorouracil + Iriniotecan + Bevacizumab 5-Fluorouracil + Cisplatin 5-Fluorouracil + Streptozocin
CAPOX regimens	Capecitabine + Oxaliplatin
	Capecitabine + Oxaliplatin + Bevacizumab
	Capecitabine + Oxaliplatin + Trastuzumab Capecitabine + Oxaliplatin + Panitumumab
ECX / EOX	Capecitabine + Cisplatin + epirubicin
	Capecitabine + Oxaliplatin + epirubicin
FEC	5-Fluorouracil + Epirubicin + Cyclophosphamide 5-Fluorouracil + Doxorubicin + Cyclophosphamide
FOLFOX regimens	5-Fluorouracil + Oxaliplatin + Folinic acid
	5-Fluorouracil + Oxaliplatin + Folinic acid + Bevacizumab
	5-Fluorouracil + Oxaliplatin + Irinotecan + Folinic acid
FLOT / DOC	5-Fluorouracil + Oxaliplatin + Docetaxel Capecitabine + Oxaliplatin + Docetaxel
Chemoradiotherapy regimens	Radiotherapy + 5-FU + Mitomycin
	Radiotherapy + Capecitabine
	Radiotherapy + Capecitabine + Mitomycin
	Radiotherapy + Capecitabine + Cisplatin
	Radiotherapy + Capecitabine + Oxaliplatin

Supplementary Table 2: Hazard ratios (HR) from Cox regression model for progression-free survival and overall survival accounting for matching by stratification for matched groups.

***DPYD* variant allele**	**Cox regression**			**Cox regression stratified for matching**		
	Progression-free survival					
	HR	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**
DPYD variant carrier	1.23	0.92 - 1.63	.159	1.76	1.25 - 2.48	.001*
*DPYD**2A	1.04	0.57 - 1.92	.899	0.86	0.42 - 1.76	.679
c.2846A>T	1.30	0.57 - 2.93	.535	2.48	0.92 - 6.67	.073
c.1236G>A	1.43	1.02 - 2.00	.036*	2.03	1.34 - 3.05	<.001*
	Overall survival					
DPYD variant carrier	0.95	0.69 - 1.31	.770	0.94	0.65 - 1.35	.736
*DPYD**2A	0.76	0.37 - 1.58	.465	0.47	0.19 - 1.15	.097
c.2846A>T	0.97	0.39 - 2.44	.953	1.40	0.50 - 3.92	.520
c.1236G>A	1.17	0.82 - 1.69	.385	1.16	0.77 - 1.77	.477

Supplementary Table 3: Hazard ratios (HR) from Cox-regression model for progression-free survival calculated by multivariable Cox-regression analysis including the matching variables.

Variable	Pooled *DPYD* variants (N=156)			*DPYD**2A (N=44)			c.2846A>T (N=21)			c.1236G>A (N=91)		
***DPYD*-status**	**HR**	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**
Wild-type (N=775)	-	-	-	-	-	-	-	-	-	-	-	-
DPYD variant	1.18	0.95 - 1.46	.135	0.96	0.64 - 1.44	.859	0.96	0.55 - 1.71	.900	1.57	1.21 - 2.04	< .001*
Gender												
Men	-	-	-	-	-	-	-	-	-	-	-	-
Women	0.93	0.76 - 1.12	.430	0.99	0.83 - 1.18	.894	0.91	0.74 - 1.13	.400	0.97	0.80 - 1.19	.800
Treatment regimen												
Cap / 5-FU monotherapy	-	-	-	-	-	-	-	-	-	-	-	-
Cap / 5-FU other	0.90	0.64 - 1.28	.570	0.99	0.65 - 1.49	.946	1.01	0.69 - 1.47	.965	0.99	0.69 - 1.42	.968
CAPOX regimens	0.62	0.45 - 0.84	.002*	0.89	0.74 - 1.07	.200	0.58	0.42 - 0.80	.001*	0.62	0.45 - 0.85	.003*
ECX / EOX	0.70	0.39 - 1.25	.224	0.93	0.60 - 1.44	.737	0.66	0.35 - 1.23	.190	0.90	0.49 - 1.64	.725
FEC	0.22	0.08 - 0.61	.004*	0.78	0.45 - 1.36	.379	0.23	0.09 - 0.65	.005*	0.22	0.08 - 0.62	.004*
FLOT / DOC	0.74	0.18 - 3.11	.684	0.93	0.17 - 5.10	.930	0.70	0.17 - 2.97	.633	0.86	0.20 - 3.61	.834
FOLFOX regimens	0.88	0.60 - 1.30	.533	0.95	0.66 - 1.38	.806	0.78	0.52 - 1.19	.248	0.95	0.64 - 1.42	.816
Chemoradiotherapy	0.61	0.44 - 0.86	.004*	0.91	0.75 - 1.10	.322	0.61	0.42 - 0.87	.007*	0.64	0.45 - 0.91	.012*
Age	1.00	0.99 - 1.01	.740	1.00	0.99 - 1.01	.920	1.00	0.99 - 1.01	.510	1.00	0.99 - 1.01	.713
Stage of cancer												
Local	-	-	-	-	-	-	-	-	-	-	-	-
Locally advanced	1.47	1.07 - 2.01	.018*	1.05	0.88 - 1.26	.574	1.68	1.17 - 2.41	.005*	1.53	1.10 - 2.13	.028*
Metastatic	6.55	4.80 - 8.92	< .001*	1.52	1.25 - 1.84	< .001*	7.76	5.39 - 11.2	< .001*	6.97	5.00 - 9.70	< .001*
Primary tumor type												
Breast	-	-	-	-	-	-	-	-	-	-	-	-
Colorectal	0.67	0.47 - 0.94	.020*	0.90	0.74 - 1.08	.256	0.69	0.48 - 1.00	.052	0.67	0.47 - 0.96	.028*
Gastric	1.62	0.94 - 2.80	.0083*	1.07	0.72 - 1.57	.747	1.71	0.94 - 3.09	.078	1.55	0.88 - 2.72	.013*
Ovarian	17.1	3.89 - 75.2	< .001*	0.00	0.00 - inf	.998						
Pancreatic	1.57	0.99 - 2.48	.054	1.29	0.78 - 2.13	.321	1.96	1.17 - 3.29	.011*	1.63	1.01 - 2.63	.047*
Other	1.01	0.68 - 1.48	.971	0.97	0.75 - 1.26	.834	1.09	0.71 - 1.67	.680	0.89	0.59 - 1.34	.577

Supplementary Table 4: Hazard ratios (HR) from Cox-regression model for overall survival calculated by multivariable Cox-regression analysis including the matching variables.

Variable	Pooled *DPYD* variants (N=156)			*DPYD**2A (N=44)			c.2846A>T (N=21)			c.1236G>A (N=91)		
***DPYD*-status**	**HR**	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**	**HR**	**95% CI**	***P* value**
Wild-type (N=775)	-	-	-	-	-	-	-	-	-	-	-	-
DPYD variant	0.95	0.74 - 1.22	.652	0.68	0.42 - 1.10	.119	0.66	0.35 - 1.26	.207	1.25	0.94 - 1.68	.128
Gender												
Men	-	-	-	-	-	-	-	-	-	-	-	-
Women	0.98	0.79 - 1.20	.825	0.95	0.76 - 1.19	.683	0.93	0.74 - 1.17	.540	1.01	0.82 - 1.25	.916
Treatment regimen												
Cap / 5-FU monotherapy	-	-	-	-	-	-	-	-	-	-	-	-
Cap / 5-FU other	0.71	0.49 - 1.03	.071	0.69	0.47 - 1.02	.065	0.70	0.46 - 1.05	.082	0.70	0.47 - 1.03	.067
CAPOX regimens	0.55	0.40 - 0.76	< .001*	0.50	0.36 - 0.70	< .001*	0.51	0.36 - 0.72	< .001*	0.55	0.39 - 0.77	< .001*
ECX / EOX	1.00	0.55 - 1.82	.996	1.17	0.63 - 2.17	.628	0.86	0.45 - 1.64	.653	1.21	0.65 - 2.24	.550
FEC	0.28	0.09 - 0.90	.032*	0.29	0.09 - 0.94	.039*	0.30	0.09 - 0.96	.042*	0.30	0.09 - 0.96	.043*
FLOT / DOC	1.32	0.31 - 5.59	.706	1.28	0.30 - 5.43	.740	1.15	0.27 - 4.91	.846	1.40	0.33 - 5.97	.648
FOLFOX regimens	1.05	0.71 - 1.55	.821	0.99	0.66 - 1.50	.965	0.94	0.62 - 1.42	.764	1.10	0.73 - 1.65	.660
Chemoradiotherapy	0.56	0.39 - 0.80	.001*	0.56	0.39 - 0.82	.003*	0.57	0.39 - 0.84	.004	0.61	0.42 - 0.88	.009*
Age	1.01	1.00 - 1.01	.173	1.01	1.00 - 1.01	.290	1.00	0.99 - 1.01	.465	1.01	1.00 - 1.01	.174
Stage of cancer												
Local	-	-	-	-	-	-	-	-	-	-	-	-
Locally advanced	1.68	1.16 - 2.43	.006*	1.77	1.18 - 2.64	.005*	1.94	1.28 - 2.93	.002	1.78	1.21 - 2.62	.003*
Metastatic	7.33	5.11 - 10.5	< .001*	8.42	5.64 - 12.6	< .001*	8.76	5.79 - 13.3	< .001*	8.20	5.59 - 12.0	<.001*
Primary tumor type												
Breast	-	-	-	-	-	-	-	-	-	-	-	-
Colorectal	0.96	0.67 - 1.37	.821	1.00	0.68 - 1.46	.999	1.00	0.67 - 1.47	.982	1.01	0.70 - 1.48	.841
Gastric	2.35	1.33 - 4.17	.003*	2.46	1.34 - 4.49	.004*	2.73	1.47 - 5.05	.001*	2.69	1.49 - 4.85	.001*
Ovarian	8.77	2.10 - 36.7	.003*	7.80	1.02 - 59.8	.048*						
Pancreatic	2.88	1.79 - 4.65	< .001*	3.15	1.91 - 5.20	< .001*	3.24	1.93 - 5.43	.001*	3.12	1.90 - 5.14	.001*
Other	1.71	1.14 - 2.55	.009*	1.83	1.18 - 2.83	.007*	1.88	1.19 - 2.96	.006*	1.59	1.03 - 2.46	.036*

Supplementary Table 5: Hazard ratios (HR) from Cox regression model for progression-free survival and overall survival by matched-pair Cox regression analysis using matching strategy that allows for one or two matches when less than three matches are available.

DPYD variant allele	N	Cox regression			Cox regression with robust standard errors			Cox regression stratified for matching		
		Progression-free survival						Progression-free survival		
		HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P*
DPYD variant carriers	128	1.22	0.95 - 1.57	.118	1.22	1.01 - 1.47	.034*	1.79	1.31 - 2.45	< .001*
*DPYD**2A	32	0.97	0.56 - 1.66	.900	0.97	0.60 - 1.55	.885	0.95	0.50 - 1.79	.872
c.2846A>T	16	1.23	0.59 - 2.57	.590	1.23	0.79 - 1.91	.370	2.00	0.80 - 5.00	.139
c.1236G>A	78	1.40	1.03 - 1.91	.033*	1.40	1.10 - 1.77	.006*	2.12	1.44 - 3.13	<.001*
		Overall survival						Overall survival		
DPYD variant carriers	128	0.98	0.74 - 1.30	.898	0.98	0.79 - 1.21	.866	0.98	0.71 - 1.37	.911
*DPYD**2A	32	0.66	0.34 - 1.28	.221	0.66	0.41 - 1.06	.086	0.48	0.22 - 1.07	.073
c.2846A>T	16	0.86	0.37 - 2.02	.733	0.86	0.42 - 1.77	.685	1.22	0.47 - 3.18	.677
c.1236G>A	78	1.15	0.82 - 1.61	.411	1.15	0.88 - 1.50	.296	1.19	0.80 - 1.77	.396

Supplementary Table 6: Hazard ratios (HR) from Cox regression model for progression-free survival and overall survival calculated by matched-pair Cox regression analysis using 1:2 matching ratio.

DPYD variant allele	N	Cox regression			Cox regression with robust standard errors			Cox regression stratified for matching		
		Progression-free survival						Progression-free survival		
		HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value
DPYD variant carriers	109	1.17	0.89 - 1.56	.265	1.17	0.95 - 1.46	.144	1.67	1.17 - 2.38	.005*
*DPYD**2A	24	0.96	0.51 - 1.83	.908	0.96	0.58 - 1.61	.885	0.73	0.33 - 1.62	.435
c.2846A>T	17	0.92	0.43 - 1.97	.836	0.92	0.55 - 1.55	.763	1.21	0.48 - 3.03	.690
c.1236G>A	67	1.48	1.05 - 2.10	.026*	1.48	1.15 - 1.92	.003*	2.30	1.46 - 3.63	<.001*
		Overall survival						Overall survival		
DPYD variant carriers	109	0.94	0.69 - 1.28	.693	0.94	0.74 - 1.19	.608	0.84	0.57 - 1.21	.360
*DPYD**2A	24	0.75	0.35 - 1.61	.460	0.75	0.47 - 1.21	.235	0.40	0.13 - 1.22	.110
c.2846A>T	17	0.65	0.27 - 1.55	.331	0.65	0.31 - 1.35	.246	0.81	0.30 - 2.19	.683
c.1236G>A	67	1.18	0.81 - 1.71	.393	1.18	0.88 - 1.57	.269	1.04	0.66 - 1.63	.877

Supplementary Table 7: Hazard ratios (HR) from Cox regression model for progression-free survival and overall survival calculated by matched-pair Cox regression analysis using 1:3 matching ratio and a maximum difference of 2 years between start of treatment.

DPYD variant allele	N	Cox regression			Cox regression with robust standard errors			Cox regression stratified for matching		
		Progression-free survival						Progression-free survival		
		HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value
DPYD variant carriers	73	1.18	0.85 - 1.63	.320	1.18	0.94 - 1.48	.160	1.61	1.09 - 2.36	.016*
*DPYD**2A	14	0.97	0.45 - 2.06	.927	0.97	0.55 - 1.69	.901	0.93	0.40 - 2.17	.859
c.2846A>T	12	1.18	0.50 - 2.80	.704	1.18	0.73 - 1.90	.491	2.13	0.76 - 5.95	.148
c.1236G>A	48	1.28	0.88 - 1.87	.191	1.28	0.96 - 1.72	.095	1.64	1.04 - 2.58	.033*
		Overall survival						Overall survival		
DPYD variant carriers	73	1.00	0.71 - 1.42	.988	1.00	0.78 - 1.29	.983	1.04	0.69 - 1.54	.864
*DPYD**2A	14	0.72	0.29 - 1.76	.468	0.72	0.40 - 1.28	.262	0.60	0.22 - 1.65	.320
c.2846A>T	12	1.03	0.41 - 2.60	.950	1.03	0.50 - 2.11	.936	1.54	0.54 - 4.40	.422
c.1236G>A	48	1.14	0.77 - 1.70	.514	1.14	0.84 - 1.55	.396	1.13	0.71 - 1.79	.614

Supplementary Table 8: Toxicity and dose-modification of matched patients (only including *DPYD* variant carriers and wild-types from the Alpe-DPD study1)

	N	No toxicity	Grade 1 toxicity	Grade 2 toxicity	Grade ≥ 3 toxicity	No dose-modification	Dose escalation	Dose reduction
		No. (%)				No. (%)		
DPYD variant carriers	52	6 (11.5)	12 (23.1)	16 (30.8)	18 (34.6)	37 (71.2)	5 (9.6)	10 (19.2)
Matched wild-types	156	40 (25.6)	29 (18.6)	62 (39.7)	25 (16.0)	118 (75.6)	5 (3.2)	33 (21.2)
*DPYD**2A	9	0	4 (44.4)	3 (33.3)	2 (22.2)	6 (66.7)	2 (22.2)	1 (11.1)
Matched wild-types	27	11 (40.7)	5 (18.5)	8 (29.6)	3 (11.1)	22 (81.5)	2 (7.4)	3 (11.1)
c.2846A>T	9	1 (11.1)	2 (22.2)	0	6 (66.7)	4 (44.4)	1 (11.1)	4 (44.4)
Matched wild-types	27	2 (7.4)	10 (37.0)	10 (37.0)	5 (18.5)	23 (85.2)	0	5 (18.5)
c.1236G>A	33	5 (15.2)	6 (18.2)	13 (39.4)	9 (27.3)	25 (75.8)	2 (6.1)	6 (18.2)
Matched wild-types	99	24 (24.2)	14 (14.1)	44 (44.4)	17 (17.2)	75 (75.8)	3 (3.0)	21 (21.2)

References:

1. Henricks LM, Lunenburg CATC, de Man FM, *et al: DPYD* genotype-guided dose individualisation of fluoropyrimidine therapy in patients with cancer: a prospective safety analysis. Lancet Oncol 19:1459-1467, 2018

Chapter 7

Predictors of severe fluoropyrimidine-induced toxicity in older adults with cancer with *DPYD* wild-type

Submitted

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Abstract

Background: Despite the implementation of *DPYD* genotype-guided dosing, approximately 1 in 3 patients receiving fluoropyrimidine-containing chemotherapy continues to experience severe toxicity. While clinical studies have demonstrated a favorable tolerance among highly selected fit older adults, real-world studies have shown an increased risk of toxicity.

Objective: To identify predictors of severe toxicity or treatment deintensification in older *DPYD* wild-type adults receiving fluoropyrimidine-containing chemotherapy.

Methods: Patients wild-type for four tested *DPYD* variants, aged ≥65 years, who participated in a prospective clinical trial investigating genotype-guided individualized fluoropyrimidine dosing, were eligible for the study. The association between tumor-, treatment-, and patient-related characteristics and the occurrence of severe toxicity (grade ≥3, CTCAE v5.0) was analyzed in univariate and multivariate logistic regression analyses. The same analyses were performed for a composite endpoint of severe toxicity or treatment deintensification (including dose reduction, cycle delay or discontinuation).

Results: A total of 311 patients were included. Median age was 71.2 years and 58.8% were male. Grade ≥3 toxicity occurred in 23.2% of patients. In multivariate analysis, none of the characteristics studied were significantly associated with the occurrence of grade ≥3 toxicity, but a trend towards increased toxicity was observed patients treated with a combination of cytotoxic cancer therapy agents (polychemotherapy), a reduced starting dose, or low BMI. The composite endpoint occurred in 41.2% of patients and was inversely associated with the use of low dose monotherapy in multivariate analysis.

Conclusion: Despite *DPYD* genotype-based dosing, grade ≥3 toxicity and treatment deintensification frequently occur in elderly treated with fluoropyrimidine chemotherapy. The use of polychemotherapy, starting at a reduced dose of polychemotherapy and low BMI appear to show a trend with toxicity and decreased tolerance, but larger studies are needed to confirm this association.

Introduction

For over six decades, fluoropyrimidine-containing chemotherapy has been widely used in various solid tumors, such as breast cancer and gastrointestinal cancers. However, approximately 30% of patients experience severe fluoropyrimidine-associated toxicity, including mucositis, diarrhea, hand-foot syndrome, and bone marrow toxicity.[1-3] Severe fluoropyrimidine-related toxicity may be the result of a deficiency of the main catabolic enzyme dihydropyrimidine dehydrogenase (DPD) which is encoded by the *DPYD* gene and occurs in almost 8% of the general Dutch cancer population[4]. Pre-therapeutic screening of four *DPYD* variant alleles and subsequent dose-individualization reduces the incidence of severe fluoropyrimidine-related toxicity,[4,5] yet still 23% of *DPYD* wild-type patients experience severe toxicity and 17% of patients discontinue fluoropyrimidine treatment due to adverse events.[4]

In clinical trials, the tolerance of fluoropyrimidine-containing chemotherapy among older patients is comparable to that of younger patients.[6-8] However, it is important to note that elderly patients participating in clinical trials are highly selected and generally in good physical condition, which may not accurately represent for the broader population of older patients in daily clinical practice. In real world settings, older adults often experience more toxicity associated with fluoropyrimidines such as severe diarrhea, stomatitis, and infection.[7,9] Furthermore, van Beek *et al.* demonstrated that dose reductions were more frequent among patients ≥70 years, compared to younger patients, which could serve as a surrogate marker for lower grade toxicities with a relevant negative impact on the quality of life.[7]

Given the increased vulnerability of older adults to severe fluoropyrimidine-related toxicity, the ability to predict the risk of toxicity, in this expanding population becomes increasingly important in clinical practice. Over the past decade, 64% of new cancer diagnoses in the Netherlands were observed in patients ≥65 years.[10] Several studies evaluating various chemotherapy types and regimens have demonstrated that comorbidities and polypharmacy are associated with severe chemotherapy-related toxicity.[11,12] Consequently, the identification of predictors for fluoropyrimidine-associated toxicity would facilitate treatment adaptation and enhance individual treatment outcomes for this frequently utilized group of chemotherapeutic agents.

This study aims to identify patient-, treatment-, and tumor- characteristics associated with severe fluoropyrimidine-associated toxicity or treatment deintensification in *DPYD* wild-type patients aged ≥65 years who participated in a prospective trial.

Methods

Study design

This is a retrospective observational cohort study that utilized data derived from a prospective clinical study (NCT02324452) investigating the use of *DPYD*-guided dosing of fluoropyrimidines in cancer patients in 17 hospitals in the Netherlands. A detailed description and primary results of this study have been previously reported.[4] Briefly, cancer patients were prospectively screened for the four most clinically relevant *DPYD*

variant alleles. A predefined dose reduction was applied prior to start of fluoropyrimidine treatment in patients identified as *DPYD* variant allele carriers, and the impact of this dose reduction on toxicity was compared to that observed in *DPYD* wild type cancer patients.[4] For the current study, data from six hospitals (three academic and three non-academic) of the original 17 participating study sites was used.

Study population

DPYD wild-type patients aged 65 years and over were included in the present study. All participants had provided written informed consent for the initial study, which included a statement on the use of data for future studies related to the initial one. The Medical Ethical Committee Leiden Den Haag Delft (METC-LDD) has stated that the Medical Research Involving Human Subjects Act (WMO) did not apply and that a separate judgement for ethical approval was not required. Approval for the study, including the extraction of additional information from medical records, was obtained from the respective boards of all six hospitals involved.

Data sources and collection

Data on patient characteristics, tumor characteristics, treatment characteristics, toxicity, and treatment deintensification were extracted from the original dataset, where this information was prospectively collected. Patients received capecitabine or fluorouracil, either as monotherapy or in combination with other anticancer agents. Additional patient characteristics obtained from the medical records were collected before start of treatment, with a time frame not exceeding 3 months prior to treatment initiation. These variables included the number of comorbidities, the number of concomitant drugs, serum creatinine (μmol/L), and serum albumin (g/L).

Study outcomes

The primary study outcome was the incidence of grade ≥3 fluoropyrimidine toxicity, assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 5.0 (NCI CTC-AE v5.0).[13] The secondary study outcome was the composite endpoint that included grade ≥3 toxicity and/or treatment deintensification during the first four treatment cycles. Treatment deintensification was a dichotomous outcome defined as the presence or absence of dose reduction, cycle delay, cycle interruption and/or treatment discontinuation after the start of the first cycle. Cycle delay was an increased interval between two treatment cycles and cycle interruption was not completing or interrupting the intended number of days of consecutive chemotherapy treatment in one cycle. Both cycle delay and cycle interruption were registered by the treating oncologist and were extracted from the original dataset.

Statistical analyses

Collected data on patient-, tumor- and treatment-related characteristics were summarized as mean or median for continuous variables and numbers (percentage) for categorical variables. The associations between characteristics and grade ≥3 toxicity or treatment deintensification were examined using univariate logistic regression to calculate the odds ratio (OR) with 95% confidence intervals (95% CI).

Patients were categorized into three groups based on their age at start of the treatment (65-69, 70-74, and ≥75 years). Treatment was categorized as monotherapy or combination

chemotherapy. In routine Dutch practice, the initial dose of monotherapy with capecitabine is 2000 - 2500 mg/m^2/ day on days 1-14 within a 21-day cycle. However, when capecitabine is used as a radiosensitizer in combination with radiotherapy, lower doses of 1650 mg/m^2/day are administered specifically on radiotherapy days. For our analysis, we defined the cut-off for normal/high dose range as ≥1800 mg/m^2/day for capecitabine, lower doses were considered low dose range. For combination chemotherapy regimens, a relative dose intensity of ≥80% of the standard chemotherapy regimen was considered a full dose and <80% a reduced dose. Both dose range and dose intensity apply to the dose at the start of the first treatment cycle. BMI was categorized into three groups (<18.5, 18.5- <25, and ≥25 kg/m^2)[14] and the number of comorbidities was grouped into three groups (0-1, 2-3, and ≥4 comorbidities). Polypharmacy was defined as the concurrent use of ≥5 pharmaceutical preparations for systemic use and inhaled drugs that were not related to the cancer treatment. The Cockroft-Gault-formula was used to calculate an estimation of the glomerular filtration rate (eGFR) to classify renal function (30-49 mL/min or ≥50 mL/min) and serum albumin was used to determine the presence of hypoalbuminemia (serum albumin ≤34 g/L).

Unknown interactions between variables may be present. For example, although not written in the study protocol, oncologists might have started chemotherapy with a lower dose intensity in older, more frail patients, and polypharmacy is likely more present in patients with a high number of comorbidities. To correct for possible unknown interactions between variables, all variables were used in the multivariate logistic regression models for both endpoints as well. A p-value of <0.05 was considered statistically significant. All analyses were performed using IBM SPSS version 28.

Results

In total, 311 *DPYD* wild-type patients aged ≥65 years were included. Median age was 71.0 years (interquartile range (IQR) 67-75) and 58.8% was male. Capecitabine was used in 80.1% of the patients and 43.7% was treated with monotherapy of a fluoropyrimidine agent. The mean BMI was 26.3 kg/m^2 (standard deviation (SD) 4.2), 2.3% of patients were underweight (BMI <18.5 kg/m^2) and 58.2% were overweight (BMI ≥25 kg/m^2). Polypharmacy was present in 37.3% of patients and 24.8% had 4 or more comorbidities. Renal function was moderately reduced (eGFR 30-49 mL/min) in 5.8% of patients and hypoalbuminemia (serum albumin ≤34 g/L) was present in 5.1% of patients. All available patient characteristics are shown in Table 1.

Grade ≥3 toxicity occurred in 23.2% of patients. In Table 2, patient characteristics, and the proportion of patients with grade ≥3 toxicity are presented, as well as the results of the univariate and multivariate analysis. Both the univariate and multivariate analysis showed no significant association with grade ≥3 toxicity for any of the variables. However, a trend towards increased risk of severe fluoropyrimidine-related toxicity was seen in the multivariate analysis for the use of polychemotherapy, a reduced starting dose, and BMI <18.5 kg/m^2.

Table 1: Patient characteristics

Characteristic	Categories	N=311
Age, years	Median (IQR))	71.0 (67-75)
	65-69 years	132 (42.4%)
	70-74 years	92 (29.6%)
	75+ years	87 (28.0%)
Sex	Male	183 (58.8%)
	Female	128 (41.2%)
Primary tumor type	Colorectal	202 (65.0%)
	Breast	29 (9.3%)
	Gastric	24 (7.7%)
	Genitourinary	21 (6.8%)
	Oesophageal	9 (2.9%)
	Pancreas	8 (2.6%)
	Other[‡]	13 (4.2%)
	Missing	5 (1.6%)
Cancer stage	Local	55 (17.7%)
	Locally advanced	156 (50.2%)
	Metastasized	96 (30.9%)
	Missing	4 (1.3%)
Drug	Capecitabine	249 (80.1%)
	5-fluorouracil	62 (19.9%)
Chemotherapy regimen	Monotherapy	136 (43.7%)
	Dose range normal/ high	47 (15.1%)
	Dose range low	89 (28.6%)
	Polychemotherapy[†]	175 (56.3%)
	Full dose	163 (52.4%)
	Reduced dose	12 (3.9%)
Concurrent radiotherapy	No	185 (59.5%)
	Yes	126 (40.5%)
BMI	Mean (95% CI)	26.3 (25.8-26.8)
	Underweight (<18.5 kg/m^2)	7 (2.3%)
	Normal (18.5 - <25 kg/m^2)	119 (38.3%)
	Overweight (≥25.0 kg/m^2)	181 (58.2%)
	Missing	4 (1.3%)
Number of comorbidities	Number of comorbidities (mean (95% CI))	2.3 (2.1-2.5)
	0-1	125 (40.2%)
	2-3	108 (34.7%)
	≥4	77 (24.8%)
	Missing	1 (0.3%)
Polypharmacy	Yes (≥5 concomitant drugs)	112 (36.0%)
	No	189 (60.8%)
	Missing	10 (3.2%)
eGFR	30-49 ml/min	18 (5.8%)
	≥50 ml/min	289 (92.9%)
	Missing	4 (1.3%)
Albumine	Hypoalbuminemia (≤34 g/l)	16 (5.1%)
	Normal (35-53 g/l)	264 (84.9%)
	Missing	31 (10.0%)

Abbreviations: IQR - interquartile range; BMI - body mass index; 95% CI - 95% confidence interval; eGFR - estimated glomerular filtration rate [‡]head and neck, skin, lung, vulvar, galbladder; [†]regimen of a fluoropyrimidine in combination with one or more other chemotherapeutical agents First course dose range low = capecitabine: 2dd <900mg/m^2. First course monotherapy with fluorouracil was never reduced. Dose intensity of first course polychemotherapy was considered reduced when <80% of the usual schedule was administered.

Table 2: Analysis of associations between patient characteristics and occurrence of grade ≥3 toxicity

		Univariate analysis		Multivariate analysis	
	Toxicity (%)	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
All	23.2				
Age			0.795		0.766
65-69 years	24.2	1.00 (ref)			
70-74 years	20.7	0.81 (0.43 - 1.55)		0.80 (0.40 - 1.61)	
75+ years	24.1	0.99 (0.53 - 1.87)		1.04 (0.51 - 2.11)	
Sex			0.518		0.460
Female	25.0	1.00 (ref)			
Male	21.9	0.84 (0.49 - 1.43)		0.81 (0.46 - 1.432)	
Cancer stage			0.962		0.981
Local	21.8	1.00 (ref)			
Locally advanced	22.4	1.04 (0.49 - 2.18)		0.88 (0.39 - 1.95)	
Metastasized	25.1	1.19 (0.54 - 2.63)		0.83 (0.34 - 2.02)	
Missing	25.0	1.19 (0.11 - 12.55)		0.79 (0.07 - 9.33)	
Drug			0.905		0.533
Capecitabine	23.3	1.00 (ref)			
5-fluorouracil	22.6	0.96 (0.49 - 1.87)		0.77 (0.34 - 1.75)	
Chemotherapy regimen			0.080		0.116
Monotherapy	18.4	1.00 (ref)			
Polychemotherapy	26.9	1.63 (0.94 - 2.82)		1.72 (0.88 - 3.38)	
Dose range capecitabine/5-FU			0.867		0.418
Normal/high dose	19.1	1.00 (ref)			
Low dose	18.0	0.925 (0.37 - 2.29)		0.77 (0.41 - 1.45)	
Dose intensity polychemotherapy			0.601		0.300
Full dose	26.4	1.00 (ref)			
Reduced dose	33.3	1.40 (0.40 - 4.87)		1.61 (0.67 - 3.96)	
BMI			0.434		0.547
Underweight (<18.5 kg/m^2)	42.9	2.13 (0.45- 10.05)		2.03 (0.37 - 11.17)	
Normal (18.5 - <25 kg/m^2)	26.1	1.00 (ref)			

		Univariate analysis		Multivariate analysis	
	Toxicity (%)	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Overweight (≥25.0 kg/m^2)	20.4	0.73 (0.42 - 1.26)		0.72 (0.40 - 1.32)	
Missing	25.0	0.95 (0.10 - 9.44)		n/a	
Number of comorbidities			0.472		0.871
0-1	20.8	1.00 (ref)			
2-3	21.3	1.03 (0.55 - 1.94)		1.01 (0.51 - 2.02)	
≥4	29.9	1.62 (0.85 - 3.11)		1.35 (0.61 - 2.98)	
Polypharmacy (≥5 drugs)			0.522		0.780
No	21.2	1.00 (ref)			
Yes	26.8	1.36 (0.79 - 2.35)		1.22 (0.64 - 2.33)	
Missing	20.0	0.93 (0.19 - 4.56)			
Renal function			0.576		0.896
eGFR ≥50 ml/min	22.5	1.00 (ref)			
eGFR 30-49 ml/min	33.3	1.72 (0.62 - 4.77)		n/a	
Missing	25.0	0.87 (0.09 - 8.51)		n/a	
Serum albumine			0.7437		0.395
Normal (35-53 g/l)	22.0	1.00 (ref)			
Hypoalbuminemia (≤34 g/l)	25.0	1.18 (0.37 - 3.81)		1.08 (0.32 - 3.66)	
Missing	32.3	1.69 (0.75 - 3.79)		1.83 (0.77 - 4.34)	

Abbreviations: 5-FU - fluorouracil; BMI - body mass index; eGFR - estimated glomerular filtration rate; n/a - not applicable

Table 3: Analysis of association between patient characteristics and occurrence of grade ≥3 toxicity and/or treatment deintensification (≤4 cycles)

		Univariate analysis		Multivariate analysis	
	Outcome (%)	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
All	41.2				
Age			0.888		0.921
65-69 years	41.7	1.00 (ref)			
70-74 years	39.1	0.90 (0.52 - 1.55)		1.01 (0.54 - 1.87)	
75+ years	42.5	1.04 (0.60 - 1.79)		1.13 (0.60 - 2.14)	
Sex			0.087		0.156
Female	46.9	1.00 (ref)			
Male	37.2	0.67 (0.42 - 1.06)		0.69 (0.41 - 1.15)	
Cancer stage			0.040		0.819
Local	34.5	1.00 (ref)			
Locally advanced	36.5	1.09 (0.57 - 2.08)		0.85 (0.41 - 1.75)	
Metastasized	53.1	2.15 (1.08 - 4.26)		1.02 (0.46 - 2.26)	
Missing	25.0	0.63 (0.06 - 6.49)		0.40 (0.03 - 5.17)	
Drug			0.311		0.747
Capecitabine	42.6	1.00 (ref)			
5-fluorouracil	35.5	0.74 (0.42 - 1.32)		0.89 (0.42 - 1.86)	
Chemotherapy regimen			0.021		0.083
Monotherapy	33.8	1.00 (ref)			
Polychemotherapy	46.9	1.73 (1.09 - 2.74)		1.70 (0.93 - 3.09)	
Dose range capecitabine/5-FU			<0.001		<0.001
Normal/high dose	57.4	1.00 (ref)			
Low dose	21.3	0.20 (0.09 - 0.43)		0.28 (0.16 - 0.49)	
Dose intensity polychemotherapy			0.413		0.113
Full dose	46.0	1.00 (ref)			
Reduced dose	58.3	1.64 (0.50 - 5.39)		1.95 (0.85 - 4.46)	
BMI			0.738		0.934
Underweight (<18.5 kg/m^2)	57.1	1.78 (0.38 - 8.30)		1.76 (0.32 - 9.86)	
Normal (18.5 - <25 kg/m^2)	42.9	1.00 (ref)			
Overweight (≥25.0 kg/m^2)	39.2	0.86 (0.54 - 1.38)		1.06 (0.62 - 1.82)	
Missing	50.0	1.33 (0.18 - 9.79)		n/a	
Number of comorbidities			0.568		0.914
0-1	40.0	1.00 (ref)			
2-3	38.0	0.92 (0.54 - 1.56)		0.95 (0.51 - 1.75)	
≥4	48.1	1.39 (0.78 - 2.46)		1.06 (0.62 - 1.82)	
Polypharmacy (≥5 drugs)			0.732		0.703
No	39.7	1.00 (ref)			
Yes	42.9	1.14 (0.71 - 1.83)		1.02 (0.56 - 1.83)	

		Univariate analysis		Multivariate analysis	
	Outcome (%)	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Missing	50.0	1.52 (0.43 - 5.43)		1.88 (0.43 - 8.21)	
Renal function			0.318		0.997
eGFR ≥50 ml/min	40.1	1.00 (ref)			
eGFR 30-49 ml/min	50.0	0.33 (0.03 - 3.84)		n/a	
Missing	75.0	0.22 (0.02 - 2.18)		n/a	
Serum albumine			0.755		0.745
Normal (35-53 g/l)	40.5	1.00 (ref)			
Hypoalbuminemia (≤34 g/l)	50.0	1.47 (0.53 - 4.03)		1.48 (0.48 - 4.54)	

Abbreviations: 5-FU - fluorouracil; BMI - body mass index; eGFR - estimated glomerular filtration rate; n/a - not applicable

The composite endpoint of grade ≥3 toxicity and/or treatment deintensification (dose reduction, cycle delay, cycle interruption, discontinuation, or a combination of these) occurred in 41.2%. Cancer stage (P=0.040), chemotherapy regimen (monotherapy or polychemotherapy) (OR 1.73; 95% CI 1.09-2.74; P=0.021), and low dose range (for monotherapy) (OR 0.20; 95% CI 0.09-0.43; P<0.001) were associated with the combined endpoint in the univariate analysis (Table 3). For cancer stage, this was mainly driven by the metastasized stage which exhibited a two times higher odd ratio compared to the local and locally advanced stages. In the multivariate analysis, only the association with dose range remained significant (OR 0.28; 95% CI 0.16-0.49; P<0.001 for low dose). A trend towards treatment deintensification was seen for the use of polychemotherapy, a reduced starting dose, and BMI <18.5 kg/m^2. The results of the univariate and multivariate analyses are shown in Table 3.

We also performed subgroup analysis for patients without concurrent radiotherapy, as these patients received low dose fluoropyrimidines. While the overall results remained largely unchanged, several notable trends were observed in the multivariate analyses for toxicity. Trends were seen for drug, reduced starting dose, BMI <18.5 kg/m^2 or BMI ≥25.0 kg/m^2, and ≥4 comorbidities. Additionally, for the composite endpoint trends were evident in the multivariate analysis for drug type, reduced starting dose, BMI <18.5 kg/m^2, number of comorbidities, and hypoalbuminemia. The results are described in Supplementary tables S1 and S2.

Discussion

This study is one of the first studies focusing on identifying predictors for severe toxicity in older patients receiving fluoropyrimidine therapy. We demonstrated that 23.2% of *DPYD* wild type patients older than 65 years experienced severe fluoropyrimidine-related toxicity and an additional 18% of patients that did not experience severe (grade ≥3) toxicity still required treatment deintensification. Our analysis for predictors of severe fluoropyrimidine-related toxicity revealed that older patients with a lower BMI, treated with polychemotherapy and a reduced starting dose trended towards higher occurrence of toxicity.

Several previous studies have investigated the incidence of severe fluoropyrimidine-related toxicity in different patient populations, with reported rates ranging from 12-53%.[4,6,9] Studies specifically focusing on older patients found similar ranges from 22-62% of severe toxicity, and rates of treatment deintensification ranging from 26-57%.[15-19] In our study, incidence of severe toxicity was relatively low, as we included only *DPYD* wild-type patients, who are at lower risk of toxicity.[20-23] Also, monotherapy was relatively frequently prescribed compared to previous studies[7], which could explain the lower toxicity rate. Monotherapy is generally associated with lower toxicity than polychemotherapy.[20,21,23] For example, a previous study showed that severe fluoropyrimidine-related toxicity occurred only in 12% of older patients treated with capecitabine monotherapy.[24]

We observed no significant difference in the incidence of severe toxicity across older age subgroups. This is in line with the results of a study by Feliú *et al.*, in which no significant difference in the incidence of severe capecitabine toxicity was observed between patients over 65 years of age and those aged 70 or 80 years and over.[24] Similarly, D'Andre *et al.* also reported no significant difference in severe toxicity between different age groups in four randomized clinical trials with more fit patients than a real-world population.[9]

The composite endpoint of grade ≥3 toxicity and/or treatment deintensification was observed in 41% of patients in our study, considering the impact of lower grade toxicity as well. We hypothesized that the presence of grade 2 toxicity, such as hand-foot syndrome or mucositis, in older patients undergoing fluoropyrimidine-based chemotherapy may significantly impact their independence and quality of life to the extent that they may opt to discontinue treatment at full dosage. This decision may be confounded by the presence of comorbidities and concomitant use of multiple medications, resulting in treatment mitigation even in cases of low-grade toxicity. Our findings suggest that low-grade toxicity should not be overlooked in older adults receiving chemotherapy, as it may have significant clinical relevance in addition to severe toxicity. In a previous real-world study conducted in the Netherlands, dose adjustments due to capecitabine-induced toxicity were reported in 83% of patients over 70 years of age.[7] Of note, *DPYD*-guided dosing was not yet in clinical practice during that study, and patients with upfront dose reductions were excluded from the analysis. Additionally, a higher proportion of patients received polychemotherapy (68%) compared to our study (56%). These differences may have contributed to a higher incidence of toxicity in the study described by van Beek *et al.*, resulting in more frequent dose reductions compared to our study. In the X-ACT trial, dose modifications (dose reduction, delay, or interruption) were required in 61% and 65% of patients ages ≥70 years for fluorouracil and capecitabine respectively.[25] Patients in the X-ACT trial received monotherapy with a fluoropyrimidine agent and the high dose of capecitabine 2500 mg/m^2 daily was used, whereas in our study, 44% of patients received monotherapy and the average dose was lower. In our study, a relatively large proportion of patients received concurrent radiotherapy, in which capecitabine is given in a low dose as a radiosensitizer. As such, capecitabine causes severe toxicity only in 14% of patients and leads to some form of treatment deintensification in up to 10% of *DPYD* wild type carriers (dose reductions 4%; interruptions 5%; prematurely stopped 10%).[26]

Patients who started with a reduced dose in a polychemotherapy schedule, did not experience more severe toxicity, but they did experience more often de-intensification of treatment, possibly because of initial frailty. In our study, 4% of patients started treatment with a reduced dose, presumably as precautionary measure from the oncologist in more frail patients. In a real-world study, other investigators found that 15% of fit patients received chemotherapy undertreatment and 33% of frail patients were overtreated. Grade 3-5 toxicity occurred significantly less in undertreated patients (24%) compared to appropriately treated patients (31%) or overtreated patients (40%), but treatment de-intensification was not assessed.[11] In an additional analysis, we tested whether certain subgroups of patients - e.g. those with lower BMI or higher age - were more likely to receive a reduced starting dose but we did not find any associations (data not shown). Our analysis revealed a trend towards higher rates of severe toxicity and treatment deintensification in older patients with lower BMI, the use of polychemotherapy, or full-dose monotherapy with fluoropyrimidines. However, these associations did not reach statistical significance. Patients with a BMI <18.5 kg/m^2 seemed to experience twice as much toxicity as patients with normal BMI and nearly three times more than those with a BMI ≥25 kg/m^2. Underweight patients may have less functional reserve in that they may suffer more severe consequences of vomiting, diarrhea, and decreased food intake that are caused by chemotherapy. Hurria *et al.* also found a lower BMI to be associated with increased toxicity, although that was only shown for a BMI ≤26.5 kg/m^2 compared to BMI >26.5 kg/m^2.[27]

Exposing underweight patients to chemotherapy appears to put them in a high risk of severe toxicity.[28,29] Of note, it was recently demonstrated that decreased BMI was associated with increased risk of developing fluoropyrimidine-induced cardiotoxicity.[30] The observation of a higher incidence of severe toxicity in patients undergoing polychemotherapy is consistent with our expectations, as the use of polychemotherapy is known to be associated with increased risk of adverse events.[20-23]

The retrospective nature of this study presents a significant limitation, as it restricted the variables for analysis. Furthermore, no geriatric assessment data were available to assess frailty of patients. Frailty is a commonly recognized age-related condition that stems from the progressive deterioration of multiple organ systems, leading to a reduced ability to withstand stressors such as chemotherapy. This is thought to be caused by the complex interplay between biological, psychological, and social factors and has been associated with adverse health outcomes.[27,31,32] The incorporation of geriatric assessments would have provided valuable information tailored to the needs of older patients. This has previously been confirmed by two randomized clinical trials in which it was studied whether toxicity could be reduced by geriatric assessment-guided treatment decisions in patients ≥70 years treated with chemotherapy.[33,34] Both studies showed that grade ≥3 toxicity could significantly be reduced by using personalized treatment in older patients treated with chemotherapy based on the outcome of geriatric assessments without affecting the one-year survival.[33,34] Unfortunately, the availability of such data was limited. Future research should prioritize the inclusion of geriatric assessments, preferably in a prospective study, to obtain a comprehensive understanding of potential predictive factors for severe toxicity in older patients treated with fluoropyrimidines. Furthermore, previous studies have shown that tolerance of fluoropyrimidines among elderly is

comparable to that of younger patients, possibly due to the inclusion of relatively fit elderly in clinical trials.[6-8] The prevalence of severe fluoropyrimidine toxicity within elderly patients our study (23.2%) implies a potential parallel with the complete cohort of the Alpe-DPD study in which the prevalence of severe toxicity was 23%. Therefore, inclusion of real-world data without selection of elderly patients based on WHO status or physical condition could provide valuable information in future studies. In addition, the relatively small sample size may have affected the power to detect significant associations between potential predictive factors and severe fluoropyrimidine-related toxicity in older patients. Hence, the findings of this study should be interpreted with caution and future prospective studies with larger samples sizes are needed to confirm the demonstrated trends towards a higher occurrence of severe toxicity and establish more robust evidence.

Conclusion

Despite *DPYD* genotype-guided dosing, which has been shown to reduce fluoropyrimidine-associated toxicity, a substantial proportion of older adults treated with fluoropyrimidines still experience severe toxicity and treatment deintensification. Although our study most likely did not have sufficient power to identify significant associations between patient-related predictors and toxicity, we observed negative trends for BMI, polychemotherapy, and use of reduced dose fluoropyrimidine-treatment. Future real-world studies, preferably prospective, are needed to investigate these predictive factors. Additionally, these studies should include specific geriatric parameters and the tolerability of low-grade toxicity to gain a better understanding of the complex interaction between aging, cancer, and fluoropyrimidine-related toxicity and ultimately improve the efficacy and safety of fluoropyrimidine treatment in older adults.

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Supplemental - Chapter 7

Table S1: Subgroup analysis of associations between patient characteristics and occurrence of grade ≥3 toxicity in patients without concurrent radiotherapy (N=185)

		Univariate analysis		Multivariate analysis	
	Toxicity (%)	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
All	25.9				
Age			0.966		0.770
65-69 years	25.0	1.00 (ref)			
70-74 years	26.9	1.11 (0.50 - 2.48)		1.05 (0.42 - 2.62)	
75+ years	26.4	1.08 (0.49 - 2.38)		1.41 (0.54 - 3.71)	
Sex			0.739		0.468
Female	27.2	1.00 (ref)			
Male	25.0	0.89 (0.46 - 1.73)		0.75 (0.35 - 1.12)	
Cancer stage			0.842		0.972
Local	25.0	1.00 (ref)			
Locally advanced	27.8	1.15 (0.37 - 3.59)		0.83 (0.24 - 2.84)	
Metastasized	24.2	0.96 (0.31 - 2.93)		0.81 (0.23 - 2.82)	
Missing	50.0	3.00 (0.16 - 57.37)		1.41 (0.06 - 33.34)	
Drug			0.136		0.089
Capecitabine	28.0	1.00 (ref)			
5-fluorouracil	14.3	0.43 (0.14 - 1.30)		0.34 (0.10 - 1.18)	
Chemotherapy regimen			0.354		0.104
Monotherapy	21.2	1.00 (ref)			
Polychemotherapy	27.8	1.44 (0.67 - 3.09)		2.37 (0.84 - 6.71)	
Dose range capecitabine/5-FU			0.931		0.229
Normal/high dose	20.9	1.00 (ref)			
Low dose	22.2	1.08 (0.19 - 6.12)		0.53 (0.18 - 1.50)	
Dose intensity polychemotherapy			0.512		0.463
Full dose	21.6	1.00 (ref)			
Reduced dose	20.0	1.54 (0.42 - 5.61)		1.60 (0.46 - 5.62)	
BMI			0.439		0.281
Underweight (<18.5 kg/m^2)	28.4	2.52 (0.47 - 13.42)		3.01 (0.41 - 22.44)	
Normal (18.5 - <25 kg/m^2)	50.0	1.00 (ref)			
Overweight (≥25.0 kg/m^2)	22.1	0.72 (0.36 - 1.42)		0.57 (0.26 - 1.25)	
Missing	33.3	1.26 (0.11 - 14.59)		n/a	
Number of comorbidities			0.258		0.541
0-1	22.2	1.00 (ref)			
2-3	21.7	0.97 (0.42 - 2.22)		0.96 (0.37 - 2.46)	
≥4	36.5	2.02 (0.92 - 4.45)		1.85 (0.68 - 5.08)	

		Univariate analysis		Multivariate analysis	
	Toxicity (%)	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Polypharmacy (≥5 drugs)			0.576		0.643
No	23.3	1.00 (ref)			
Yes	30.1	1.42 (0.72 - 2.80)		1.49 (0.65 - 3.41)	
Missing	22.2	0.94 (0.18 - 4.83)		1.23 (0.19 - 7.83)	
Renal function			0.574		0.964
eGFR ≥50 ml/min	25.0	1.00 (ref)			
eGFR 30-49 ml/min	38.5	1.88 (0.58 - 6.05)		1.22 (0.30 - 5.02)	
Missing	25.0	1.00 (0.10 - 9.87)		n/a	
Serum albumine			0.327		0.324
Normal (35-53 g/l)	24.2	1.00 (ref)			
Hypoalbuminemia (≤34 g/l)	25.0	1.05 (0.27 - 4.06)		1.11 (0.25 - 4.81)	
Missing	40.0	2.09 (0.79 - 5.50)		2.32 (0.77 - 6.95)	

Abbreviations: 5-FU - fluorouracil; BMI - body mass index; eGFR - estimated glomerular filtration rate; n/a - not applicable

Table S1: Subgroup analysis of associations between patient characteristics and occurrence of grade ≥3 toxicity in patients without concurrent radiotherapy (N=185)

		Univariate analysis		Multivariate analysis	
	Outcome (%)	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
All	53.0				
Age			0.724		0.690
65-69 years	51.2	1.00 (ref)			
70-74 years	57.7	1.30 (0.64 - 2.62)		1.40 (0.62 - 3.15)	
75+ years	50.9	0.99 (.49 - 1.98)		1.03 (0.46 - 2.34)	
Sex			0.225		0.266
Female	58.0	1.00 (ref)			
Male	49.0	0.70 (0.39 - 1.25)		0.68 (0.35 - 1.34)	
Cancer stage			0.989		0.920
Local	50.0	1.00 (ref)			
Locally advanced	542	1.18 (0.44 - 3.19)		0.78 (0.25 - 2.42)	
Metastasized	52.7	1.12 (0.42 - 2.94)		0.79 (0.26 - 2.41)	
Missing	50.0	1.00 (0.06 - 18.30)		0.34 (0.01 - 8.48)	
Drug			0.020		0.087
Capecitabine	56.7	1.00 (ref)			
5-fluorouracil	32.1	0.36 (0.15 - 0.85)		0.42 (0.15 - 1.13)	
Chemotherapy regimen			0.422		0.507
Monotherapy	57.7	1.00 (ref)			
Polychemotherapy	51.1	0.77 (0.40 - 1.47)		1.33 (0.57 - 3.10)	

		Univariate analysis		Multivariate analysis	
	Outcome (%)	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Dose range capecitabine/5-FU			0.381		0.006
Normal/high dose	60.5	1.00 (ref)			
Low dose	44.4	0.52 (0.12 - 2.23)		0.29 (0.12 - 0.70)	
Dose intensity polychemotherapy			0.391		0.398
Full dose	50.0	1.00 (ref)			
Reduced dose	63.6	1.75 (0.49 - 6.29)		1.59 (0.54 - 4.64)	
BMI			0.865		0.929
Underweight (<18.5 kg/m^2)	66.7	1.77 (0.31 - 10.20)		1.53 (0.20 - 11.90)	
Normal (18.5 - <25 kg/m^2)	53.1	1.00 (ref)			
Overweight (≥25.0 kg/m^2)	51.6	0.94 (0.52 - 1.70)		0.85 (0.43 - 1.69)	
Missing	66.7	1.77 (0.15 - 20.27)		n/a	
Number of comorbidities			0.552		0.423
0-1	51.4	1.00 (ref)			
2-3	48.3	0.89 (0.45 - 1.76)		0.84 (0.38 - 1.87)	
≥4	61.5	1.51 (0.73 - 3.13)		1.75 (0.71 - 4.35)	
Polypharmacy (≥5 drugs)			0.979		0.595
No	52.4	1.00 (ref)			
Yes	53.4	1.04 (0.57 - 1.90)		1.12 (0.54 - 2.32)	
Missing	55.6	1.13 (0.29 - 4.47)		2.34 (0.45 - 12.10)	
Renal function			0.551		0.986
eGFR ≥50 ml/min	51.8	1.00 (ref)			
eGFR 30-49 ml/min	61.5	1.49 (0.47 - 4.74)		1.13 (0.28 - 4.51)	
Missing	75.0	2.79 (0.29 - 27.40)		n/a	
Serum albumine			0.905		0.676
Normal (35-53 g/l)	52.3	1.00 (ref)			
Hypoalbuminemia (≤34 g/l)	58.3	1.28 (0.39 - 4.20)		1.75 (0.45 - 6.84)	
Missing	55.0	1.12 (0.44 - 2.85)		0.85 (0.29 - 2.46)	

Abbreviations: 5-FU - fluorouracil; BMI - body mass index; eGFR - estimated glomerular filtration rate; n/a - not applicable

Chapter 8

Predictors of poor treatment tolerability in older patients receiving fluoropyrimidine-based chemotherapy: results from the Alpe2U study

Interim analysis

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Abstract

Introduction: Although fluoropyrimidines are widely prescribed to older patients, studies investigating predictors for chemotherapy intolerance in older patients are scarce. Therefore, we aimed to identify predictors of poor treatment tolerability in older patients receiving fluoropyrimidine-based chemotherapy.

Methods: Patients aged ≥70 years who received fluoropyrimidine-based chemotherapy were selected from the prospective, multicentre, non-randomized Alpe2U study (NCT04194957). Before treatment initiation, participants underwent a geriatric assessment investigating the somatic, nutritional, functional, and mental domain. Predictors of the composite endpoint "poor treatment tolerability", defined as either Common Toxicity Criteria Adverse Events (CTCAE) grade 3-5 chemotherapy-related toxicity, dose reduction or treatment discontinuation within the first two cycles, were analysed using uni- and multivariable logistic regression models.

Results: Of the 194 included patients, median age was 75 (interquartile range 73-79) years and the most common tumour types were colorectal (60%) and esophagogastric (19%) cancer. Most patients (89%) received capecitabine-based chemotherapy. Poor treatment tolerability within the first two cycles was seen in 31% of patients. In multivariable analysis, associations with poor treatment tolerability were found for deficits in 3-4 geriatric domains compared with 0 deficits (odds ratio (OR) 4.03, 95% confidence interval (CI) 1.09 - 14.97, P=0.037) and for combination chemotherapy (OR 2.83, 95% CI 1.31-6.09, P=0.008).

Conclusion: Having deficits in multiple geriatric domains and combination chemotherapy were predictors of poor treatment tolerability within the first two cycles in older patients treated with fluoropyrimidine-based chemotherapy. These findings highlight the importance of a geriatric assessment before fluoropyrimidine administration to estimate risk of treatment intolerance.

Introduction

Fluoropyrimidines, such as capecitabine and 5-fluorouracil (5-FU), are prescribed for a wide variety of tumour types, including colorectal, gastric, breast, and head- and neck cancer. Despite extensive research on the safety of fluoropyrimidines showing that upfront *DPYD*-guided dose-individualization significantly reduces the incidence of severe fluoropyrimidine-related toxicity, severe toxicity remained present in 23% of patients,[1] suggesting that the occurrence of toxicity can only partially be traced back to *DPYD*-genotype. Future studies should therefore focus on identifying other risk factors for toxicity.

Approximately 35-55% of the patients receiving fluoropyrimidines are aged 70 years or older, and this proportion will further increase due to our aging population.[2] Due to functional deficits, multimorbidity and renal, hepatic, or bone marrow dysfunction, older patients may be at increased risk of developing severe fluoropyrimidine-related toxicity.[3] These toxicities result in high rates of hospitalization, early treatment discontinuation and declined quality of life (QoL) and physical functioning in the older population.[4-6] A pre-treatment geriatric assessment can aid clinicians in identifying individuals at high risk of developing chemotherapy-related toxicity.[7,8] While previous studies have shown associations between geriatric characteristics and toxicity,[9-11] these studies included patients treated with a variety of chemotherapy types. Given the substantial variation in toxicity rates across chemotherapy regimens,[12,13] studies focusing solely on patients receiving the same chemotherapy base and toxicity associated with this chemotherapy could provide more accurate risk estimates.

To date, large prospective studies specifically focusing on geriatric predictors for toxicity in older patients receiving fluoropyrimidine-based chemotherapy are scarce. We therefore aimed to identify predictors of poor treatment tolerability in older patients treated with fluoropyrimidine-based chemotherapy.

Methods

The design, study population, and results of the Alpe2U study (NCT04194957) are described in chapter 4 of this thesis. Briefly, Patients with a pathologically confirmed malignancy and an indication for fluoropyrimidine treatment were recruited from 15 Dutch hospitals between January 13, 2020, and July 1, 2022. *DPYD* wild-type patients with an elevated pre-treatment uracil concentration (>16 ng/mL) and patients carrying a *DPYD* variant allele were treated with reduced dose of 50%. The Alpe2U study was approved by the medical ethical committee of the Netherlands Cancer Institute - Antoni van Leeuwenhoek and all patients provided informed consent before enrolment. The current analysis investigates possible predictors of poor treatment tolerability in older patients treated with fluoropyrimidine-based chemotherapy. Patients carrying a *DPYD* variant allele (*DPYD**2A, c.1236G>A, c.2846A>T, or c.1679T>G) or with an elevated uracil concentration were excluded as they received an upfront dose-reduction.

Geriatric assessment

Before treatment initiation, participants aged ≥70 years who agreed to undergo a geriatric

assessment, were asked to complete the Geriatric 8 (G8) questionnaire, 6-item Cognitive Impairment Test (6-CIT), Katz Activities of Daily Living (ADL), Lawton Instrumental ADL and Patient Health Questionnaire-2 (PHQ-2) by telephone. The G8 questionnaire was specifically developed for older patients with cancer and consists of eight items on food intake, weight loss, mobility, neuropsychological problems, body mass index (BMI), number of daily drugs, self-perception of health and age.[14] Scores range from 17 (not at all impaired) to 0 (heavily impaired), and scores ≤14 are considered impaired. The 6-CIT involves three tests of temporal orientation, two tests of attention and one test of short-term memory.[15] It is scored out of 28, with scores >7 indicating an abnormal test. Functional status was assessed by the 6-item Katz ADL, with scores higher than 1 (loss of dependency in at least 1 activity) indicating ADL dependence.[16] The level of independence on shopping, telephone, housekeeping, food preparation, transport, medication, and finances was assessed with the Lawton IADL.[17] The 2-item PHQ-2 screens for anxiety and depression, with a cut-off ≥3 points.[18] Data on patient-, tumour- and treatment characteristics were obtained from the case report forms.

Based on the outcomes of the geriatric assessment, we calculated the cumulative number of geriatric domains with a deficit. In line with previous studies, we defined the following four geriatric domains: the somatic, nutritional, functional, and mental domain.[19,20] The somatic domain was considered abnormal if polypharmacy was present, defined as having 4 or more daily medications (derived from the G8 questionnaire). Information on comorbidity was not available. We scored the nutritional domain impaired if the patient had unintentional weight loss in the last 3 months or a BMI <18.5. Functional status was abnormal in case of an impaired Katz ADL or Lawton IADL. The mental domain was impaired if either the PHQ-2 screening, or the 6-CIT screening was abnormal.

Study endpoints

In older patients, not only severe toxicity but also low-grade toxicity often leads to dose reductions or early discontinuation.[21] To take into account these low-grade toxicities, we designed a composite endpoint, "poor treatment tolerability", defined as either the occurrence of grade 3-5 fluoropyrimidine-related toxicity (as defined by the NCI Common Terminology Criteria for Adverse Events (CTCA v5.0)[22]), treatment discontinuation due to toxicity or dose reduction within the first two chemotherapy cycles or, in case of chemoradiotherapy, during the first two weeks of treatment.[8] Secondary endpoints were grade 3-5 toxicity, early treatment discontinuation, dose reduction and dose delay in the first two cycles or, in case of chemoradiotherapy, during the first two weeks of treatment.

Statistics

Descriptive characteristics were reported using frequencies and proportions for categorical variables and means and standard deviation (SD) or medians and interquartile range (IQR) for continuous variables. We imputed missing questionnaires from the geriatric assessment using multiple imputations, assuming that data were missing at random,[23] with chained equations, based on the pooled results of 20 imputed sets. For each imputed variable, imputation models were applied that included nine correlated predictors of a missing geriatric assessment, identified using univariable logistic regression.

Primary and secondary outcomes were visually depicted and stratified for treatment type (dose-reduced capecitabine monotherapy, full-dose capecitabine monotherapy, fluoropyrimidine with concurrent radiotherapy -with or without mitomycin C- and combination chemotherapy). Uni- and multivariable logistic regression models were used to identify possible predictors of the composite primary endpoint of poor treatment tolerability. Predefined geriatric parameters, including the G8 score, functional status, cognitive and psychosocial deficits, and polypharmacy, as well as the cumulative number of impaired geriatric domains, were correlated with treatment tolerability. Clinically relevant predictors and variables that reached a p-value <0.1 were added in a multivariable logistic model. We calculated the odds ratio (OR) and the corresponding 95% confidence interval (CI). To assess the influence of the imputed questionnaires in the logistic regression models, a sensitivity analyses was performed in which patients with imputed questionnaires were excluded. In an additional sensitivity analysis, we stratified treatment outcomes by the cumulative number of impaired geriatric domains. All analyses were performed in SPSS v25 and a p-value of <.05 was considered statistically significant.

Results

Baseline characteristics

Between January 2020 and July 2022, 214 patients aged 70 years and older receiving fluoropyrimidine-based chemotherapy were included. After exclusion of *DPYD* variant allele carriers and patients with pretreatment serum uracil level of >16 ng/mL, 194 patients were eligible for the analysis (Figure S1 Appendix). Median age was 75 (IQR 73-79) years and 108 (56%) were males (Table 1). Most common tumour types were colorectal (60%) and esophagogastric (19%) cancer. Ninety-six patients (50%) had metastatic disease. Most patients (89%) were treated with a capecitabine-based regimen. Combination chemotherapy was given in 52% and concurrent radiotherapy in 39 (21%) patients.

Baseline geriatric assessment

A baseline geriatric assessment was done in 151 of the 194 eligible patients. In the 43 patients without a geriatric assessment, scores were imputed. Baseline patient- and tumour characteristics could predict which patients had a missing geriatric assessment (Table S1). We imputed the missing questionnaires using multiple imputation with all variables shown in Table S1. After multiple imputation, polypharmacy was observed in 47% and an impaired G8 was seen in 53% of the patients (Table 2). ADL dependence was reported in 13% and IADL dependence in 28%. Nineteen percent had cognitive impairments and 15% had symptoms of anxiety or depression. Half of the patient population had no or one impaired geriatric domain and were consequently considered non-frail. The other half had either two (28%) or three to four (22%) impaired geriatric domains and were therefore considered to be frail.

Treatment outcomes

Poor treatment tolerability within the first two cycles was seen in 60 (31%) patients (Figure S2). Twenty-seven (14%) patients developed grade 3-5 chemotherapy-related toxicity, which was mostly non-haematological and consisted predominantly of gastrointestinal toxicity (12%) (Appendix Table S1). Twenty-two (11%) patients discontinued treatment

due to toxicity, of whom 47% experienced no greater than grade 2 toxicity. Discontinuation due to disease progression was seen in three patients (2%). Dose reduction was performed in 32 (17%) and dose delay in 26 (13%) patients. Poor treatment tolerability was seen in 40% of patients treated with combination chemotherapy versus 21% in reduced-dose monotherapy, 28% in full-dose monotherapy and 18% in those treated with chemoradiotherapy (P=.047). Moreover, patients treated with combination chemotherapy more often had dose reductions (26% in combination chemotherapy group versus 5% in reduced-dose monotherapy, 11% in full-dose monotherapy and 8% in chemoradiotherapy, P=.013) and dose delay (20% in combination chemotherapy group versus 0% in reduced-dose monotherapy, 15% in full-dose monotherapy and 3% in chemoradiotherapy, P=.014).

Table 1: Patient and tumor characteristics (N=194)

Variable	Categories	N (%)
Age	Median, years (IQR)	75 (73-79)
	70-74 years	84 (43)
	75-79 years	64 (33)
	≥80 years	46 (24)
Sex	Male	108 (56)
	Female	86 (44)
Tumour type	Colorectal	117 (60)
	Esophagogastric	37 (19)
	Breast	19 (10)
	Other†	21 (11)
Disease stage	Local	42 (22)
	Locally Advanced	56 (29)
	Metastatic	96 (50)
Type of treatment regimen	Capecitabine monotherapy (with (N=17) or without targeted therapy (N=50))	67 (35)
	Capecitabine + RT (with (N=8) or without mitomycin C (N=24)	32 (17)
	Capecitabine + oxaliplatin (with (N=14) or without bevacizumab (N=56))	70 (36)
	Capecitabine + other anticancer drugs	4 (2)
	5-FU + RT (with (N=5) or without mitomycin (N=2))	7 (4)
	5-FU + oxaliplatin and folinic acid (with (N=1) or without bevacizumab (N=13))	14 (7)
Starting dose capecitabine monotherapy (without RT)	≥1900 mg/m² per day	47 (24)
	<1900 mg/m² per day	19 (10)
WHO*	0	94 (49)
	1	79 (41)
	2	15 (8)
BMI*	Mean (SD)	25.7 (4.0)
	<18.5 kg/m²	4 (2)
	18.5-24.9 kg/m²	87 (45)
	25-30 kg/m²	74 (38)
	>30 kg/m²	27 (14)

†other tumour types included pancreatic cancer, bladder cancer, anal cancer, vulvar carcinoma, unknown primary tumour and several rare tumour types. *Unknown WHO status N=6, unknown BMI N=2. Abbreviations: BMI: Body Mass Index, IQR: interquartile range, RT: radiotherapy.

Predictors for poor treatment tolerability

Table 3 shows logistic regression models analyzing predictors of poor treatment tolerability. In the univariable analysis, only receiving combination chemotherapy (OR 2.39; 95% CI 1.27 - 4.52, P=.007) was significantly associated with poor treatment tolerability compared to monotherapy. A deficit in three or four geriatric domains in comparison with no deficits was non-significantly associated with treatment tolerability (OR 3.09; 95% CI 0.97-9.87, P=.057). After adjusting for age, sex, disease stage and concurrent radiotherapy, deficits in three or four geriatric domains (OR 4.03; 95% CI 1.09-14.97, P=.037) and combination chemotherapy (OR 2.83; 95% 1.31-6.09, P=.008) were independently associated with poor treatment tolerability (Figure 1). No significant associations between the individual geriatric tests and treatment tolerability were found.

Table 2: Baseline geriatric characteristics

Geriatric variable	Categories	All patients, geriatric assessment not imputed: N, (%)	All patients, geriatric assessment imputed* (%)
Polypharmacy (≥4 medicines)	No	64 (45)	53%
	Yes	79 (55)	47%
	Unknown	51	
Geriatric-8 (G8)	Not impaired	64 (46)	47%
	Impaired	76 (54)	53%
	Unknown	54	
6-CIT	Not impaired	128 (88)	81%
	Impaired	18 (12)	19%
	Unknown	48	
Activities of Daily Living (ADL)	Independent	146 (97)	87%
	Dependent	5 (3)	13%
	Unknown	43	
Instrumental Activities Of Daily Living (IADL)	Independent	115 (77)	72%
	Dependent	34 (23)	28%
	Unknown	45	
Patient Health Questionnaire-2	Not impaired	136 (91)	85%
	Impaired	14 (9)	15%
	Unknown	44	
Deficits in geriatric domains±	0	34 (18)	20%
	1	48 (25)	30%
	2	41 (21)	28%
	3 or 4	22 (10)	22%
	Unknown	51	

*Multiple imputation was used to account for the missing geriatric assessments, under a missing at random assumption. Imputation of 20 datasets was performed using chained equations. Variables included in the imputation process were: 1) age, 2) sex, 3) tumour type, 4) disease stage, 5) polychemotherapy, 6) targeted therapy, 7) WHO performance status and 8) BMI. ±Domains represent the cumulative number of impaired geriatric domains.

In a sensitivity analysis excluding the patients with imputed geriatric questionnaires, deficits in one geriatric domain (OR 3.28; 95% CI 1.09-9.90, P=.035), three or four geriatric domains (OR 5.17; 95% CI 1.46-18.28, P=.011) and combination chemotherapy (OR 2.27; 95% CI 1.08 - 4.75, P=.030) were associated with poor treatment tolerability (Table S3). After adjusting for age, sex, disease stage, concurrent radiotherapy and IADL dependency, deficits in one geriatric domain (OR 4.14; 95% CI 1.23-13.86, P=.021) and combination chemotherapy (OR 2.49; 95% CI 1.01-6.12, P=.047) was significantly associated with poor treatment tolerability, whereas the OR of the association between deficits in three or four geriatric domains and poor tolerability was 5.38 (95% CI 0.99-29.29, P=.051).

To further investigate the influence of deficits in geriatric domains on outcomes, we stratified treatment outcomes by the cumulative number of impaired geriatric domains. The percentage of patients with poor treatment tolerability increased from 18% in patients without impaired geriatric domains to 39% in patients with three or four impaired geriatric domains (Figure 2). The percentage of patients with either grade 3-5 toxicity or early treatment discontinuation also increased with a higher number of impaired geriatric domains.

Table 3: Associations between patient and geriatric characteristics and poor treatment tolerability within the first two treatment cycles

Variable	Category	Univariable (OR, 95% CI)	p-value	Multivariable (OR, 95% CI)	*p*-value
Age	70-74 years	Ref		Ref	
	75-79 years	1.11 (0.56 - 2.20)	.775	0.96 (0.44 - 2.12)	.925
	≥80 years	0.66 (0.29 - 1.50)	.325	0.94 (0.37 - 2.42)	.901
Sex	Male	Ref		Ref	
	Female	1.39 (0.76 - 2.57)	.288	1.64 (0.83 - 3.25)	.158
Disease stage	Local	Ref		Ref	
	Locally Advanced	1.64 (0.67 - 4.04)	.279	1.47 (0.55 - 3.90)	.441
	Metastatic	1.53 (0.67 - 3.50)	.318	1.30 (0.51 - 3.32)	.589
Radiotherapy	No	Ref		Ref	
	Yes	0.49 (0.21 - 1.14)	.098	0.57 (0.21 - 1.55)	.269
Additional chemotherapy	No	Ref		Ref	
	Yes	2.39 (1.27 - 4.52)	.007	2.83 (1.31 - 6.09)	.008
Targeted therapy	No	Ref			
	Yes	1.21 (0.54 - 2.70)	.645		
WHO status	0	Ref			
	1	0.87 (0.45 - 1.67)	.666		
	2	1.96 (0.65 - 5.92)	.232		
Geriatric-8	Not impaired	Ref			
	Impaired	1.61 (0.77 - 3.37)	.204		
6-CIT	Not impaired	Ref			
	Impaired	0.83 (0.32 - 2.14)	.696		
ADL	Independent	Ref			
	Dependent	1.40 (0.51 - 3.84)	.514		

Variable	Category	Univariable (OR, 95% CI)	p-value	Multivariable (OR, 95% CI)	*p*-value
IADL	Independent	Ref			
	Dependent	1.49 (0.68 - 3.27)	.316		
PHQ-2	Not impaired	Ref			
	Impaired	1.06 (0.42 - 2.67)	.910		
Polypharmacy	No	Ref			
	Yes	1.19 (0.57 - 2.50)	.641		
Frailty domains	0	Ref		Ref	
	1	2.43 (0.78 - 7.60)	.126	2.68 (0.77 - 9.41)	.122
	2	2.14 (0.67 - 6.84)	.201	2.54 (0.69 - 9.38)	.162
	3-4	3.09 (0.97 - 9.86)	.057	4.03 (1.09 - 14.97)	.037

Poor treatment tolerability occurred in 60 patients (31%). Data derived from the multiple imputation of missing geriatric assessments were included in the model. Although 'tumour type' could also be associated with poor treatment tolerability due to differences in chemotherapy regimen, concurrent radiotherapy, frailty characteristics and metastatic disease per tumour type, this was not added as a variable in the logistic regression. As chemotherapy regimen, radiotherapy, frailty characteristics and metastatic disease are already variables in the model and to maintain the statistical power of the logistic regression, we did not add tumour type. Abbreviations: ADL: Activities of Daily Living, IADL: Instrumental Activities of Daily Living, CI; confidence interval, OR; odds ratio, PHQ-2: Patient Health Questionnaire.

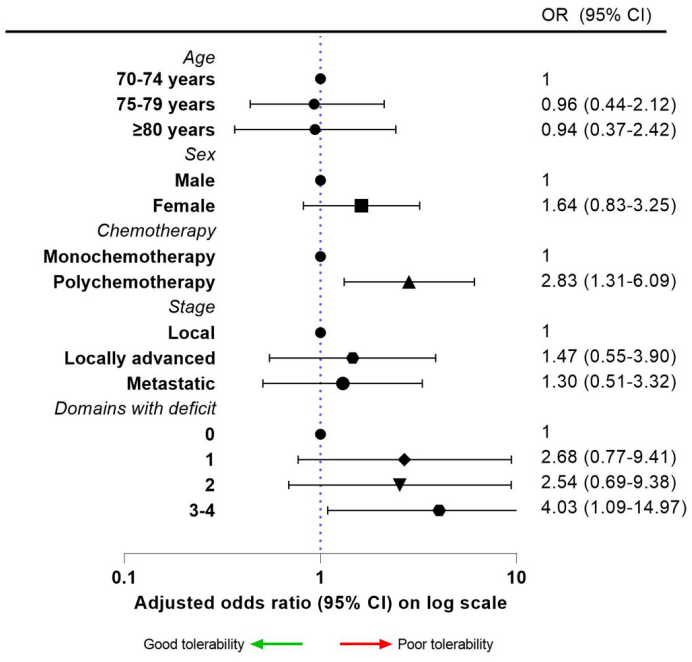

Figure 1: Forest plot of the adjusted logistic regression model to assess the association between geriatric parameters and the composite outcome of treatment tolerability. Odds ratios (OR) and 95% confidence intervals (CI) are depicted. X-axis is displayed as log scale.

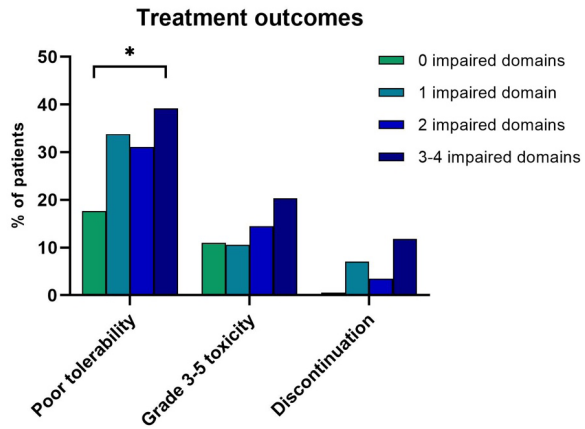

Figure 2: Treatment outcomes, stratified by cumulative number of impaired geriatric domains. "Poor tolerability" represents the percentage of patients in which the composite endpoint (either grade 3-5 toxicity, early treatment discontinuation or dose reduction within the first two treatment cycles) occurred. "Discontinuation" represents the percentage of patients that discontinued treatment within the first two cycles due to toxicity. * Represents p<0.05, derived from multivariate logistic regression, adjusted for age, sex, number of chemotherapy regimen, concurrent radiotherapy, and disease stage.

Discussion

This prospective study demonstrates that 31% of older patients receiving fluoropyrimidine-based chemotherapy had a poor treatment tolerability during the first two cycles, and this percentage increased with the accumulation of impaired geriatric domains. Deficits in three or four geriatric domains as well as receiving combination chemotherapy predicted poor treatment tolerability within the first two cycles.

The finding that the accumulation of geriatric deficits across multiple domains, rather than individual deficits, was predictive of poor treatment tolerability, is in line with the concept of frailty. Frailty is commonly defined as an age-related condition that is caused by the cumulative deterioration across multiple organ system. This multifactorial decline in physiologic reserve and organ systems results in decreased resistance to stressors such as chemotherapy.[24-26] As deficits in geriatric domains accumulate, patients become more susceptible to adverse events, while the individual particulars of each deficit are of less importance. A previous study by Hamaker *et al.* also found that the number of geriatric deficits was predictive of grade 3-4 chemotherapy-related toxicity.[27] Similarly, a Deficit Accumulation Frailty Index, derived by calculating all frailty deficits in older patients receiving chemotherapy, was associated with poor treatment tolerability in a study by Cohen and colleagues.[28] Various other studies focusing solely on the association between separate geriatric domains and chemotherapy tolerability did not find any association,[29-31] supporting the idea that a multi-domain assessment rather than single domain deficits detects those at risk of poor chemotherapy outcomes.

The gold standard for evaluating geriatric domains and frailty is a comprehensive geriatric assessment (CGA): a multidimensional process that includes systematically assessing the physical, somatic, cognitive, mental and social functioning, and formulating and evaluating an integrated care plan.[32] The paradigm of CGA can provide oncologists and patients with a basis for selecting those at risk of poor outcomes and making individualized treatment decisions, by objectively and systematically integrating geriatric impairments in oncologic care.[33] One possible reason for not widely implementing a routine CGA before fluoropyrimidine-based chemotherapy administration in daily practice is that it is time-consuming. A prediction tool for chemotherapy intolerance, integrating only the most predictive parts of the CGA, might therefore be a more efficient and practical approach to estimate risk of treatment intolerance. The Cancer and Aging Research Group (CARG) developed a model with various parameters from the geriatric assessment which predicts severe chemotherapy-related toxicity in older patients with cancer.[9,34] The CARG tool unfortunately has a poor performance in Dutch older patients treated with chemotherapy, possibly due to differences in patient populations and treatment regimens between the Netherlands and the United States,[35] where the tool was developed.[36] Validation of the tool in other cohorts with various tumour types yielded similar results.[37-39] Future studies should therefore focus on developing new - or updating existing - toxicity prediction tools to support the decision making in older patients requiring fluoropyrimidine-based chemotherapy.

When specifically considering fluoropyrimidine-related toxicity, we found a relatively low rate of grade 3-5 toxicity (14%), particularly in patients receiving monotherapy and in

non-frail patients. The toxicity rate was similar in Alpe2U participants aged <70 years (12%). Two non-randomized trials of Feliu and colleagues found similar toxicity rates in Spanish older patients with metastatic colorectal cancer treated with first-line capecitabine monotherapy (N=51, 82% WHO 0-1, grade 3-5 toxicity 12%)[40] or first-line capecitabine combined with oxaliplatin (N=68, 98% WHO 0-1, grade 3-5 toxicity 28%.[41] In contrast, other randomized trials[29,42-45] and a real-word study[46] designed to investigate fluoropyrimidine monotherapy in older patients found higher rates of grade 3-5 toxicity (ranging from 22-62%), dose reduction (range 26-57%) and discontinuation due to toxicity (range 7-28%). Other studies investigating capecitabine-based combination therapy also found higher rates of grade 3-5 toxicity (range 43-66%), dose reduction (range 40-59%) and toxicity-related discontinuation (range 16-43%).[47-50]

This discrepancy in findings may be due to patient selection, as our study population consisted of relatively fit older patients, which was expected as a WHO £ 2 was inclusion criteria of the Alpe2U study and was underscored by an impaired G8 score in only half of the population, while other studies recruited a larger proportion of patients with a worse performance status or impaired G8 score. According to the protocol, study participants without the 4 tested *DPYD* variant alleles or pretreatment elevated serum uracil concentrations had to be treated with standard dosage of chemotherapy. As a result, frailer patients in whom the treating physician preferred upfront dose reduction may have been underrepresented in our study. Moreover, even though previous studies have shown that severe toxicity, especially hematological toxicity, mainly occurs within the first two chemotherapy cycles,[51,52] a subset of patients might have developed toxicity after the second cycle.[53,54] One important reason for only monitoring toxicity in the first two treatment cycles was that treatment tolerability will not yet be influenced by disease progression and disease-related clinical deterioration. The relatively low rate of early-onset severe toxicity might suggest that fluoropyrimidine-based treatment was generally well tolerated in our study population. Yet, around half of the patients who discontinued treatment did this due to grade 1-2 toxicity, suggesting that low-grade toxicity might be clinically relevant in older patients and should be considered when studying treatment tolerability.

Initially, one may assume that older patients have a higher incidence of toxicity due to potential differences in pharmacokinetics (PK), resulting in increased exposure to 5-FU and ultimately leading to a greater risk of severe toxicity. However, very limited research has been performed comparing exposure to fluoropyrimidines in older patients, with inconclusive results.[55] Two comparative studies of 5-FU and capecitabine PK parameters between patients aged <65 years and ≥65 years and <75 years ≥75 years did not reveal any significant differences, respectively.[56,57] These findings suggest that severe toxicity in older patients is rather multifactorial, with factors such as frailty possibly contributing more than PK. A comparison of PK parameters between frail and non-frail individuals would be of interest to assess whether there is a pharmacological explanation for the higher risk of severe toxicity in frail patients.

In frail patients with multiple impaired geriatric domains, toxicity risk could be reduced by adapting treatment plans, for example by performing upfront dose reduction or prescribing less toxic chemotherapy regimens. Several trials showed that upfront dose

reduction of chemotherapy in older patients with frailty resulted in less toxicity, dose reductions or treatment discontinuation and an increased quality of life, without compromising survival.[29,58,59] On the other hand, non-frail patients without any impaired geriatric domains predominantly had a good treatment tolerability, suggesting that these patients might be safely treated with fluoropyrimidine-based chemotherapy. Yet, we did not gather information on the effect of fluoropyrimidine-based chemotherapy on quality of life and functional decline. A previous cohort study investigating older patients receiving chemotherapy showed that, even in older patients without any geriatric deficits, 60% either had a declined quality of life or physical functioning or died one year after chemotherapy initiation, irrespective of the occurrence of grade 3-5 toxicity.[6] Thus, even in non-frail older patients without geriatric deficits, clinicians should carefully weigh the benefits of chemotherapy against the risk of deteriorated quality of life and physical functioning, especially in a palliative setting. By identifying frailty in a standardized way, this will become easier for treating clinicians.

The uniqueness of this study lies in its multicenter prospective design, the composite endpoint that combines the most relevant outcomes for older patients treated with chemotherapy and the relatively large sample size. This study addresses a very large but frequently understudied population. While it is important to note that very frail older patients may have been underrepresented, our cohort is more representative of real-world clinical practice compared to pivotal trials and the results can be extrapolated to a large proportion of older patients treated with fluoropyrimidine-based chemotherapy.

The findings should be also interpreted in the context of its limitations. Inclusion of all older patients receiving fluoropyrimidine-based chemotherapy led to a heterogenous study population with various tumour types and treatment regimens, potentially influencing toxicity rates. However, we took into account this heterogeneity by adjusting for these confounders in the analyses. Additionally, a subset of patients did not undergo a baseline geriatric assessment and the results suggests that frail patients were more likely to have a missing assessment. To solve this issue, multiple imputation was employed to account for individuals with missing geriatric questionnaires. In addition, a sensitivity analysis was performed in which patients with imputed scores were excluded to assess the influence of the imputed questionnaires. Results of this analysis were comparable to the models in which missing scores were imputed. This suggests that imputation of the patients without GA did not significantly influence the outcome. Third, due to a relatively low event rate of poor tolerability, the statistical power of the univariable logistic regression model may be modest. Last, we did not gather data on the social domain, which is considered an important geriatric domain, or on comorbidity.

Conclusion

Poor treatment tolerability during the first two cycles occurred in 31% of patients, and this percentage increased with the accumulation of impaired geriatric domains. Deficits in three or four geriatric domains and receiving combination chemotherapy were predictors of poor treatment tolerability, highlighting the importance of a geriatric assessment before fluoropyrimidine initiation to estimate risk of treatment intolerance.

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Supplemental - Chapter 8

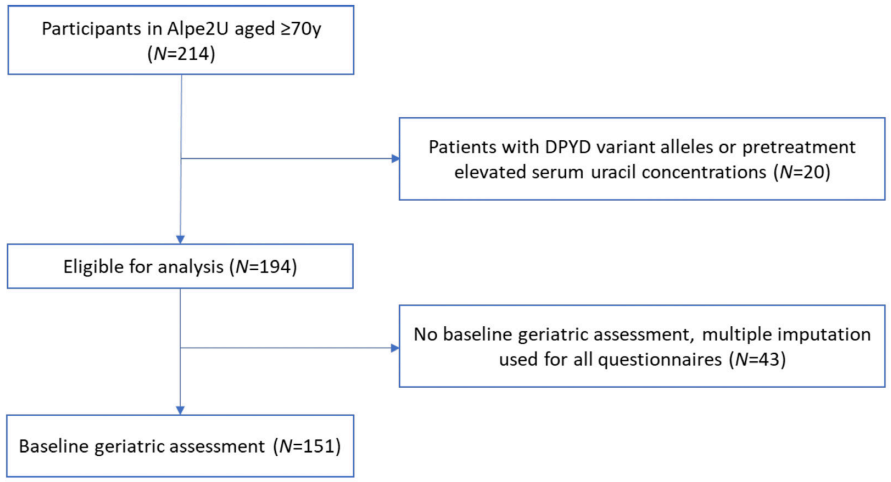

Figure S1: Flow diagram of included patients

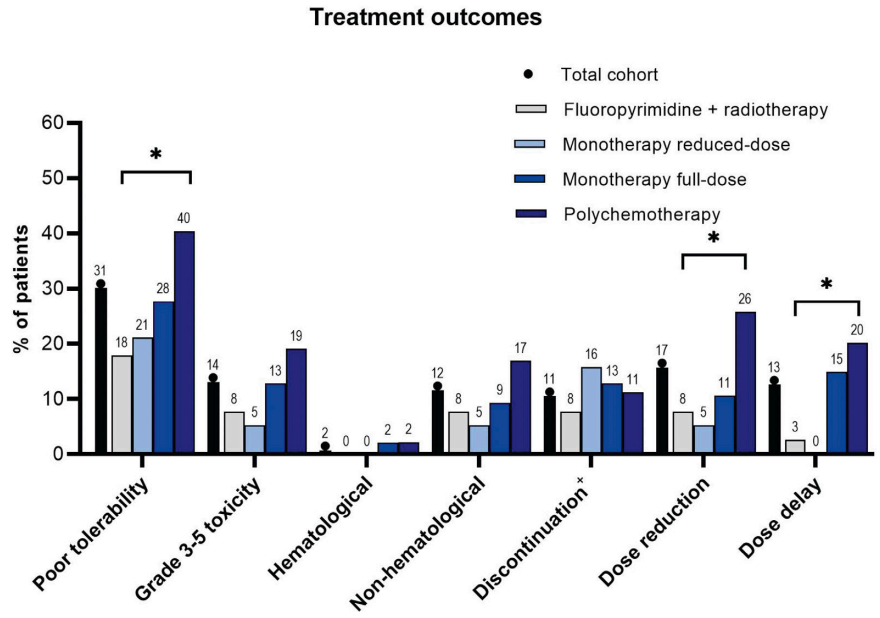

Figure S2: Treatment outcomes in the first two cycles of fluoropyrimidine, stratified for treatment dosage and type (reduced-dose fluoropyrimidine monotherapy (<1900 mg/m² per day) (N=19), full-dose fluoropyrimidine monotherapy (≥1900 mg/m² per day) (N=47), fluoropyrimidine with concurrent radiotherapy (with or without mitomycin) (N=39) and fluoropyrimidine combined with other chemotherapy agents (N=89)). Haematological and non-haematological toxicity represent only grade 3-5 toxicity. *Represent a significant p-value using chi-square test between the four treatment groups. Rates of poor tolerability (P=0.047), dose reduction (P=0.013) and dose delay (P=0.014) significantly differed between the four treatment groups. *Discontinuation represents the percentage of patients who discontinued due to toxicity within the first two treatment cycles. Of these patients, 47% stopped due to grade 1-2 toxicity and 53% due to grade 3-4 toxicity.

Table S1: Associations between baseline characteristics and a missing geriatric assessment

Variable	Categories	OR (95% CI)	*p*-value
Age	70-74 years	Ref	
	75-79 years	4.68 (1.98 - 11.04)	.000
	≥80 years	2.60 (0.97 - 7.02)	.058
Sex	Male	Ref	
	Female	1.81 (0.92 - 3.59)	.088
Tumour type	Colorectal	Ref	
	Oesophageal	4.24 (1.38-13.04)	.012
	Gastric	0.77 (0.21 - 2.84)	.690
	Breast	2.83 (0.99 - 8.08)	.052
	Other	1.94 (0.67 - 5.61)	.221
Disease stage	Local	Ref	
	Locally Advanced	1.09 (0.38 - 3.14)	.878
	Metastatic	1.86 (0.73 - 4.67)	.191
Concurrent radiotherapy	No	Ref	
	Yes	1.45 (0.65-3.21)	.364
Additional chemotherapy	No	Ref	
	Yes	0.66 (0.34 - 1.32)	.243
Targeted therapy	No	Ref	
	Yes	2.13 (0.93 - 4.86)	.073
WHO performance status	0	Ref	
	1	2.49 (1.19 - 5.24)	.016
	2	1.43 (0.36 - 5.72)	.614
	Unknown	2.86 (0.48 - 17.11)	.250
BMI	19-25	Ref	
	<19	4.89 (1.00-23.86)	.050
	25-30	0.86 (0.39 - 1.87)	.695
	30+	1.28 (0.47 - 3.51)	.627

Associations between baseline characteristics and having a missing geriatric assessment were analyzed with univariate logistic regression models. *Abbreviations*: CI; confidence interval, OR; odds ratio.

Table S2: Toxicity outcomes

	Grade 3-5 toxicity, N (%)
Non-hematological	
Diarrhea	8 (4.1)
Nausea/vomiting	4 (2.0)
Fatigue	0 (0)
Sensory neuropathy	3 (1.5)
Anorexia	0 (0)
Mucositis	0 (0)
Cardiac symptoms	1 (0.5)
Malaise	2 (1.0)
Abdominal pain	0 (0)
Infection (without neutropenia)	0 (0)
Hand-food syndrome	2 (1.0)
Constipation	0 (0)
Hemorrhage	2 (1.0)
AKI/dehydration	2 (1.0)
Syncope	0 (0)
Electrolyte disorder	1 (0.5)
Hematological	
Anemia	1 (0.5)
Febrile neutropenia	2 (1.0)
Neutropenia	1 (0.5)
Purpura	0 (0)
Total	**29 (14.6)**

Toxicity was recorded during the first two treatment cycles. *Abbreviations*: AKI; acute kidney injury

Table S3: Sensitivity analysis: Associations between characteristics and poor treatment tolerability within the first two treatment cycles, only including patients with a baseline geriatric assessment (N=151)

Variable	Category	Univariable (OR, 95% CI)	*p*-value	Multivariable (OR, 95% CI)	*p*-value
Age	70-74 years	Ref		Ref	
	75-79 years	1.03 (0.58-2.91)	.517	1.13 (0.43-2.43)	.804
	≥80 years	0.47 (0.17-1.28)	.139	0.60 (0.19-1.88)	.376
Sex	Male	Ref		Ref	
	Female	1.68 (0.82 -3.40)	.154	1.74 (0.76-3.99)	.190
Disease stage	Local	Ref		Ref	
	Locally Advanced	1.63 (0.60 - 4.45)	.337	1.69 (0.55-5.27)	.364
	Metastatic	1.45 (0.57- 3.70)	.442	1.24 (0.42-3.66)	.703
Radiotherapy	No	Ref		Ref	
	Yes	0.33 (0.11 - 1.01)	.051	0.50 (0.13-1.92)	.499
Additional chemotherapy	No	Ref		Ref	
	Yes	2.27(1.08 - 4.75)	.030	2.49 (1.014-6.12)	.047
Targeted therapy	No	Ref			
	Yes	1.26 (0.47 - 3.36)	.649		
WHO status	0	Ref			
	1	0.72 (0.33 - 1.58)	.416		
	2	2.33 (0.68 - 7.97)	.176		
Geriatric-8	Not impaired	Ref			
	Impaired	1.86 (0.87 - 3.97)	.110		
	Incomplete (N=11)	2.04 (0.52 - 7.98)	.305		
6-CIT	Not impaired	Ref			
	Impaired	0.68 (0.21 - 2.19)	.514		
	Incomplete (N=5)	1.58 (0.25 - 9.82)	.625		
ADL	Independent	Ref			
	Dependent	1.65 (0.27- 10.24)	.590		
IADL	Independent	Ref		Ref	
	Dependent	2.08 (0.69- 4.63)	.074	1.69 (0.52-5.46)	.381
	Incomplete (N=2)	2.97 (0.18-48.94)	.447	7.63 (0.39-147.89)	.179
PHQ-2*	Not impaired	Ref			
	Impaired	0.96 (0.28 - 3.24)	.960		
Polypharmacy	No	Ref			
	Yes	0.80 (0.39 - 1.65)	.540		
	Not asked (N=8)	1.32 (0.29 - 6.07)	.721		
Frailty domains	0	Ref		Ref	
	1	3.28 (1.09-9.90)	.035	4.14 (1.23-13.86)	.021
	2	2.27 (0.71-7.33)	.169	2.44 (0.63-9.45)	.198
	3-4	5.17 (1.46-18.28)	.011	5.38 (0.99-29.29)	.051

Poor treatment tolerability occurred in 44 patients (29%). Patients without a baseline geriatric assessment were excluded for this sensitivity analysis. *Abbreviations*: ADL: Activities of Daily Living, IADL: Instrumental Activities of Daily Living, CI; confidence interval, OR; odds ratio, PHQ-2: Patient Health Questionnaire. *Only one PHQ-2 questionnaire was incomplete, therefore the odds ratio is not shown in the table.

Part III – Bioanalysis in support of fluoropyrimidine-based chemotherapy

Chapter 9

A review of the bioanalytical methods for the quantitative determination of capecitabine and its metabolites in biological matrices

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Abstract:

Capecitabine is an anticancer agent and is the oral prodrug of 5-fluorouracil (5-FU). In this study, an ultra-high performance liquid chromatography coupled to turbo ion spray tandem mass spectrometry (UPLC-MS/MS) method was developed and validated to quantify capecitabine and its metabolites including 5'-deoxy-5-fluorocytidine (5'-dFCR), 5'-deoxy-5-fluorouridine (5'-dFUR), 5-FU, and fluoro-β-alanine (FBAL) in lithium heparinized human plasma. Analytes were extracted by protein precipitation, chromatographically separated by Acquity UPLC HSS T3 column with gradient elution, and analyzed with a tandem mass spectrometer equipped with an electrospray ionization source. Capecitabine and 5'-dFCR were quantified in positive ion mode and 5'-dFUR, 5-FU, and FBAL were quantified in negative ion mode. The total chromatographic run time was 9 min. Stable isotopically labeled internal standards were used for all analytes. The assay was validated over the range from 25.0 to 2,500 ng/mL for capecitabine, 10.0 to 1,000 ng/mL for 5'-dFCR, 5'-dFUR, and 5-FU and 50 to 5,000 ng/mL for FBAL in human plasma. Validation results have shown the developed assay allows for reliable quantitative analysis of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL in plasma samples.

Introduction:

Capecitabine (Xeloda®) is an oral fluoropyrimidine-based chemotherapeutic agent indicated for the treatment of several malignancies including colon cancer, colorectal cancer and breast cancer.[1] Additionally, capecitabine is used for the treatment of gastric, pancreatic and head and neck cancer.[2] Capecitabine is an inactive prodrug that is rapidly absorbed from the gastrointestinal tract and enzymatically converted into the active agent 5-fluorouracil (5-FU) through three metabolic steps.[2-4] Thereupon, 5-FU is inactivated by dihydropyrimidine dehydrogenase (DPD) mainly in the liver to 5,6-dihydro-5-fluorouracil (5-FUH2) which is further metabolized into α-fluoro-β-ureidopropionic (FUPA) and α-fluoro-β-alanine (FBAL). Eventually, three metabolites are formed intracellularly which are ultimately responsible for the anticancer effect of capecitabine (complete metabolism is depicted in Figure 1).

Pharmacokinetic parameters of capecitabine and its metabolites are characterized by substantial inter-individual variability which is likely caused by the variability in activity of enzymes involved in the metabolism of capecitabine.[3,5] Therefore, bioanalytical assays for the quantitative determination and therapeutic drug monitoring of capecitabine and its metabolites are imperative and could improve the safety and efficacy of treatment with capecitabine and are essential in support of clinical pharmacological studies with capecitabine and 5-FU.[3,6] The simultaneous analysis of capecitabine and its metabolites can be challenging due to broad concentration ranges and varying polarity. Capecitabine has a long carbon chain and shows lipophilicity. During biotransformation the polarity of the metabolites gradually increase and become more hydrophilic.[7] Plasma concentrations of the metabolites 5'-deoxy-5-fluorocytidine (5'-dFCR) and 5'-deoxy-5-fluorouridine (5'-dFUR) are similar to the plasma concentration of capecitabine. However, plasma concentrations of 5-FU and its converted products are significantly lower (Figure 2).[8] Large differences in retention time can be expected due the differences in polarity between capecitabine and its metabolites, which could pose a challenge for simultaneous analysis particularly using liquid chromatography (LC).[3,9] Furthermore, high selectivity is pivotal using mass spectrometry (MS) detection as the molecular weights of 5'-dFCR and 5'-dFUR differ by only one mass unit and therefore should be separated during chromatography. In addition, the high hydrophilicity of 5-FU, 5-FUH2, FUPA and FBAL hampers its isolation from a biological matrix.

Several assays have been developed for the quantitative determination of capecitabine and its metabolites. This has shown to be troublesome due to the aforementioned challenges. Thus, the aim of this review is to give an overview and discuss published methods for the bioanalysis of capecitabine and its metabolites. Bioanalytical assays for the determination of capecitabine and its metabolites are based on a chromatographic separation method combined with either MS or ultraviolet (UV) detection, preceded by a sample pretreatment procedure. In this review the three components of the bioanalytical method (sample pre-treatment, separation and detection) will be discussed separately. Moreover, the strengths and weaknesses of the different methods will be discussed. Table 1 gives an overview of the published bioanalytical methods for the determination of capecitabine and its metabolites.

Sample pre-treatment

Generally, biological samples (e.g., plasma, urine and tissues) cannot be analyzed directly and sample pre-treatment is required to remove endogenous compounds such as lipids, proteins and salts, which usually interfere with chromatographic separation of the analytes. Furthermore, using MS detection these compounds can pollute the system and influence the ionization efficiency of the mass spectrometer and therefore the sensitivity of the method. Hence, sample pre-treatment is an important step in the quantitative determination of capecitabine and its metabolites. The most applied techniques to remove interfering endogenous substrates are liquid-liquid extraction (LLE), solid-phase extraction (SPE) and protein precipitation (PP).

Liquid-liquid extraction

LLE is a sample preparation technique based on the solubility of a compound in 2 different immiscible solvents. LLE is cheap and easily executed compared to SPE, but a disadvantage is the difficulty of automatizing LLE, which is readily possible for SPE. LLE is best suited for lipophilic analytes that are extracted from an aqueous matrix to an apolar organic matrix. LLE as sample pre-treatment for capecitabine and its metabolites is possible, however is complicated due to the differences in physiochemical properties between analytes. Which can lead to the extraction of endogenous compounds. Zufia *et al.* (2004) reported the use of a mixture of ethyl acetate and acetonitrile (4:1, v/v) after sample acidification with orthophosphoric acid to simultaneously extract capecitabine, 5'-dFUR, 5-FU and 5-FUH2 from plasma. Whereas the pKa (amine-group) of these compounds lie close to 8, which is the pKa of 5-FU.[10] To ensure that the analytes exist in neutral form during extraction, given that they are weak organic acids, sample acidification was applied. A mean recovery of 90.34% ± 9.48, 90.47% ± 8.95, 95.24% ± 3.96 and 91.5% ± 3.31 were obtained for capecitabine, 5'-dFUR, 5-FU and 5-FUH2, respectively.[11]

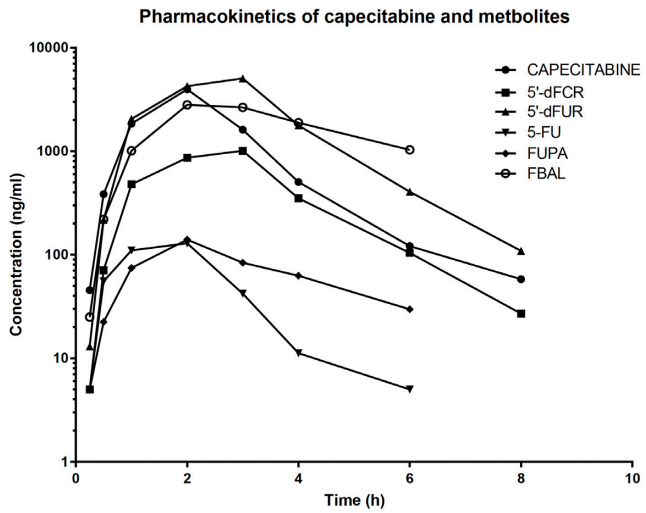

Figure 2: Representative of plasma concentration-time curves of capecitabine 5'-dFCR, 5'-dFUR, 5-FU, FUPA and FBAL in a patient with colorectal cancer treated with 1500 mg capecitabine.. Abbreviations: 5'-dFCR, 5'-deoxy-5-fluorocytidine; 5'-dFUR, 5'-deoxy-5-fluorouridine; 5-FU, 5-fluorouracil; FBAL, α-fluoro-β-alanine; FUPA, α-fluoro-β-ureidopropionic

Table 1: Mass spectrometer settings for the quantification of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU and FBAL in human plasma.

Analyte	Matrix/ Tissue	Species	Sample (µl or mg)	Sample preparation	Detection method	(Internal) Standard	Runtime (min)	Refer-ence
Capecitabine	Plasma	Human	500	On-line SPE	MS	5'-CUDR	24	17
5'-dFCR								
5'-dFUR								
Capecitabine	Plasma	Human	250	LLE	MS	Fluvastatin	8	34
5-FU						$[^{15}N_2]$-5-FU		
Capecitabine	Plasma	Human	500	LLE	UV	Tegafur	30	11
5'-dFUR								
5-FU						5-BU		
5-FUH_2								
Capecitabine	P/L/T	H or M	50	PP	MS	Ex: Capecitabine	12	20
5'-dFCR						Ex: 5'-dFCR		
5'-dFUR						Ex: 5'-dFUR		
5-FU						Ex: 5-FU		
Capecitabine	P/S/B	M or R	-	PP	UV	Ex: Capecitabine	10	18
5'-dFCR						Ex: 5'-dFCR		
5'-dFUR						Ex: 5'-dFUR		
5-FU						Ex: 5-FU		
Capecitabine	Plasma	Human	500	SPE	MS	$[^2H_8]$-capecitabine	14	14
5'-dFCR						3-Methyl-uridine		
5'-dFUR								
5-FU						$^{15}N_2$]-5-FU		
Capecitabine	Plasma	Human	50	LLE	MS	$[^2H11]$-capecitabine	4,5	4
5-FU						$[^{13}C_1,^{15}N_2]$-5-FU		
FBAL				SPE		$[^{13}C_3]$-FBAL		
Capecitabine	Plasma	Human	1000	SPE	UV	Ex: Capecitabine	6,5	16
Capecitabine	Plasma	Human	500	LLE	MS	Carbamazapine	15	9
5'-dFCR						5-CldUrd		
5'-dFUR								
5-FU						$[^{13}C_1,^{15}N_2]$-5-FU		
Capecitabine	Plasma	Human	200	PP	MS	Fludarabine	15	21
5'-dFCR						5-chlorouracil		
5'-dFUR								
5-FU								
5-FUH_2								
5'-dFCR	Plasma	Human	1000	SPE	UV	Ex: 5'-dFCR	30	15
5'-dFUR						Ex: 5'-dFUR		
Capecitabine	Plasma	Human	100	PP	MS	$[^2H_{11}]$-capecitabine	9	6
5'-dFCR						$[^{13}C_1,^{15}N_2]$-5'-dFCR		
5'-dFUR						$[^{13}C_1,^{15}N_2]$-5'-dFUR		

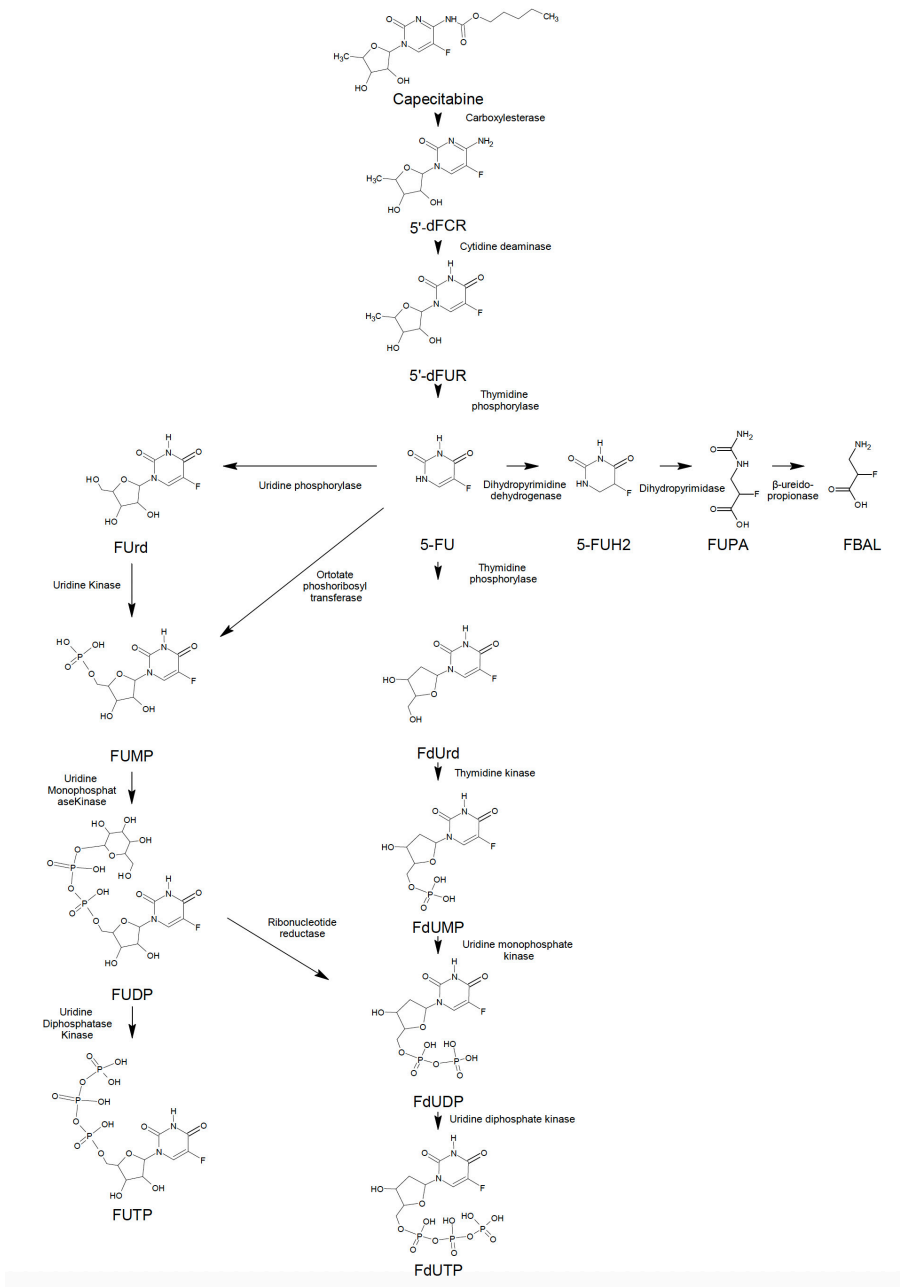

Figure 1: Metabolism of capecitabine. Abbreviations: 5'-dFCR, 5'-deoxy-5-fluorocytidine; 5'-dFUR, 5'-deoxy-5-fluorouridine; 5-FU, 5-fluorouracil; 5-FUH2, 5,6-dihydro-5-fluorouracil; FBAL, α-fluoro-β-alanine; FdUDP, 5-Fluoro-2'-deoxyuridine 5'-diphosphate; FdUMP, 5-fluoro-2'-deoxyuridine 5'-monophosphate; FdUrd, 5-fluoro-2'-deoxyuridine; FdUTP, 5-fluoro-2'-deoxyuridine 5-'triphosphate; FUDP, 5-fluorouridine 5'-diphosphate; FUMP, 5-fluorouridine 5'-monophosphate; FUPA, α-fluoro-β-ureidopropionic; FUrd, 5-fluorouridine; FUTP, 5-fluorouridine 5'-triphosphate

Analyte	Matrix/ Tissue	Species	Sample (µl or mg)	Sample preparation	Detection method	(Internal) Standard	Runtime (min)	Refer-ence
5-FU	Plasma	Human	100	PP	MS	$[^{13}C_1,^{15}N_2]$-5-FU	5	6
5-FUH_2						$[^{13}C_1,^{15}N_2]$-FUH_2		
FUPA						$[^{13}C_3]$-FUPA		
FBAL						$[^{13}C_3]$-FBAL		
Capecitabine	Plasma	Human	1000	LLE	UV	Voriconazole	8	12
Capecitabine	Plasma	Human	100	PP	MS	$[^{2}H_{11}]$-capecitabine	10,5	3
5'-dFCR						$[^{13}C_1,^{15}N_2]$-5'-dFCR		
5'-dFUR						$[^{13}C_1,^{15}N_2]$-5'-dFUR		
5-FU						$[^{13}C_1,^{15}N_2]$-5-FU		
Capecitabine	Serum	Human	100	PP	UV	Gemcitabine	-	19
Capecitabine	Plasma	Human	100	LLE	MS	Fludarabine	5	7
5'-dFCR								
5'-dFUR								
5-FU						5-Chlorouracil		
5-FdUrd						Fludarabine		
5-FUH_2						5-Chlorouracil		

Abbreviations: 5'-dFCR, 5'-deoxy-5-fluorocytidine; 5'-dFUR, 5'-deoxy-5-fluorouridine; 5-BU, 5-Bromouracil; 5-CldUrd, 5-Chloro- 2-deoxyUridine; 5-CUDR, 5-chloro-2'-deoxyuridine; 5-FdUrd, 5-fluoro-2'-deoxyuridin;5-FU, 5-Fluorouracil; 5-FUH2, 5,6-dihydro-5-fluorouracil; B, bile; Ex, external; FBAL, α-fluoro-β-alanine; FUPA, α-fluoro- β-ureidopropionic; H, human; L, liver; LLE, liquid-liquid extraction; M, mouse; MS, mass spectrometry; PP, protein precipitation; R, rabbit; S, serum; SPE, solid phase extraction; T, tumor; UV, ultra violet.

Interestingly, in Piorkowska *et al.* (2014) a similar extraction method is reported using a mixture of ethyl acetate and acetonitrile (4:1, v/v) but without sample acidification. Acidification was not deemed necessary because the assay was developed to only determine capecitabine concentrations (no metabolites) and could therefore be simplified. This allowed for a simplification (leaving out the acidification) of the extraction and a reduction of the extraction solvent volume.[12] A drawback is that the obtained recovery is lower compared to the method including acidification (67.6-71.2% vs. 90.3%). In Licea-Perez *et al.* (2009) a method was reported in which capecitabine and 5-FU were extracted and separated from FBAL by LLE using a mixture ethyl acetate and acetonitrile (8:3, v/v). The significant differences in polarity between capecitabine, 5-FU and FBAL lead to difficulties in simultaneous extraction of all analytes and poor retention of 5-FU and FBAL in reversed phase chromatography. A derivatization step with dansyl chloride was introduced to overcome these difficulties.[4] After extraction the organic phase, containing 5-FU and capecitabine, were transferred to a 96-well plate and dansyl-chloride in acetonitrile and sodium bicarbonate were added and vortex mixed for 3 minutes and incubated for 30 min. Further purification of capecitabine and 5-FU was achieved by a second extraction with tertiary-methyl-butyl ether (tBME). The aqueous phase, containing FBAL, was incubated with dansyl-chloride in acetone and sodium bicarbonate. Further purification was achieved by utilizing SPE. A clear drawback of this method is that an extra step is introduced which complicates the sample pretreatment and prolongs the duration of sample pretreatment.[4] Wang *et al.* (2019) reported that they tested several separation methods (SPE and PP) and extraction fluids, but that the use of a one-step

liquid-liquid extraction of capecitabine, 5-'dFCR, 5'-dFUR, 5-fluoro-2'-deoxyuridine (FdUrd), 5-FU and 5-FUH2 by ethyl acetate and isopropanol (19:1, v/v) gave optimal extraction recovery (59.3% to 90.2%).[7]

Solid-phase extraction

SPE is a commonly used sample preparation technique by which analytes that are dissolved in a liquid matrix are separated from other compounds based on chemical and physical characteristics. Usually, the analyte is retained on a SPE cartridge, after which the interfering compounds are removed by a washing step. Thereafter, the analyte is eluted from the solid phase cartridge using an elution solvent. SPE can be applied in a wide range of analyses, due to the availability of many different types of cartridges and solvents. Nevertheless, the differences in polarity between capecitabine and its metabolites make the choice of cartridge and eluent challenging.[13] Salvador *et al.* (2006) describes a SPE method using an Atoll XWP extraction cartridge which allowed for simultaneous extraction of capecitabine and metabolites (5'-dFCR, 5'-dFUR and 5-FU) with a high recovery (>90%), but not the smaller more polar metabolites of 5-FU. The Atoll XWP cartridge consists out of polystyrene divinylbenzene, which shows similar chemistry to C18 columns.[14] Licea-Perez *et al.* (2009) reported a SPE method with the use of an Oasis MAX 96 column for the extraction of FBAL out of the aqueous phase after an initial LLE step.[4] Buchner *et al.* (2013) and Farkouh *et al.* (2010) applied Oasis HLB cartridges for the separation of the analytes from the matrix.[15,16] The Oasis HLB is an all-purpose polymeric reversed-phased column. A practical advantage of this cartridge over others is that these columns maintain their interaction capacities, without the need to keep the phases moisturized. Furthermore, the Oasis HLB column also can retain polar analytes while having both hydrophilic and lipophilic properties. A disadvantage of SPE is that it is labor-intensive and costly compared to LLE and PP. This is unfavorable for assays intended for use in a routine clinical setting (e.g. therapeutic drug monitoring). A way to reduce the workload of SPE is by utilizing on-line SPE, which has been described by Xu *et al.* (2003) as a sample pre-treatment method.[17] On-line sampling brings sample handling back to a minimum, improving the sample throughput and reproducibility. The major advantage of SPE is that it often results in a clean final extract compared to LLE and PP, due to the SPE cartridges being very efficient for the removal of interfering endogenous substances.

Protein precipitation

Due to its simplicity and good recovery rates PP is frequently chosen as sample pre-treatment in bioanalytical procedures. It involves the addition of a protein precipitating solvent, subsequent homogenizing and centrifugation, after which, the clear supernatant with analytes can be used further for analysis. Generally, methanol, acetonitrile or a mixture with either of these organic solvents is used for PP. For this review mixtures of methanol and water, trichloroacetic acid (TCA) or mixtures containing acetonitrile were found for PP. Dhananjeyan *et al.* (2007), Deng *et al.* (2015) and Thorat *et al.* (2018) used methanol as the precipitating solvent.[3,18,19] Dhananjeyan *et al.* (2007) reported a single-step PP method using a mixture of methanol-water (50:50, v/v), by which recoveries of >85% were obtained for capecitabine, 5'-dFCR, 5'-dFUR and 5-FU from biological matrices which include mouse plasma, mouse serum and rabbit bile, without having to make concessions on the assay's selectivity or specificity.[18] Guichard *et al.* (2005) reported two separate single step PP methods for the extraction of capecitabine, 5-'dFCR, 5'-FUR and

5-FU from human and mouse plasma, human tumor tissue and mouse liver tissue. Acetonitrile was used to extract the analytes from plasma. It was shown that mouse plasma contains high concentrations of carboxylesterase, which converts capecitabine to 5'-dFCR. To prevent this conversion samples were thawed on ice. A mixture of ammonium acetate: acetonitrile (1:3, v/v) was used to extract the analytes from the tissues.[20] Thorat *et al.* (2018) observed a higher efficiency of extraction with methanol then with acetonitrile or a mixture of acetonitrile and methanol.[19] Vainchtein *et al.* (2010) tested several sample pre-treatment methods including LLE and PP but high degradation of FUH2 was found when evaporating the supernatant. To prevent an evaporation step and to efficiently precipitate the plasma proteins, 10% (v/v) trichloroacetic acid (TCA) in water was finally used.[21] Deenen *et al.* (2013) developed two separate assays, one assay for the quantitative determination of capecitabine, 5'-dFCR and 5'-dFUR and another assay for 5-FU, 5-FUH2, FUPA and FBAL (Deenen, 2013). For sample pre-treatment the same PP procedure as described by Vainchtein *et al.* (2010) was considered and tested, but not deemed suitable due to the acid environment catalyzing the conversion of ^{2}H11-capecitabine (stable isotope) into 5'-dFUR. Instead, a pH-neutral solution of methanol and acetonitrile (1:1, v/v) was selected as precipitating solvent. For the 5-FU assay, proteins were precipitated with acetonitrile in an acetonitrile-plasma ratio of 4:1 (v/v).[6,21] During pre-validation experiments of 5-FU assay it was seen that the sensitivity of 5-FUH2 significantly increased using a HybridSPE-phospholipid technology (PPT) cartridge filter, which removes phospholipids from the extract. This increased the 5-FUH2 signal significantly, which led to a gain of sensitivity of factor 50. Therefore, 5-FUH2 was quantified after filtration, whereas 5-FU, FUPA and FBAL were quantified without filtration.[6] A disadvantage of PP is that it yields less clean samples compared to SPE and LLE, which can cause matrix effect (ionization of co-eluting components in the matrix) during detection with MS. This was shown by Wang *et al.* whom have tested both LLE and PP and reported that LLE could remove interfering endogenous components at the greatest extent and subsequently showed less of a matrix effect compared to PP.[7] Nevertheless, PP can still be an excellent option for sample pre-treatment of capecitabine and its metabolites. If a very sensitive assay and low lower limit of quantification is required, clean extracts and therefore SPE or LLE are preferred. On the contrary, if adequate sensitivity can be reached by using PP as sample pre-treatment, PP is preferred due to easy applicability and low cost.

Microextractions

Although LLE, SPE are widely used, these methods can be laborious or consume a great amount of organic solvent. Currently, more attention is being paid to the development of sample pre-treatment methods that are more efficient and environmentally friendly.[22,23] A novel sample pre-treatment method that has gained popularity is microextraction. Extraction techniques are classified as a microextraction if the volume of the extracting phase is very small in relation to the volume of the sample.[24] Based on the extraction phase a microextraction method can be classified as solid-phase microextraction (SPME) or liquid-phase microextraction (LPME). The extraction phase in SPME can be either a tube design or a fiber design. The tube or the fiber are exposed to the sample and the analytes are removed from matrix. The difference between SPE is that the objective of SPME is never that of exhaustive extraction, in contrary to SPE.[25] LPME usually makes use of a hollow fiber (HF) which is dipped into a water immiscible solvent, such as 1-octanol. This solvent fills the pores of the HF, after which the inner lumen of the hollow fiber is

filled with an acceptor solution. The HF is placed in the sample and extracts the analytes from the sample.[24,26] Recently, it was shown that by modifying HF with sorbents that they can be used for solid-phase microextraction, thereby combining SPME and LPME which is named solid-liquid-phase microextraction (SLPME).[27] Forough *et al.* reported the development of a HF-SLPME method for the simultaneous extraction of capecitabine and 5-FU. In this method HFs of which the lumen was embedded with silver nano-particles were introduced to strengthen the absorption capacity and provide and extra way for solute transport of the LPME. Therefore, combining both LPME and SPME. In this case mobile composition of matter No. 41 (MCM-41) was used due to its mesoporous characteristics and anchored to silver nano-particles due to their specific interaction with selected organic functional groups. By using this method both capecitabine and 5-FU could be extracted from the plasma with a high recovery and therefore could be considered as sample pre-treatment for capecitabine and its metabolites.[28]

Chromatography

Liquid chromatography (LC) is the main separation method utilized for the bio-analysis of capecitabine and its metabolites. Separation of analytes from structurally related endogenous compounds is an important step since these can interfere in analyte detection.[13] Most of the assays discussed in this review use high pressure liquid chromatography (HPLC), which is the most common type of chromatography. Licea-Perez *et al.* were the only ones to report the use of ultra-high pressure liquid chromatography (UHPLC). UHPLC utilizes smaller particles in column packing and a higher pressure which reduces the run time and improves the resolution. A disadvantage of the higher pressure utilized in UHPLC is the reduced column life due to the higher pressure.[29] An important difference between assays utilizing UV detection and MS detection is that the MS assays use the chromatography step primarily to separate the analytes from any matrix components. Whereas most LC-UV assays use chromatography to separate the analytes, internal standards (ISs) or potential metabolites. Due to the selectivity of the MS, co-eluting peaks do not necessarily cause interference with the detection of the analytes.[13] Yet, co-eluting endogenous components in the matrix can affect the quantitation of the analytes due to matrix effect. Therefore, if matrix effect occurs chromatographic conditions or run time can be adjusted to enhance the chromatographic separation between the component causing the matrix effect and the analyte.[30,31] While capecitabine is a lipophilic compound (Log P=0.84, as calculated by Benet *et al.* (2011) using the method of Leo[32,33]), the metabolites are more hydrophilic. These compounds elute rapidly from reversed-phase columns even with mobile phases containing a low percentage of organic content, which complicates the development of a chromatographic method to simultaneously analyze these compounds.[14,17] Normal phase or ion-exchange chromatography are not easy applicable with MS due to incompatible organic solvents and modifiers being used here. Moreover, additives to the mobile phase such as phosphates and strong acids as trifluoroacetic acid (TFA) are undesirable because they contaminate and reduce the MS signal significantly.[13] Furthermore, to ensure stable retention times on a HPLC column the pH of the mobile phase should preferably be approximately 2 units above or below the pKa of the analytes to assure that they are >99% unionized. The most common components of the mobile

phase for capecitabine and its metabolites are acetonitrile, methanol, formic acid and ammonium acetate (see Table 1). Moreover, the majority of the chromatographic runs were carried out in a gradient mode allowing for simultaneous analysis of capecitabine and its more polar metabolites. Several stationary phases have been exploited in the assays described in this review (see Table 1). Most assays employed a reversed phase column with C18 modified material. Siethoff *et al.* (2004) describe the use of a column switching method. Two different columns, possessing different polarities were used to analyze capecitabine and 5-FU simultaneously.[34] A Hypercarb (porous grafitic carbon) column to simultaneously quantify capecitabine, 5′-dFCR, 5′-dFUR, 5-FU and 5-FUH2 was used by Vainchtein *et al.* (2010). This column showed unique separation properties and can retain a wide range of small polar and non-polar compounds, allowing for the simultaneous analysis of capecitabine and subsequently formed more polar metabolites.[21] But over time and upon frequent application of the assay it was seen, however, that the signal intensity decreased, which led to significant loss of sensitivity. Therefore, a new assay was developed utilizing a different type of chromatography. Two different assays were developed due to differences in polarity of capecitabine, 5′-dFCR and 5′-dFUR on the one hand compared to 5-FU and its more polar metabolites on the other hand. Standard reversed phase chromatography was used for capecitabine, 5′-dFCR and 5′-dFUR. Hydrophilic interaction liquid chromatography was utilized for 5-FU. 5-FUH2, FUPA and FBAL due to the more polar nature of these analytes.[6,21] Run times of the described assays vary between approximately 5 and 30 min, depending on the analytes being analyzed. Several assays described in this review have shown that run time higher than 10 min are not necessary (See Table1). Which allows for high-throughput analysis of capecitabine in a routine clinical setting.

Thin layer chromatography

Interestingly, Thorat *et al.* (2010) reported the use of high-performance thin layer chromatography (HPTLC) using a TLC plate pre-coated with silica gel to rapidly and easily determine capecitabine concentrations.[19] The TLC plates were analyzed using a densitrometric scanner and the lower limit of quantification was 250 ng/ml. The accuracy ranged from 96.0% to 102.7% and intraday precision and inter-day precision were 12.6% and 13.9% or less at each quality control level, respectively. The method was developed and applied for therapeutic drug monitoring purposes in serum for capecitabine alone and in combination with oxaliplatin. It was shown that developed HPTLC method was sufficiently sensitive and accurate for this purpose. TLC has the advantage of being cheaper, easier and quicker, due to being able to analyze multiple samples simultaneously compared to other established analytical methods.[19] A major disadvantage of TLC is that it cannot be coupled easily with other techniques such as mass spectrometry which leads to a higher detection limit compared to other assays utilizing other techniques. Furthermore, TLC plates have a limited length which limits the length of separation, TLC is an open system which can be influenced by exogenous factors such as temperature and humidity. It can be concluded that LC is the preferred separation method compared with TLC. Yet, the described HPTLC method by Thorat *et al.* can still be a viable option if proper equipment for LC is not available or a simple and fast method for the quantification of capecitabine is needed.

Detection

MS is a sensitive and selective detector which is suitable for detection of a large range of compounds. Triple quadrupole (TQ) MS is the most applied and first choice in the quantitative analysis of capecitabine and its metabolites.[13] To be able to detect a compound with MS the analyte needs to be, negatively or positively ionized. The most applied technique for the ionization of capecitabine and its metabolites is by electrospray ionization (ESI). In Licea-Perez *et al.* (2009) a variant of ESI is described in which heated gas is used for desolvation of the eluent, by a turbo ion spray (TIS).[4] Another form of ionization is atmospheric pressure chemical ionization (APCI) which is described by Montagne *et al.*, using both positive and negative modes.[9] Both ESI and APCI were considered but best signals were acquired with APCI, which also showed less of a matrix effect and decreased chemical background noise.[9] Furthermore, it was shown by Deenen *et al.* that 5-FUH2 is influenced by the presence of phospholipids in the matrix, which reduced the sensitivity of 5-FUH2 significantly.[6] It is known that phospholipids may influence the signal by causing matrix effect by either signal enhancement or suppression.[27,35] Therefore, the presence of phospholipids should be kept to a minimum or separated from the influenced analytes during chromatography. After ionization the ions enter the mass spectrometer and ion selection and detection take place. Detection can be executed by means of selecting the molecular ion in selective ion monitoring (SIM) or multiple reaction monitoring (MRM) modes. Operating in MRM allows for the identification and detection of the analyte by means of both the parent ion and typical fragment product ion for TQ platforms. A higher sensitivity and selectivity is reached by MRM in comparison to SIM.[13] Fragmentation of capecitabine and its metabolites was described extensively by Deenen *et al.* (2013) and Vainchtein *et al.*[6,21] (depicted in Table 2).

The most abundant product ion of capecitabine is a product ion with a *m/z* of 174 in Vainchtein *et al.* (2010) and 130 in Deenen *et al* (2013). This difference in *m/z* of 44 can be explained by the loss of a sugar moiety and a pentane chain as described in Vainchtein *et al.* (2010) instead of the pentanoic acid described in Deenen *et al* (2013). The reported fragmentation of 5'-dFUR, 5-FU and 5-FUH2 was similar in both articles. The *m/z* of the product ion of 5'-dFUR was 108, corresponding with the loss of a sugar moiety and the fluorine atom. The product ions of 5-FU and 5-FUH2 had a *m/z* of 42 and 83 which corresponds with the loss of a formamide moiety or fluoroethane moiety.[6,21] Additionally, the fragmentation of FUPA and FBAL were described by Deenen *et al.* (2013). The most abundant product ion of FUPA and FBAL had a *m/z* of 106 and 86, respectively. This corresponds with the loss of a formamide moiety for FUPA and a fluoroethane moiety for FBAL. Deng *et al.* (2015) reported the use of a polarity switching method between ESI- and ESI+ in a single run.[3] This method was chosen due to the chemical noise found when analyzing 5'-dFCR, 5'-dFUR and 5-FU in ESI+ mode compared to ESI-. Capecitabine was analyzed under ESI+.[3] Several papers referred to in this review have applied UV detection as detection method. Due to different absorption maxima of capecitabine and its metabolites the setting of the wavelength must be well considered. Capecitabine exhibits UV absorption maxima at approximately 214, 241 and 305 nm. While the exhibited UV absorption maxima of 5'-dFCR and 5-FUH2 is at 285 nm and 205 nm, respectively. 5-FU and 5'-dFUR exhibit a maximum at approximately 205 nm and 266 nm. Zufia *et al.* (2004) described a method in which multiple wavelengths were monitored

to detect capecitabine, 5'-dFUR, 5-FU and 5-FUH2. Dhanajeyan *et al.* (2007) detected capecitabine, 5'-dFCR, 5'-dFUR and 5-FU at a wavelength of 254 nm, at this wavelength capecitabine and the measured metabolites showed near equal absorption. Piorkowska *et al.* (2014) and Farkouh *et al.* (2014) both described an assay which only quantifies capecitabine and set the wavelength at 305 nm.[11,12,16,18] Drawbacks of the described assays utilizing UV detection are the relatively low sensitivity for pharmacokinetic studies in humans[16,18] and the long runtime seen in Zufia *et al.*[11] In contrary to the mentioned assays, the assay developed by Piorkowska *et al.* (2014) has satisfactory sensitivity for application in pharmacokinetic studies in humans and has an acceptable runtime (8 min), but only quantifies capecitabine.[12] UV detection is thus a viable option for the quantification of capecitabine, but if quantification of the metabolites is needed MS detection is to be preferred due to its higher sensitivity.

Internal standards

Differences in sample pre-treatment, instrumental related parameters or experimental conditions can cause variations in concentrations detected. The variations can be corrected by using either an external standard (ExS) or internal standard (IS).[13] This is especially important with MS detection where the signal can be variable and differ due to matrix effect, whereas the signal in UV detection is more stable. Three assays described in this review used an ExS to correct for the variations.[15,18,20] Although it is well known that ISs reduce the effect of interfering matrix components, minimizes sample processing errors and the variability of detection, ISs are not always required.[36] ISs standards are preferred due to being more accurate. Most of the described assays use ISs that are structurally related to the analytes. Examples of these are 5-bromouracil (5-BU) and 5-chlorouracil (5-CU). The use of 5-BU in LC-MS/MS was described by Vainchtein *et al.* (2010) but showed a reasonably high ion suppression, due to elution along with other endogenous plasma components. For that reason, 5-CU was chosen over 5-BU.[21] Furthermore, prescribed drugs such as fluvastatin, carbamazepine, voriconazole, gemcitabine and fludarabine have been used as IS[7,9,12,19,34] It can be questioned if using prescribed drugs, especially commonly prescribed drugs such as fluvastatin, carbamazepine and voriconazole should be used as an IS, due to the limitation in the applicability of the assay. Depending on the use of the assay prescribed drugs can be used as an IS in for example pharamacology studies in healthy volunteers, but are not suited for clinical use such as therapeutic drug monitoring.[37] The most ideal IS for MS is a stable isotopically labeled identical isomer, due to its identical behavior to the analyte in terms of matrix effect.[31,38] Yet until recently stable isotopically labeled ISs of capecitabine and its metabolites were unavailable. Salvador *et al.* (2006) were the first to describe the use of stable isotopically labeled ^{2}H8-capecitabine as IS.[14] In the years thereafter Deenen *et al.* (2013) and Deng *et al.* (2015) both described methods using ^{2}H11-capecitabine as IS.[3,6] A disadvantage reported by Deenen et al. (2013) is the rapid conversion of ^{2}H11-capecitabine into 5'-dFUR in an acid environment. Storage of plasma samples at 2-8 °C that were processed with TCA for a couple of hours resulted in an unacceptable increase in 5'-dFUR of more than 25%, whereas the concentration of capecitabine decreased with a similar amount. A pH-neutral solution of methanol and acetonitrile (1:1, v/v) was used in which stability of ^{2}H11-capecitabine was sufficient.[6] Regarding stable isotopically

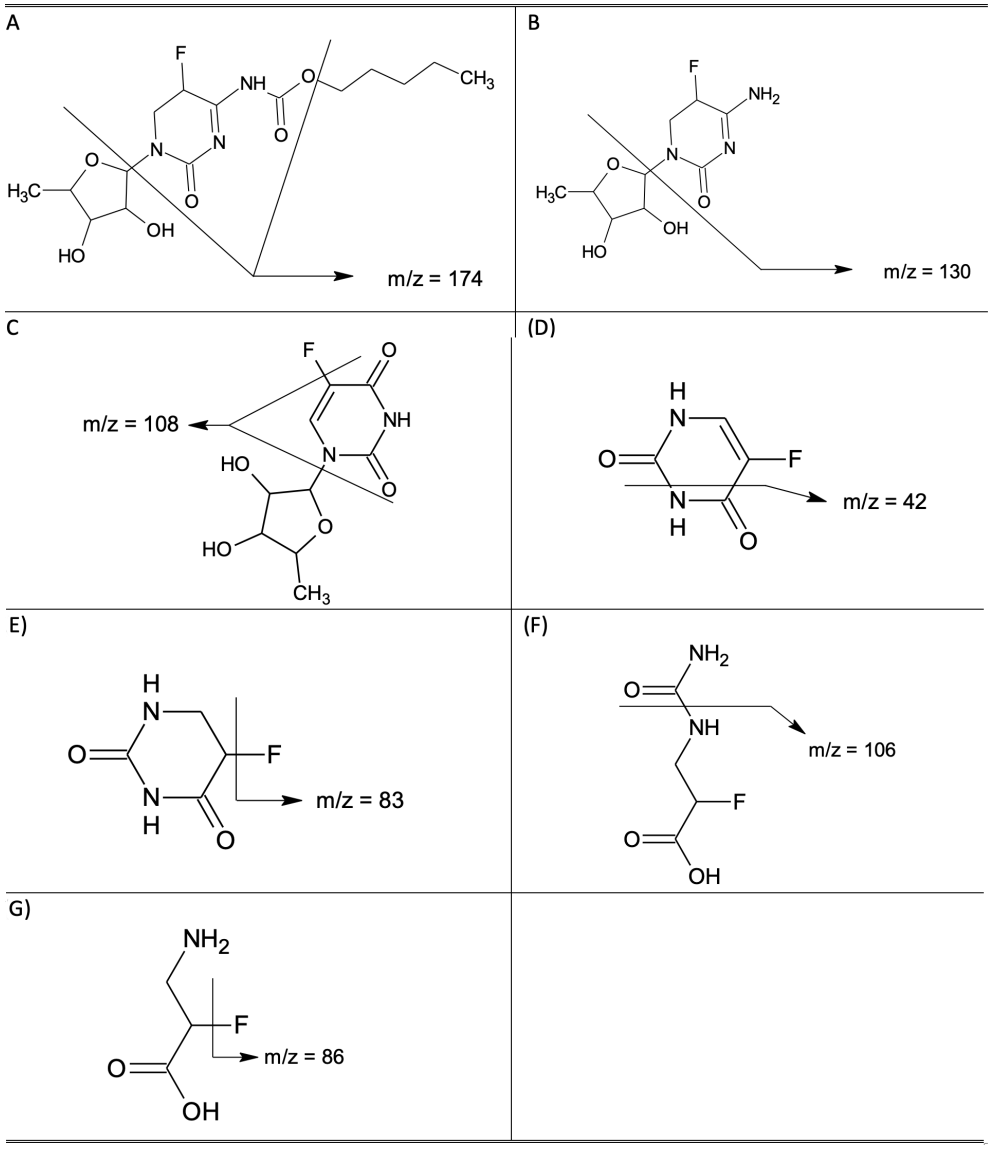

Table 2: Fragmentation of capecitabine and metabolites. (A) = Capecitabine, (B) = 5'-dFCR, (C) = 5'-dFUR, (D) = 5-FU, (E) = 5-FUH2, (F) = FUPA, (G) = FBAL

labeled ISs, ^{13}C or ^{15}N are the preferred labeled ISs. The retention time of deuterium (^{2}H) labeled isotopes can differ slightly compared to analyte due to the deuterium isotope effect. Deuterated ISs can elute slightly earlier on a reverse phase chromatography system due to being less lipophilic than the analyte, which can lead to an inadequate correction of the variations.[38] As far as we know this problem has not been reported for ^{13}C and ^{15}N labeled ISs which have the same retention time as the analyte.

Intracellular metabolites - FdUMP, FUTP and FdUTP

Capecitabine is ultimately metabolized into and 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP), 5-fluorouridine 5'-triphosphate (FUTP), and 5-fluoro-2'-deoxyuridine 5'triphosphate (FdUTP) (5-FU nucleotides).[2,6] FdUMP inhibits thymidylate synthase (TS), which leads to accumulation of deoxyuridine triphosphate (dUTP) and exhaustion of deoxythymidine triphosphate (dTTP) which leads to an intracellular metabolic imbalance. This interferes with DNA synthesis and repair, ultimately leading to apoptosis. FUTP and FdUTP are incorporated in RNA and DNA, which disrupts normal RNA processing and function and leads to DNA damage.[2,7,39] These nucleotides are not included in assays for the quantification of capecitabine or 5-FU as they are formed intracellularly and are not emerging in plasma. However, since these nucleotides are held responsible for the anticancer activity and toxicity, quantification of intracellular concentrations could be interesting. Through the years several assays for the quantification for FdUMP, FUTP and FdUTP have been described. Nonetheless, most assays were not applicable for clinical use for various reasons (e.g., the use of radioactive-labeled nucleotides or lack of sensitivity).[40,41] To date and to our knowledge only Derissen *et al.* (2015) have reported the development of an LC-MS/MS assay that can quantify FdUMP, FUTP and FdUTP intracellularly, in a clinical setting.[42] Quantification of FdUMP, FUTP and FdUTP in peripheral blood mononuclear cells (PBMCs) by cell lysis and subsequent extraction with methanol was described. A common obstacle in the quantification of nucleotides is the interference by other structurally related endogenous nucleotides. Sufficient separation of analytes and endogenous compounds during chromatography was achieved by using a Biobasic anion exchange column and a gradient in which the amount of ammonium acetate and the pH were gradually decreased and increased, respectively. This LC-MS/MS assay showed to be applicable for the measurement of intracellular FUTP concentrations after administration of capecitabine to patients. For the measurement of FdUTP and FdUMP a higher sensitivity is needed.[42]

Analytical challenges and future perspectives

Analysis of capecitabine and its metabolites has some difficulties. The major challenge is the simultaneous extraction, chromatographic separation and detection of capecitabine and its metabolites due to the large differences in polarity. To date no assay has been reported which can measure capecitabine, its metabolites and those of 5-FU simultaneously. Vainchtein *et al.* (2010) has reported an assay which was capable of quantifying capecitabine and its metabolites, 5-FU and 5-FUH2. But it was not robust enough and upon frequent application of this assay signal intensity decreased, which led to a significant loss of sensitivity.[21] Deenen *et al.* (2013) developed a method, which consisted of two separate assays, to quantify capecitabine and its 'large' metabolites and 5-FU and its subsequent 'small' metabolites. Furthermore, attention should be paid to sample pre-treatment due to the influence of phospholipids on the signal intensity of 5-FUH2. Another challenge faced in the bioanalysis of capecitabine and its metabolites is the quantification of these analytes from tumor tissue. Tumor tissue analysis is challenging compared to liquid matrices such as plasma due to difficulty of tissue sampling and the heterogeneous nature of tumor tissue. Therefore, aspects such as sample collection,

tissue homogenization, extractability from the tissue and stability should be carefully considered.[43] It was shown by Guichard *et al.* that quantification of capecitabine and it metabolites in tumor tissue is possible with a sensitivity comparable to previous published work.[20,34,44] Another interesting issue is that quality controls which are prepared by spiking the control matrix with the test analyte do not always mimic the activity of the analyte in samples from treated patients. Samples from treated patients can differ from the control matrix due to various reasons including incurred instability, matrix effect and sample inhomogeneity.[45] To ensure reproducibility of the measured concentrations, repeated measurement of analytes within a selected sample set of treated patients in separate runs on different days is advised. This is also known as incurred sample reanalysis (ISR) and is mostly applied during drug discovery stage.[45,46] Most assays described in this review did not perform any kind of ISR strategy. Therefore, reproducibility cannot be certainly be assured and should be considered during the development stage of the assay. Improvement in the quantification of capecitabine and metabolites could be the development of an integrated assay which can also quantify 5-FU and all its metabolites simultaneously. Furthermore, more efficient and environmentally friendly sample pre-treatment methods such as microextractions should be considered. In addition, future research should consider the possibility of simultaneously quantifying capecitabine and its metabolites and concomitant drugs which could stimulate the use of the assays for therapeutic drug monitoring of capecitabine. As was shown by Thorat *et al.*, whom have quantified capecitabine and oxaliplatin using one assay.[19] Moreover, development of an assay able to quantify all cytotoxic intracellular 5-FU nucleotides should be considered which could provide valuable information concerning the pharmacokinetics of the active metabolites. This information could potentially be used to assess the exposure to treatment with fluoropyrimidines and to assess the risk of developing severe fluoropyrimidine-related toxicity in patients. Subsequently, it should be kept in mind that this method is heavily dependent on the number of metabolites being released from the cell matrix, which can differ and can be difficult to assess.

Conclusion

HPLC methods coupled to UV detection have been successfully applied for the quantification of capecitabine and its metabolites in biological matrices. However, LC coupled to tandem MS is now the preferred method for determining capecitabine in biological samples due to improved selectivity and sensitivity. This allows for better assessment of the pharmacokinetic parameters of capecitabine and metabolites. LLE, SPE and PP are described as sample pre-treatment. PP was the most reported technique due to being easy and fast to perform. LLE and SPE were described but are labor intensive and costly, but yield cleaner extracts compared to PP. Differences in polarity between capecitabine and metabolites raise problems in the simultaneous bioanalysis of these compounds. Due to these differences sample pre-treatment and chromatographic separation are complicated. An 'all-in-one' system fulfilling all bioanalytical validation requirements, remains to be developed. Furthermore, the measurement of the active metabolites of capecitabine is complicated by their intracellular formation. The intracellular metabolites are present in the cells of patients in very low concentrations, therefore high sensitivity is required.

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Chapter 10

Development of a UPLC-MS/MS assay for the quantitative determination of capecitabine, 5'-deoxy-5-fluorocytidine (5'-dFCR), 5'-deoxy-5-fluorouridine (5'-dFUR), 5'-fluorouracil (5-FU), and α-fluoro-β-alanine (FBAL)

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Abstract:

Capecitabine is an anticancer agent and is the oral prodrug of 5-fluorouracil (5-FU). In this study, an ultra-high performance liquid chromatography coupled to turbo ion spray tandem mass spectrometry (UPLC-MS/MS) method was developed and validated to quantify capecitabine and its metabolites including 5'-deoxy-5-fluorocytidine (5'-dFCR), 5'-deoxy-5-fluorouridine (5'-dFUR), 5-FU, and fluoro-β-alanine (FBAL) in lithium heparinized human plasma. Analytes were extracted by protein precipitation, chromatographically separated by Acquity UPLC HSS T3 column with gradient elution, and analyzed with a tandem mass spectrometer equipped with an electrospray ionization source. Capecitabine and 5'-dFCR were quantified in positive ion mode and 5'-dFUR, 5-FU, and FBAL were quantified in negative ion mode. The total chromatographic run time was 9 min. Stable isotopically labeled internal standards were used for all analytes. The assay was validated over the range from 25.0 to 2,500 ng/mL for capecitabine, 10.0 to 1,000 ng/mL for 5'-dFCR, 5'-dFUR, and 5-FU and 50 to 5,000 ng/mL for FBAL in human plasma. Validation results have shown the developed assay allows for reliable quantitative analysis of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL in plasma samples.

Introduction:

5-Fluorouracil (5-FU) and capecitabine (Xeloda®) are fluoropyrimidine-based chemotherapeutic agents used in the treatment of several solid tumors.[1] 5-FU is administered intravenously, whereas capecitabine is the pro-drug of 5-FU and is administered orally. Capecitabine is converted through three enzymatic steps into 5-FU, which in its turn is taken up by the cell and metabolized into the active intracellular metabolites. Capecitabine is converted to 5'-deoxy-5-fluorocytidine (5'-dFCR) by carboxyl esterase, upon which it is converted to 5'-deoxy-5-fluorouridine (5'-dFUR) and 5-FU by cytidine deaminase and thymidine phosphorylase, respectively.[2,3] Approximately 80-90% of the administered dose of 5-FU is catabolized by dihydropyrimidine dehydrogenase (DPD) primarily in the liver.[4] DPD converts 5-FU into the inactive metabolite 5,6-dihydro-5-fluorouracil (5-FUH2). Thereupon, 5-FUH2 is converted into α-fluoro-β-ureidopropionic (FUPA) and α-fluoro-β-alanine (FBAL) by dihydropyrimidinase and β-ureidopropionase, respectively (See Figure 1).[5]

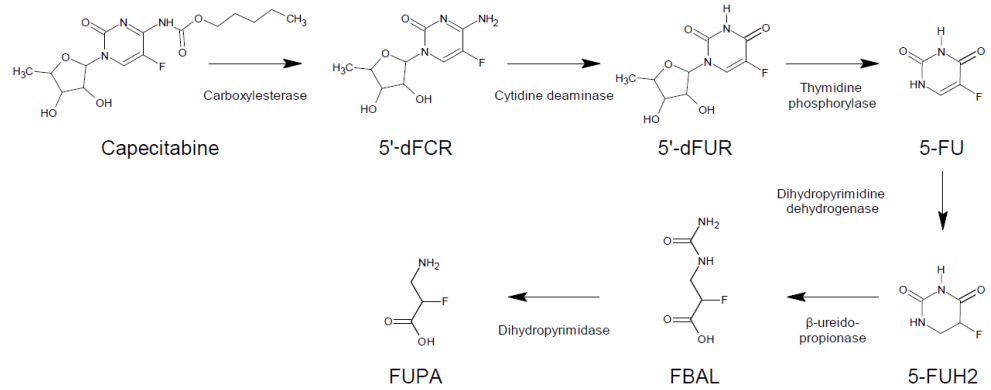

Figure 1: Metabolism of capecitabine. Abbreviations: 5'-dFCR: 5'-deoxy-5-fluorocytidine, 5'-dFUR: 5'-deoxy-5-fluorouridine, 5-FU: 5-Fluoruracil, 5-FUH2: 5,6-dihydro-5-fluorouracil CAP: Capecitabine, FBAL: α-fluoro-β-alanine, FUPA: α-fluoro-β-ureidopropionic. *Abbreviations*: 5'-dFCR: 5'-deoxy-5-fluorocytidine, 5'-dFUR: 5'-deoxy-5-fluorouridine, 5-FU: 5-Fluoruracil, CAP: Capecitabine, FBAL: α-fluoro-β-alanine.

A small fraction of 5-FU is phosphorylated intracellularly to active metabolites which are incorporated into RNA or DNA which interferes with normal RNA function and causes DNA damage, respectively.[6,7] The main side-effects attributed to fluoropyrimidines are mucositis, gastrointestinal side effects and, hand-foot syndrome.[8,9] Bioanalytical assays for the quantitative determination of capecitabine and its metabolites are necessary in support of clinical pharmacological studies and could also be used for therapeutic drug monitoring. Over the years, multiple liquid chromatography (LC) methods have been developed for the analysis of capecitabine and its metabolites.[10] LC was combined with either ultraviolet (UV)[11-16] or mass spectrometric[17-25] detection. However, none of these assays can quantify capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL simultaneously in a single assay due to the differences in physiochemical properties. Deenen *et al.*[24] reported the quantification of these metabolites but this method consists of two separate assays, one for the quantification of capecitabine, 5'-dFCR and, 5'-dFUR and another assay to determine 5-FU, 5-FUH2, FUPA and FBAL.[24] Herein we describe the development and

validation of a selective and sensitive LC-MS/MS for the simultaneous quantification of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL in human plasma. This assay validation has been based on the EMA and FDA guidelines on bioanalytical validation[26,27] and is applied in pharmacokinetic studies with capecitabine.

Investigations, results, and discussion

Chromatography

In one of our previously described quantification methods capecitabine and its metabolites 5'-dFCR, 5'-dFUR, 5-FU, and 5-FUH2 could be measured in plasma using a hypercarb (porous graphitic carbon) column. This column was not suited for measuring large batches of samples. The chromatography deteriorated and signal intensity decreased in time, which led to a significant loss of sensitivity. Furthermore, the batch-to-batch variability of the packed columns was considerable.[23] In another report, two separate assays were needed due to the physicochemical differences between capecitabine and its hydrophilic metabolites.[24] Capecitabine has a long carbon chain and shows lipophilicity. However, during biotransformation, the polarity of the metabolites gradually increases and become more hydrophilic.[10] Therefore we decided to develop a new assay using in which capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL can be separated and measured in a single run. Reversed-phase chromatography was applied using an Acquity UPLC HSS T3 column. Using this column in combination with gradient allowed for an adequate analyte retention (even for the most hydrophilic metabolite FBAL) and separation allowing for multiplexing of all analytes. The total run time was 9 min and typical retentions of capecitabine, 5'-dFUR, 5'-dFCR, 5-FU, and FBAL were 6.47, 5.14, 4.91, 3.10, and 1.12 minutes, respectively. Due to this multiplex approach only one assay is needed to quantify capecitabine and metabolites, allowing for a higher throughput of samples. The assay proved to be very stable and robust, also when extensively used for a period of 1.5 years.

Sample pre-treatment

Sample pre-treatment and analytical run time had to be efficient for rapid and high-throughput quantification of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL in human plasma. Protein precipitation was chosen as the sample pre-treatment procedure to ensure high sample throughput. A mixture of methanol and acetonitrile (50:50, v/v) was successfully applied and added to the plasma in a ratio of 1:3. However, only protein precipitation was not sufficient to achieve acceptable sensitivity for FBAL. Therefore, an extra step was introduced in which after protein precipitation supernatants were evaporated under a gentle stream of nitrogen, reconstituted in mobile phase A and transferred to a 96-well plate. This step concentrates the sample by factor 3, which increases the signal intensity and allows for a lower LLOQ. The use of a 96-well plate allows for the analysis of large batches of samples. During validation, it was found that 5-FUH_2 converted to FUPA during evaporation. Therefore, this method is not suitable for the quantification of 5-FUH_2, and FUPA. This conversion did not affect the data integrity of the other analytes.

Mass spectrometry

To optimize the source settings and the analyte-specific conditions flow injection analysis was applied. To obtain the largest signal-to-noise ratios, the mass spectrometer was utilized in the negative mode for quantification of 5'-dFUR, 5-FU, and FBAL and in the positive mode for 5'-dFCR and capecitabine. Optimized detector and analyte specific settings are presented in Table 1. Representative chromatograms of blank QC sample, QC LLOQ sample, and study sample are shown in Figure 2.

Validation

Calibration standards, including standards without internal standard nor analytes (double blank), a standard only containing an internal standard (blank) were analyzed at the beginning and the end of 3 separate analytical runs. Linear regression was applied (area ratio of 5-FU and FBAL vs the concentration) with a weighting factor of $1/x^2$, where x is the concentration of the analyte. The calibration data of capecitabine, 5'-dFUR, and 5'-dFCR quadratic fits were applied with a weighting factor of $1/x$. The assay was validated over the range from 25.0 to 2,500 ng/mL for capecitabine, 10.0 to 1,000 ng/mL for 5'-dFCR, 5'-dFUR, and 5-FU and 50 to 5,000 ng/mL for FBAL in human plasma. For all compounds at all concentration levels, deviations of measured concentrations from nominal concentrations ranged from -13.4% and 5.8%, with a maximum coefficient of variation (CV) value of 9.7%, and 8.2% for concentrations above LLOQ and at LLOQ, respectively. Correlation coefficients were 0.9964 or better for all compounds.

Table 1: Mass spectrometer settings for the quantification of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU and FBAL in human plasma

Analyte	Precursor ion (m/z)	Product ion (m/z)	Ion spray Voltage (V)	Dwell (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
Capecitabine	360.0	243.9	5000	50	106	10	40	12
Capecitabine-d11	371.1	254.8	5000	50	106	10	40	12
5'-dFCR	246.0	130.0	5000	25	71	10	44	18
5'-dFCR-$^{13}C^{15}N_2$	249.0	133.0	5000	25	71	10	44	18
5'-dFUR	244.9	128.8	-4500	25	-100	-10	-22	-13
5'-dFUR-$^{13}C^{15}N_2$	246.9	130.8	-4500	25	-100	-10	-22	-13
5-FU	128.9	42.1	-4000	20	-70	-10	-22	-7
5-FU-$^{13}C^{15}N_2$	131.8	43.8	-4000	20	-70	-10	-22	-7
FBAL	105.9	85.9	-4000	30	-25	-10	-12	-5
FBAL$^{13}C_3$	108.8	88.7	-4000	30	-25	-10	-12	-5

Abbreviations: 5'-dFCR, 5'-deoxy-5-fluorocytidine; 5'-dFUR, 5'-deoxy-5-fluorouridine; 5-FU, 5-Fluoruracil; CE, Collision energy; CXP, Collision exit potential; DP, Declustering potential; EP, Entrance potential; FBAL, α-fluoro-β-alanine; V, voltage.

Five replicates of each validation sample concentration (LLOQ, low, mid and high) in human lithium heparinized plasma in three separate analytical runs were used to analyze and validate the accuracy and precision of this method. The intra-run bias should be within ±20% for the LLOQ and within ±15% for the other concentrations. A one-way ANOVA was used to estimate the intra- and inter-assay precision. The assay performance data (inter-assay accuracies and precisions) for capecitabine and its metabolites are

listed in Table 2.

The intra-assay accuracies ranged from 17.8% to 16.9%, and from 3.8% to 8.5% at LLOQ and at higher concentrations, respectively. The maximum intra-assay precision was 0.4% and 12.0% at LLOQ and at the higher levels. Ultimately, the accuracy and precision for all compounds were within the acceptance criteria.

Analytes were separately spiked in control lithium heparinized plasma at their ULOQ concentration to assess cross-analyte interference. To assess potential interferences from internal standards separate samples were prepared by spiking control lithium heparinized plasma at the assay concentration. The relative interference for all analytes was ≤20% except for the following ULOQ samples: capecitabine (342.6% of the LLOQ area of 5′-dFCR and 37.0% of 5′-dFUR) and 5′-dFCR (897.6% of the LLOQ of DFUR). The impact of the interferences has been investigated by calculating the maximum tolerable ratio's (interference ≤20%).

The ratios for capecitabine/5′-dFCR, capecitabine/5′-dFUR, and 5′-dFCR/5′-dFUR were 14.6, 135, and 2.22, respectively. The ratios were calculated in clinical samples (N=2148) obtained in several clinical studies and for the interference of capecitabine-5′-dFCR, capecitabine-5′-dFUR, and 5′-dFCR-5′-dFUR 99.8%, 100%, and 98.0% of the samples were within the maximum tolerable ratios. Based on this we demonstrated that cross-analyte interference was acceptable for these analytes. The relative interference for the internal standards were all ≤5.0% and within the criteria.

*Inter-run precision could not be calculated (mean square between group is less then mean square within group). *Abbreviations*: 5′-dFCR, 5′-deoxy-5-fluorocytidine; 5′-dFUR, 5′-deoxy-5-fluorouridine; 5-FU, 5-Fluoruracil; FBAL, α-fluoro-β-alanine.

To analyze for the presence of endogenous interferences six different batches of double blanks and samples were separately spiked at their LLOQ. The LLOQ samples of capecitabine, 5′-dFCR, 5′-dFUR, 5-FU, and FBAL were all within ±20% of their nominal concentrations in at least 4 out of 6 bathes. No interferences from endogenous material at the retention time of the analytes were observed in the blanks. Therefore, the tests for endogenous interferences were considered acceptable.

To assess the matrix effect (ion suppression), six batches of individual blank matrices at low and high concentrations in singular were investigated. The matrix effect was calculated for each lot of matrices by calculating the ratio of the peak area in the presence of matrix (measured by analyzing blank matrix spiked after extraction with analyte), to the peak area in absence of matrix (working solution of the analyte). The absolute matrix effect ranged from 2 to 60% signal reduction at both tested levels. However, SIL-ISs were able to correct for this (N=6, RSD ranging from 0.4 - 2.2%). From data it can be concluded that the matrix effect of different matrix batches does not influence on the accuracy and precision of the method.

To determine carry-over two double blank samples were injected after an ULOQ sample in at least 3 validation runs. The response in the first 2 blank matrices at the retention time of the analytes and the internal standards was less than 1.6% (0.3% for the IS) of the mean response at the LLOQ for tested analytes. In conclusion, the carry-over was

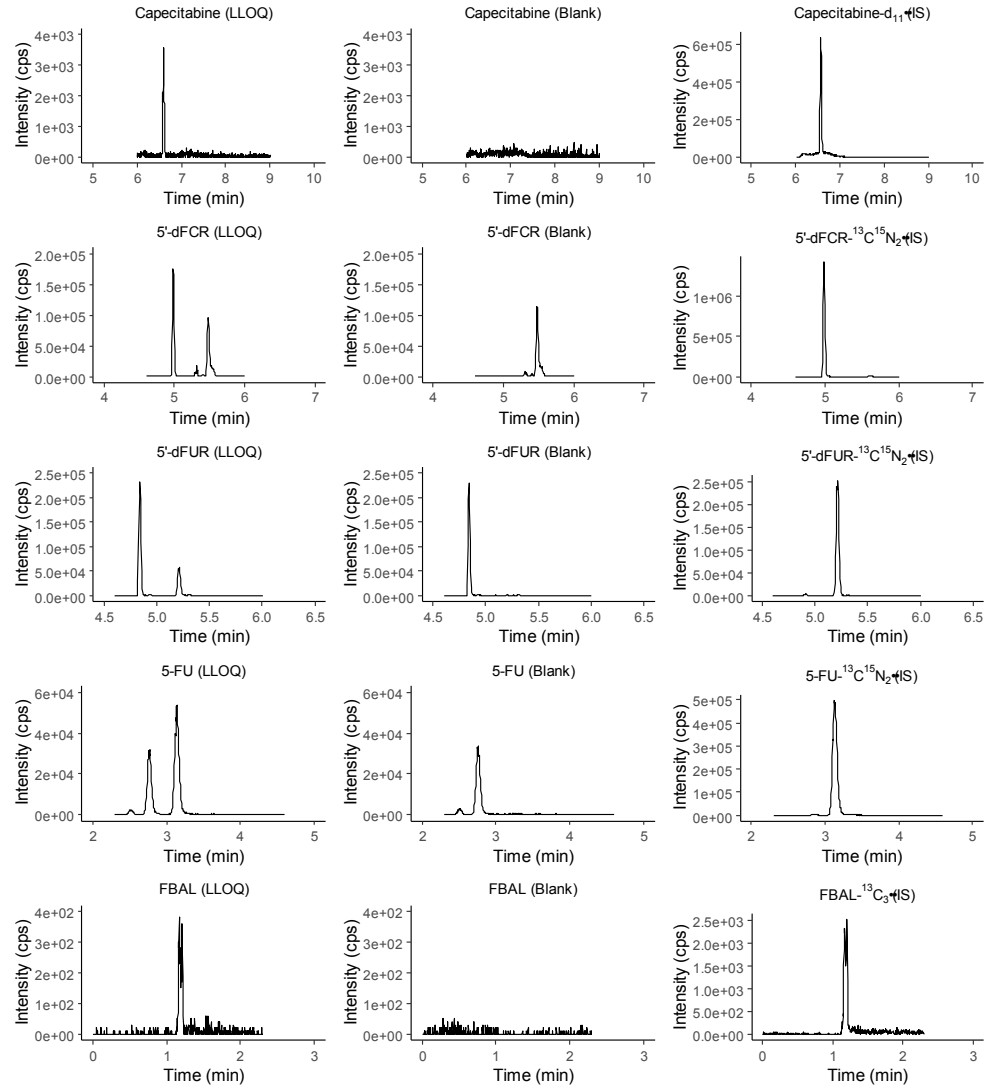

Figure 2: Representative UPLC-MS/MS chromatogram from blank human samples, spiked plasma samples at the LLOQ of capecitabine, 5′-dFCR, 5′-dFUR, 5-FU, FBAL, and spiked plasma with their internal standards (IS), respectively.

satisfactory.

To assess the stability, several experiments were performed. Analytes were considered stable if the determined concentration did not deviate more than ±15% from the nominal concentrations. Previously, benchtop stability of capecitabine and its metabolites in plasma was shown at ambient temperature for at least 6 hours.[24] Table 3 shows the results of the stability experiments. The deviation after three freeze (-70°C) /thaw (room temperature) cycles was within ±15% of the nominal concentrations. The stability of the final extract was evaluated after 10 days at 2-8°C in processed human lithium heparinized plasma samples. The deviation for low concentrations was less than or equal to 5.8% of

the nominal concentration for all analytes and the CV was less than or equal to 4.4%. The stability of the dried extract was evaluated after 7 days at 2-8°C and the deviation for CAP, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL at both the low and high concentrations were less than or equal to 6.5%. Therefore, it is concluded that samples are stable in dry extract plasma samples after at least 7 days at 2-8°C for capecitabine, 5'-dFUR, 5-FU, and FBAL and at least 20 days at 2-8°C for 5'-dFCR. The long-term stability was studied for up to 5 months at -70°C and showed that the deviation was less or equal to 8.7% of the nominal concentration for all analytes, indicating that the samples are stable when stored for at least 5 months under these conditions.

Table 2: Assay performance data for the quantification of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU and FBAL in human lithium heparinized plasma. Accuracy and precision were established in 5 replicates of each validation sample concentration in 3 separate analytical runs.

Compound	Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	Inter-assay accuracy (%)	Intra-assay accuracy (%)	Inter-assay precision (%)	Intra-assay precision (%)
Capecitabine	25.0	21.8	± 13.0	≤ 17.8	± 6.2	≤ 5.2
	90.0	91.6	± 1.8	≤ 3.4	± 0.9	≤ 3.0
	625	628	± 0.5	≤ 1.5	± 0.4	≤ 2.5
	1500	1473	± 1.8	≤ 2.7	± 1.2	≤ 1.9
5'-dFCR	10	10.34	± 3.5	≤ 16.9	± 11.2	≤ 2.0
	37.5	38.5	± 2.7	≤ 8.5	± 4.9	≤ 2.9
	250	257.0	± 2.8	≤ 5.0	± 2.7	≤ 3.6
	625	651	± 4.4	≤ 5.3	-*	≤ 2.7
5'-dFUR	10	9.1	± 8.5	≤ 13.1	± 4.3	≤ 5.5
	37.5	37.17	± 0.9	≤ 3.8	± 2.5	≤ 2.2
	250	259	± 3.6	≤ 4.2	-*	≤ 4.1
	625	657	± 5.1	≤ 5.8	-*	≤ 2.0
5-FU	10	9.5	± 4.9	≤ 6.3	± 2.3	≤ 2.6
	37.5	38.15	± 1.7	≤ 2.7	± 0.4	≤ 2.8
	250	258.9	± 3.5	≤ 4.3	± 0.5	≤ 2.4
	625	634.2	± 1.5	≤ 1.9	-*	≤ 2.9
FBAL	50.0	47.0	± 6.1	≤ 13.4	± 9.7	≤ 12.0
	187.5	188	± 0.1	≤ 1.4	-*	≤ 7.3
	1250	1291	± 3.3	≤ 7.5	± 2.9	≤ 7.3
	3125	3236	± 3.6	≤ 7.0	± 3.4	≤ 5.4

Clinical application

The presented method was successfully used in support of a large clinical trial (NCT02324452),[28] where patients were treated with fluoropyrimidine-based chemotherapy to determine plasma concentrations of capecitabine, 5'-dFUR, 5'-dFCR, 5-FU, and FBAL. Plasma lithium-heparin samples were collected after approval by the medical ethical committee and informed consent of all patients. Plasma samples were taken on day 1 prior to treatment with fluoropyrimidines (pre-dose), and after 0.25, 0.5, 1, 2, 3, 4, 6, and 8 hours after administration. A representative plasma concentration-time curve from pharmacokinetic analyses of a colorectal cancer patient treated with 1500 mg capecitabine

Table 3: Stability of capecitabine and its metabolites tested under various conditions

Matrix	Condition	Compound	Initial concentration	Measured concentration	DEV (%)	CV (%)
Plasma	-70°C, 5 months	Capecitabine	90.0 1500	97.8 1500	8.7 -1.1	2.2 4.3
		5'-dFCR	37.5 625	38.6 640	2.9 2.3	2.9 2.9
		5'-dFUR	37.5 625	37.9 631	1.0 0.9	2.9 1.2
		5-FU	37.5 625	38.0 644	1.2 3.1	0.9 1.3
		FBAL	187.5 3125	197.0 3277	5.1 4.9	6.6 1.2
Final extracts	2-8°C, 10 days	Capecitabine	90.0 1500	95.2 1537	5.8 2.4	4.4 4.4
		5'-dFCR	37.5 625	38.2 636	1.8 1.8	0.2 2.2
		5'-dFUR	37.5 625	36.8 634	-1.8 1.4	1.8 2.7
		5-FU	37.5 625	37.2 634	-0.9 1.4	1.3 0.9
		FBAL	187.5 3125	192.7 3193	2.8 2.2	1.6 1.2
Dry extracts	2-8°C, 20 days	Capecitabine	90.0 1500	94.0 1573	2.6 4.9	1.8 3.2
		5'-dFCR	30.0 750	30.8 702	2.6 -6.4	2.4 1.7
	2-8°C, 7 days	5'-dFUR	37.5 625	38.7 665	3.2 6.5	0.7 3.1
		5'-FU	37.5 625	37.3 646	-0.5 3.4	1.7 2.1
		FBAL	187.5 3125	184.0 3327	-1.9 6.5	6.6 5.1
Freeze (-70°C) / Thaw (RT)	3 cycles	Capecitabine	90.0 1500	101.3 1545	12.6 3.0	1.9 2.2
		5'-dFCR	37.5 625	39.1 660	4.3 5.6	3.5 3.0
		5'-dFUR	37.5 625	38.2 627	1.7 0.3	2.5 3.0
		5'-FU	37.5 625	37.4 627	-0.2 0.4	1.6 1.3
		FBAL	187.5 3125	190.5 3265	1.6 4.5	3.5 1.6

Abbreviations: 5'-dFCR, 5'-deoxy-5-fluorocytidine; 5'-dFUR, 5'-deoxy-5-fluorouridine; 5-FU, 5-Fluoruracil; CV, Coeffience of variance; DEV, Deviation; FBAL, α-fluoro-β-alanine; RT, room temperature.

is presented in Figure 3. Pharmacokinetic data of capecitabine and its metabolites has the potential the be used for the monitoring and even dose-individualization of fluoropyrimidine-based chemotherapy. It has been shown that 5-FU plasma

concentrations are correlated to the onset of severe fluoropyrimidine-related toxicity.[29,30] In addition, exposure to FBAL has shown to be associated with the incidence of severe (grade 3-4) capecitabine-induced diarrhea.[31] These data show the value of pharmacokinetic follow-up of patients treated with fluoropyrimidine-based chemotherapy and the utility for the simultaneous quantification of capecitabine, 5'-dFUR, 5'-dFCR, 5-FU, and FBAL.

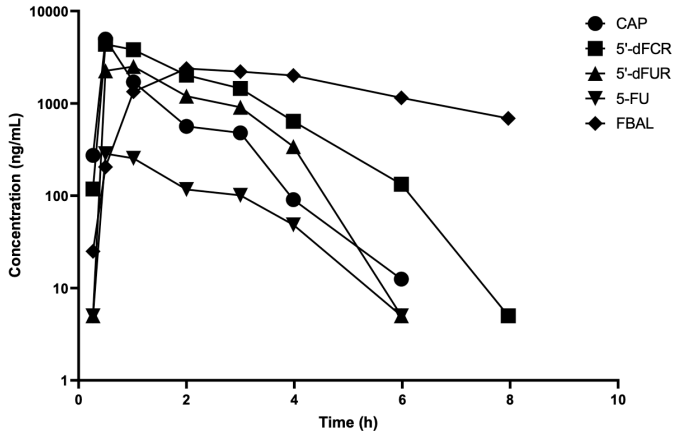

Figure 3: Representative pharmacokinetic curves of capecitabine and its metabolites in a colorectal cancer patient treated with 1500 mg capecitabine.[28] *Abbreviations*: 5'-dFCR: 5'-deoxy-5-fluorocytidine, 5'-dFUR: 5'-deoxy-5-fluorouridine, 5-FU: 5-Fluoruracil, CAP: Capecitabine, FBAL: α-fluoro-β-alanine.

Conclusions

An accurate, sensitive, and robust UPLC-MS/MS assay for the quantification of capecitabine, 5'-dFUR, 5'-dFCR, 5-FU and FBAL in human plasma was developed and validated. Sample pretreatment consists of protein precipitation in combination with evaporation and reconstitution. The validated concentrations are from 25 to 2,500 ng/mL for capecitabine, from 10 to 1,000 ng/mL for 5'-dFCR, 5'-dFUR and 5-FU and from 50 to 5,000 ng/mL for FBAL. Stable isotope labeled internal standards were used for all analytes. The stability of all analytes was adequate at 2-8°C at -70°C and after 3 free/thaw cycles. Examined assay validation parameters fulfilled the acceptance criteria of the US Food and Drug Administration.[26,32] The developed assay allows for robust and reliable quantitative analysis of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL in plasma samples and has shown its clinical applicability.

Experimental

Chemicals and materials

Capecitabine ($C_{15}H_{22}FN_3O_6$), 5'-dFCR ($C_9H_{12}FN_3O_4$), 5'-dFUR ($C_9H1_1FN_2O_5$), 5-FU ($C_4H_3FN_2O_2$), FBAL ($C_3H_6FNO_2$), Capecitabine-d_{11} ($C_{15}H_{11}FN_3O_6$-$^2H_{11}$), 5'-DFCR-$^{13}C^{15}N_2$ ($C_8H_{12}FNO_4$-$^{13}C^{15}N_2$), 5'-DFUR-$^{13}C^{15}N_2$, ($C_8H_{11}FO_5$-$^{13}C^{15}N_2$), 5-FU-$^{13}C^{15}N_2$ ($C_3H_3FO_2$-$^{13}C^{15}N_2$), FBAL-$^{13}C_3$ (H_6FNO_2-$^{13}C_3$) were purchased from Toronto Research Chemicals Inc. (North York, TO, Canada). Acetonitrile (Supra-Gradient grade and ULC/MS grade), formic acid (ULC/MS grade), water (ULC/MS-grade), and methanol (Supra-Gradient grade) were purchased from Biosolve Ltd. (Valkenswaard, The Netherlands). Blank human lithium heparinized plasma was purchased from Bioreclamation (Hicksville, NY, USA).

Preparation of stock and working solutions

Two sets of stock solutions for capecitabine and its metabolites, which were used for the calibration standards and quality control samples, were prepared from two independent weightings. Both were prepared by weighing approximately 1.0 mg of each analyte and adding an appropriate amount of water to obtain a 1.0 mg/mL stock solution. The working solutions were prepared separately by further diluting the stock solutions in water. Eight working solutions were prepared containing all analytes. Working solutions were prepared with concentrations ranging from 500 ng/mL to 50,000 ng/mL for capecitabine, 200 ng/mL to 20,000 ng/mL for 5'-dFCR, 5'-dFUR, and 5-FU, and 1,000 ng/mL to 100,000 ng/mL for FBAL. The stock solutions for the quality control samples were diluted with water to obtain 5 working solutions. Working solutions were prepared with concentrations of 500, 1,500, 12,500, 37,500, and 50,000 ng/mL for capecitabine, 200, 600, 5,000, 15,000, and 20,000 ng/mL for 5'-dFCR, 5'-dFUR, and 5-FU, and 1,000, 3,000, 25,000, 75,000, and 100,000 ng/mL for FBAL. IS stock solutions were prepared by weighing approximately 1.0 mg of each IS. For capecitabine-d11 and FBAL-$^{13}C_3$ an appropriate amount of water is added to obtain stock solutions of 1.0 mg/mL. For 5'-DFCR-$^{13}C^{15}N_2$, 5'-DFUR-$^{13}C^{15}N_2$, and 5-FU-$^{13}C^{15}N_2$ an appropriate amount of dimethyl sulfoxide (DMSO) was added to obtain a 1.0 mg/mL separately prepared stock solutions. IS working solutions were made by diluting the IS stock solutions in water. A final concentration of 3,750 ng/mL was obtained for capecitabine--d11, 1,500 ng/mL for 5'-DFCR-$^{13}C^{15}N_2$, 5'-DFUR-$^{13}C^{15}N_2$ and 5-FU-$^{13}C^{15}N_2$, and 7,500 ng/mL for FBAL-$^{13}C_3$. All stock and working solutions were stored at -20°C.

Preparation of Calibration Standards and Quality Control Samples

Both calibration standards and quality control samples were prepared by adding 200 µL of the appropriate working solution to 3800 µL in control lithium heparinized plasma, followed by short vortex mixing. Calibration standards were prepared at concentrations of 2,500, 1,800, 1,250, 625, 250, 125, 62.5, 25 ng/mL for capecitabine, 1000, 750, 500, 250, 100, 50, 25 and 10 ng/mL for 5'-dFCR, 5'-dFUR and 5-FU, 5000, 3750, 2500, 1250, 500, 250, 125 and 50 ng/mL for FBAL. For capecitabine, the quality control samples were prepared at concentrations of 1,875, 625, 75, and 25 ng/mL. The working solutions for the quality control samples were further diluted to concentrations of 750, 250, 30, and 10 ng/mL for 5'-dFCR, 5'-dFUR, and 5-FU. The quality control samples were prepared at concentrations of 3750, 1250, 150, and 50 ng/mL for FBAL by adding 200 µL of the appropriate working solution to 3800 µL in control lithium heparinized plasma. Both calibrations standards and quality control samples were stored in replicates of 300 µL in 2.0 mL tubes at -70°C until use.

Sample preparation

To 300 µL of sample aliquots, 20 µL of the internal standard working solution (Using stable isotope labeled (SIL) internal standards) for 5'-DFCR-$^{13}C^{15}N_2$, 5'-DFUR-$^{13}C^{15}N_2$, 5-FU-$^{13}C^{15}N_2$ and in H2O for Capecitabine-d11, and FBAL-$^{13}C_3$) was added. Subsequently, proteins were precipitated with 900 µL of methanol:acetonitrile 1:1 (v/v), followed by short vortex mixing, 10 min of automatic shaking at 1,250 rpm, and centrifuging at 14,000 rpm for 10 min at room temperature. Thereupon, the supernatant was evaporated in the

turbo evaporator. Afterward, the dry extract was dissolved in 100 µL of 0.1% formic acid in water. This was followed by short vortex mixing and centrifuging at 14,000 rpm at 4°C. The supernatant was then transferred to a vial with an insert or to a capped 96 wells plate.

Liquid chromatography - mass spectrometry

Capecitabine and its metabolites were separated using an Acquity UPLC HSS T3 column, 150 mm x 2.1 mm ID, particle size 1.8 µm (Waters Corp., Milford, MA, USA) protected with a 0.2 µm filter at a temperature of 30°C. Chromatographic separation was achieved by using a gradient consisting of mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) at a constant flow rate of 300 µL/min. The gradient started for the first 2.50 min with 100% mobile phase A after which the proportion of mobile phase B increased linearly to 90% until 7.50 min. At t=7.5 min, the column was brought back to its original state of 100% mobile phase A. The total run time was 9.0 min. Detection of the analytes was performed on a quadruple trap mass spectrometer with a Turbo Ion Spray Interface (Q-trap 5500 triple quadrupole, Sciex, Framingham, M, USA). Detection was performed in the negative ion mode for FBAL, 5-FU, and 5'-DFUR and positive ion mode for 5'-DFCR and CAP. Nebulizing gas, turbo gas, collision gas, and curtain gas were set to 50, 50, 6, and 40 arbitrary units, respectively. The source temperature was 600°C. Other mass spectrometric settings are shown in Table 1.

Validation

The validation of the assay was based on the current EMA and FDA guidelines on bioanalytical method validation.[26,27] The following validation parameters were assessed: calibration model, accuracy and precision, specificity and selectivity, matrix effect, carry-over, and stability. This assay has been used successfully in support of a large clinical trial (NCT02324452)[28] thereby showing clinical applicability.

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Chapter 11

Assay performance and stability of uracil and dihydrouracil in clinical practice

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Abstract

Purpose: Measurement of endogenous uracil (U) is increasingly being used as dose-individualization method in the treatment of cancer patients with fluoropyrimidines. However, instability at room temperature (RT) and improper sample handling may cause falsely increased U levels. Therefore, we aimed to study the stability of U and dihydrouracil (DHU) to ensure proper handling conditions.

Methods: Stability of U and DHU in whole blood, serum, and plasma at RT (up to 24 hours) and long-term stability (≥7 days) at -20°C were studied in samples from 6 healthy individuals. U and DHU levels of patients were compared using standard serum tubes (SSTs) and rapid serum tubes (RSTs). The performance of our validated UPLC-MS/MS assay was assessed over a period of 7 months.

Results: U and DHU levels significantly increased at RT in whole blood and serum after blood sampling with increases of 12.7% and 47.6% after 2 hours, respectively. A significant difference (P=0.0036) in U and DHU levels in serum were found between SSTs and RSTs. U and DHU were stable at -20°C at least 2 months in serum and 3 weeks in plasma. Assay performance assessment fulfilled the acceptance criteria for system suitability, calibration standards, and quality controls.

Conclusion: A maximum of 1h at RT between sampling and processing is recommended to ensure reliable U and DHU results. Assay performance tests showed that our UPLC-MS/MS method was robust and reliable. Additionally, we provided a guideline for proper sample handling, processing and reliable quantification of U and DHU.

Introduction

Fluoropyrimidines, including 5-fluorouracil (5-FU), and its oral prodrugs capecitabine, tegafur and S-1, play a vital part in the treatment of several solid tumors and are estimated to be used in two millions patients annually.[1-3] Although fluoropyrimidines have been used for several decades and are reasonably well tolerated by patients, severe toxicity remains a substantial clinical problem which can result in early treatment discontinuation, hospital admission and even death.[1-5] Severe fluoropyrimidine-related toxicity is often caused by a deficiency of the main catabolic enzyme dihydropyrimidine dehydrogenase (DPD) resulting in high exposure to 5-FU due to less capacity to convert active 5-FU into inactive metabolites.[2,6] DPD is encoded by the gene *DPYD* and single nucleotide polymorphisms in *DPYD* have been related to reduced DPD enzyme activity and increased risk of severe fluoropyrimidine-related toxicity.[7,8] Pre-therapeutic screening of the *DPYD* gene and subsequent dose-reductions in *DPYD* variant allele carriers have importantly reduced the risk of severe fluoropyrimidine-related toxicity.[9,10] Despite this success, severe fluoropyrimidine-related toxicity still occurs in approximately 23% of patients who are non-carriers for the four *DPYD* variants currently being screened for.[10]

Alongside *DPYD*-genotyping several other methods have been studied to establish the presence of DPD deficiency, mainly aimed towards the measurement of endogenous uracil (U) and dihydrouracil (DHU) levels.[11-13] Considering that U is converted by DPD into DHU, similar to 5-FU, it is hypothesized that the concentration of U or the DHU/U-ratio can be used as a surrogate for the DPD enzyme activity with high levels of U or low ratio's being indicative of DPD deficiency and predictive of severe fluoropyrimidine-induced toxicity.[11-14] In line with this thought, it has been shown that pretreatment U levels are associated with an increased risk of severe fluoropyrimidine-related toxicity.[11,15,16] However, U is an endogenous substance and large variability in measured U concentrations have been found between cohorts and hospitals, most likely as result of pre-analytical factors.[17] Previous research has also shown that U levels are influenced by food-intake, circadian rhythm, fluoropyrimidine-therapy and renal impairment.[14,18-21] In addition, stability experiments performed during the development of quantification methods for U and DHU have shown that both U and DHU are highly unstable at room temperature (RT) with substantial increases in concentration, indicating that both U and DHU are also being formed after blood sampling.[22-25] This phenomenon could be the result of enzymes involved in uracil metabolism which are still active in whole blood after sampling at RT. U is formed from uridine and deoxy-uridine by uridine phosphorylase and thymidine phosphorylase, respectively.[18,22,24] Activity of these enzymes after blood sampling could lead to ex vivo formation of U resulting in an increase of the measured U concentration. This increase in concentration, indicates that specific and standardized sample handling and processing are required to ensure reliable results and subsequently accurate assessment of DPD deficiency.[22-26] However, the extent to which U and DHU levels are impacted at RT differ between studies ranging from +5.0% to +27.2% after 1hour and +22.0% to +52.2% after 2 hours dependent on the matrix.[24] The main conclusion from these studies is that blood samples taken for the measurement of pre-treatment U levels should be processed quickly, although strict guidelines are scarce. Interestingly, DPD phenotyping by measurement of pretreatment U levels is nowadays mandatory for anyone treated with fluoropyrimidines in France and is also used in Belgium whom both

also have provided guidance for sample handling (Supplementary Table 1).[27,28] Furthermore, recently Maillard *et al.* recommended to reduce the time between sampling and centrifugation to 1h as after 1,5h the uracil concentration significantly exceeded ±15% accepted bioanalytical variation (+23.4%).[29] The aim of our study was to further establish a strict and extensive guideline for sample handling and processing to ensure reliable pretreatment U levels. Afterwards, this guideline was implemented in support of a large clinical trial (The Alpe2U-study, NCT04194957) in which dosing was based on pretreatment U levels.

Methods

Stability experiments were performed on blood samples taken from both healthy individuals and from cancer patients treated with fluoropyrimidines in the Netherlands Cancer Institute - Antoni van Leeuwenhoek in the Alpe2U-study.[10] Data has been collected from patients participated in the Alpe2U study from February 2020 to August 2022.

Stability of uracil and dihydrouracil in whole blood, serum, and plasma

Blood samples were collected from 6 healthy individuals to study the stability of U and DHU in different matrices and under different storage conditions (Table 1) to mimic situations that could occur in routine clinical care. Stability of U and DHU was studied in whole blood, serum and plasma. Stability in whole blood was assessed by collecting 5 blood samples of 3.5 mL using standard serum tubes (BD Vacutainer® SST™ Tubes) and storing these blood samples at RT for 0.5h, 1h, 2h, 4h, and 24h to resemble the situation in which a sample was left at RT after sampling. Of note, whole blood coagulates within 0.5h when using serum tubes, forming a blood clot resulting in a whole blood / serum matrix which will be referred to as whole blood. Stability in serum was assessed by collecting two blood samples per individual N=6) using SSTs of 3.5 mL which were processed after 0.5h at RT. One of these serum samples was kept at RT after which aliquots of 300 µl serum were taken at 0.5h, 1h, 2h, 4h, and 24h to resemble the situation in which a sample was centrifuged according protocol but afterwards left at RT. The other serum sample was divided over 5 Eppendorf tubes of 2.0 mL from which one sample was analyzed directly after processing and the other four after 7 days, 3 weeks, 2 months and 21 months stored at -20°C to assess long-term stability. Stability in plasma was assessed by taking one blood sample of 10 mL of each individual using a lithium heparin tube (BD Vacutainer® Heparin Tubes) which was directly centrifuged after sampling. The obtained plasma was divided over 5 Eppendorf tubes of 2.0 mL of which one was directly analyzed after processing. The four other samples were analyzed after storage of 7 days, 1 month, 2 months, and 6 months at -20°C to assess long-term stability. All blood samples were centrifuged at 4°C at 3300 rpm (1960 g) after which serum or plasma was obtained for further analyses.

Standard serum tubes vs. rapid serum tubes

The stability of U and DHU in patients treated with fluoropyrimidines was assessed by comparing concentrations in standard serum tubes (BD Vacutainer® SST™ Tubes) and rapid serum tubes (BD Vacutainer® Rapid Serum Tubes) (Table 1). Two blood samples per patient were collected from 31 patients treated with fluoropyrimidines before start of treatment of which the standard serum tube (3.5 mL) which was processed after at least 0.5h and maximum of 1h at RT and one rapid serum tube (8.5 mL) which was processed after at least 5 min and maximum of 15 min at RT. Samples were centrifuged at 3300 rpm (1960 g) at 4°C after which serum was obtained and directly frozen at -20°C. Aliquots of 300 µl serum were taken to measure U and DHU concentrations.

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Table 1: Overview of samples taken per healthy individuals and storage conditions for stability testing of U and DHU.

	Matrix	Type of sampling tube	N (per individual)	Amount of blood sampled per tube	Storage condition	Time points
Short-term stability (max. 24h)	Whole blood	BD Vacutainer® SST™ Tubes	5	3.5 mL	RT	0.5h, 1h, 2h, 4h, 24h
	Serum		1	8.5 mL		
Long term stability (≥ 7 days)	Serum	BD Vacutainer® SST™ Tubes	1	8.5 mL	-20°C	0.5h, 7 days, 3 weeks, 2 months, 6 months, 21 months
	Plasma	BD Vacutainer® Heparin Tubes	1	10 mL		0 min, 7 days, 3 weeks, 2 months, 6 months, 21 months
Standard serum tubes vs. Rapid serum tubes	Serum	BD Vacutainer® SST™ Tubes	1	3.5 mL	RT	Min 0.5h - max 60 min
	Serum	BD Vacutainer® Rapid Serum Tube	1	8.5 mL		Min 5 min - max 15 min

Abbreviations: DHU, Dihydrouracil; h, hours; RT, Room temperature; SST, Serum separator tube; U, Uracil, Standard serum tubes vs. rapid serum tubes

Sample analysis

Aliquots of 300 µl serum and plasma were taken to measure U and DHU concentrations. Analytes were extracted using protein precipitation by adding 900 µl methanol:acetonitrile (50:50, v/v). After a 10 sec vortex spin, samples were shaken for 10 min in an automatic shaker. Subsequently, samples were centrifuged at 14,000 rpm (18626 g) at room temperature. Clear supernatants were collected an evaporated under a stream of nitrogen gas at 40°C for 45 min. Afterwards, dry extracts were obtained and reconstituted with 100 µl of 0.1% formic acid in water, vortex mixed, and centrifuged at 14,000 rpm for 10 min at 4°C. All samples were measured in duplicate using a validated rapid and sensitive ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) assay. A volume of 5 µl was injected into the UPLC-MS/MS system. Analytes were

chromatographically separated using an Acquity UPLC system (T3 column with gradient elution) (Waters, Milford, MA, USA) and analyzed with QTrap 5500 triple quadrupole spectrometer (Sciex, Framingham, MA, USA) equipped with an electrospray ionization source as described in detail by Jacobs *et al.*[22]

Assay performance

In addition to the stability experiments, the analytical performance of the used quantification method for U and DHU during was assessed over a period of 7 months (March 2022 to August 2022). This was done by performing and evaluating a system suitability test (SST) before starting an analytical run or a check run. SST solution was prepared by adding 10 µl of DHU working solution (100,000 ng/mL), 10 µl U working solution (10,000 ng/mL) and 100 µl internal standard working solution DHU/U (10,000 ng/mL stable isotope labeled (SIL) DHU-$^{13}C4^{15}N_2$ and 1,000 ng/mL SIL U-$^{13}C4^{15}N_2$) to 10 mL water in a polypropylene (PP) tube of 15 mL resulting in final concentrations of 100 ng/mL DHU, 10 ng/mL U, 100 ng/mL SIL DHU,10 ng/mL SIL U. To ensure adequate system suitability the covariance of variation (CV) should be less than or equal to 10.0% of the area ratio analyte/IS and the signal to noise ratio (S/N) of all analytes should be greater than 10. Furthermore, calibration standards, spiked QC samples in dialyzed plasma and a QC sample in the bio matrix were measured in every run to ensure adequate assay performance. Calibration standards consist of five non-zero standards, a standard spiked with only IS and a blank sample and were prepared in formic acid 0.1% in water and were validated over a range of 4 to 20 ng/mL. QC samples were prepared in dialyzed human plasma to remove the endogenously present U and DHU. The dialysis of the control human plasma is described in Jacobs *et al.*[22] Thereupon, QC samples were spiked with concentrations of U and DHU at 10 ng/mL and 100 ng/mL and measured in triplo, respectively. The QC sample in biomatrix was prepared by obtaining a blood sample from 2healthy individuals using standard serum tubes (BD Vacutainer® SST™ Tubes). These samples were centrifuged after 0.5h at RT at 3300 rpm (1960 g) at 4°C for 10 minutes to obtain serum which was pooled. Aliquots of 300 µL were taken and measured 5 times to determine the measured concentration. The remaining serum was filled in aliquots of 300 µL and frozen at -20°C. During every run an aliquot of the reference standard was also measured to compare to the measured concentration. To meet the acceptance criteria 4 of 5 calibration samples, 2 of 3 spiked QC mid samples in dialyzed plasma, and the QC sample in biomatrix should be within ±15% of the measured.

Statistical analysis

Descriptive statistics including median, mean, and standard deviation (SD) were used to describe the change in concentration of U and DHU under the tested condition. To assess the stability of the analytes the ratio of measured concentrations at the stated time points and the reference concentration (T=0 for plasma or T=0.5h for serum and whole blood) were calculated and multiplied by 100 to obtain a percentage. An interval of ± 15% was considered an acceptable variation in concentration from the reference concentration. After testing for normality of distribution according to the Shapiro-Wilk normality groups were statistically compared using either Wilcoxon signed-rank test of medians (non-parametric) or student's t-test (parametric). When samples of the same patient were compared tests were paired. For analyses, p-values <0.05 were considered statistically significant. All statistical analyses were performed using R v3.6.3.[30]

3 - Results

3.1 - Assay performance

The CVs of the system suitability tests ranged from 0.7% to 8.9% for U and 1.6% - 5.5% for DHU and were within the acceptance criteria of 10.0% and signal-to-noise ratio was > 10 for all runs. The deviations of the calibration standards, the spiked mid QC samples in dialyzed plasma and the QC sample in biomatrix are shown in Figure 1. The deviations of the calibration standards ranged from -5.83% to 5.0 % and -2.5% to 4.0% for U and DHU, respectively. The deviation of the spiked mid QCs in dialyzed plasma ranged from -5.0% to 15.0 and -7.0% to 13.0% for U and DHU, respectively. Both Calibration standards and spiked mid QCs of both U and DHU fulfilled the acceptance criteria with no deviations larger than 15% at all concentration levels during all runs. The U and DHU concentrations of the QC sample in biomatrix deviated more than ±15.0% once during this time period for both analytes (on separate occasions).

3.2 - Stability of uracil and dihydrouracil in whole blood, serum and plasma

The measured concentrations of U and DHU are shown in supplementary Table 2. The relative concentrations in % compared to T=0.5h are depicted in Figure 2 and Table 2. The mean U concentration increased rapidly at RT when stored as whole blood and even more pronounced when stored as serum, with increases of 12.7% and 47.6% after 2hours, respectively. However, one individual (S1) in the serum stability test had a substantially lower U (2.47 ng/mL and 56.05 ng/mL) and DHU concentration at T=0.5h compared to following time points (Table 2 and supplementary Table 3). DHU concentration also increased over time, however less substantial when compared to U, with increases of 50% and 30% after 24 hours stored as whole blood and serum, respectively. Interestingly, U and DHU concentration stored in both whole blood and in serum at T=0.5h were markedly lower compared to the concentration in plasma at T=0 (supplementary table 2). DHU/U-ratio slowly decreased during the first 4hours and significantly decreased after 24h4hours (Supplementary Table 3).

3.3 - Long term stability

Long-term stability was assessed by storing plasma and serum at -20°C for prolonged periods of time (≥ 7 days, supplementary Table 4). Both U and DHU concentration increased over time, however, less pronounced to when kept at RT. In addition, mean deviations were within the accepted deviation of ± 15% from baseline after 2 months except for plasma with a deviation of +20.4% (Supplementary table 4).

3.4 - Standard serum tube vs. rapid serum tube

Samples taken with standard serum tube and rapid serum tube were available from 31 patients who were treated with fluoropyrimidines. U concentration was significantly lower in the rapid serum tube samples compared to the standard serum tube samples with mean U levels of 10.14 ng/mL and 10.51 ng/mL (P=0.0036, Figure 3), respectively. Mean DHU levels in SSTs and RSTs were 111.7 ng/mL and 108.5 ng/mL (P=0.012, Figure 3), respectively.

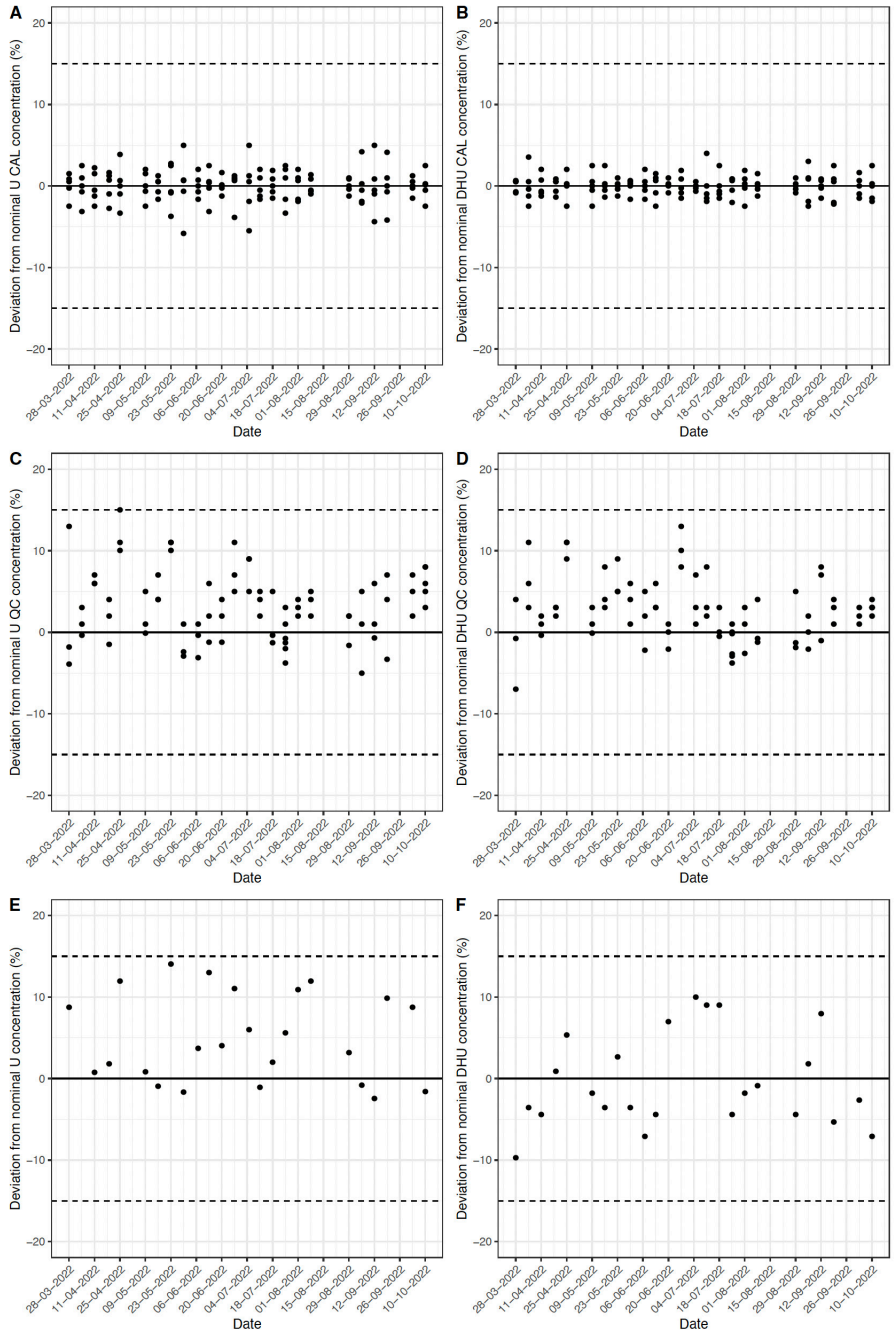

Figure 1: Overview of the deviation from the nominal U and DHU concentration of the calibration standards (A and B), spiked QC mid samples in dialyzed plasma (C and D), and the QC sample in biomatrix (E and F). *Abbreviations*: CAL, Calibration standards; DHU, Dihydrouracil; U, Uracil; QC, Quality control

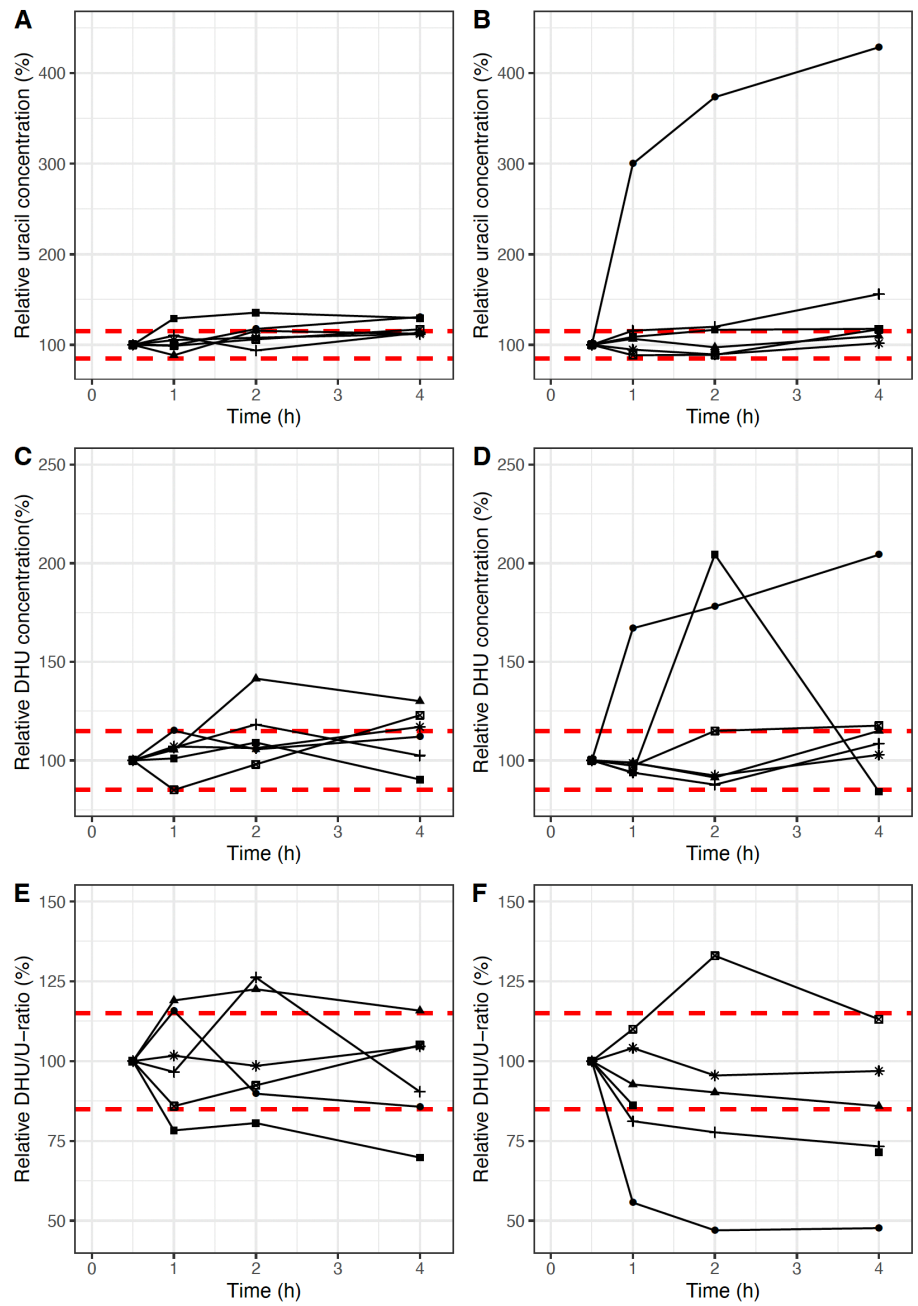

Figure 2: Concentrations of uracil (U) and dihydrouracil (DHU) and the DHU/U-ratio in % compared to T=0.5h in both whole blood and serum between T=0.5h toT=4h. Red dotted line shows accepted ±15% variation. A) Relative uracil concentration (%) in whole blood, B) Relative uracil concentration in serum, C) Relative DHU-concentration in whole blood, D) Relative DHU concentration in serum, E) Relative DHU/U-ratio in whole blood, F) Relative DHU/U-ratio in serum. *Abbreviations*: DHU, Dihydrouracil; h, hours; U, Uracil.

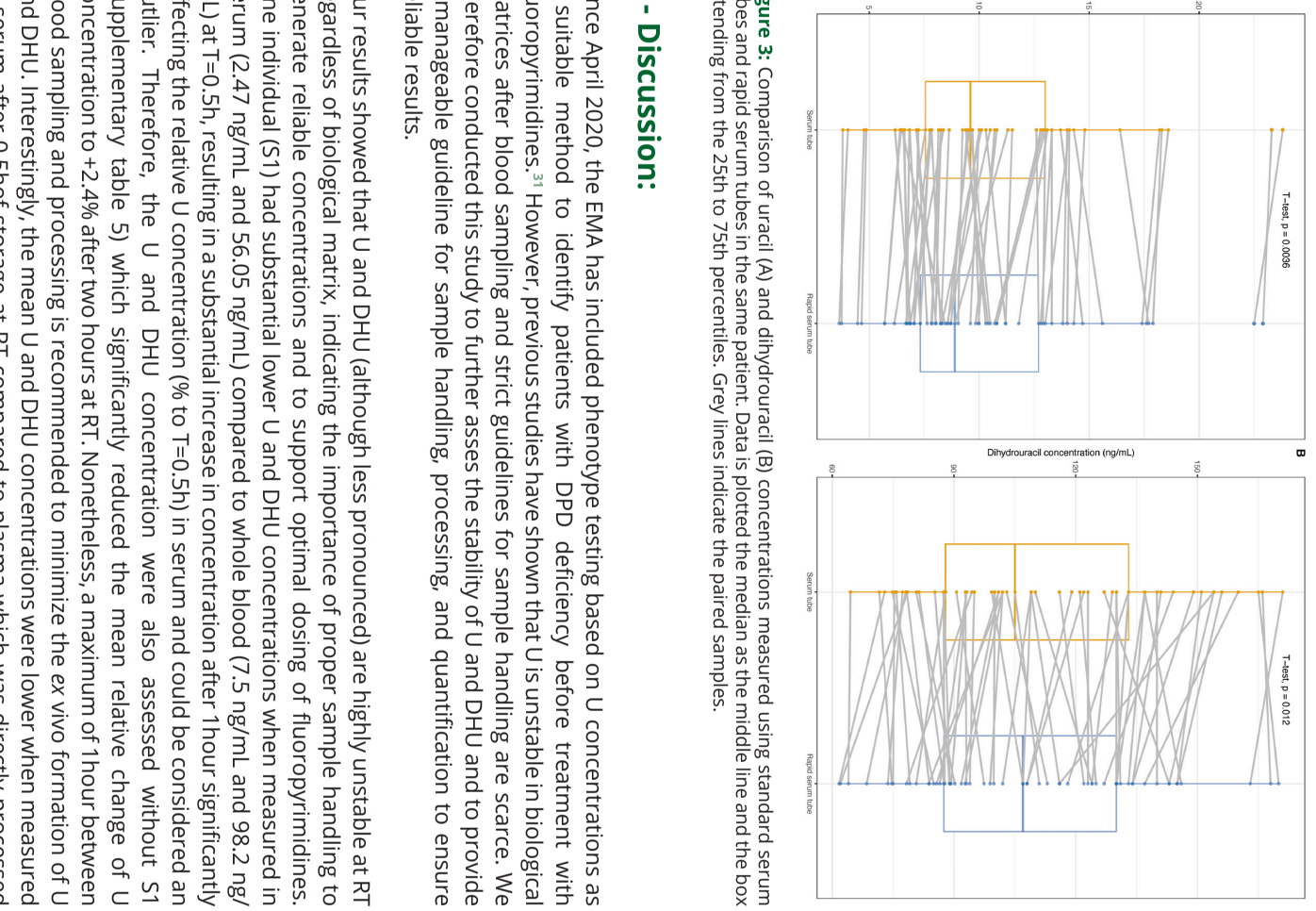

Figure 3: Comparison of uracil (A) and dihydrouracil (B) concentrations measured using standard serum tubes and rapid serum tubes in the same patient. Data is plotted the median as the middle line and the box extending from the 25th to 75th percentiles. Grey lines indicate the paired samples.

4 - Discussion:

Since April 2020, the EMA has included phenotype testing based on U concentrations as a suitable method to identify patients with DPD deficiency before treatment with fluoropyrimidines.[31] However, previous studies have shown that U is unstable in biological matrices after blood sampling and strict guidelines for sample handling are scarce. We therefore conducted this study to further asses the stability of U and DHU and to provide a manageable guideline for sample handling, processing, and quantification to ensure reliable results.

Our results showed that U and DHU (although less pronounced) are highly unstable at RT regardless of biological matrix, indicating the importance of proper sample handling to generate reliable concentrations and to support optimal dosing of fluoropyrimidines. One individual (S1) had substantial lower U and DHU concentrations when measured in serum (2.47 ng/mL and 56.05 ng/mL) compared to whole blood (7.5 ng/mL and 98.2 ng/mL) at T=0.5h, resulting in a substantial increase in concentration after 1 hour significantly affecting the relative U concentration (% to T=0.5h) in serum and could be considered an outlier. Therefore, the U and DHU concentration were also assessed without S1 (supplementary table 5) which significantly reduced the mean relative change of U concentration to +2.4% after two hours at RT. Nonetheless, a maximum of 1 hour between blood sampling and processing is recommended to minimize the *ex* vivo formation of U and DHU. Interestingly, the mean U and DHU concentrations were lower when measured in serum after 0.5hof storage at RT compared to plasma which was directly processed after blood sampling. However, this difference was still within the accepted interval of ±15% and could also be attributed to bioanalytical variation. In addition, U and DHU

Table 2: Concentration of uracil (U) and dihydrouracil (DHU) over time in % to T=0.5h in whole blood and serum at room temperature.

Analyte	Matrix	Time point	Concentration (% of T=0.5h)						Mean of concentration (% of T=0.5h)	Mean deviation (SD)	Relative standard deviation (RSD)
			S1	S2	S3	S4	S5	S6			
U	Whole blood	0.5h	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
		1h	99.47	88.48	129.04	110.16	98.93	105.26	105.22	13.75	13.06
		2h	117.53	115.45	135.48	93.73	106.07	107.89	112.69	13.98	12.40
		4h	130.73	112.26	129.49	113.44	117.08	111.84	119.14	8.70	7.31
		24h	391.33	308.32	380.70	289.02	357.68	229.82	326.15	61.96	19.00
	Serum	0.5h	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
		1h	299.80	106.59	108.75	115.63	88.49	94.69	135.66	81.01	59.72
		2h	373.28	97.28	116.56	119.90	88.90	89.38	147.55	111.38	75.49
		4h	428.34	109.85	117.67	155.94	117.19	101.77	171.79	127.06	73.96
		24h	971.66	341.71	320.19	295.47	270.52	177.43	396.17	287.65	72.61
DHU	Whole blood	0.5h	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
		1h	115.07	105.39	101.01	106.46	85.02	107.05	103.33	10.06	9.74
		2h	105.60	141.46	109.08	118.22	98.00	106.22	113.10	15.35	13.57
		4h	112.02	130.03	90.31	102.42	122.89	117.01	112.45	14.35	12.76
		24h	175.15	154.52	151.26	141.29	148.73	132.37	150.55	14.43	9.59
	Serum	0.5h	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
		1h	166.99	98.89	93.83	93.80	97.29	98.60	108.23	28.87	26.68
		2h	178.06	91.32	204.40	87.71	114.95	92.31	128.12	50.51	39.42
		4h	204.28	114.99	84.16	108.55	117.62	102.80	122.07	42.00	34.41
		24h	216.77	107.47	142.94	95.34	119.74	98.95	130.20	45.77	35.15

Abbreviations: DHU, Dihydrouracil; S, Subject; SD, Standard deviation; U, uracil.

concentrations remained stable over prolonged periods when stored at -20°C of time indicating that samples can easily be transported to other laboratories when frozen at -20°C. Of note, DHU/U-ratio seemed to be more stable over time with deviations of only 1.7% and 3.2% after 2hours in whole blood and serum compared to both U and DHU, respectively. Notably, recently it was shown that U levels were less stable when centrifuged at 4°C compared to RT. However, no clear explanation was given, and further research is needed.[32] Comparison of SSTs with RSTs has shown that the U concentration was significantly lower in RSTs. This was expected as these tubes can be processed almost immediately after sampling, preventing *ex vivo* conversion of uridine and deoxy-uridine to U. However, the absolute difference in concentration was small (~0.36 ng/mL) which suggests that the advantage of using RSTs compared to SSTs is limited. Especially, considering that immediate sample processing is not always possible in routine clinical care. Notwithstanding, when pretreatment U levels are utilized as a dose-individualization method this small absolute difference could result in misclassification of patients as being DPD deficient when their U level is close to the threshold of 16 ng/mL described in literature.[11,17]

In addition to the performed stability experiments we also assessed the performance of our UPLC-MS/MS assay over a period of 7 months in support of a large clinical trial. In this study blood samples for quantification of U and DHU were taken using serum tubes and were processed within 1hour after sampling and were frozen immediately after processing as was concluded from our stability experiments. Samples from other hospitals were send on dry ice and were measured within 1 week of sampling. Assay performance assessment fulfilled the acceptance criteria and showed that the quantification method was robust and reliable. Therefore, we have no reason to believe that possible differences in U and DHU were caused by bioanalytical errors.

A potential way to overcome the instability of U and DHU at RT could be the use of inhibitors of enzymes involved in the metabolism of U. The increase of uracil could potentially be halted by adding inhibitors of uridine phosphorylase and thymidine phosphorylase to prevent the conversion of uridine and deoxy-uridine to U and an inhibitor of DPD to prevent the conversion from U to DHU, resulting in stable uracil levels. However, this warrants further research.

5 - Conclusion

We can conclude from these experiments that U concentrations increase rapidly over time when kept at RT showing the difficulty of clinical implementation of U DPD deficiency testing. Preferred is to process these samples directly after blood sampling to minimize the increase in U, and to a lesser extent DHU, concentration. A maximum of 1hour at RT between sampling and processing is recommended. Based on our experiments and previous research we provided a guide (Table 3) in which critical pre-analytical factors have been taken into account to ensure proper sample handling, processing, and reliable quantification and could be immediately used in clinical practice. This is, to our knowledge, the first extensive guideline for sample handling, processing and quantification of U and DHU samples and could potentially reduce the number of patients being wrongly classified as DPD deficient and subsequently reduce sub optimal treatment.

Table 3: Guideline for handling and processing of blood samples for the quantification of uracil and dihydrouracil.

Steps		Recommendation	Specification
Step 1	Sampling tube/ matrix	- BD Vacutainer® Rapid Serum Tube - BD Vacutainer® SST™ Tubes - BD Vacutainer® Heparin Tubes	- Rapid serum tubes are recommended if quick processing (within 15 min of blood sampling) of the sample is possible. - Otherwise, standard serum tubes will suffice. - Lithium heparin tubes can be used however, higher concentrations were found at T=0 compared to standard serum tubes at T=0.5h.
Step 2	Blood sampling	- Between 08:00 and 10:00 am - In fasted state (>8 h since last food-intake)	- To minimize influence of circadian rhythm - To minimize influence of food-effect
Step 3	Sample handling & Processing	- Maximum of 1hour at ambient temperature in whole blood between blood sampling and centrifugation - Centrifuge at 3,300 rpm (1960 g) for 10 min at 4°C. - Transfer at least 1.0 mL of the supernatant (serum or plasma) to a 2.0 mL Eppendorf tube	- To minimize ex vivo forming or uracil and dihydrouracil - Preferably centrifuge at 4°C to minimize ex vivo formation or uracil and dihydrouracil
Step 4	Storage conditions	Freeze immediately at a maximum of -20°C Maximum storage time at -20°C: Serum: 2 months Plasma: 3 weeks	To minimize ex vivo formation or uracil and dihydrouracil
Step 5	Thawing	Thaw samples on ice-water (maximum of 2h) and vortex-mix each sample for approximately 10 sec.	To minimize ex vivo formation or uracil and dihydrouracil
Step 6	Sample processing	Centrifuge at 3,000 rpm (855 g) for 3 min at 4°C	Preferably centrifuge at 4°C to minimize ex vivo formation or uracil and dihydrouracil
Step 7	Sample processing	Transfer an aliquot of 300 µl into 2.0 mL reaction tubes and add 20 µl of internal standard working solution[22]	
Step 8	Sample pre-treatment & Quantification	- Extract analytes using protein precipitation by adding methanol:acetonitrile (50:50) - Remaining sample preparation and quantification according to method published by Jacobs et al. using a rapid and sensitive UPLC-MS/MS assay[22]	

Abbreviations: Rpm, rounds per minute; Sec, Seconds; SST, Serum separator tube; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry

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Supplemental - Chapter 11

Supplementary tables:

Table 1: Overview of guidelines used for sample processing of blood samples for measurement of uracil and dihydrouracil in France and Belgium.

Conditions	France[1]	Belgium[2]
Sampling tube	Without separating gel with anti-coagulant	Not specified
Maximum time between blood sampling and processing	RT < 1.5 h	RT < 1.5 h
	4°C < 4h	4°C < 4h
Temperature during centrifugation	4°C	4°C
Processing after centrifugation	Obtained plasma should be frozen immediately (temperature not specified)	Obtained plasma should be frozen immediately (-20°C)
Transportation	Not specified	Plasma samples should be kept at -20°C

Abbreviations: RT, Room temperature.

Table 2: Absolute uracil (U) and dihydrouracil (DHU) concentrations in whole blood, serum, and plasma at room temperature

Analyte	Matrix	Time point	Concentration (ng/mL)						Mean uracil concentration (ng/mL)
			S1	S2	S3	S4	S5	S6	
U	Plasma	0 h	7.93	6.38	6.67	6.22	8.10	13.0	8.05
	Whole blood	0.5h	7.50	6.73	5.60	6.70	9.31	11.40	7.87
		1h	7.46	5.96	7.22	7.38	9.21	12.00	8.20
		2h	8.82	7.77	7.58	6.28	9.88	12.30	8.77
		4h	9.81	7.56	7.25	7.60	10.90	12.75	9.31
		24h	29.35	20.75	21.30	19.35	33.30	26.20	25.04
	Serum	0.5h	2.47	7.36	6.34	5.86	9.69	11.30	7.17
		1h	7.41	7.85	6.90	6.77	8.57	10.70	8.03
		2h	9.20	7.16	7.39	7.02	8.61	10.10	8.25
		4h	9.22	8.09	7.46	9.13	11.35	11.50	9.46
		24h	10.58	25.15	20.30	17.30	26.20	20.05	19.93
DHU	Plasma	0 h	100.75	105.40	76.0	82.20	103.20	123.0	98.43
	Whole blood	0.5h	98.20	91.90	89.25	86.70	94.80	120.50	96.89
		1h	113.00	96.85	90.15	92.30	80.60	129.00	100.32
		2h	103.70	130.00	97.35	102.50	92.90	128.00	109.08
		4h	110.00	119.50	80.60	88.80	116.50	141.00	109.40
		24h	172.00	142.00	135.00	122.50	141.00	159.50	145.33
	Serum	0.5h	56.05	99.05	87.45	97.65	93.95	143.00	96.19
		1h	93.60	97.95	82.05	91.60	91.40	141.00	99.60
		2h	99.80	86.90	178.75	85.65	108.00	132.00	115.18
		4h	114.50	88.40	73.60	106.00	110.50	147.00	106.67
		24h	121.50	131.50	125.00	93.10	112.50	141.50	120.85

Abbreviations: DHU, Dihydrouracil; S, subject; U, uracil.

Table 3: Ratio of uracil (U) and dihydrouracil (DHU) over time and in % to t=0.5h in whole blood and serum at room temperature.

Matrix	Time point (h)	DHU/U-ratio						Mean DHU/U-ratio
		S1	S2	S3	S4	S5	S6	
Plasma	0	12.7	16.5	11.4	13.2	12.7	9.4	12.7
Whole blood	0.5	13.1	13.7	15.9	12.9	10.2	10.6	12.7
	1h	15.1	16.3	12.5	12.5	8.8	10.8	12.7
	2h	11.8	16.7	12.8	16.3	9.4	10.4	12.9
	4h	11.2	15.8	11.1	11.7	10.7	11.1	11.9
	24h	5.9	6.8	6.3	6.3	4.2	6.1	5.9
Serum	0.5	22.7	13.5	13.8	16.7	9.7	12.7	14.8
	1h	12.6	12.5	11.9	13.5	10.7	13.2	12.4
	2h	10.8	12.1	24.2	13.0	12.9	12.1	14.2
	4h	10.8	11.6	9.9	12.2	11.0	12.3	11.3
	24h	5.1	4.8	6.2	6.0	5.2	6.8	5.7
		DHU/U-ratio (% to T=0.5h)						Mean DHU/U-ratio (% to t=0.5h)
Whole blood	0.5	100	100	100	100	100	100	100
	1h	115.7	119.0	78.3	96.6	85.9	101.7	99.6
	2h	89.8	122.5	80.6	126.2	92.4	98.5	101.7
	4h	85.7	115.8	69.8	90.4	105.0	104.6	95.2
	24h	44.8	50.1	39.8	48.9	51.6	57.6	47.1
Serum	0.5	100	100	100	100	100	100	100
	1h	55.7	92.7	86.2	81.2	110.0	104.1	88.3
	2h	47.0	90.2	175.4	77.7	133.0	95.5	103.2
	4h	47.7	85.9	71.5	73.3	113.1	96.9	81.4
	24h	22.3	35.9	44.6	36.0	53.1	54.0	41.0

Abbreviations: DHU, Dihydrouracil; S, subject; U, uracil.

Table 4: Long term stability of uracil (U) and dihydrouracil (DHU) at -20°C in plasma and serum in % compared to T=0 (plasma) and t=0.5h (serum).

Analyte	Matrix	Time point (months)	Concentration (% of T=0)						Mean of concentration (% of T=0)	Mean deviation (SD)	Relative standard deviation (RSD)
			S1	S2	S3	S4	S5	S6			
U	Plasma	0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
		0.25	85.81	111.69	106.82	97.11	96.73	104.23	100.40	9.17	9.13
		0.75	97.35	111.37	165.67	102.89	94.94	95.77	111.33	27.31	24.53
		2	114.94	122.67	166.42	112.54	105.25	100.38	120.37	23.85	19.82
		6	146.28	140.00	-*	135.21	105.93	111.54	127.79	17.94	14.04
		21	138.71	143.61	-*	129.10	127.24	-*	134.67	7.80	5.79
	Serum	0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
		0.25	101.64	94.58	107.03	100.63	86.23	110.78	100.15	8.81	8.79
		0.75	128.68	107.45	118.01	81.66	85.51	120.59	106.98	19.39	18.13
		2	133.95	104.67	111.44	102.19	87.36	106.86	107.75	15.21	14.12
		6		101.15	126.74	122.26	125.84	116.67	118.53	10.49	8.85
DHU	Plasma	0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
		0.25	99.75	98.15	107.30	92.46	99.71	112.60	101.66	7.16	7.10
		0.75	114.14	107.69	113.62	112.77	95.98	113.41	109.60	7.08	6.73
		2	107.69	103.89	118.03	105.96	102.23	113.82	108.60	6.11	6.09
		6	100.89	87.62	-*	107.91	95.49	110.16	100.42	9.21	9.21
		21	111.17	102.94	-*	121.29	114.83	-*	112.56	7.66	7.60
	Serum	0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
		0.25	89.62	98.35	102.17	100.11	95.06	89.04	95.73	5.48	5.72
		0.75	104.76	113.62	123.97	102.81	107.58	96.58	108.22	9.53	8.81
		2	102.86	121.34	113.27	116.88	97.80	104.79	109.49	9.08	8.29
		6	-*	101.29	123.56	99.24	114.72	153.68	118.50	22.05	18.61

Abbreviations: DHU, Dihydrouracil; S, subject; SD, standard deviation; U, uracil. * Not enough plasma or serum was available to quantify U and DHU.

Table 5: Concentration of uracil (U) and dihydrouracil (DHU) over time in % to t=0.5h in serum at room temperature without subject 1 (outlier).

Analyte	Matrix	Time point	Concentration (% of T=0.5h)					Mean of concentration (% of T=0.5h)	Mean deviation (SD)	Relative standard deviation (RSD)
		S1	S2	S3	S4	S5	S6			
U	Serum	0.5h	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
		1h	106.59	108.75	115.63	88.49	94.69	102.83	11.01	10.71
		2h	97.28	116.56	119.90	88.90	89.38	102.40	14.87	14.52
		4h	109.85	117.67	155.94	117.19	101.77	120.48	20.85	17.31
		24h	341.71	320.19	295.47	270.52	177.43	281.07	63.77	22.69
DHU		0.5h	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
		1h	98.89	93.83	93.80	97.29	98.60	96.48	2.51	2.60
		2h	91.32	204.40	87.71	114.95	92.31	118.14	49.41	41.82
		4h	114.99	84.16	108.55	117.62	102.80	105.62	13.31	12.60
		24h	107.47	142.94	95.34	119.74	98.95	112.89	19.25	17.05

Abbreviations: DHU, Dihydrouracil; S, subject; SD, standard deviation; U, uracil.

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Conclusions and perspectives

Conclusions and perspectives

Fluoropyrimidine-based chemotherapy has been used for over half a century in the treatment of multiple types of cancers including gastrointestinal, breast, and head and neck cancers. While potentially effective, severe toxicity remains a serious clinical problem affecting ~20-30% of patients treated with a fluoropyrimidine-based treatment regimen, despite the extensive experience and research regarding dose-individualization of fluoropyrimidines.[1,2] In this thesis, we aim to optimize the treatment of fluoropyrimidine-based chemotherapy by novel dose-individualization strategies, improved understanding of clinical outcomes in *DPYD* variant carriers and older patients treated with fluoropyrimidines, and improved quantification of fluoropyrimidines and improved understanding of the stability of uracil.

Dose-individualization strategies for fluoropyrimidine-based chemotherapy

As shown in the first part and chapter 1 of this thesis, several dose-individualization strategies have been studied to reduce the onset of severe fluoropyrimidine-related toxicity. The most established dose-individualization strategy is based on pre-therapeutic genotyping of the *DPYD* gene. This gene encodes for the main catabolic enzyme of fluoropyrimidines, dihydropyrimidine dehydrogenase (DPD), and single nucleotide polymorphisms (SNPs) (c.1236G>A, *DPYD**2A, c.2846A>T, and c.1679T>G) in *DPYD* have been strongly related to the onset of severe fluoropyrimidine-related toxicity.[3–5] There is convincing clinical evidence that dose-reductions in patients carrying one of these variant alleles significantly reduces the onset of severe fluoropyrimidine-related toxicity.[5,6] However, despite the improved safety severe toxicity remains present in approximately 23% of patients, not carrying one of these four *DPYD* variants, treated with a genotype-adjusted dose of fluoropyrimidines.[5] This suggests that additional factors, possibly other *DPYD* genetic variants and/or variants affecting other genes involved in fluoropyrimidine metabolism, may contribute to severe toxicity. Therefore, in chapter 2 we sought to identify potential biomarkers of severe fluoropyrimidine-related toxicity risk in a patient population that did not carry one of the four well-characterized risk alleles to *DPYD* by sequencing of the *DPYD* gene and genome-wide association study (GWAS). *DPYD*-sequencing of 1,103 patients revealed 24 non-synonymous *DPYD* variants of which 7 were predicted to be deleterious. Furthermore, after GWAS of 942 patients none of the individual SNPs achieved genome-wide significance, however, 5 genetic variants were suggestive of association with severe toxicity. This suggests that at a population level it is not likely that besides the four established *DPYD* variants, genetic variants either inside or outside *DPYD* have a clinically relevant contribution to severe fluoropyrimidine-related toxicity in patients treated with fluoropyrimidines. Moreover, the four established *DPYD* variants are especially relevant for Caucasians, as most genetic studies are performed in patients of this ethnic origin, as was our study.[3–5,7] Results from previous studies have shown that patients of non-Western descent carry different and possibly clinically relevant variants in the *DPYD* gene compared to Western patients. For that reason, additional studies in populations with greater ancestral diversity are needed to identify other possible deleterious *DPYD* variant alleles and to improve the safety of fluoropyrimidines in populations of non-Western descent.

Severe toxicity can only be partially traced back to genetic alterations in *DPYD*, therefore other dose-individualization strategies are needed to further improve the safety of fluoropyrimidine-based chemotherapy, as reviewed in Chapter 1. A promising strategy is the use of pretreatment uracil levels. Uracil is an endogenously present pyrimidine base used in RNA and is metabolized, similar to 5-FU, by DPD into dihydrouracil.[8,9] Therefore, uracil is thought to be a proxy for DPD activity, with elevated levels or uracil being reflective of a DPD deficiency and predictive of increased risk of severe fluoropyrimidine-related toxicity. Subsequently, patients with elevated uracil levels could be treated with a reduced dose of fluoropyrimidines, similar to *DPYD* variant allele carriers, to prevent severe toxicity. The major upside of using pretreatment uracil levels is that DPD deficient patients which were not able to be identified with *DPYD*-genotyping can potentially be identified. While the evidence for genotype-directed dosing of fluoropyrimidines is substantial, the level of evidence supporting pretreatment uracil levels to predict DPD activity in clinical practice is limited. Notwithstanding this, uracil-based phenotyping is now recommended by the European Medicines Agency (EMA) and used in clinical practice in various countries in Europe.[10–12] Interestingly, in our study described in Chapter 3, pretreatment uracil levels in 955 patients treated with fluoropyrimidines did not correlate with DPD enzyme activity in peripheral blood mononuclear cells (PBMCs) or with severe fluoropyrimidine-related toxicity. Substantial issues were identified concerning the use of pretreatment uracil in clinical practice, including large variation between study center differences in measured pretreatment uracil levels, most likely as a result of pre-analytical factors. These factors include the instability of uracil in whole blood at room temperature, the circadian rhythm of DPD, and food intake. The stability of uracil has further been studied and is described in Chapter 11. Nonetheless, we conducted a large clinical trial, described in Chapter 4, in which pretreatment uracil levels were used as a dose-individualization strategy for *DPYD* wild-type patients taking critical pre-analytical factors into account. Patients without any of the four established *DPYD* variant alleles but with increased pretreatment uracil levels (> 16 ng/mL) were treated with a reduced fluoropyrimidine dose of 50%. During the interim-analysis the magnitude of impairment on DPD enzyme activity in these patients was assessed by pharmacokinetic analysis and measurement of the DPD enzyme activity in PBMCs. Non-compartmental analysis of 19 *DPYD* wild-type patients with increased uracil levels treated with a reduced dose of 50% revealed a markedly lower area-under-the-curve (AUC) of 179.2 ng*h/mL compared to 381 ng*h/mL from reference literature in which patients were treated with a full dose of fluoropyrimidines.[13] Mean exposure to capecitabine and other metabolites including 5'-dFCR, 5'-dFUR, and FBAL was also substantially lower compared to reference values from literature[13], suggesting that mean drug exposure was inadequate. Exposure to 5-FU was approximately 50% of the reference AUC obtained from Deenen *et al.* which is in line with the applied dose-reduction of 50%, suggesting that uracil-guided dosing on average resulted in undertreatment and is not suitable for phenotype-guided dosing of fluoropyrimidines. As a result of the interim-analysis, the study protocol was adapted and the dose reduction in patients with elevated uracil levels was halted. Patient inclusion continued, however, all with elevated uracil levels were treated with a full dose of fluoropyrimidines to be able to compare exposure to 5-FU and other metabolites with the patients previously treated with a reduced dose as an internal control. Pharmacokinetic analysis of two patients with elevated uracil levels treated with a full dose showed that

mean exposure to 5-FU was comparable with the reference AUC0-8h (341.2 ng*h/mL). In addition, no correlation between uracil levels and DPD enzyme activity was found. These results are in line with our previous study described in chapter 3, which also described a lack of correlation between pretreatment uracil levels, severe fluoropyrimidine-related toxicity, and DPD enzyme activity. As mentioned before, it was hypothesized that this was caused by pre-analytical factors. However, in this study, blood sampling for measurement of pretreatment uracil levels was standardized, blood samples were taken between 08:00 and 10:00 in a faster state and processed within 1 hour of blood sampling to minimize the influence of these factors. Considering these factors were taken into account, it can be questioned whether elevated pretreatment uracil levels are associated with increased exposure to 5-FU and increased risk of severe fluoropyrimidine-related toxicity in a similar manner as *DPYD* variant alleles. Thus, we currently do not recommend the use of uracil-guided dose-individualization for fluoropyrimidine-based treatment regimens. The results of our study contrast with previous findings that showed a clear association between elevated pretreatment uracil levels and increased risk of fluoropyrimidine-related toxicity. These findings have been the basis for the EMA's recommendation to include DPD phenotyping based on pretreatment uracil levels as a suitable method for identifying patients with DPD deficiency.[9,14,15] Based on the results of this study, this recommendation is worrying, as patients with increased uracil levels may be receiving inadequate doses of fluoropyrimidines, which could negatively impact treatment outcomes.

While pretreatment uracil levels could potentially improve the safety of the fluoropyrimidine-based treatment in DPD deficient patients, this is not the case for patients without a DPD deficiency, of whom ~20% still experience severe toxicity.[5,9] This highlights the need for dose-individualization strategies taking other factors in to account which may also increase the risk of severe fluoropyrimidine-related toxicity. Previous research has shown that patient- and treatment-related factors such as sex, body composition, age, body surface area (BSA), type of fluoropyrimidine-based treatment regimen and renal function have been associated with early onset of severe toxicity.[2,16–18] The presence of multiple of these risk factors may predispose patients to high risks of early severe toxicity. As was described in Chapter 5, a nomogram was constructed based on data from two large clinical trials of 2,147 patients treated with capecitabine with age, sex, BSA, treatment regimen and creatinine levels as predictors of severe capecitabine-induced toxicity. Internal validation of this nomogram demonstrated a good discriminative ability for prediction of severe capecitabine-related toxicity. This suggests that the model can accurately predict the probability of severe fluoropyrimidine-related toxicity in *DPYD* wild-type patients treated with capecitabine-based treatment regimen. In addition, by using easily measured or obtainable variables this nomogram can be easily used by clinicians in clinical practice. Ideally, this would result in a two-step approach in which the dose of capecitabine would be based on the predicted risk of severe toxicity by the nomogram. The dose would then be titrated upwards if treatment is well tolerated to ensure maximal safe exposure. Because this nomogram was only internally validated, confirmatory studies, including external and prospective validation, are needed before clinical implementation.

Clinical outcomes of fluoropyrimidine-based chemotherapy

While previous research has shown that progression-free survival (PFS) and overall survival (OS) were not negatively impacted by an initial dose reduction of 50% in *DPYD**2A variant carriers[19], this has not yet been shown for c.2836A>T and c.1236G>A carriers treated with a dose reduction of 25%. As described in the study described in chapter 6, the effectiveness of fluoropyrimidine treatment after dose reduction was compared for *DPYD* variant carriers as a group and the individual *DPYD* variants (*DPYD**2A, c.2846A>T, and c.1236G>A). The results of this study showed that there are no significant differences in PFS and OS between the pooled group of *DPYD* variant carriers and matched wild-type patients, suggesting that *DPYD*-guided dose-individualization can likely be performed safely without compromising effectiveness. In addition, no negative impact on survival was found for *DPYD**2A variant carriers confirming previous research that a 50% dose reduction can safely be applied.[19] Carriers of c.2846A>T were found to trend towards shorter PFS, although not significant, and were hampered by lower power due to the small sample size. Therefore, additional (prospective) studies are required to confirm these results. Interestingly, a consistent shorter PFS was found for c.1236G>A variant carriers treated with dose reduction of 25%. In view of the known large variation of DPD enzyme activity and exposure to 5-FU in c.1236G>A variant carriers[5], there is a strong rationale that a dose reduction of 25% may not be beneficial for all c.1236G>A variant carriers. To prevent subtherapeutic levels of 5-FU, early dose escalations on an individual basis after a reduced dose of fluoropyrimidines in c.1236G>A variant carriers in the absence of severe toxicity are highly recommended. This is especially important given the larger dose reduction of 50% currently being recommended by the Clinical Pharmacogenetics Implementation Consortium.[20]

As described in chapter 7 and 8, the majority of patients treated with fluoropyrimidines are aged 70 years and older, and these number will most likely increase due to our aging population. Despite these changing demographics, older patients are mostly underrepresented in clinical trials studying fluoropyrimidines.[21] These patients are at increased risk of severe fluoropyrimidine-related toxicity due to functional deficits, multimorbidity, and renal, hepatic, and/or bone marrow dysfunction.[22] In our study in Chapter 7, patients characteristics which are potentially associated with severe toxicity in older patients were retrospectively studied. A trend for the occurrence of severe fluoropyrimidine-related toxicity in older patients with lower BMI, increased number of comorbidities, the use of polychemotherapy or full dose monotherapy with 5-FU. However, these associations did not reach the level of significance. These results suggest that older underweight patients may have less functional reserve in that they may suffer more from severe consequences of vomiting, diarrhea, and decreased food intake that are caused by fluoropyrimidines. Furthermore, the presence of comorbidities and/or use of multiple drugs potentially reduces treatment tolerance, including tolerance of low grade (0-2) toxicity, resulting in treatment mitigation. Thus, potentially also impairing treatment efficacy. Future studies should investigate lower grades of toxicity that may impact treatment adherence and quality of life. In addition to patient characteristics, pretreatment geriatric assessments can aid clinicians in specifically identifying elderly patients at high risk of developing treatment toxicity.[23] However, large studies specifically focusing on predicting toxicity in older patients receiving fluoropyrimidine-based chemotherapy are scarce. In our study in chapter 8, patients aged 70 or older treated

with fluoropyrimidines were asked to complete several geriatric assessments, including Geriatric 8 questionnaire, 6-item cognitive impairment test, Katz Activities of Daily Living (ADL), Lawton Instrumental ADL and Patient Health Questionnaire-2 by telephone before treatment initiation. Based on the geriatric assessments the cumulative number of geriatric domains (the somatic, nutritional, functional, and mental domain) with a deficit was calculated. This study demonstrated that one-third of older patients receiving fluoropyrimidine-based chemotherapy had a poor treatment tolerability during the first two treatment cycles. Strong predictors of poor treatment tolerability within the first two treatment cycles were deficits in three or more geriatric the geriatric domains and polychemotherapy. Individual geriatric assessments did not predict severe toxicity. These results highlight the importance of multiple geriatric assessments to gain a complete picture of older patient's health status and risk of severe toxicity. These assessments doses could be used to personalize treatment with fluoropyrimidines in older patients whom have deficits in multiple geriatric domains and further optimize fluoropyrimidine-based chemotherapy. However, better understanding of the geriatric domains, including the social domain, and prospective studies in which dosing is adjusted accordingly are needed before clinical implementation.

Bioanalysis in support of fluoropyrimidine-related chemotherapy

Pharmacokinetic parameters of capecitabine and its metabolites are characterized by a substantial inter-individual variability which is likely caused by the variability in activity of different enzymes involved in the metabolism of capecitabine.[24] Bioanalytical assays for the quantitative determination of capecitabine and its metabolites are therefore imperative and essential in support of clinical pharmacological studies with fluoropyrimidines. Quantifying both capecitabine and its metabolites simultaneously presents a challenge due to their broad concentration ranges and varying polarities. While multiple analytical methods have been described in Chapter 9, none have been reported to effectively measure all analytes. Further research is needed to develop an assay that can overcome these difficulties. Our study in Chapter 10 presents the development of an accurate, sensitive, and robust UPLC-MS/MS assay for quantifying capecitabine and its metabolites in lithium heparinized plasma. Sample pretreatment involves protein precipitation, evaporation, and reconstitution. However, during method validation, we observed that 5-FUH2 converted into FUPA during evaporation, rendering the method unsuitable for the simultaneous quantification of both analytes. Despite this limitation, our assay is the first to successfully quantify capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL simultaneously. Further development of an 'all-in-one' system that meets all bioanalytical validation requirements is necessary.

In addition to quantifying fluoropyrimidines, measuring pretreatment uracil and dihydrouracil levels are of interest as well. As described in chapter 1, 3, and 4 pretreatment uracil levels have the potential to improve the safety of fluoropyrimidine-based chemotherapy. However, pre-analytical factors such as instability at room temperature and improper sampling handling and storage can lead to falsely increased uracil levels. Chapter 11, describes a study that investigates the assay performance and stability of uracil and dihydrouracil in whole blood, serum, and plasma under various storage conditions. The study found that uracil concentrations increased rapidly at room temperature, regardless of the matrix, highlighting the challenges associated with

implementing uracil DPD deficiency testing in clinical settings. Ideally, samples should be processed immediately after sampling, but this is of impractical in a clinical setting. To ensure reliable results, we recommend a maximum of 1 hour at room temperature between blood sampling and processing. Our assay performance met acceptance criteria, demonstrating the reliability and robustness of our method. We therefore have no reason to believe that difference in uracil or dihydrouracil were caused by bioanalytical errors. Our study provides a strict guideline that considers pre-analytical factors to ensure proper sample handling, processing, and reliable quantification. This guideline could be implemented in future research and clinical practice. Additionally, the use of inhibitors of enzymes involved in uracil metabolism may mitigate uracil instability, with uridine phosphorylase and thymidine phosphorylase inhibitors preventing the conversion of uridine and deoxy-uridine to uracil, and a DPD inhibitor preventing the conversion of uracil and dihydrouracil, resulting in stable uracil levels. However, further research is required to investigate this potential solution.

Future perspectives

The studies in this thesis suggest that the onset of severe fluoropyrimidine-related toxicity cannot be attributed to a single factor. Therefore, dosing strategies specifically focusing on a single factor can only improve the safety of fluoropyrimidines to a certain extend. Ideally, multiple risk factors and/or dosing strategies would be combined into a dosing algorithm using a multiparametric approach. *DPYD* status would play a pivotal role in this algorithm as the level of evidence of its association with severe toxicity is substantial. However, more research is needed to establish if other or rare *DPYD* variants or possibly other genetic factors should be included. Based on our studies, uracil currently would not have a place in this algorithm as no direct association between uracil and DPD enzyme activity, exposure to 5-FU or severe toxicity could be found. However, other phenotypic approaches, such as measurement of the DPD enzyme activity could significantly contribute to the predictive ability of this algorithm and should be considered. In addition, patient characteristics (including geriatric assessments for elderly) and therapy-related factors should be considered as these are relatively easily obtainable and have been associated with the onset of severe toxicity. However, it is crucial to bear in mind that the abovementioned risk factors do not have a similar effect on severe toxicity, therefore each factor included in the algorithm should have a corresponding weight, depending on its impact on toxicity. Developing an algorithm incorporating the mentioned risk factors and dosing strategies would necessitate gathering a vast amount of data, which poses a significant challenge. The collection and analysis of genetic and clinical data from a large and diverse population, along with robust statistical methods, would be required to ensure accuracy and reliability of the algorithm. Nonetheless, with the increasing availability of real-world evidence, the development of such an algorithm is becoming increasingly feasible and could potentially predict the majority of severe fluoropyrimidine-related toxicity.

However, the challenge of accurately predicting severe toxicity is not the only obstacle to optimizing fluoropyrimidine therapy. Dose-individualization based on a predicted probability of severe toxicity poses a significant challenge, as exemplified by the shorter PFS in c.1236G>A variant carriers after treatment with a reduced dose. Further research is required to gain a more comprehensive understanding of the heterogeneity in *DPYD*

variant carriers and of the influence of dose reductions on toxicity and treatment outcomes. This knowledge could further inform the development of personalized dosing strategies for fluoropyrimidine-based chemotherapy, leading to improved patient safety and treatment outcomes. Moreover, pharmacokinetically-guided follow up, particularly after an initial dose reduction, could serve as a valuable strategy to monitor patients for potential adverse events but also for potential subtherapeutic treatment and facilitate dose adjustments in subsequent cycles.

Fluoropyrimidines represent a vital class of anticancer agents that have served as a cornerstone treatment for numerous indications for many years. Despite their longstanding use, these drugs are expected to continue as a cornerstone treatment in the forthcoming years. However, given the ~2 million patients that are treated annually worldwide with fluoropyrimidines, it is clear that treatment optimization within this patient group is imperative. Our results demonstrated that treatment with fluoropyrimidines can be enhanced in both safety and efficacy; however, it also underscores the need for continued efforts to optimize their use.

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Conclusions and perspectives

Summary

Summary

Fluoropyrimidines are among the most commonly used anticancer drugs used for multiple types of solid tumors. The studies described in this thesis are focused on improving the safety of fluoropyrimidine-based chemotherapy by individualized dosing based on dihydropyrimidine dehydrogenase activity (DPD) and pretreatment uracil concentrations.

Part I - Dose-individualization of fluoropyrimidine-based chemotherapy

In *chapter 1*, we reviewed the available strategies to personalize the dose of fluoropyrimidine-based chemotherapy to prevent severe fluoropyrimidine-related toxicity in clinical practice. The described dose-individualization strategies are based on genotyping of the *DPYD* gene, DPD phenotyping, pharmacokinetically-guided dosing, patient characteristics at baseline, and multiparametric approaches. We evaluated the current evidence on clinical validity and utility of the different strategies, and discussed the advantages and limitations of these methods when used in clinical practice and provide our perspective on the future of dose-individualization of fluoropyrimidine-based chemotherapy.

In *chapter 2*, we described a study in which we sought to identify potential biomarkers of severe fluoropyrimidine-related toxicity in a patient population that did not carry any of the four well-characterized risk alleles in *DPYD* (*DPYD**2A, c.1236G>A, c.2846A>T, and c.1679T>G). To identify additional genetic variants in *DPYD* we sequenced the exon of *DPYD* and classified *DPYD* variants based on both in silico and *in vitro* tools. Association with severe fluoropyrimidine-related toxicity was assessed using a matched-pair analysis. To identify variant outside of *DPYD* we performed a genome-wide association study (GWAS). Association with severe fluoropyrimidine-related toxicity was assess by logistic, Cox, and ordinal regression analyses. Clinical data including baseline characteristics and toxicity data were derived from patients included in the Alpe-DPD study (clinicaltrial.gov identifier NCT02324452). Twenty-four non-synonymous, frameshift, and splice site *DPYD* variants were detected in ten of 1,103 patients. Seven of these variants (c.1670C>T, c.1913T>C, c.1925T>C, c.506delC, c.731A>C, c.1740+1G>T, and c.763-2A>G) were predicted to be deleterious. The carriers of either of these variants showed a trend towards a 2·14-fold (95% CI, 0·41-11·3, P=0·388) increased risk of severe toxicity compared to matched controls (N=30). After GWAS of 942 patients, no individual single nucleotide polymorphisms achieved genome-wide significance (P≤5x10-8), however, five variants were suggestive of association (p<5x10-6) with severe toxicity. Our results from *DPYD* exon sequencing and GWAS analysis suggest that at population level, testing for single markers in addition to the four established *DPYD* variants, currently has limited value in improving fluoropyrimidine toxicity prediction.

In *chapter 3*, we described a study in which we aimed to determine the value of pretreatment uracil levels in predicting DPD deficiency and severe fluoropyrimidine-related toxicity. *DPYD*-guided dosing has shown to improve safety of fluoropyrimidine-based chemotherapy. However, severe toxicity remains in ~23% of patients not carrying

*DPYD**2A, c.1236G>A, c.2846A>T, or c.1679T>G. Endogenous plasma uracil is converted into dihydrouracil (DHU) by DPD, the concentration of uracil in plasma is thought to be a proxy for DPD activity, with (exceptionally) elevated levels of endogenous plasma uracil being reflective of a (complete) DPD deficiency and therefore predictive of increased risk for severe toxicity. We determined the pretreatment uracil levels in 955 patients, and assessed to correlation with DPD enzyme activity in peripheral blood mononuclear cells (PBMCs) and severe fluoropyrimidine-related toxicity. Interestingly, uracil levels did not correlate with DPD enzyme activity nor were elevated uracil levels predictive of severe fluoropyrimidine-related toxicity. Moreover, we identified substantial issues concerning the use uracil in clinical practice, including large between-center differences in measured uracil levels, most likely a result of differences in pre-analytical sample handling and processing. We therefore urge that robust clinical validation should first be performed before pretreatment plasma uracil levels are used in clinical practice as part of a dosing strategy for fluoropyrimidines.

In *chapter 4,* we described a large prospective clinical trial which studied whether the risk of fluoropyrimidine-related toxicity can be reduced by uracil-guided dose-individualization of fluoropyrimidine-based chemotherapy. Uracil levels were measured for all patients prior to treatment with fluoropyrimidine-based treatment regimens. According to standard of care in the Netherlands, all patients were genotyped for *DPYD**2A, c.1236G>A, c.2846A>T, and c.1679T>G variant alleles. Patients not carrying any of the mentioned *DPYD* variant alleles were considered *DPYD* wild-type and were treated based on pretreatment uracil levels. *DPYD* wild-type patients with elevated uracil levels (>16 ng/mL) and all *DPYD* variant allele carriers received an initial dose reduction of 50%. Toxicity in *DPYD* wild-type patients with elevated uracil levels was compared to historical cohort of *DPYD* wild-type patients with elevated uracil levels which were treated with a full dose of fluoropyrimidines. In addition, pharmacokinetic parameters in *DPYD* wild-type patients with elevated uracil levels were investigated and compared to reference values from literature. A total, 612 evaluable patients were enrolled, of whom 22 (3·6%) were *DPYD* wild-type patients with elevated uracil levels and 46 (7·5%) were *DPYD* variant allele carriers. Two of the 22 wild-type patients with elevated uracil levels received a full fluoropyrimidine-dose at start of treatment and were therefore excluded from analysis. The incidence of severe fluoropyrimidine-related toxicity in *DPYD* wild-type patients with elevated uracil levels treated with a reduced dose was significantly lower compared to the historical cohort of *DPYD* wild-type patients with elevated uracil levels treated with a full dose (4/20 [20%] vs 6/14 [43%], P=0·029). Pharmacokinetic analysis of these patients showed that exposure to 5-FU was substantially lower (179.2 ng*h/mL) compared to reference values (381 ng*h/mL) from a historical cohort treated with a full dose. Furthermore, no correlation (R^2=0·014, P=0·64) between uracil levels and DPD enzyme activity was found. Pretreatment uracil levels as an indicator for DPD enzyme activity, accompanied by a dose reduction of 50% in *DPYD* wild-type patients with elevated uracil levels, results in an inadequate exposure to 5-FU in our study population and thus should not be recommended to be used for dose-individualization of fluoropyrimidine-based chemotherapy. More research is needed to investigate whether the proposed threshold of uracil and if this is associated with decreased functionality of the DPD enzyme and subsequently predictive of severe fluoropyrimidine-related toxicity, but in the meantime, we strongly encourage *DPYD* genotype-guided dosing.

In *chapter 5*, we described the development of a predictive model based on patient-related and treatment-related factors aimed at estimating the risk of developing severe toxicity when treated with capecitabine-based treatment regimens. Data from patients from two large clinical trials including 1463 and 913 cancer patients treated with capecitabine was used to develop the predictive model (nomogram). Based on previous findings in multivariable analyses, age, sex, body surface area, treatment regimen, and creatinine level were used for model development. All these variables were included in the model irrespective of their statistical significance in the univariable or multivariable regression. Multivariable logistic regression was used to for the development of the nomogram. All predefined predictors used in the nomogram were available for 1745 patients. Age, sex, and type of treatment regimen were strong predictors of toxicity with increasing risk of severe toxicity with age (per 10 years an increase in OR of 1.17, 95% CI 1.04 - 1.32, P=0.01) and male sex having a decreased risk of developing severe toxicity (OR 0.68, 95% CI 0.49 - 0.95, P=0.02). The model's discriminative ability, as measured by the concordance index, was 0.68 (95% CI 0.64-0.71), which indicates good discriminative ability for prediction of severe toxicity. Ideally, this nomogram could be used in patients who are identified as *DPYD* wild-type after *DPYD* genotyping to predict the probability of developing severe fluoropyrimidine-related toxicity. Subsequently, the dose could be individualized if the probability exceeds a predefined threshold (e.g., 40%). The developed nomogram includes readily available parameters could be a helpful tool for clinicians to assess the risk of developing severe fluoropyrimidine-related toxicity in patients treated with capecitabine.

Part II - Clinical outcomes of fluoropyrimidine-based chemotherapy

In *chapter 6*, we described a retrospective matched-pair analysis to compare the progression-free survival (PFS) and overall survival (OS) between *DPYD* variant allele carriers treated with a reduced dose and *DPYD* wild-type patients treated with full fluoropyrimidine dose. Data from a prospective multicenter study (NCT02324452) in which *DPYD* variant carriers received a 25% (c.1236G>A and c.2846A>T) or 50% (*DPYD**2A and c.1679T>G) reduced dose and data from *DPYD* variant carriers treated with a similarly reduced dose of fluoropyrimidines identified during routine clinical care, was obtained. In each matched group (pooled *DPYD* variant carriers and the individual *DPYD**2A, c.2846A>T and c.1236G>A carriers) each *DPYD* variant carrier was matched to three unique *DPYD* wild-type controls from the Alpe-DPD study. Matching was performed based on gender, age (±10 years), primary tumor type (colorectal, breast, gastric, other), stage of cancer (local, locally advanced, or metastatic), and treatment regimen. Survival analyses were performed using Kaplan-Meier estimates and Cox regression. In total, 156 *DPYD* variant carriers and 775 *DPYD* wild-type controls were available for analysis. Sixty-one c.1236G>A, 25 *DPYD**2A, 13 c.2846A>T and -when pooled- 93 *DPYD* variant carriers could each be matched to three unique *DPYD* wild-type controls. Cox regression analysis showed no statistically significant difference in PFS for the 93 pooled *DPYD* variant carriers compared to their matched wild-type controls (HR, 1.23; 95% CI 1.00 - 1.51, P=.053), but significantly shorter PFS in c.1236G>A variant carriers (HR, 1.43; 95% CI 1.10 to 1.86, P=.007). No statistically significant difference in PFS was found between 25 *DPYD**2A and

13 c.2846A>T variant carriers and matched wild-type controls. OS was not negatively impacted by *DPYD*-guided dosing in all matched groups. Notably, both c.1236G>A and c.2846A>T carriers still experienced significantly more severe fluoropyrimidine-induced toxicity after a 25% dose reduction compared to wild types treated with a full dose.11 Apparently, the 25% dose reduction was not sufficient to protect all the variant carriers from developing severe toxicity and therefore more research is needed to explain the heterogeneity of the impact on DPD enzyme activity in *DPYD* variant allele carriers. Meanwhile, close monitoring with early dose modifications, escalation when possible and reduction when necessary, based on toxicity is recommended when treating c.1236G>A variant carriers with a reduced fluoropyrimidine starting dose.

Despite *DPYD* genotype-based dosing, grade ≥3 toxicity and treatment mitigation frequently occur with cancer treated with fluoropyrimidine-based chemotherapy. In *chapter 7,* we retrospectively studied older adults (≥ 65 years) treated with fluoropyrimidine-based chemotherapy to identify predictors of grade ≥3 toxicity or treatment mitigation. The association between tumor-, treatment-, and patient-related characteristics and the occurrence of grade ≥3 toxicity or the composite endpoint of grade ≥3 toxicity or treatment mitigation (dose reduction, cycle delay discontinuation) were analyzed using univariate and multivariate logistic regression analyses. A total of 311 patients were included with a median age of 71.2 years were included. Grade ≥3 toxicity occurred in 23.2% of patients. In the multivariate analysis, none of the characteristics studied were significantly associated with the occurrence of grade ≥3 toxicity, but trends towards increased toxicity were observed for female sex, the use of polychemotherapy, low BMI, increased number of comorbidities, and reduced renal function. The composite endpoint occurred in 41.2% of patients and was inversely associated with the use of reduced dose range of (capecitabine) monotherapy in multivariate analysis. Female sex, the use of polychemotherapy, starting at a reduced dose of polychemotherapy, low BMI, and hypoalbuminemia appeared to be associated with decreased tolerance, but this study likely lacked the power to establish a significant association.

In *chapter 8*, we prospectively studied older adults treated with fluoropyrimidine-based chemotherapy to identify predictors for poor treatment tolerability. Patients aged ≥70 years with a malignancy who received fluoropyrimidine-based chemotherapy were selected from the prospective, multicenter, non-randomized Alpe2U-study (NCT04194957). Before treatment initiation, participants underwent a geriatric assessment investigating the somatic, nutritional, functional, and mental domain. Predictors of the composite endpoint "poor treatment tolerability", defined as either Common Toxicity Criteria Adverse Events (CTCAE) grade 3-5 chemotherapy-related toxicity, dose reduction or treatment discontinuation within the first two cycles, were analyzed using uni- and multivariable logistic regression models. In total. 194 patients were included with a median age of 75 and the most common tumor types were colorectal (60%) and esophagogastric (19%). The majority of patients (89%) were treated with capecitabine-based chemotherapy. Poor treatment tolerability within the first two cycles was seen in 31% of patients. In the multivariable analysis, associations with poor treatment tolerability were found for deficits in 3-4 geriatric domains compared with 0 deficits (odds ratio (OR) 4.03, 95% CI 1.09-14.97, P=0.037) and polychemotherapy (OR 2.83, 95% CI 1.31-6.09, P=0.008). These results indicate that having deficits in multiple

geriatric domains and polychemotherapy are associated with poor treatment tolerability within the first two treatment cycles. The findings highlight the importance of geriatric assessment before fluoropyrimidine initiation to estimate the risk of treatment intolerance.

Part III - Bioanalysis in support of fluoropyrimidine-based chemotherapy

In *chapter 9*, we reviewed the published methods for quantification of capecitabine and its metabolites. The review focused on the sample pretreatment, chromatography and detection and discusses the choice of internal standards and analytical problems encountered during the analysis of capecitabine and its metabolites in biological matrices. The major challenge in the quantification of capecitabine and its metabolites are the simultaneous extraction and analysis due to the large differences in polarity of the analytes.

In *chapter 10*, we described the development and validation of an ultra-high performance liquid chromatography coupled to turbo ion spray tandem mass spectrometry (UPLC-MS/MS) method to quantify capecitabine and its metabolites including 5'-deoxy-5-fluorocytidine (5'-dFCR), 5'-deoxy-5-fluorouridine (5'-dFUR), 5-FU, and fluoro-β-alanine (FBAL) in lithium heparinized human plasma. Analytes were extracted by protein precipitation, chromatographically separated by Acquity UPLC HSS T3 column with gradient elution and analyzed with a tandem mass spectrometer equipped with an electrospray ionization source. Capecitabine and 5'-dFCR were quantified in positive ion mode and 5'-dFUR, 5-FU and FBAL were quantified in negative ion mode. Total chromatographic run time was 9 min. Stable isotopically labeled internal standards were used for all analytes. The assay was validated over the range from 25.0 to 2,500 ng/mL for capecitabine, 10.0 to 1,000 ng/mL for 5'-dFCR, 5'-dFUR, and 5-FU and 50.0 to 5,000 ng/mL for FBAL in human plasma. Validation results have shown the developed assay allows for reliable quantitative analysis of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU and FBAL in plasma samples.

In *chapter 11*, we studied the stability of uracil and assessed the assay performance of our validated UPLC-MS/MS method which quantifies uracil and dihydrouracil. Previous research has shown that uracil is highly unstable at room temperature in whole blood and improper sample handling may result in falsely increased uracil levels, subsequently resulting in patients being misclassified as DPD deficient. Therefore, we performed stability by collecting whole blood, serum, and plasma from 6 healthy volunteers, which were left at room temperature for 0.5h, 1h, 2h, 4, and 24h. Furthermore, long-term stability at -20°C in plasma and serum from healthy volunteers and a comparison of U and dihydrouracil (DHU) levels using standard serum tubes and rapid serum tubes for patient samples were studied. In addition, performance of the used UPLC-MS/MS was assessed over period of 7 months in support of our clinical trial described in chapter 4. Uracil levels significantly increased at room temperature in whole blood and serum with increases of 12.7% and 47.6% after 2 hours, respectively. Furthermore, a significant difference in uracil level was found between standard serum tubes and rapid serum tubes (10.51 ng/mL vs. 10.14 ng/mL, P=0.0036). Long term stability was at least adequate

up until 3 weeks and 2 months at -20°C in plasma and serum, respectively. Assay performance assessment fulfilled the acceptance criteria for system suitability, calibration standards, quality controls (QCs), and the QC in biomatrix. Our results were in line with previous studies and showed a rapid increase of uracil when blood samples were left at room temperature. We therefore recommend a maximum of 1 hour at room temperature between blood sampling and sample processing to ensure stable and reliable uracil levels. Assay performance tests showed that our quantification method was robust and reliable. Additionally, we provided a guideline for proper sample handling, processing and reliable quantification of U based on our own experiments and literature which can be applied in clinical practice to ensure reliable results.

Nederlandse samenvatting

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Fluoropyrimidines behoren tot de meest gebruikte antikankergeneesmiddelen voor verschillende soorten solide tumoren. De studies beschreven in dit proefschrift zijn gericht op het verbeteren van de veiligheid van fluoropyrimidines door middel van geïndividualiseerd doseren op basis van de activiteit van dihydropyrimidine dehydrogenase (DPD) en de uracil concentratie voor start van de behandeling.

Deel I - Dosisindividualisatie van fluoropyrimidines

In *hoofdstuk 1* hebben we de beschikbare strategieën geëvalueerd om de dosis van fluoropyrimidines te personaliseren om ernstige fluoropyrimidine-gerelateerde toxiciteit in de klinische praktijk te voorkomen. De beschreven dosisindividualisatie strategieën zijn gebaseerd op genotypering van het *DPYD* gen, DPD-fenotypering, farmacokinetisch gestuurde dosering, patiëntkarakteristieken voor start van de behandeling en multiparametrische benaderingen. We hebben het huidige bewijs met betrekking tot de klinische validiteit en bruikbaarheid van de verschillende strategieën geëvalueerd en bediscussiëren de voor- en nadelen van deze methodes bij gebruik in de klinische praktijk. We geven ook onze visie op de toekomst van dosisindividualisatie van fluoropyrimidines en welke aanvullende informatie nodig is om de verschillende strategieën te kunnen implementeren in de klinische praktijk.

Hoofdstuk 2 beschrijft een klinische studie waarin er getracht wordt potentiële bio-markers voor ernstige fluoropyrimidine-gerelateerde toxiciteit te identificeren in een patiëntenpopulatie die geen van de vier goed gekarakteriseerde risicovarianten in *DPYD* (*DPYD**2A, c.1236G>A, c.2846A>T en c.1679T>G) met zich mee dragen. Om aanvullende genetische varianten in *DPYD* te identificeren, hebben we de exon van *DPYD* gesequenced en *DPYD*-varianten geclassificeerd op basis van zowel *in silico*- als *in vitro* tools. De associatie met ernstige fluoropyrimidine-gerelateerde toxiciteit werd beoordeeld met behulp van een matched-pair analysis. Genetische variatie buiten het *DPYD* gen werden onderzocht met behulp van een genome-wide association study (GWAS). De associatie met ernstige fluoropyrimidine-gerelateerde toxiciteit werd onderzocht met behulp van logistische, Cox en ordinale regressie. De klinische data, waaronder baseline karakteristieken en toxiciteit, zijn afkomstig van patiënten die hebben deelgenomen aan de Alpe-DPD studie (clinicaltrial.gov identifier: NCT02324452). In totaal werden in 10 van de 1,103 patiënten die genetisch gescreend zijn, 24 non-synonymous frameshift en splice site *DPYD*-varianten geïdentificeerd. Zeven van de geïdentificeerde varianten (c.1670C>T, c.1913T>C, c.1925T>C, c.506delC, c.731A>C, c.1740+1G>T en c.763-2A>G) werden geclassificeerd als potentieel schadelijk. In de dragers van deze varianten was het risico op het ontwikkelen van ernstige aan fluoropyrimidine gerelateerde bijwerkingen 2.14-maal (95% CI, 0.41-11.3, P=0.388) groter in vergelijking met gematchte controle patiënten. Na GWAS-analyse van 942 patiënten werd er geen enkel individueel single nucleotide polymorfisme gevonden dat significant (p≤5x10-8) geassocieerd was met ernstige toxiciteit, echter zijn er wel 5 genetische varianten geïdentificeerd met een suggestieve associatie (p≤5x10-6) met ernstige toxiciteit. De resultaten van *DPYD* exon sequencing en GWAS-analyse suggereren dat het testen van individuele markers naast de vier goed gekarakteriseerde *DPYD*-varianten momenteel beperkte waarde heeft bij het voorspellen fluoropyrimidine-toxiciteit op populatieniveau.

In *hoofdstuk 3* is een klinische studie beschreven gericht op het achterhalen van de waarde van uracil in het voorspelen van DPD-deficiëntie en ernstige fluoropyrimidine-gerelateerde toxiciteit. Het doseren van fluoropyrimidines op geleide van *DPYD*-gen heeft aangetoond de veiligheid van fluoropyrimidines significant te kunnen verbeteren. Echter, ondanks de verbetering van de veiligheid ervaart ~23% van de patiënten die geen van de vier *DPYD*-varianten (*DPYD**2A, c.1236G>A, c.2846A>T, or c.1679T>G) met zich meedraagt ernstige toxiciteit. Endogeen uracil wordt in het lichaam omgezet naar dihydrouracil (DHU) door DPD, en de concentratie van uracil wordt beschouwd als een proxy voor DPD-activiteit. Verhoogde concentraties duiden op een (volledige) DPD-deficiëntie en worden als voorspellend gezien voor een verhoogd risico op ernstige toxiciteit. In deze studie is de uracil concentratie voor start van de behandeling bepaald bij 955 patiënten en de correlatie ervan met DPD-enzymactiviteit in perifere bloed mononucleaire cellen (PBMCs) en ernstige fluoropyrimidine-gerelateerde toxiciteit geëvalueerd. Uracil concentraties vertoonden geen correlatie met DPD-activiteit, noch voorspelden verhoogde uracil concentraties ernstige fluoropyrimidine-gerelateerd toxiciteit. Bovendien zijn er aanzienlijke problemen geconstateerd met het gebruik van uracil in de klinische praktijk, waar grote verschillen concentratie tussen centra in uracil concentratie zijn geïdentificeerd. Dit is hoogstwaarschijnlijk het gevolg van verschillen in pre-analytische monster verwerking. Robuuste klinische validatie wordt sterk aanbevolen voordat uracil concentraties in de klinische praktijk worden toegepast als doseringsstrategie voor fluoropyrimidines.

In *hoofdstuk 4* wordt een prospectieve klinische studie beschreven waarin wordt onderzocht of het risico op ernstige fluoropyrimidine-gerelateerd toxiciteit gereduceerd kan worden door middel van op uracil geleide dosis-individualisatie van fluoropyrimidines. Voorafgaand aan de start van de behandeling met fluoropyrimidines werd bij alle patiënten de uracil concentratie gemeten en de het *DPYD*-genotype bepaald. Patiënten zonder een *DPYD*-variant (*DPYD**2A, c.1236G>A, c.2846A>T, or c.1679T>G) werden beschouwd als *DPYD*-wildtype en behandeld op basis van de uracil concentratie. *DPYD*-wildtype patiënten met een verhoogde uracil concentratie (>16 ng/mL) en alle *DPYD*-variant dragers werden behandeld met 50% gereduceerde dosering. Toxiciteit bij *DPYD*-wildtype patiënten met een verhoogde uracil concentratie werd vergeleken met een historisch cohort van *DPYD*-wildtype patiënten met een verhoogde uracil concentratie die werden behandeld met een volledige dosis fluoropyrimidines. In aanvulling daarop werden farmacokinetische parameters onderzocht bij *DPYD*-wildtype patiënten met een verhoogde uracil concentratie en vergeleken met referentiewaarden uit de literatuur. In totaal zijn er 612 evalueerbare patiënten includeert waarvan er 22 (3,6%) *DPYD*-wildtype patiënten waren met een verhoogde uracil concentratie en 46 (7.5%) *DPYD*-variant dragers. Twee van de 22 wildtype patiënten met een verhoogde uracil concentratie werden behandeld met een volledige dosis fluoropyrimidines bij aanvang van de behandeling en werden daarom uit de analyse gesloten. De incidentie van ernstige fluoropyrimidine-gerelateerde toxiciteit bij *DPYD* wild-type patiënten met een verhoogde uracil concentratie die behandeld werden met gereduceerde dosering in vergelijking met het historische cohort van *DPYD*-wildtype patiënten met een verhoogde uracil concentratie

die behandeld werden met een volledige dosis (4/20 [20%] vs. 6/14 [43%], P=0.029). Farmacokinetische analyse van deze patiënten toonde aan dat de blootstelling van 5-FU aanzienlijk lager was (179.2 ng*h/mL) in vergelijking met referentiewaarden (381 ng*h/mL) uit een historisch cohort behandeld met een volledige dosis. Verder werd er geen correlatie gevonden (R^2=0.014, P=0.64) gevonden tussen uracil concentratie en DPD-enzymactiviteit. Uracil concentratie als een indicator voor DPD-enzymactiviteit, in combinatie met een dosisverlaging van 50% bij *DPYD*-wildtype patiënten met een verhoogde uracil concentratie, resulteert in onvoldoende blootstelling aan 5-FU in onze onderzoekspopulatie en wordt op dit moment niet aanbevolen als dosis-individualisatie strategie voor behandeling met fluoropyrimidines. Meer onderzoek is nodig om te bepalen of de voorgestelde drempelwaarde van uracil geassocieerd is met een verminderde functionaliteit van het DPD-enzym en of uracil voorspellend is voor ernstige fluoropyrimidine-gerelateerde toxiciteit. In de tussentijd wordt doseren op geleide van het *DPYD*-genotype sterk aanbevolen.

In *hoofdstuk 5* wordt de ontwikkeling van een model beschreven die het risico op ernstige capecitabine-gerelateerd toxiciteit kan voorspellen aan de hand van patiënt- en behandeling-gerelateerde karakteristieken. Het model is ontwikkeld aan de hand van patiënt- en behandelkarakteristieken. Gegevens van patiënten uit twee grote klinische onderzoeken, waaronder 1463 en 913 kankerpatiënten behandeld met capecitabine, werden gebruikt om het model (nomogram) te ontwikkelen. Op basis van eerdere bevindingen in multivariate analyses werden leeftijd, geslacht, lichaamsoppervlakte, behandelingsregime en creatininespiegel gebruikt voor de ontwikkeling van het model. Al deze variabelen werden opgenomen in het model, ongeacht hun statistische significantie in de univariate of multivariate regressie. Multivariate logistische regressie werd gebruikt voor de ontwikkeling van het nomogram. Alle vooraf gedefinieerde voorspellers die in het nomogram werden gebruikt, waren beschikbaar voor 1745 patiënten. Leeftijd, geslacht en het behandelingsregime waren sterke voorspellers van toxiciteit, waarbij het risico op ernstige toxiciteit toenam met de leeftijd (per 10 jaar een toename in OR van 1.17, 95% CI 1.04-1.32, P=0.01) en mannelijk geslacht een verminderd risico had op het ontwikkelen van ernstige toxiciteit (OR 0.68, 95% CI 0.49-0.95, P=0.02). De onderscheidende capaciteit van het model, gemeten aan de hand van de concordence-index was 0.68 (95% CI 0.64-0.71), wat duidt op een goede onderscheidende capaciteit voor de voorspelling van ernstige toxiciteit. Idealiter zou dit nomogram kunnen worden gebruikt bij patiënten die na *DPYD*-genotypering worden geïdentificeerd als *DPYD*-wildtype, om de waarschijnlijkheid van het ontwikkelen van ernstige fluoropyrimidine-gerelateerd toxiciteit te voorspellen. Vervolgens zou de dosis geïndividualiseerd kunnen worden als de waarschijnlijkheid op ernstige toxiciteit een vooraf gedefinieerd drempel overstijgt (bijv. 50%). Het beschreven nomogram omvat gemakkelijk beschikbare parameters en kan een nuttig instrument zin voor clinici op het risico op het ontwikkelen van ernstige fluoropyrimidine-gerelateerd toxiciteit te beoordelen bij patiënten behandeld met capecitabine.

Deel II - Klinische uitkomsten van behandeling met fluoropyrimidines

In *hoofdstuk 6* wordt een retrospectieve matched-pair studie beschreven waarin de progressievrije overleving (PFS) en algehele overleving (OS) wordt vergeleken tussen *DPYD*-variant dragers behandeld met een gereduceerde dosering en *DPYD*-variant dragers behandeld met een volledige dosering fluoropyrimidines. Gegevens werden verkregen uit een prospectieve multicenter studie (NCT02324452) waarin *DPYD*-variant dragers met een 25% (c.1236G>A en c.2846A>T) of een 50% (*DPYD**2A en c.1679T>G) gereduceerde dosering werden behandeld en uit de klinische praktijk van *DPYD*-variant dragers die met dezelfde gereduceerde dosering zijn behandeld. In elke gematchte groep (gepoolde *DPYD*-variant dragers en de individuele *DPYD**2A, c.2846A>T en c.1236G>A dragers) werd elke *DPYD*-variant drager gematcht met drie unieke *DPYD*-wildtype controle patiënten uit de Alpe-DPD studie. Patiënten werden gematcht op basis van geslacht, leeftijd (± 10 jaar), primair tumortype (darm-, borst-, maag- en overige kanker), kankerstadium (lokaal, lokaal gevorderd en gemetastaseerd) en behandelingsregime. Overlevingsanalyses werden uitgevoerd met behulp van Kaplan-Meier schattingen en Cox-regressie. In totaal waren er 156 *DPYD* variantdragers en 775 *DPYD*-wildtype controle patiënten beschikbaar voor analyse. Eenenzestig c.1236G>A, 25 *DPYD**2A, 13 c.2846A>T en - wanneer gepoold - 93 *DPYD* variantdragers konden elk worden gematcht met drie unieke *DPYD*-wildtype controle patiënten. Cox-regressie toonde geen statistisch significant verschil in PFS voor de gepoolde 93 *DPYD* variantdragers in vergelijking met de gematchte *DPYD*-wildtype controle patiënten (HR 1.23; 95% CI 1.00 - 1.51, P=0.053). Een significant kortere PFS werd gevonden bij c.1236G>A variantdragers (HR 1.43, 95% CI 1.10 - 1.86, P=0.007). Er werd geen statistisch significant verschil in PFS gevonden tussen *DPYD**2A en c.2846A>T en gematchte *DPYD*-wildtype controles. OS werd niet negatief beïnvloed door het op *DPYD*-genotype geleide doseren in alle groepen. Opmerkelijk genoeg ervaarden zowel c.1236G>A als c.2846A>T dragers nog steeds significant meer ernstige fluoropyrimidine-geïnduceerde toxiciteit na een dosisreductie van 25% in vergelijking met *DPYD*-wildtype controle patiënten behandeld met een volledige dosis. Dit suggereert dat de 25% dosisreductie niet voldoende is om alle variant dragers te beschermen tegen het ontwikkelen van ernstige toxiciteit en er meer onderzoek nodig is om heterogeniteit van impact op de DPD-enzymactiviteit bij *DPYD*-variantdragers te verklaren. In de tussentijd wordt aanbevolen om c.1236G>A variant dragers nauwlettend te monitoren met vroege dosisaanpassingen, escalatie indien mogelijk en verlaging indien nodig, op geleide van toxiciteit bij de behandeling met een verlaagde startdosering van fluoropyrimidines.

Deel III - Bioanalyse ter ondersteuning van de behandeling met fluoropyrimidines

In *hoofdstuk 9* hebben we de beschikbare literatuur met betrekking tot kwantificatie methoden voor capecitabine en bijbehorende metabolieten uiteengezet. Dit review is gericht op de monstervoorbewerking, chromatografische scheiding en detectie en bediscussieerd de interne standaarden en analytische problemen die men tegenkomt bij de analyse van capecitabine en metabolieten in biologische matrices. De grootste

uitdaging in de kwantificatie van capecitabine en bijbehorende metabolieten is de gelijktijdige extractie en analyse door grote verschillen in polariteit van de analieten.

In *hoofdstuk 10* beschrijven we de ontwikkeling en validatie van een ultra-high performance liquid chromatografie gekoppeld aan turbo ion spray tandem massa spectrometrie (UPLC-MS/MS) methode voor de kwantificatie van capecitabine, 5'-deoxy-5-fluorocytidine (5'-dFCR), 5'-deoxy-5-fluorouridine (5'-dFUR), 5-FU en fluoro-β-alanine (FBAL) in lithium heparine humaan plasma. De analieten zijn geëxtraheerd met behulp van eiwitprecipitatie, chromatografisch gescheiden met behulp van een Acquity UPLC HSS T3 kolom met gradiënt elutie en geanalyseerd door een tandem massa spectrometer uitgerust met een electrospray ionisatie bron. Capecitabine en 5'-dFCR werden gekwantificeerd in een positieve ion modus en 5'-dFUR, 5-FU en FBAL werden gekwantificeerd in een negatieve ion modus. De totale chromatografische looptijd was 9 minuten. Stabiele isotopisch gelabelde interne standaarden werden gebruikt voor alle analieten. De assay is gevalideerd over een range van 25.0 tot 2,500 ng/mL voor capecitabine, 10.0 tot 1,000 ng/mL vor 5'-dFCR, 5'-dFUR en 5-FU en van 50.0 tot 5,000 ng/mL voor FBAL in humaan plasma. De resultaten van de validatie hebben aangetoond dat de ontwikkelde assay robuust is en betrouwbaar is voor de kwantitatieve analyse van capecitabine, 5'-dFCR, 5'-dFUR, 5-FU en FBAL in plasma monsters.

In *hoofdstuk 11* hebben we de stabiliteit van uracil en dihydrouracil onderzocht, evenals de evaluatie van de prestatie van onze gevalideerde UPLC-MS/MS methode voor de kwantificatie van deze verbindingen. Voorgaand onderzoek heeft aangetoond dat uracil in volbloed op kamertemperatuur zeer instabiel is. Onjuiste monstervoorbewerking kan resulteren in fout-positieve verhoogde concentraties van uracil wat kan leiden tot een verkeerde classificatie van een patiënt als "DPD-deficiënt". Om hier meer inzicht in te krijgen hebben we stabiliteitsonderzoek uitgevoerd door volbloed, serum en plasma te verzamelen van 6 gezonde vrijwilligers verzameld, die gedurende 0.5 uur, 1 uur, 2 uur, 4 uur en 24 uur op kamertemperatuur werden bewaard. Aanvullend is de lange termijn stabiliteit bij -20°C in plasma en serum van gezonde vrijwilligers onderzocht, en zijn de concentraties van uracil en dihydrouracil vergeleken bij het gebruik van standaard serumbuizen en "rapid" serum buizen. Bovendien werd de prestatie van de gebruikte UPLC-MS/MS methode ter ondersteuning van onze klinische studie (zoals beschreven in *hoofdstuk 4*) geëvalueerd gedurende een periode van 7 maanden. De uracil concentratie nam significant toe bij kamertemperatuur in zowel volbloed als serum, met toenames van 12.7% en 47.6% na 2 uur. Bovendien werd een er een significant verschil gevonden in uracil concentratie tussen standaard serumbuizen en "rapid" serum buizen (10.51 ng/mL vs. 10.14 ng/mL, P=0.0036). Lange termijn stabiliteit bij 20°C was ten minste adequaat tot 3 weken in plasma en 2 maanden in serum. De evaluatie van de prestatie van onze methode voldeed aan de acceptatiecriteria voor systeemgeschiktheid, kalibratie-standaarden, kwaliteitscontroles (QCs) en QC in biomatrix. Onze resultaten waren in lijn met eerdere studies en toonden een snelle toename van uracil wanneer bloedmonsters bij kamertemperatuur werden bewaard. We raden daarom een maximum van 1 uur bij kamertemperatuur aan tussen bloedafname en monsterverwerking om stabiele en betrouwbare uracil concentraties te waarborgen. De evaluatie van de prestatie van onze methode heeft aangetoond dat onze methode robuust en betrouwbaar was. Aan de hand van literatuur en de resultaten van dit onderzoek hebben we richtlijnen opgesteld

voor het omgaan met de monsters, de monstervoorbewerking en kwantificatie van uracil, die kunnen worden toegepast in de klinische praktijk om betrouwbare resultaten te waarborgen.

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Authorship contribution

Preface
Author's contribution: The general research question and its general scientific perspective were proposed by my promotors and co-promotors. I delineated the research objectives, described how it fits in current scientific literature and described its potential impact. I wrote the first draft of the preface and implemented the input and feedback of my supervisory team.

Chapter 1: Individualized Dosing of Fluoropyrimidine-Based Chemotherapy to Prevent Severe Fluoropyrimidine-Related Toxicity: What are the Options?
Author's contribution: I proposed the design of the review, performed the literature search, drafted an initial version of the manuscript, and implemented the contribution of the co-authors and external reviewers up to final publication.

Chapter 2: Discovering novel germline genetic variants outside and inside *DPYD* related to the onset of severe fluoropyrimidine related toxicity
Author's contribution: I contributed to the conception and design of the study, and contributed to the interpretation of the study results. I wrote the first draft of the manuscript and implemented the contributions of the co-authors.

Chapter 3: Dihydropyrimidine Dehydrogenase Phenotyping Using Pretreatment Uracil: A Note of Caution Based on a Large Prospective Clinical Study
Author's contribution: I performed the data-analysis and visualization, and contributed to the interpretation of study results. I contributed to the writing of the first draft of the manuscript and provided comments on subsequent versions of the manuscript. I also contributed to the implementation of the contribution of the co-authors and external reviewers up to the final publication.

Chapter 4: Dose-individualization of fluoropyrimidine-based chemotherapy based on pretreatment uracil levels - A safety and pharmacokinetic analysis form the Alpe2U study
Author's contribution: I proposed and contributed to the conception and design of the study, contributed to the execution of the clinical trial, performed the data-analysis, and contributed to the interpretation of the study results. I wrote the first draft of the manuscript and implemented the contribution of co-authors.

Chapter 5: Prediction of severe toxicity in patients treated with capecitabine-based anticancer regimens
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Chapter 6: Survival of cancer patients with *DPYD* variant alleles after reduced-dose fluoropyrimidine-based chemotherapy - A matched-pair analysis
Author's contribution: I proposed the design of the study, contributed to the data collection, performed the data analysis, and contributed to the interpretation of the

study results. I wrote the first draft of the manuscript and implemented the contribution of co-authors and external reviewers up to final publication.

Chapter 7: Identifying potential predictors of severe fluoropyrimidine-based chemotherapy in elderly

Author's contribution: I contributed to the conception and design of the study, and contributed to the data collection and interpretation of the study results. I contributed to the writing of the first draft of the manuscript, contributed to the implementation of the contribution of the co-authors.

Chapter 8: Geriatric parameters influencing severe fluoropyrimidine-induced toxicity

Author's contribution: I contributed to the conception and design of the study, and contributed to the data collection and interpretation of the study results. I contributed to the writing of the first draft of the manuscript, contributed to the implementation of the contribution of the co-authors.

Chapter 9: A review of the bioanalytical methods for the quantitative determination of capecitabine and its metabolites in biological matrices

Author's contribution: I contributed to the conception and design of the review, performed the literature search, drafted an initial version of the manuscript, and implemented the contribution of the co-authors and external reviewers up to final publication.

Chapter 10: Development of a UPLC-MS/MS assay for the quantitative determination of capecitabine, 5'-deoxy-5-fluorocytidine (5'-dFCR), 5'-deoxy-5-fluorouridine (5'-dFUR), 5'-fluorouracil (5-FU), and α-fluoro-β-alanine (FBAL)

Author's contribution: I contributed to conception of the study and the interpretation of the results of this study. I wrote the first draft of the manuscript, and implemented the contribution of the co-authors and external reviewers up to final publication.

Chapter 11: Assay performance and stability of uracil and dihydrouracil in clinical practice

Author's contribution: I contributed to the conception and design of the study, performed the data analysis, and contributed to the interpretation of the study results. I wrote the first draft of the manuscript, and implemented the contribution of the co-authors and external reviewers up to final publication.

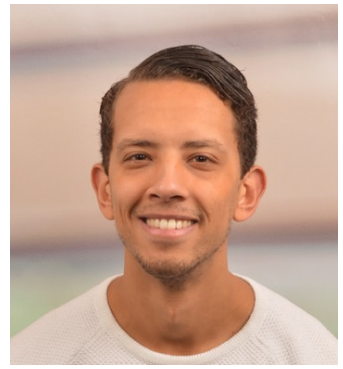
Conclusions and perspectives

I wrote the first draft of the conclusions and perspectives and implemented the input and feedback from my supervisory team.

Authorship contribution

Curriculum Vitae

Curriculum Vitae



Jonathan Emanuel Knikman was born on May 29th, 1993, in Brunssum and grew up in Dordrecht, The Netherlands. After graduating from secondary school at the Insula College in Dordrecht, he went on to study Pharmacy at the University of Utrecht in 2011. As part of his master program, he did a six-month internship at the Erasmus Medical Center in Rotterdam under the supervision of Prof. dr. Patricia van den Bemt, investigating the adherence, quality of life and patient satisfaction between vitamin K anticoagulant and direct oral anticoagulant users. In 2018, Jonathan obtained his master's degree in Pharmacy (PharmD). In May 2018, Jonathan started his PhD research at the division of Clinical Pharmacology and the department of Pharmacy & Pharmacology of the Netherlands Cancer Institute – Antoni van Leeuwenhoek hospital, under the supervision of Prof. dr. Jos H. Beijnen, Prof. dr. Henk-Jan Guchelaar, and Dr. Annemieke Cats. His thesis focused on the treatment optimization of fluoropyrimidine-based chemotherapy. As of June 2023, he started a researcher position as assistant professor at the Julius Center for Health Sciences & Primary Care. Here his research is focused on the treatment optimization of antihypertensive therapy.

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Dankwoord

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