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## **DPD screening to prevent toxicity in fluoropyrimidine treated patients**

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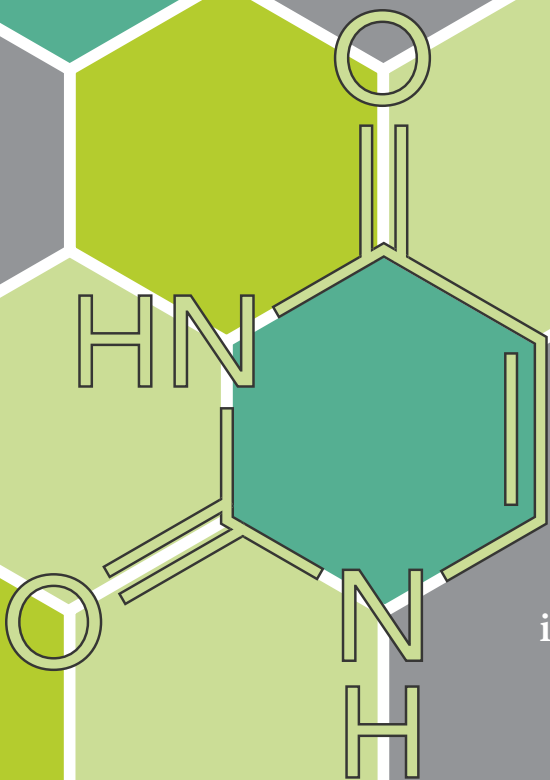


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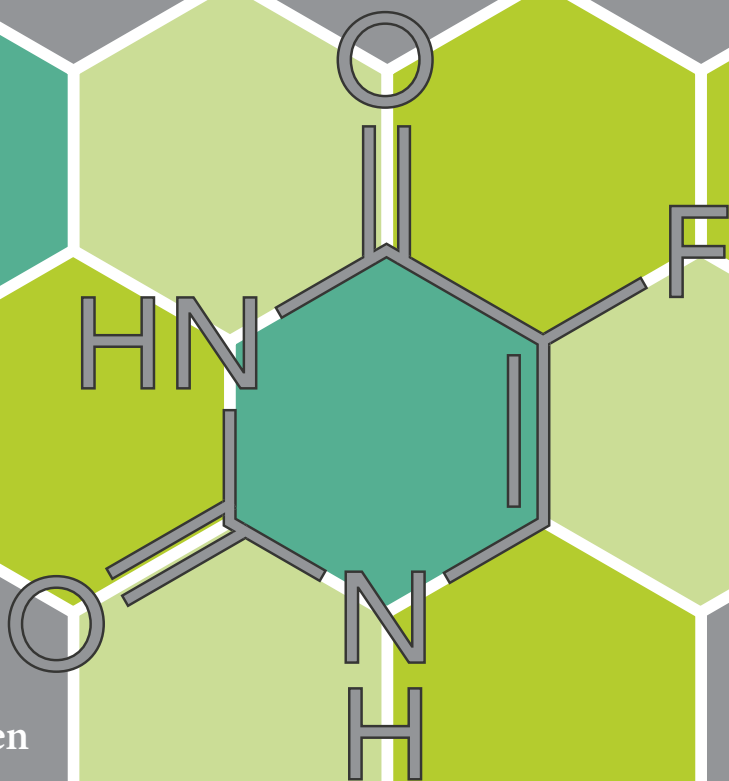
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DPD screening  
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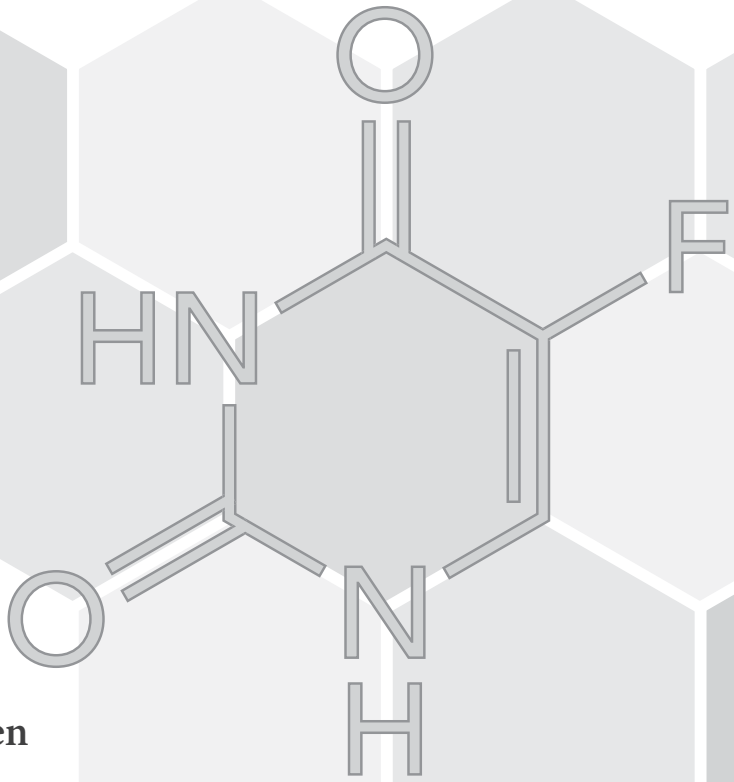


Maurice  
van Staveren





DPD screening  
to prevent **TOXICITY**  
in fluoropyrimidine  
treated patients



Maurice  
van Staveren

The research presented in this thesis was performed at the departments of Clinical Pharmacy and Toxicology of Leiden University Medical Center, Leiden, The Netherlands.

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# **DPD screening to prevent toxicity in fluoropyrimidine treated patients**

## **Proefschrift**

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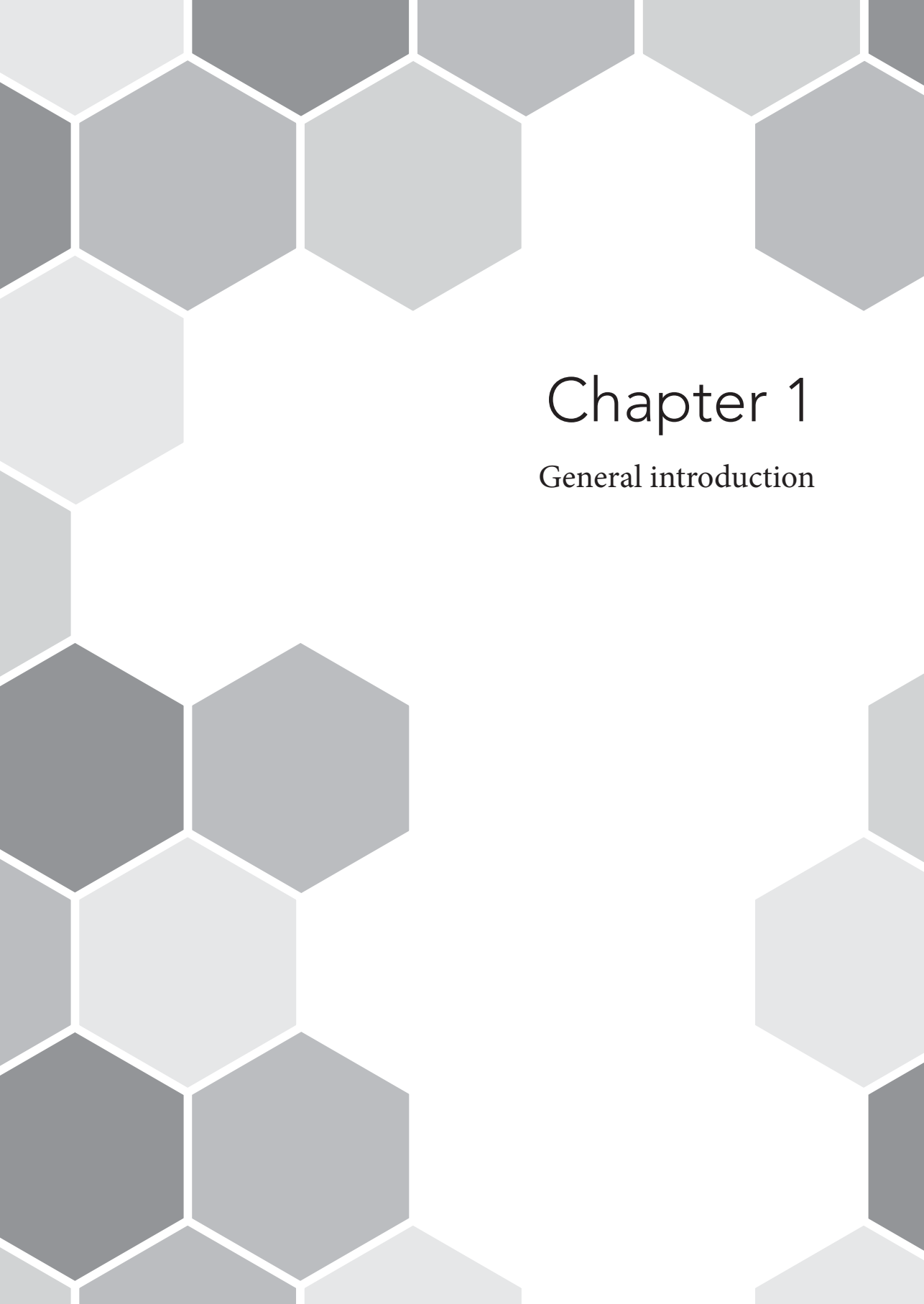
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# Chapter 1

General introduction

## Fluoropyrimidines in the treatment of cancer

Fluoropyrimidines have been applied in the treatment of cancer for decades and are extensively used worldwide. The most known fluoropyrimidine is 5-fluorouracil (5-FU). 5-FU was developed in 1954 after the discovery that liver tumours absorbed radioactive uracil more readily than normal liver cells. Previously, Charles Heidelberger discovered that fluorine in fluoroacetic acid inhibited the vital enzyme aconitase that catalyzes the stereo-specific isomerization of citrate to isocitrate via cis-aconitate in the tricarboxylic acid cycle. Based on this finding that a fluorine atom causes a profound alteration in biological effect, and the fact that uracil in tumours is incorporated into RNA, Heidelberger substituted a hydrogen atom in the uracil molecule for a fluorine atom with the idea that if 5-FU were to have biological activity, it should block DNA synthesis [1]. These two scientific results led to the development of 5-FU as an anticancer drug [2].

In a clinical setting, 5-FU is administered intravenously, as a bolus or prolonged infusion or as a topical cream and orally. The intravenous and oral presentations of 5-FU are widely used in the treatment of a range of cancers such as breast cancer, cancers of the gastrointestinal and urogenital tract and head and neck cancer. Topical 5-FU is used in the treatment of actinic keratosis and Bowen's disease. Meta-analysis of the efficacy of intravenous continuous infusion of 5-FU compared with bolus administration in advanced colorectal cancer showed that continuous infusion is superior compared to bolus infusion in terms of tumour response and resulted in a slightly increase in overall survival [3]. The morbidity associated with indwelling catheters and infusion pumps and patient inconvenience with regard to the length of continue infusion schemes of 5-FU led to the development of the oral fluoropyrimidines. Oral administration of 5-FU results in wide intra- and interpatient variability in 5-FU plasma levels [4]. The oral fluoropyrimidines can be divided into 3 groups, 5-FU prodrugs; 5-FU combined with a DPD inhibitor, and 5-FU prodrugs combined with a DPD inhibitor [5]. The most frequently used 5-FU prodrug is capecitabine. This drug is first converted to 5-deoxyfluorocytidine in the liver by carboxylesterase and then converted to doxifluridine by cytidine deaminase, which is found in liver, plasma, and tumour tissue. The toxic intermediary doxifluridine is then converted to 5-FU by thymidine phosphorylase, that is more abundant in tumours than in normal tissue, resulting in tumour 5-FU concentrations that far exceed plasma levels and produce greater antineoplastic effects with lower toxicity [6, 7]. The availability of capecitabine resulted in the Netherlands in a massive shift from intravenously therapy to oral therapy in the treatment of colorectal

cancer. In the Netherlands, approximately 90% of all fluoropyrimidine-containing therapy contains capecitabine. Published cost-effectiveness analysis regarding the treatment of stage III colon cancer shows that capecitabine is less costly and more effective than 5-FU treatment [8, 9], which indicates that huge cost savings are established in the treatment of colon cancer.

## Fluoropyrimidine mechanism of action and toxicity

*In vivo*, 5-FU is partly converted intracellular to the active metabolites fluorodeoxyuridine monophosphate (FdUMP) and fluorouridine triphosphate (FUTP) [10]. FUTP disrupts RNA synthesis and FdUMP inhibits thymidylate synthase (TS), which is the key enzyme in the *de novo* synthesis of thymidylate that is necessary for DNA replication and repair. TS catalyzes the reductive methylation of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (TMP). The TS protein functions as a dimer with one nucleotide-binding site. The 5-FU metabolite FdUMP binds to this nucleotide-binding site forming a stable complex and thereby blocking the binding of dUMP, which results in inhibition of TMP synthesis [11, 12].

The metabolite FUTP is incorporated into RNA leading to dysfunction [3]. In cancer cell lines a correlation was observed between FUTP misincorporation into RNA and loss of clonogenic potential [13, 14]. Several *in vitro* studies indicated that 5-FU misincorporation disrupt many aspects of RNA processing leading to profound effects on cellular metabolism and viability [10]. With intravenous administration, the length of infusion is correlated with the mechanism of action of 5-FU since the mechanism of action of bolus administration is mainly inhibition of TS, while continuous infusion is cytotoxic by misincorporation into RNA [15]. These two mechanisms of action lead to differences in type of toxicity. The results of a meta-analysis showed that, with 5-FU bolus, hematologic toxicity was more frequent than with continuous infusion (31% and 4%,  $p < 0.0001$ ). On the other hand, continuous infusion resulted in higher incidence of hand-foot syndrome compared with bolus infusion (13% and 34%,  $p < 0.0001$ ) [3]. Incidence and proportions of all other toxicities were identical for bolus and continuous infusion. Independent prognostic factors were age, sex, and performance status for nonhematologic toxicities, performance status, and treatment for hematologic toxicities, and age, sex, and treatment for hand-foot syndrome.

## Fluoropyrimidine metabolism

The rate-limiting enzyme in 5-FU catabolism is dihydropyrimidine dehydrogenase (DPD). More than 80% of the amount of 5-FU administered is normally catabolized primarily in the liver where DPD is abundantly expressed [16, 17]. DPD is also present in normal and tumour cells. 5-FU is converted by DPD into 5,6-dihydrofluorouracil (DHFU). Subsequently, DHFU is degraded into fluoro- $\beta$ -ureidopropionic acid (FUPA) and fluoro- $\beta$ -alanine (FBAL). In general, fluoropyrimidines are tolerated well although approximately 10% of the patients treated with fluoropyrimidines suffer from CTC grade III or IV toxicity. Interindividual variability in the activity of DPD can be the cause of this severe toxicity. DPD is encoded by *DPYD* and polymorphisms in *DPYD* have shown to be related to toxicity in colorectal patients treated with capecitabine and 5-FU [18-20]. In patients with a near complete DPD deficiency this can even lead to death [21]. Knowledge of the clinical impact of reduced DPD activity on the pharmacokinetics and pharmacodynamics of fluoropyrimidines may lead to dose individualized therapy. Therapeutic drug monitoring of 5-FU has been shown to result in reduced intra- and inter-individual variability in 5-FU plasma levels and pharmacokinetic guided dose adjustments of 5-FU-containing therapy results in a significantly improved efficacy and tolerability [22]. In addition, pharmacokinetic Michaelis-Menten models allows the use a limited sampling strategy and offer the opportunity to predict a priori the 5-FU plasma concentrations in patients receiving adapted doses of 5-FU [23].

## Aim and outline of this thesis

The general aim of this thesis is to study the use of an oral uracil loading dose as probe for DPD deficiency in cancer patients treated with fluoropyrimidines and to develop a test procedure that is suitable to be incorporated broadly into daily practice in hospital care. With regard to this latter aspect, it will be studied if a prospective DPD testing strategy can be successfully incorporated into routine clinical healthcare as a standard procedure for all patients using a fluoropyrimidine.

In **CHAPTER 2** a review is presented of studies that describe predictive tests developed for screening for DPD. This chapter outlines the status of methods for testing for DPD deficiency and their use in daily practice.

In order to perform clinical tests, uracil as an investigational medicinal product must be approved by the competent authorities since is not registered for human use. The document

required for this approval is called 'Investigational Medicinal Product Dossier' (IMPD) and includes summaries of information related to the quality, manufacture and control of the Investigational Medicinal Product, data from non-clinical studies and data from its clinical use. **CHAPTER 3** is the Investigational Medical Product Dossier of uracil for the oral uracil loading test that was used for approval by the Medical Ethical committees of clinical trials described in Chapter 5, 6 and 7.

In **CHAPTER 4** the pharmacokinetics of the oral uracil loading dose in healthy volunteers and cancer patients is studied. Part of this study is a dose finding strategy in order to determine which uracil dose is optimal. This study can be considered as a phase 1 study in order to determine optimal dose, safety and compare results between healthy volunteers and patients. In this study oral uracil is administered in dosages of 500 and 1000 mg/m<sup>2</sup> Body Surface Area (BSA) to healthy volunteers. This study is important to determine the most effective dose and to determine if disease status will influence the results of the uracil loading test.

**CHAPTER 5** describes a study in which the oral uracil loading dose is administered to colorectal cancer patients with and without metastasis, all with normal DPD status. This study is performed to investigate if presence of metastases will influence the pharmacokinetics of oral uracil. Since the objective of the oral uracil loading dose is that it will be used prospectively in all patients with different types of cancer, disease status ideally does not influence pharmacokinetics. This is the first study in which the potential effect of metastatic disease on uracil pharmacokinetics in colorectal cancer patients will be investigated.

The EURABEL2 study is described in **CHAPTER 6**. The aim of this study is to develop a limited sampling strategy, to detect decreased uracil elimination in patients with a DPD deficiency and to perform a more in-depth quantitative compartmental pharmacokinetic analysis of uracil plasma concentrations.

In this study patients with toxicity will be included and divided in two groups based on the results of the measurement of DPD activity in peripheral blood mononuclear cells (PBMCs), which in this study is considered to be the gold standard. The performance of the oral uracil loading dose in the two groups will be compared with the results of the gold standard and specificity and sensitivity will be calculated.

In **CHAPTER 7** the results are described of a study that evaluated the clinical acceptance and adherence of a prospective *DPYD* genotyping strategy that was implemented at Leiden University Medical Center. The objective of this genotyping strategy is that all patients who

have an indication for first time treatment with 5FU or CAP are routinely prospectively screened for the presence of four pathogenic variants to prevent 5FU related toxicity caused by *DPYD* genetic variations.

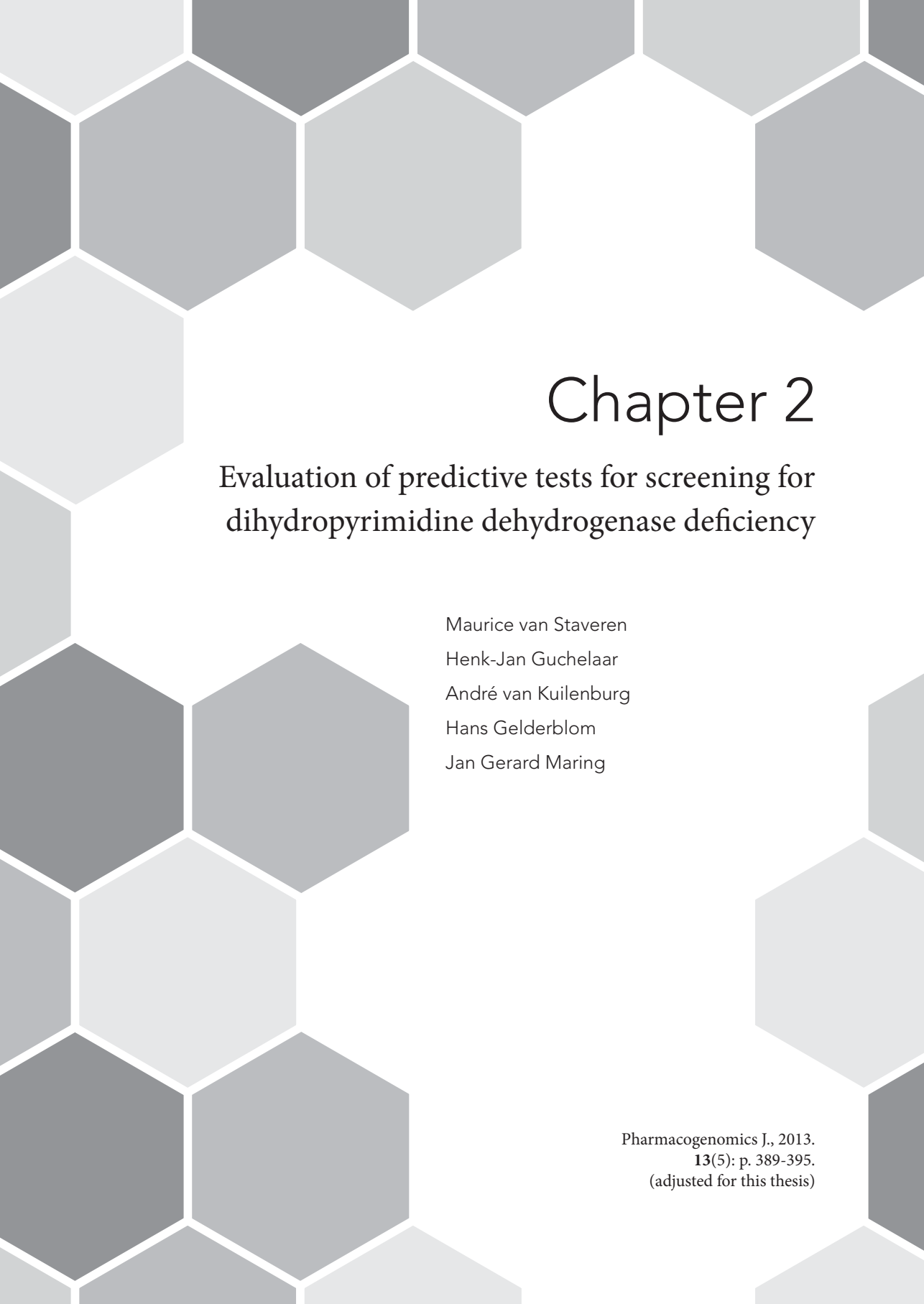
This thesis ends with a general discussion and future perspectives in **CHAPTER 8**. A summary of this thesis is presented in **CHAPTER 9**.

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# Chapter 2

## Evaluation of predictive tests for screening for dihydropyrimidine dehydrogenase deficiency

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Pharmacogenomics J., 2013.  
13(5): p. 389-395.  
(adjusted for this thesis)

## ABSTRACT

5-fluorouracil (5-FU) is rapidly degraded by dihydropyrimidine dehydrogenase (DPD). Therefore, DPD deficiency can lead to severe toxicity or even death following treatment with 5-FU or capecitabine. Different tests based on assessing DPD enzyme activity, genetic variants in *DPYD* and mRNA variants have been studied for screening for DPD deficiency, but none of these are implemented broadly into clinical practice. We give an overview of the tests that can be used to detect DPD deficiency and discuss advantages and disadvantages of these tests.

## INTRODUCTION

The fluoropyrimidine 5-fluorouracil (5-FU) and its prodrug capecitabine are the cornerstone of treatment of numerous types of cancer. The use of fluoropyrimidines is associated with numerous side effects such as myelosuppression, hand-foot syndrome, mucositis, diarrhea and occasionally cardiac toxicity.

After parenteral administration of 5-FU, 70–90% of the parent drug is degraded by dihydropyrimidine dehydrogenase (DPD) [1-5]. A small proportion of patients develop extreme toxicity after administration of a fluoropyrimidine due to a partial or complete DPD deficiency and hence a strongly reduced capacity to degrade 5-FU [6-10]. In case of complete DPD deficiency 5FU treatment may even result in a lethal outcome [11]. It was initially estimated that in 3–5% of Caucasians the activity of DPD is strongly reduced due to (epi)genetic variations in the gene encoding DPD [4, 12]. However, this percentage has been disputed since there is still no consensus on the definition of DPD deficiency and therefore the incidence of DPD deficiency reported in numerous studies is strongly dependent on the method used to assess DPD deficiency [13] and the cut off level chosen to define DPD deficiency [5].

Prospective testing for DPD deficiency in patients might prevent DPD deficient patients from severe toxicity or even death. In this review, we describe current methods for determination of DPD deficiency. We discuss the potential and limitations of these tests for routine clinical use. In addition, we have defined recommendations that can help successful implementation of a preemptive testing strategy to predict fluoropyrimidine related toxicity.

## METHODS

To identify studies describing diagnostic tests to detect DPD deficiency a systematic MEDLINE, EMBASE, Web of Science and Cochrane search was conducted using the following combination of (MESH) terms: “dihydropyrimidine dehydrogenase deficiency”, “dpd deficiency”, “Familial Pyrimidinemia”, “uracil”, “capecitabine”, “dihydrouracil dehydrogenase (nadp)”, “dihydrouracil dehydrogenase”, “dihydropyrimidine dehydrogenase”, “dihydrouracil dehydrogenase (nad+)” and “dpd”. The unique hits collected from these databases were selected and further limited to English language papers from 1980 to December 2015. Papers describing studies aimed at testing DPD activity and/or DPD

deficiency in volunteers or patients were selected. Cross-references were identified from bibliographies from the selected studies. Only articles that describe the complete performance of the test were included, reviews and papers describing in vitro studies including DPD activity in cancer cells or studies in animals were neglected. The collected publications were divided into three categories; describing tests aimed at assessing DPD enzyme activity, genetic variants in *DPYD* and mRNA variants.

## RESULTS

### Tests aimed at assessing (surrogates for) DPD enzyme activity

Several tests have been described to assess the activity of the enzyme DPD.

#### *DPD activity in peripheral blood mononuclear cells (PBMCs)*

The majority of DPD is reported to be in the liver [1, 14] but DPD activity in other tissues such as lymphocytes contribute to metabolism of fluoropyrimidines as well [15]. The liver DPD activity in patients revealed a strong correlation with DPD activity in peripheral blood mononuclear cells (PBMCs) [16]. The mean DPD activity in PBM cells of patients with a partial DPD deficiency, is approximately 48% of that observed in the normal population due to heterozygosity for a pathological mutation [17]. The methodology of the test includes incubating isolated lymphocytes with radioactive labeled 5-FU or thymine after which the degradation products are measured by high-performance liquid chromatography (HPLC) with a radioisotope flow detector [2, 18-20]. A HPLC-electrospray tandem mass spectrometry (HPLC MS/MS) method has also been developed [17, 21]. The use of a porous graphitic carbon (PBC) column [22] results in a HPLC process that is highly pH stable compared to the reversed-phase C-18 column and the detection limit was at least similar to the C-18 columns with considerably shorter analysis time. This method was validated (Table 2.1). Evaluation of the stability of DPD in PBMCs indicated that the DPD activity decreased approximately 50% upon freezing, but was stable for at least 1 month [16]. The stability of radioactive 5-FU and its metabolite dihydrofluorouracil (DHFU) in the reaction mixture was found to be stable for three months and not affected by at least three freeze-thawing cycles [19]. The use of an on-line radioisotope flow detector makes this method very useful as a semi-automated radioassay but the specific equipment that is necessary might hamper the implementation on a broad scale. The correlation between DPD activity in PBMCs and

**Table 2.1 Pharmacokinetic parameters of the two study groups with standard deviation between**

| Study                      | N                                                         | Reference method                  | Recovery                                             | Sensitivity | Specificity | Inter-assay variability  | Linear | Intra-assay variability  |
|----------------------------|-----------------------------------------------------------|-----------------------------------|------------------------------------------------------|-------------|-------------|--------------------------|--------|--------------------------|
| Mattison et al. [24]       | 58 volunteers                                             | DPD in PBMC Genotyping with DHPLC | ND                                                   | 100%        | 96%         | 'reproducible'           | ND     | <5%                      |
| Mattison et al. [25]       | 23 volunteers, 8 cancer patients                          | DPD in PBMCs                      | ND                                                   | ND          | ND          | ND                       | ND     | ND                       |
| Mattison et al. [26]       | 258 volunteers                                            | DPD in PBMCs                      | ND                                                   | 86%         | 99%         | ND                       | ND     | ND                       |
| Cunha-Junior [27]          | 33 cancer patients                                        | Dihydrouracil/uracil ratio        | ND                                                   | 75%         | 85%         | ND                       | ND     | ND                       |
| Johnson et al. [19]        | 100 volunteers, 80 cancer patients                        | n.a.                              | > 95%                                                | ND          | ND          | < 8% CV                  | yes    | < 6.5% CV                |
| Liem et al. [22]           | 132 volunteers                                            | n.a.                              | 99 ± 2%, 3% CV                                       | ND          | ND          | < 4% CV                  | yes    | < 3%                     |
| Lostia et al. [21]         | 39 cancer patients                                        | HPLC-UV                           | ND                                                   | ND          | ND          | 0.7–5.6% CV <sup>a</sup> | yes    | 8.3–1.4% CV <sup>a</sup> |
| Van Kuilenburg [17]        | 28 cancer patients, 1 patient with verified DPYD mutation | Radioactive Thymine by HPLC MS/MS | 94.4–102.4% <sup>c</sup><br>99.4–101.6% <sup>b</sup> | ND          | ND          | 1.7–4.7% CV              | yes    | 1.0–3.2% CV              |
| Van Kuilenburg et al. [18] | 2 cancer patients unknown number healthy volunteers       | n.a.                              | ND                                                   | ND          | ND          | 9% CV                    | yes    | 5% CV                    |

Table 2.1 Continued

| Study                  | N                                                             | Reference method                     | Recovery                                                                         | Sensitivity | Specificity | Inter-assay variability                                         | Linear | Intra-assay variability                                         |
|------------------------|---------------------------------------------------------------|--------------------------------------|----------------------------------------------------------------------------------|-------------|-------------|-----------------------------------------------------------------|--------|-----------------------------------------------------------------|
| Bi et al. [29]         | 4 patients participating in a phase 1 trial                   | n.a.                                 | (relative recovery compared to water)<br>95.8 ± 2.0% 1 µM<br>95.5 ± 10.9% 100 µM | ND          | ND          | 6.73%, 6.95%, 5.96% (RSD 0.27, 25 and 250 µM)                   | ND     | 7.04%, 5.40%, 1.24% (RSD 0.27, 25 and 250 µM)                   |
| Garg et al. [32]       | 23 cancer patients, blank plasma healthy volunteers.          | n.a.                                 | 93–100% Uracil <sup>d</sup><br>95–99% UH2 <sup>d</sup>                           | ND          | ND          | 0.2–7% RSD Uracil <sup>e</sup><br>1.2–9.9% RSD UH2 <sup>e</sup> | yes    | 0.8–7% RSD uracil <sup>e</sup><br>2.6–9.7% RSD UH2 <sup>e</sup> |
| Kristensen et al. [41] | 68 CRC patients, 100 healthy controls                         | IVS+1G>A mutation 5-FU plasma levels | ND                                                                               | 87%         | 93%         | ND                                                              | ND     | ND                                                              |
| Ciccolini et al. [34]  | 30 blank plasma samples, one case report                      | U/UH2 ratio                          | 90%                                                                              | ND          | ND          | 10.6%, 12%, 12.8% 20 ng/mL, 75 ng/mL, 375 ng/mL 5-FU            | yes    | 12%, 2.4%, 3.3% 20 ng/mL, 75 ng/mL, 375 ng/mL 5-FU              |
| Beumer et al. [47]     | 156 plasma samples obtained from patients Plasma pool samples | LC-MS/MS                             | 93–104%                                                                          | ND          | ND          | < 2% CV                                                         | yes    | 2.1–3.9% CV                                                     |

Table 2.1 Continued

| Study                     | N                                  | Reference method                        | Recovery                                      | Sensitivity       | Specificity      | Inter-assay variability             | Linear | Intra-assay variability                    |
|---------------------------|------------------------------------|-----------------------------------------|-----------------------------------------------|-------------------|------------------|-------------------------------------|--------|--------------------------------------------|
| di Paolo et al. [43]      | 25 CRC patients                    | DPD in PBMC                             | 85% 5-FU<br>81% 5-FDHU                        | ND                | ND               | 5.28–9.44% 5FU<br>5.00–9.03% 5-FDHU | yes    | 4.91–6.05%<br>5-FU<br>4.55–6.99%<br>5-FDHU |
| Remaud et al. [40]        | 8 volunteers,<br>blank plasma      | n.a.                                    | 73 ± 2% Uracil<br>67 ± 2% UH2<br>82 ± 3% FUH2 | ND                | ND               | 0.9–2.3% U<br>0.7–5.6% UH2          | yes    | 1.3–5.3% U<br>1.3–7.1% UH2                 |
| Van Kuilenburg et al. [9] | 30 cancer patients,<br>18 controls | 5-FU loading dose                       | ND                                            | 100% <sup>f</sup> | 90% <sup>f</sup> | ND                                  | yes    | ND                                         |
| Déporte-Féty et al. [44]  | 1 volunteer, 29<br>cancer patients | DPD in PBMC<br>with radiolabeled<br>5FU | ND                                            | ND                | ND               | 2.5–8.7%                            | yes    | 9.5–3.2%                                   |

<sup>a</sup> 5-FU concentration range of 0.156; 0.195; 0.78 and 1.95 µg/mL.<sup>b</sup> Interassay recovery at low, normal and high concentration of dihydrothymine.<sup>c</sup> Intraassay recovery at low, normal and high concentration of dihydrothymine.<sup>d</sup> Concentration range of 0.05; 0.1; 0.2; 0.5; 1; and 2 µM of uracil and dihydrouracil resp.<sup>e</sup> Concentration range of 0.02; 0.05; 0.1; 0.2; 0.5; 1; and 2 µM of uracil and dihydrouracil resp.<sup>f</sup> Multiple variables  $V_{max}$  (mg/h) and  $t_{1/2}$  (h) after a single dose of 300 mg/m<sup>2</sup> 5-FU.  
n.a., not applicable; ND, not determined.

systemic 5-FU clearance has been studied but the results are contradictory. One study found a good correlation [2] but two other studies found a much poorer relation [4, 23]. Instead of radiolabeled substrates, the use of nonradiolabeled thymine as substrate was investigated [17]. Nevertheless, because of the superior sensitivity of radiochemical assays, the presence of a complete DPD deficiency can be established only with the DPD assay that use radiolabeled substrates [18, 19, 22]. The distinction between a partial and complete DPD deficiency is important to make because in the case of a partial deficiency the oncologist might consider dose reduction. Complete DPD deficiency may lead to switching to a non fluoropyrimidine containing regimen. In general, the assays to measure DPD activity in PBMCs are labor intensive and therefore expensive. For this reason it is not likely that this method is suitable to use broadly into clinical practice.

### ***Uracil breath test***

The principle of this test is that after ingestion of an aqueous solution of 2- $^{13}\text{C}$ -uracil (6 mg/kg), degradation of 2- $^{13}\text{C}$ -uracil by DPD followed by the other two enzymes of the pyrimidine degradation pathway takes place resulting in the production of  $^{13}\text{CO}_2$  [24-27] which is determined in exhaled breath using IR spectroscopy (UBiT-IR300). In DPD deficient individuals, reduced 2- $^{13}\text{C}$ -uracil catabolism results in decreased exhaled  $^{13}\text{CO}_2$  levels. The amount of  $^{13}\text{CO}_2$  present in breath samples is expressed as  $\delta$  over baseline (DOB) ratio that represents a change in the  $^{13}\text{CO}_2/^{12}\text{CO}_2$  ratio of breath samples collected before and after 2- $^{13}\text{C}$ -uracil ingestion. The breath test was evaluated in multiple studies with volunteers and cancer patients with or without (partial) DPD deficiency and has been extensively validated (Table 2.1), but sensitivity and specificity show considerable variation between the studies performed. One study compared the plasma 2- $^{13}\text{C}$ -uracil pharmacokinetics with expired  $^{13}\text{CO}_2$  in subjects with normal and reduced PBMC DPD activity [25]. The results of this study showed that  $T_{\max}$  and  $C_{\max}$  in exhaled air are not correlated to plasma  $C_{\max}$  and  $T_{\max}$ . The breath test is rapid and non-invasive since it only requires exhaled air from patients, which is collected into sealed bags that are transported to a central laboratory. The integrity of the breath collection bags was stable for up to 201 days after their initial examination. However, the transportation may delay the availability of outcome of the test result for the physician, possibly delaying the start of chemotherapy. A broad clinical use of this test is further hampered by the limited availability of the expensive 2- $^{13}\text{C}$ -uracil and the fact that the UBiT-IR<sub>300</sub> spectrophotometer that is needed for the analysis is not common available in every hospital and must be purchased specially for this test.

### **Endogenous U/DHU or DHU/U ratio**

Besides the *ex vivo* measurement of DPD activity in human cells, alternative assays have been developed including the analysis of endogenous uracil (U) and/or dihydrouracil (DHU) levels or their ratio in plasma [28-36], urine [20, 37, 38] or saliva [39]. In case of a (partial) DPD deficiency the breakdown of uracil is impaired causing elevated endogenous uracil levels and decreased dihydrouracil levels in biological fluids such as plasma or urine. U and DHU can be measured by HPLC [40] and GC [29] methods which have been validated (Table 2.1). A strong correlation was detected between the DHU/U ratio in plasma with 5-FU half-life, clearance and plasma levels [28, 31, 33]. However, it was reported that in some individuals with a normal U/DHU ratio strongly elevated 5-FU levels were measured indicating that determination of the U/DHU ratio may not always correctly reflect 5FU levels [41]. Interestingly, in one of the studies it is suggested that the U/DHU plasma ratio might only be predictive during 5-FU treatment when DPD is saturated and not at baseline when DPD is nonsaturated [35]. The stability of uracil in plasma is at least 2 months at 80°C [29]. A significant circadian rhythm in plasma and urine DHU/U ratio and DPD activity in mononuclear cells was observed in healthy subjects, but this was disrupted in patients who were continuously infused with a high dose of 5-FU [42]. The biological significance of the DPD circadian pattern to fluoropyrimidine drug treatment is obvious, since the variations in DPD between individuals can result in 5-FU concentration fluctuations that can be directly correlated with treatment toxicity. The circadian rhythm in a population of patients and volunteers should be investigated to determine threshold values for those patients who are prone to toxicity. The good stability of uracil in plasma and the fact that the HPLC equipment needed for this assay is available in most clinical laboratories, make this method suitable for broad clinical implementation.

### **5-FU Therapeutic Drug Monitoring**

The pharmacokinetics of 5-FU and its metabolite DHFU can be assessed to detect possible DPD deficiency. The determination of plasma concentrations of 5-FU and DHFU can be performed by HPLC [30, 43-45], LC-MS/MS [46] or immunoassay [47]. These methods have been validated (Table 2.1) [30, 43, 47]. Based on this principle, several investigators hypothesized that the administration of a test dose of 5-FU before the start of chemotherapy might enable identifying subjects at risk of severe treatment-related side effects [9, 43, 44, 48-51]. The test doses 5-FU investigated ranged from 250 mg/m<sup>2</sup>, 300 mg/m<sup>2</sup>, 370 mg/m<sup>2</sup> to 450 mg/m<sup>2</sup> after which 5-FU and DHFU were measured in plasma. The pharmacokinetic

results following a test dose of 250 mg/m<sup>2</sup> 5-FU were significantly different from a dose of 370 mg/m<sup>2</sup> [49], but there was no linear correlation between 5-FU and DHFU pharmacokinetics and no correlation between DPD activity and 5-FU pharmacokinetics following a dose of 250 mg/m<sup>2</sup>. In one study test doses of 300 mg/m<sup>2</sup> or 450 mg/m<sup>2</sup> were used in subjects with and without the presence of the c.1905+1G>A (IVS14+1G>A) mutation [51]. Only with the dose of 450 mg/m<sup>2</sup>, the mean AUC and clearance of 5FU was significantly different, but the terminal half-life of 5-FU measured at both test doses showed a highly significant difference. A limited sampling model based on two time points was studied in patients receiving 370 mg/m<sup>2</sup> 5-FU as an iv bolus making the test more suitable for clinical practice and less patient intensive [50]. More recently, a validated limited sampling two compartment PK model was presented based on a single sample after 300 or 450 mg/m<sup>2</sup> 5-FU. Normal and DPD deficient patients could be discriminated at 300 mg/m<sup>2</sup> [9]. In line with these results, a three compartment model for 5-FU and 5 fluoro-5,6-dihydrouracil was developed using retrospectively PK data from 127 colorectal cancer patients treated with 5-FU bolus infusion [52]. The model that was developed has significant potential to identify patients with the decreased DPD phenotype requiring earlier adjustment of the 5-FU dose. The principle of a PK modeling approach is simple and might be promising. A potential disadvantage of the test dose of 5-FU is that it might cause toxicity in severely deficient DPD patients (i.e. homozygous DPD deficient). One study showed no toxicity in 20 of 22 patients heterozygous for the c.1905+1G>A after the administration of the single doses of 5-FU 300 and 450 mg/m<sup>2</sup> [9]. However possible toxic reactions following a 5-FU test dose in severely deficient patients should be studied further in a larger population.

### ***Oral thymine administration***

Thymine (5-methyluracil) is catabolized by the same enzymatic pathway as 5-FU and was investigated as a probe to detect carriers of DPD and dihydropyrimidinase deficiencies [53]. Oral powdered thymine was administrated encapsulated in a dose of 250 mg in the morning. At pre-set points, blood samples were drawn.

Plasma and urine concentrations of thymine, dihydrothymine can be measured with the use of a validated rapid method based on LC-MS/MS [54]. The oral thymine test is at this moment no candidate to be used to detect DPD deficiency since it is only used in 12 healthy volunteers with no *DPYD* variants and not in cancer patients with or without DPD deficiency. The method has to be further developed in order to prove its potential value.

## Tests aimed at assessing genetic variants in *DPYD*, the gene encoding DPD

### *Polymerase Chain Reaction (PCR)- Denaturing High-Performance Liquid Chromatography (DHPLC)*

DPD is encoded by the *DPYD* gene and there is an increased incidence of 5-FU and capecitabine related toxicity when pathological *DPYD* variants are present [45, 55-63]. Based on the evidence from the published literature dosing recommendations for fluoropyrimidines based on genotype are recommended [64-66]. One possible method to detect *DPYD* mutations involves Denaturing High-Performance Liquid Chromatography (DHPLC) [55, 67-69]. This assay is based on temperature-depending separation of DNA containing mismatched base pairs from a pool of Polymerase Chain Reaction (PCR)-amplified DNA fragments. This method is highly sensitive and can detect heterozygous variants. Homozygous wildtype vs. homozygous mutated sequences are not really resolved by this method. To detect homozygous mutations, mixing of each sample with a wildtype sample is necessary. For sequencing samples with aberrant peak patterns it is necessary to finally determine the kind of mutation with the use of a secondary test, which will increase total cost. DHPLC can be used to detect currently unrecognized unknown sequence variations in the *DPYD* gene. The DNA is isolated out of blood obtained by a single venapuncture. It takes approximately 250 min to screen the *DPYD* gene for mutations at one temperature [67]. A drawback of the DHPLC method is that it is laborious and time consuming, and for that reason not suitable for large numbers of patients. Secondly, the equipment that is used is not available in every hospital laboratory. With this technique the 23 *DPYD* exons were screened in DNA samples of randomly selected individuals, DPD deficient patients and patients who experienced toxicity following 5-FU treatment [67-69]. Missense mutations in the *DPYD* gene will not necessary lead to reduced DPD activity. Gross et al. [69] analyzed DNA of 4 individuals with symptoms of 5-FU-related toxicity and compared the data to control samples of 157 healthy individuals. Several missense mutations were found in the DNA of the patients but only in one patient with 4 mutations a lower DPD activity in PBMCs was found. This result displays a general problem that can be found with genotyping and 5-FU related toxicity. For a good predictive value there has to be a clear correlation between genetic variants and its effect on DPD activity or the likelihood to develop toxicity. A solution to encounter this problem is to determine the impact of novel mutations found in the DPD gene on enzyme activity [69-71].

### ***Pyrosequencing***

Pyrosequencing [72-78] is based on the utilization of ATP to produce light. The pyrosequencing reaction results in the release of a pyrophosphate molecule with the sequential incorporation of bases to the DNA template. The technique is developed in to a fully automated process as a result of advanced equipment that allows genotyping of multiple samples within an hour. However, the initial investment in the equipment is around 50,000–80,000 Euro and as a result it is not available in every hospital laboratory. The technique of pyrosequencing and its role in detecting mutations in the *DPYD* gene has been extensively studied in volunteers and cancer patients. In a population of 14 individuals with a reduced DPD activity and severe 5-FU related toxicity, 57% had a molecular basis for their deficient phenotype [76]. Three *DPYD* variants, the c.1905+1G>A (IVS14+1G>A), c.2846A>T, and c.1236A>T were strongly associated with fluoropyrimidine-induced toxicity [72, 77, 78]. In a large population of 487 patients, one-third of the patients with one of the SNPs had no adverse reactions, and therefore the presence of a SNP will not automatically imply development of toxicity following 5-FU therapy. Interestingly, the absence of a SNP will also not exclude developing toxicity since in several studies patients were identified that had no SNP but still suffered from severe initial side effects as a result of a lowered 5-FU plasma clearance [73, 79, 80]. The determination of a single SNP can be easily performed in hospital settings and costs at this moment around 25 Euros per SNP. Determination of multiple clinically relevant SNPs could potentially enhance sensitivity and specificity. Pyrosequencing is used on more and more in daily practice prior to 5-FU administration despite the lack of prospective evidence of its usefulness. Like all PCR based tests, a disadvantage of pyrosequencing is that with this method only SNPs and small deletions are detected. Large genomic deletions are missed and especially those are present in the DPD gene [77].

### ***Restriction Fragment Length Polymorphism (RFLP)***

RFLP is a technique in which DNA is spliced into fragments by restriction enzymes. Following the splicing, the DNA fragments are separated by gel electrophoresis. RFLP has been used in studies that investigated the presence of the c.1905+1G>A mutation in cancer patients with 5-FU related toxicity [11, 81].

### ***Single-strand conformation polymorphisms SSCP***

SSCP is a technique that was also optimized to be useful for pharmacogenetic DPD studies, and can be used to detect DNA sequence changes. The principle of SSCP is based on changes

in the secondary structures in single-strand DNA fragments caused by a change in sequence, which are detected as alterations in fragment mobility by gel electrophoresis. The conditions of SSCP were optimized with an automated system to screen genetic polymorphisms in the *DPYD* gene and its efficacy was evaluated by using 21 DNA samples with previously characterized polymorphisms [82]. The polymorphism detection rate of this technique was 95.3%.

### **Epigenetics**

Since the observation in several studies, that *DPYD* sequence variants could not fully explain the molecular basis of DPD deficiency, it is hypothesized [83, 84] that methylation of the *DPYD* promoter might be an alternative mechanism for DPD deficiency in cancer patients. Methylation of CpG islands located in the 5'-regulatory region of the *DPYD* gene has been shown to inhibit transcription [83]. The methylation status of DNA fragments can be detected with the use of DHPLC or with pyrosequencing. One study showed a significant association between aberrant methylation of the *DPYD* promoter and DPD enzyme deficiency in 80% of DPD-deficient individuals, whereas all individuals with normal DPD enzyme activity tested negative for methylation [83]. In contrast, other studies did not confirm the role of *DPYD* promoter hypermethylation to development of severe 5-FU toxicity [85, 86]. The contradictory results of the studies investigating the role of methylation of the *DPYD* promoter make it unlikely that screening for aberrant methylation of the promoter of *DPYD* will become current practice soon.

### **Tests aimed at assessing mRNA variants encoding DPD**

DNA mutations can result in altered mRNA levels or aberrant splicing of the pre-mRNA. It is hypothesized that DPD enzyme activity is possibly correlated with DPD mRNA expression [87]. Using a reverse transcription-PCR-based assay, the exon 14 skipping mutation has been studied in patients with and without grade 3-4 toxicity following 5-FU therapy [88]. The DPD enzyme activity correlated with DPD mRNA expression in biopsy-sized tissue samples including peripheral blood mononuclear cells [89]. A disadvantage of mRNA screening is the instability of mRNA in blood, which makes it unsuitable for routinely screening since the equipment needed for mRNA screening is not widely available in every hospital. For this reason it is unlikely that this technique is suitable for prospective screening of large amounts of patients.

## DISCUSSION

### Predicting toxicity

The concept of the tests described in this review is that DPD deficiency results in decreased 5-FU clearance leading to a higher incidence of side effects. However, some patients that suffer from severe side effects caused by 5-FU show normal 5-FU pharmacokinetics or wildtype *DPYD* genotype and for this fact it is important to realize that prospective testing for DPD deficiency will not exclude all 5-FU related toxicity.

### Sensitivity and specificity

Tests at the DNA and RNA may assess the presence of certain known mutations in the *DPYD* gene. However, new and undetected mutations are missed. Analysis of the prevalence of the various mutations in the DPD gene (*DPYD*) revealed that three mutations, of which two in non coding areas, c.1905+1G>A (IVS14+1G>A), c.2846A>T (p.D949V) and c.1129-5923C>G were most commonly involved with toxicity [5, 11, 72, 77, 78, 80, 90]. Unfortunately, fluoropyrimidine toxicity is only partly explained by mutations in the coding region of *DPYD* [77]. With respect to overall toxicity, the sensitivity for screening for only the c.1905+1G>A mutation was only 5.5 % [91] and 31% for c.1905+1G>A (IVS14+1G>A), c.2846A>T (p.D949V) and c.1679T>G [80]. Until now, only coding areas and flanking intron sequences have been analyzed, though deep intron mutations might influence the splicing of *DYPD* pre-mRNA, as was demonstrated recently [77]. In the case of a test to detect DPD deficiency, sensitivity should be as high as possible. Low specificity of a test might lead to unnecessary dose reduction and non-optimal therapy in false positive cases. A solution for this might be to develop a 5-FU dose escalation model [92] but this has not been investigated yet.

### Definition of DPD deficiency

Among the tests described, there is wide variation in the definition of DPD deficiency that is used. The lack of a clear definition of DPD deficiency might hamper the implementation of prospective screening on a broad scale. With phenotyping, a patient is considered to be DPD deficient if the DPD activity is below a subjective pre-set threshold value. DPD activity measured in PBMCs has the problem that its precision largely depends on the isolated PBMC

sub-fractions of blood cell components that can be variable [93]. With genotyping studies, DPD deficiency is often defined as the presence of one or multiple SNPs.

### Cost effectiveness

The cost-effectiveness aspects of methods that might be used for prospective screening for DPD deficiency are sparsely described in the literature. In most countries hospital costs are paid by government or social healthcare systems so it is because of social-economic reasons that the increase of costs should be as low as a reasonable achievable. The prevalence of partial DPD deficiency in Caucasian population is approximately at least 3% [4, 12, 80]. As a result many patients need to be screened preemptive in order to diagnose those with DPD deficiency. The costs of prospective testing ideally must be in favor to the costs of treatment of toxicity due to DPD deficiency that is prevented by prospective screening. Recently it was shown that for *DPYD*\*2A genotype dosing of fluoropyrimidines, average total treatment cost per patient was lower for screening as compared to nonscreening, outweighing screening costs [94].

Based on the characteristics of the tests described, the technique of genotyping is more simple and less patient invasive than the phenotyping tests. The main problem with genotyping is the sensitivity and specificity. The determination of multiple SNPs with high prevalence might enhance sensitivity. Phenotyping tests for now seem to have a better correlation with the occurrence of 5-FU toxicity, but as with the genotyping strategy, sensitivity and specificity have to be established and improved.

In order to improve broad clinical implication of a diagnostic test to detect DPD deficiency, we recommend that the following points in the nearby future should be taken into account:

- To enhance sensitivity of preventing fluoropyrimidine toxicity, future studies should investigate the optimization of the genotyping strategy and if the combination with a phenotyping test will enhance further detection of DPD deficient patients.
- Following the determination of effectiveness of a test, future studies regarding this test should specific focus on the cost-effectiveness.
- A consensus definition of DPD deficiency has to be derived internationally to establish incidence of DPD deficiency and to compare study results.

- Sensitivity and specificity in relation with the prevention of fluoropyrimidine related toxicity of every test have to be determined to measure the predictive outcome of establishing toxicity following 5-FU or capecitabine containing therapy.

In conclusion, several predictive tests are available to screen patients for DPD deficiency before their first treatment with fluoropyrimidines in a rapid and possibly low invasive way. At this moment the challenge is to optimize the tests and to determine which test strategy is most suitable to predict patients at risk of developing fluoropyrimidine related toxicity caused by DPD deficiency and to incorporate preemptive screening broadly into daily routine.

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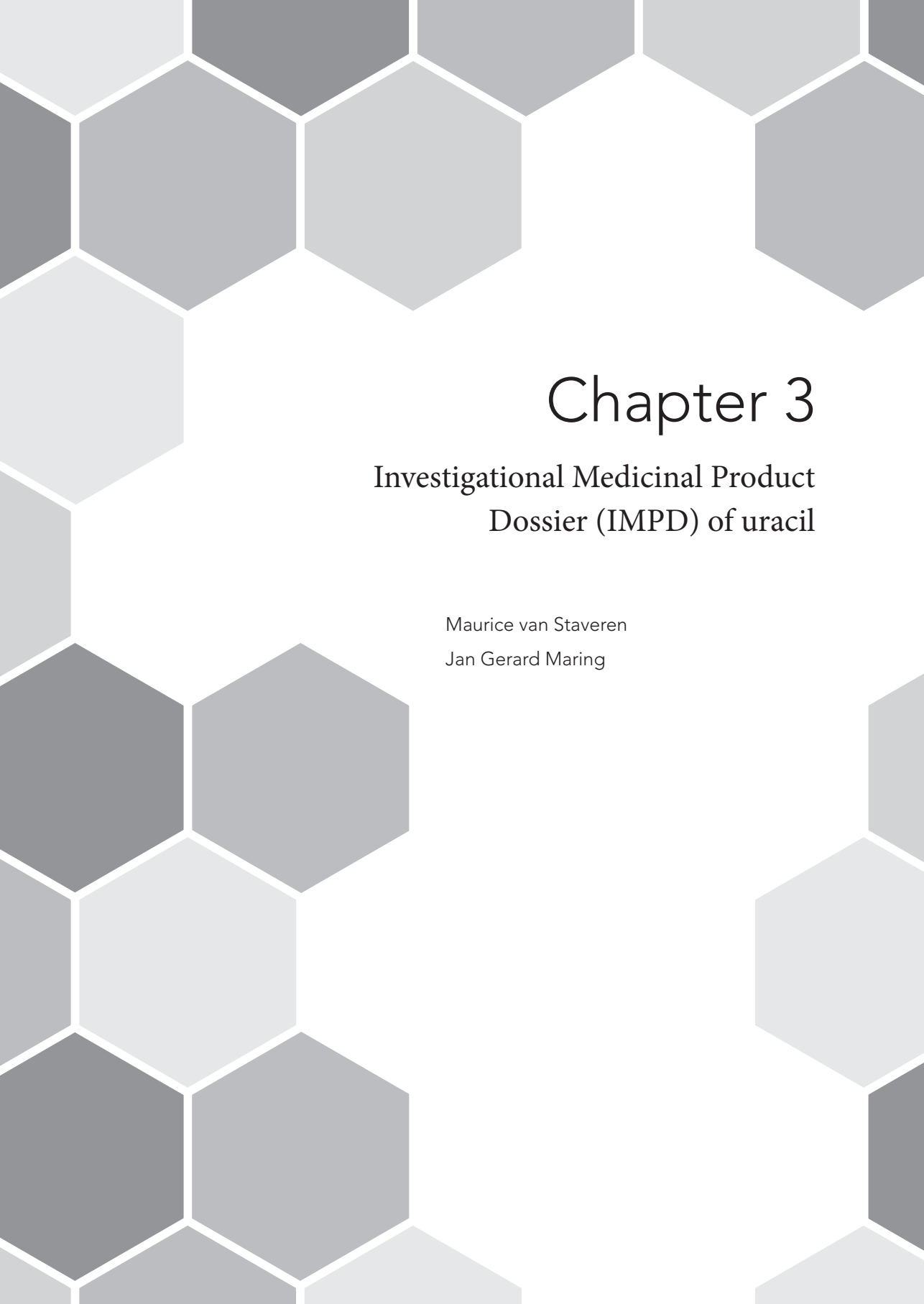
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# Chapter 3

## Investigational Medicinal Product Dossier (IMPD) of uracil

Maurice van Staveren

Jan Gerard Maring



## INTRODUCTION

The Investigational Medicinal Product Dossier (IMPD) is the basis for approval of clinical trials by the competent authorities in the EU [1]. The IMPD includes summaries of information related to the quality, manufacturing and control of the Investigational Medicinal Product, data from non-clinical studies and from its clinical use. An overall risk-benefit assessment, critical analyses of the non-clinical and clinical data in relation to the potential risks and benefits of the proposed study are essential parts of the IMPD. In certain situations, e.g. where the Investigational Medicinal Product has already been authorised as a medicinal product in one of the EU Member States or when clinical studies with the IMP have already been approved by a Member State, a simplified IMPD will be sufficient.

The Clinical Trials Directive (2001/20/EC) came into force in April 2001, harmonizing the laws, regulations and administrative provisions of the Member States relating to the implementation of Good Clinical Practice (GCP) in the conduct of clinical trials on medicinal products for human use. Member States were obliged to transform the requirements outlined in the Directive into the respective national laws by May 2004. The Directive introduced a harmonized procedure for the authorization to perform a clinical study in any one of the EU Member States. In addition, it defines the documentation to be submitted to the Ethics Committee as well as the IMPD to be submitted to the competent authority for approval. Thus, an IMPD is requested whenever the performance of a clinical study in any one of the EU Member States is intended [1].

Since uracil has no marketing authorization, an IMPD is required to perform clinical studies with the oral uracil loading dose.

## METHODS

Based on the IMPD template [2], all paragraphs were written for uracil. All tests considering impurities, assays and quality were performed by an analytical monograph with the use of liquid chromatography with UV photo diode array detection. Acceptance criteria were derived from the European Pharmacopeia General Monograph 04/2013 Substances for Pharmaceutical Use, H5.10 Control of impurities in substances for Pharmaceutical Use and H2.2.46 Chromatographic Separation Techniques. All tests were performed at the laboratory of the Hospital Pharmacy Meppel-Hoogeveen. The manufacturing of the study drug was

performed by the Hospital Pharmacy Haagse Ziekenhuizen under Good Manufacturing Practice and Good Clinical Practice conditions. Chemical data about uracil and its synthesis route was derived from the supplier and safety data was derived from published animal studies. All studies were performed with an oral powder formulation.

1. Directive, C.T. IMPD. 2006 08.08.2006; Available from: <http://www.impd.eu>.
2. Directive, E.C.T., IMPD, CCMO, Editor. 2016: Web page. p. 26.

## INVESTIGATIONAL MEDICINAL PRODUCT DOSSIER

# URACIL

3

**VERSION 4**

**MAY 2013**

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## CHEMICAL PHARMACEUTICAL AND BIOLOGICAL DATA

### 1. Introduction

This clinical trial application presents information relating to uracil oral powder in dosages of 700, 800, 900, 1000, 1100 and 1200 mg. Uracil is an endogenous compound, and is used as a substrate for phenotyping of dihydropyrimidine dehydrogenase (DPD). DPD is a key metabolizing enzyme in the metabolic pathway of 5-fluorouracil. Patients with DPD deficiency are at high risk to develop severe toxicity after treatment with 5-fluorouracil or capecitabine. Pre-chemotherapy phenotyping of DPD with uracil may be a feasible method for patient screening. Uracil has been evaluated in several clinical studies [1-6] involving healthy subjects to evaluate the safety and tolerability profile and to assess the pharmacokinetic behaviour of the compound. In one of these studies, uracil was administered orally to 12 healthy individuals in a dose of 500 mg/m<sup>2</sup>. No unintended effects or adverse effects were reported during or after the administration. In 4 of these subjects uracil was also administered in a dose of 1000 mg/m<sup>2</sup> [5]. No adverse events were reported. In addition uracil was administered to 8 patients with proven DPD deficiency in a dose of 500 mg/m<sup>2</sup>. Physical observation of the patients after administration did not reveal any unexpected side effects or unintended effects [6].

### 2.1 Chemical pharmaceutical data

#### 2.1.S Drug substance

##### 2.1.S.1 General information

Uracil is a common naturally occurring pyrimidine [7]. Uracil was originally discovered in 1900 and it was isolated by hydrolysis of yeast nuclein that was found in bovine thymus and spleen, herring sperm, and wheat germ [8]. Uracil is a planar, unsaturated compound that has the ability to absorb light [9].

##### 2.1.S.1.1 Nomenclature

Uracil

2,4-dihydropyrimidine; 2,4(1H,3H)-pyrimidinedione, 2-oxy-4-oxy pyrimidine, 2,4-pyrimidinediol

CAS number: 66-22-8

### 2.1.S.1.2 Structure

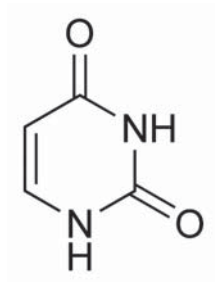


**Figure 3.1** Uracil chemical structure.

Chemical formula:  $C_4H_4N_2O_2$

Molecular mass: 112.09 g/mol

### 2.1.S.1.3 General properties

Stereochemistry: Not applicable

Description: White to slightly yellowish crystalline powder

Melting range: 335°C

Hygroscopicity: Uracil is not hygroscopic

pKa (acidic): 9.45

Solubility: Freely soluble in hot water, sparingly in cold water (100 parts of water at 25°C dissolves 0.358 part of uracil). Almost insoluble in alcohol, ether. Soluble in ammonia water and other alkalies.

## 2.1.S.2 Manufacture

### 2.1.S.2.1 Manufacturer

*Pharma Waldhof GmbH*

*Hansaallee 159*

*D-40549 Düsseldorf*

*Germany*

2.1.S.2.2 Description of manufacturing process and process controls

The uracil used in this study was synthesized by the condensation of maleic acid with urea in fuming sulfuric acid [6].



Related substances from the synthesis pathway, as supplied by the manufacturer, are listed in Table 3.1.

2.1.S.2.3 Control of materials

Table 3.1 Reagents, solvents and other materials

| Material        | Grade   | Specific test item | Possible impurity |
|-----------------|---------|--------------------|-------------------|
| dl-maleic acid  | unknown | no                 | Yes               |
| Urea            | unknown | no                 | Yes               |
| Sulphate        | unknown | no                 | Yes               |
| Methanol-sodium | unknown | no                 | Yes               |
| Ethyl formate   | unknown | no                 | Yes               |

2.1.S.2.4 Controls of critical steps and intermediates

Unknown / Not available.

2.1.S.2.5 Process validation and/or evaluation

Unknown / Not available.

2.1.S.2.6 Manufacturing process development

Unknown / Not available.

### 2.1.S.3 Characterization

#### 2.1.S.3.1 Elucidation of structure and other characteristics

Each batch of uracil is identified by infra red absorption spectrophotometry. The spectrum obtained is compared with a uracil reference standard.

#### 2.1.S.3.2 Impurities

Each batch of uracil is tested on related substances by liquid chromatography with UV photo diode array detection. The sum of areas of any peak corresponding to impurities may not be greater than 1.0%. Furthermore each batch is tested for heavy metals (< 10 ppm) and loss on drying (< 0.5%).

### 2.1.S.4 Control of drug substance

#### 2.1.S.4.1 Specification

Batches of the active ingredient will comply with the below specification (Table 3.2). Batches will be released only if the impurity profiles can be supported by available non-clinical data.

**Table 3.2 Specifications for URACIL drug substance**

| Attribute                                    | Method                                       | Acceptance criteria                                                        |
|----------------------------------------------|----------------------------------------------|----------------------------------------------------------------------------|
| Appearance<br>Dissolution, pH and appearance | Visual observation<br>0.35 g in 100 ml water | White crystalline powder<br>Conforms colour reference test<br>pH = 5.0–5.5 |
| Identification                               | IR Absorption                                | Conforms to the reference spectrum Figure 3.2                              |
| Melting point                                | PhEur                                        | 330–340°C                                                                  |
| Purity                                       |                                              |                                                                            |
| (1) Heavy metals                             | Ph.Eur., Test C                              | ≤ 20 ppm                                                                   |
| (2) Related substances                       | HPLC (UV-PDA)                                | Each: ≤ 0.3%<br>Total: ≤ 1.0%                                              |
| Water                                        | Ph.Eur., loss on drying                      | ≤ 0.5%                                                                     |
| Residue on ignition                          | PhEur                                        | ≤ 0.10%                                                                    |
| Assay                                        | HPLC                                         | 99.0 to 101.0%                                                             |

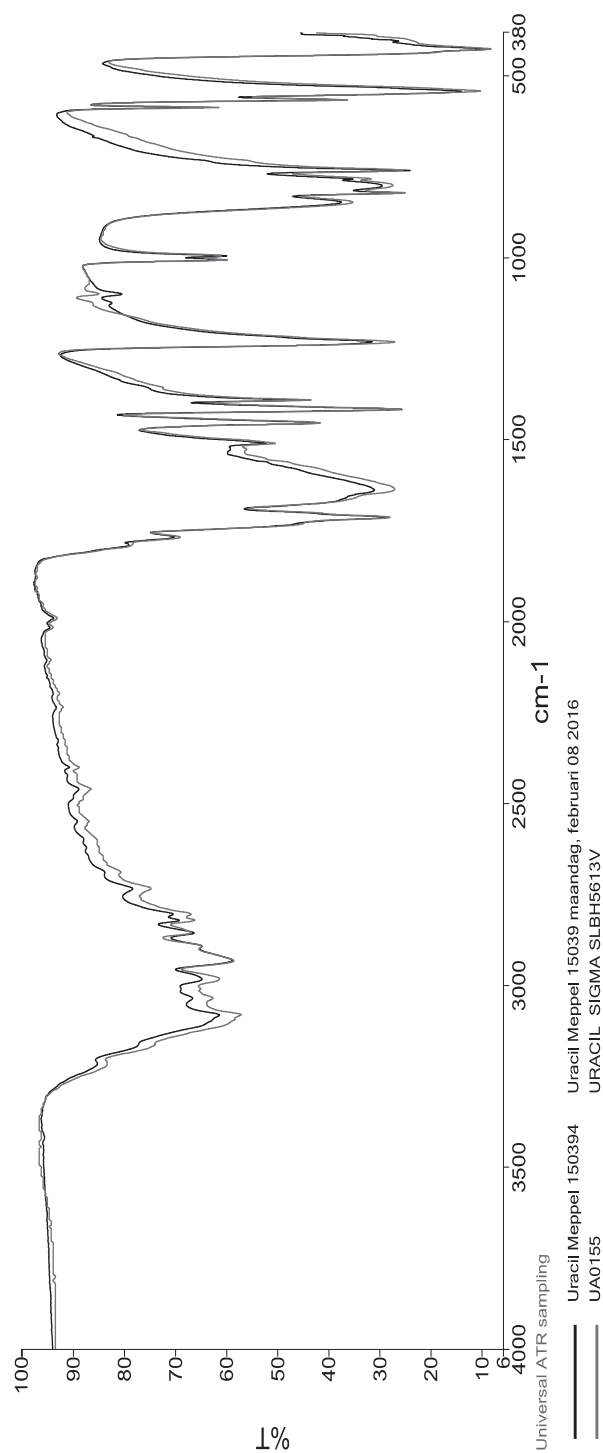
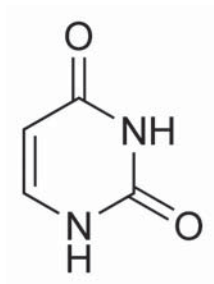


Figure 3.2 IR Absorption spectrum of two batches of uracil.

## 2.1.S.4.2 Analytical procedures

## Monograph Uracil



URACIL

Uracilum

$C_4H_4N_2O_2$   
CAS [66-22-8].

Mr112.09

## DEFINITION

Uracil contains not less than 98.0 percent and not more than the equivalent of 102.0 percent of 2,4(1H,3H)-pyrimidinone, calculated with reference to the dried basis.

## CHARACTERS

A white or almost white, crystalline powder, freely soluble in hot water, sparingly in cold water. Almost insoluble in alcohol.

## IDENTIFICATION

Examine by infrared absorption spectrophotometry (Ph.Eur. 2.2.24), comparing with the spectrum obtained with uracil reference standard.

## TESTS

**Solution S.** Dissolve 0.35 g in hot *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

**Appearance of solution.** Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY7 or Y7 (2.2.2, *Method II*).

**pH** (2.2.3). The pH of solution S is 5.0 to 5.5.

**Related substances.** Examine by liquid chromatography (2.2.29).

**Test solution.** Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 100.0 ml to obtain a solution having a known concentration of about 1 mg per ml.

*Reference solution (a).* Dilute 1.0 ml of the *test solution* to 100.0 ml with *water R*.

*Reference solution (b).* Dilute 1.0 ml of *reference solution (a)* to 10.0 ml with *water R*.

*Reference solution (c).* Dissolve 25 mg of *dihydrouracil reference standard* plus 25 mg of *5-fluorouracil reference standard* and 25 mg of *uracil reference standard* in *water R* and dilute to 100.0 ml.

The chromatographic procedure may be carried out using:

- A stainless steel column 0.250 m long and 4 mm in internal diameter packed with octadecylsilyl silica for chromatography R (5  $\mu$ m),
- As mobile phase at a flow rate of 0.8 ml per minute a mixture 99 parts 1.5 mM phosphate buffer (pH 5.8) and 1 part methanol R,
- As detector a spectrophotometer set at 205 nm.

Inject 20  $\mu$ l of *reference solution (c)*. Adjust the sensitivity of the system so that the heights of the three peaks are not less than 20 percent of the full scale of the recorder. The test is not valid unless the resolution between the first and second peak and between the second and third peak is less than 2.5. Inject 20  $\mu$ l of *test solution*, 20  $\mu$ l of *reference solution (a)* and 20  $\mu$ l of *reference solution (b)*. Continue the chromatography for three times the retention time of uracil. In the chromatogram obtained with the test solution: the sum of the areas of any peaks corresponding to impurities is not greater than the area of the principal peak in the chromatogram obtained with *reference solution (a)* (1.0 percent); the area of any peaks corresponding to dihydrouracil and 5-fluorouracil is not greater than the area of the principal peak in the chromatogram obtained with *reference solution (b)* (0.1 percent) and the sum of the areas of such peaks is not greater than three times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 percent). Disregard any peak with an area less than 0.2 times of the principal peak in the chromatogram obtained with *reference solution (b)*.

**Heavy metals** (2.4.8). Use a *platinum crucible*. 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

**Loss on drying** (2.2.32). Not more than 0.5 percent, determined on 1.000 g by drying in vacuo at 80°C for 4h.

**ASSAY**

*Test solution.* Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 100.0 ml.

*Test solution (a).* Dilute 1.0 ml of the *test solution* to 100.0 ml with *water R*.

*Reference solution.* Dissolve 0.10 g *uracil reference standard* in *water R* and dilute to 100.0 ml with the same solvent.

*Reference solution (a).* Dilute 1.0 ml of the *reference solution* to 100.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- A stainless steel column 0.250 m long and 4 mm in internal diameter packed with octadecylsilyl silica for chromatography R (5  $\mu$ m),
- As mobile phase at a flow rate of 0.8 ml per minute a mixture 99 parts 1.5 mM phosphate buffer (pH 5.8) and 1 part methanol R,
- As detector a spectrophotometer set at 266 nm.

Inject 20  $\mu$ l of *test solution (a)* and 20  $\mu$ l of *reference solution (a)*, record the chromatograms, and measure the responses for the major peaks. Calculate the quantity in mg of  $C_4H_4N_2O_2$  in the proportion of uracil taken by the formula:

$$10C(r_T/r_{RS}),$$

in which C is the concentration, in  $\mu$ g per ml, of uracil in the *reference solution*, and  $r_T$  and  $r_{RS}$  are the uracil peak responses obtained from the *test solution* and the *reference solution* respectively.

**STORAGE**

Store in tight, light-resistant containers.

**2.1.5.4.3 Validation of analytical procedures**

Standard Operating Procedure: Validation chromatographic analytical methods ZAMH, version 4.

2.1.S.4.4 Batch analyses

One batch is purchased so far for clinical testing. The batch analyses from the producer are shown below:

**Table 3.3 Results from two batch analyses uracil**

| Test                | Specifications | Result Batch 1<br>Lot# 46303900 |
|---------------------|----------------|---------------------------------|
| Assay               | Min. 98%       | 99.75%                          |
| Loss on drying      | Max. 0.5%      | 0.15%                           |
| Heavy metals        | Max. 10 ppm    | < 10 ppm                        |
| Residue on ignition | Max. 0.2%      | 0.05%                           |

2.1.S.4.5 Justification of specification

The uracil analytical monograph is partly based on the Ph.Eur. monograph of 5-fluorouracil, and partly on specifications supplied by the manufacturer.

2.1.S.5 Reference standards or materials

The reference standard, Uracil Lot 41K3648 was purchased from Sigma-Aldrich Inc. No additional recrystallization or purification was performed.

2.1.S.6 Container closure system

The uracil powder is shipped and stored in bag stored in an airtight container.

2.1.S.7 Stability

Uracil is very stable under normal temperatures and pressures. Uracil is not compatible with strong oxidizing agents. Hazardous polymerization has not been reported.

Uracil undergoes keto-enol tautomeric shifts because of its resonance structures due to the NH<sub>2</sub> substituents and OH substituents. Also because any nuclear instability the molecule may have from the lack of formal aromaticity is compensated by the cyclic-amidic stability [8]. The keto tautomer is referred to the lactam structure, while the enol tautomer is referred

to as the lactim structure. These tautomeric forms are predominant at pH = 7. The lactam structure is the most common form of uracil.

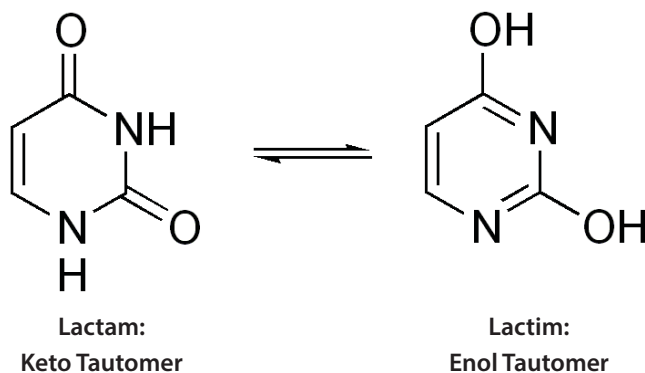


**Figure 3.3** Tautomeric forms of uracil.

## 2.1.P Medicinal product

### 2.1.P.1 Description and composition of URACIL ORAL POWDER

*The qualitative compositions of uracil oral powder filled in flasks is listed in Table 3.4.*

**Table 3.4** Qualitative composition of URACIL oral powder 700, 800, 900, 1000, 1100, and 1200 mg

| Component                    | Reference to standards | Function          |
|------------------------------|------------------------|-------------------|
| URACIL                       | Uracil Lot 41K3648     | Active ingredient |
| Brown plastic bottle, 300 mL | Ph.Eur.                | Container         |

### 2.1.P.2 Pharmaceutical development

The formulation used for the clinical trials is uracil powder without additives. In previous clinical studies the same formulation was used.

#### 2.1.P.2.1 Components of the medicinal product

The powder is filled in brown 300 ml plastic bottles without additives.

#### **2.1.P.2.2 Medicinal product**

Not applicable.

#### **2.1.P.2.3 Manufacturing process development**

No development information needs to be provided.

#### **2.1.P.2.4 Container closure system**

No development information needs to be provided.

#### **2.1.P.2.5 Microbiological attributes**

Not applicable.

#### **2.1.P.2.6 Compatibility**

Not applicable.

### **2.1.P.3 Manufacture**

#### **2.1.P.3.1 Manufacturer**

GMP manufacturing:  
Apotheek Haagse Ziekenhuizen  
Escamplaan 900  
2547 EX Den Haag

#### **2.1.P.3.2 Batch formula**

This information does not have to be provided.

#### **2.1.P.3.3 Description of manufacturing process and process controls**

The production process involves the weighing of uracil powder directly in the container. Only one dosage strength (700, 800, 900, 1000, 1100 or 1200 mg) is produced in each batch. The production takes place under GMP conditions. All containers comply with the specifications (95–105% of declared value).

**2.1.P3.4 Controls of critical steps and intermediates**

The weighing of the powder is double checked by two persons. The total batch is released by a qualified person.

**2.1.P3.5 Process validation and/or evaluation**

All equipment, documents and procedures used for manufacturing comply with GMP standards.

**2.1.P4 Control of excipients****2.1.P4.1 Specifications**

Not applicable.

**2.1.P4.2 Analytical procedures**

Not applicable.

**2.1.P4.3 Validation of analytical procedures**

Not applicable.

**2.1.P4.4 Justification of specifications**

Not applicable.

**2.1.P4.5 Excipients of human or animal origin**

Not applicable.

**2.1.P4.6 Novel excipients**

Not applicable.

2.1.P.5 Control of medicinal product

2.1.P.5.1 Specifications (s)

Clinical trial batches of URACIL oral powder 700, 800, 900, 1000, 1100 and 1200 mg will meet the following specifications.

**Table 3.5 Release and shelf-life specifications for Uracil oral powder in glass containers**

| Test item          | Method               | Acceptance criteria                                                    |
|--------------------|----------------------|------------------------------------------------------------------------|
| Description        | Visual observation   | White powder in brown coloured flask                                   |
| Identification     | IR spectrophotometry | The product IR spectrum is identical to that of the reference spectrum |
| Content Uniformity | Weighing             | Conforms to Ph. Eur.                                                   |

2.1.P.5.2 Analytical procedures

Document ID: Uracil in plasma with use of HPLC.

Document ID: Standard Operating Procedure uracil with use of (U)HPLC.

2.1.P.5.3 Validation of analytical procedures

Document ID: Validation chromatographic analytic methods ZAMH, version 4.

2.1.P.5.4 Batch analyses

Not applicable.

2.1.P.5.5 Characterization of impurities

Since the active substance is used without additives and no further processing is performed on the uracil powder, no characterization of impurities is performed in the final product.

2.1.P.5.6 Justification of specification(s)

Not applicable.

### 2.1.P.6 Reference standards

The reference standard, Uracil Lot 41K3648 was purchased from Sigma-Aldrich Inc. No additional re-crystallization or purification was performed.

### 2.1.P.7 Container closure system

The uracil powder is filled in brown plastic 300 mL bottles.

### 2.1.P.8 Stability

The stability data for the drug substance show that uracil is intrinsically very stable.

There is no indication that any degradation occurs during storage in closed polypropylene containers.

Stability of uracil solution of 3 mg/ml in water was tested at 30, 50 and 70°C for one week showing stability of concentration (vc 2.3). The shelf life of an uracil solution in water of 3 mg/ml was tested for 26 weeks showing stability of concentration (vc 1.6).

Considering the above, a shelf life of 36 months at room temperature is set for uracil powder in polypropylene bottles. The storage instruction will be to store the bottles below 30°C.

## 2.2 Non-clinical pharmacology, pharmacokinetics and toxicology

### 2.2.1 Test materials used in toxicity studies

Uracil powder.

### 2.2.2 Integrated assessment of the data package

See CAS 66-22-8 [10].

2.2.3 List of studies conducted & references

**Table 3.6** LD<sub>50</sub> values of uracil in different species [9]

| Species | Route of administration | LD <sub>50</sub> |
|---------|-------------------------|------------------|
| Rat     | oral                    | > 6 g / kg       |
| Mouse   | oral                    | > 8 g / kg       |
| Mouse   | parenteral              | 1513 mg / kg     |
| Dog     | oral                    | > 5 g / kg       |
| Rabbit  | oral                    | > 10 g / kg      |

2.2.3.1 Safety data regarding long-term (chronic) administration of uracil

Uracil was administered to male and female dogs for 3 months and to male dogs for 12 months at dose levels of 0, 210, 420, 840 and 1680 mg uracil per kg body weight per day by gavage. While there were minor differences seen in food consumption, water consumption and erythroid parameters between the 3-month and the 12-month studies, it was concluded that there were no adverse effects seen neither on these parameters nor on body weight, EKG, clinical laboratory studies and organ weights. Pathological observations did not show treatment-related effects [12].

2.2.4 GLP statement and bioanalytical methods

The non-clinical pharmacology data have been extracted from the CAS database and from peer reviewed publications.

2.3 Clinical data

2.3.1 Clinical pharmacology

Uracil is a non-toxic endogenous pyrimidine and essential part of the structure of RNA.

Uracil and 5-FU are chemically almost alike and both substances are substrates for DPD. Uracil is an endogenous pyrimidine involved in RNA synthesis and, accordingly, an excellent candidate for DPD phenotyping.

### 2.3.2 Clinical pharmacokinetics

The pharmacokinetics of uracil after oral intake has been established in human volunteers and in patients with DPD deficiency in previous studies [5, 6].

There is no data available of pharmacokinetic parameters in special populations (i.e. age and gender, race, renal insufficiency, hepatic insufficiency).

### 2.3.3 Human exposure

In a Dutch study in 12 healthy volunteers, no adverse reaction was observed after oral ingestion of 500 mg/m<sup>2</sup> and 1000 mg/m<sup>2</sup> uracil [2, 3]. Oral administration of 6 mg/kg <sup>13</sup>C labeled uracil in 255 American volunteers also did not reveal adverse reactions [1, 4].

## 2.4 Overall risk and benefit assessment

Uracil is an endogenous pyrimidine base and an essential part of the structure of RNA. The LD50 value of uracil is very high and ranges from 6–8 g/kg in rats, mice and rabbits [11]. Chronic oral administration of 1680 mg/kg uracil in dogs during 1 year appeared completely safe [12].

Uracil is commercially marketed in combination with tegafur in the pharmaceutical product UFT® (Merck). This product is registered for the treatment of colorectal cancer. The daily dose of uracil in this commercial formulation is 672 mg/m<sup>2</sup>.

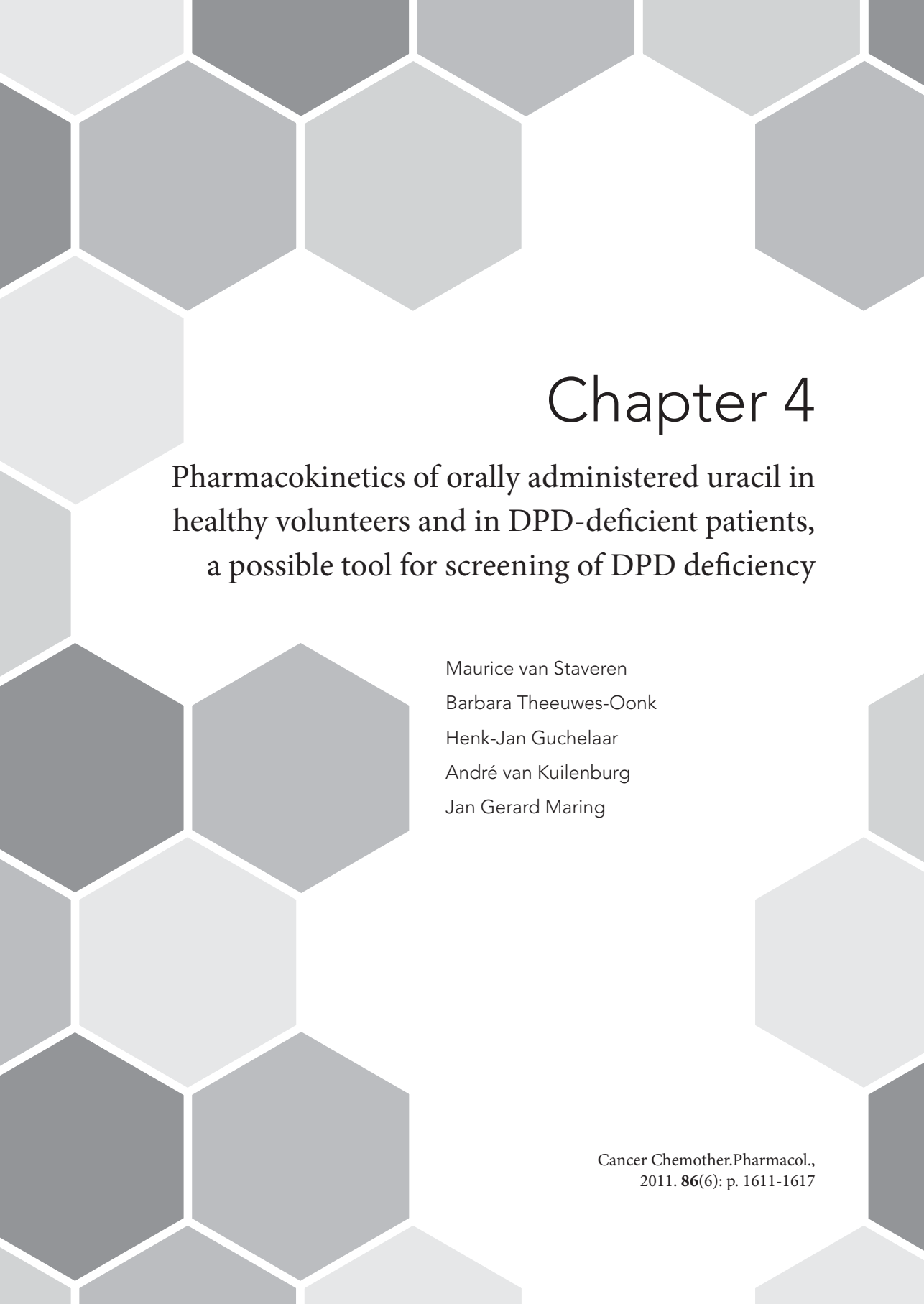
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# Chapter 4

Pharmacokinetics of orally administered uracil in healthy volunteers and in DPD-deficient patients, a possible tool for screening of DPD deficiency

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## ABSTRACT

**Purpose:** Dihydropyrimidine dehydrogenase (DPD) deficiency can lead to severe toxicity in patients treated with standard doses of 5-fluorouracil (5-FU). Oral uracil administration and subsequent measurement of uracil and dihydrouracil (DHU) plasma concentrations might detect patients with DPD deficiency. This study compares the pharmacokinetics (PK) of uracil and DHU after oral uracil administration in subjects with normal and deficient DPD status.

**Methods:** Five hundred milligrams of uracil per metre square was administered orally to 11 subjects with normal DPD status and to 10 subjects with reduced DPD activity. Repeated administration ( $n = 3$ ) of this dose was performed in 4 subjects and 1,000 mg uracil/m<sup>2</sup> was administered to 4 subjects to assess intra-individual variation and linearity of pharmacokinetics.

**Results:** In subjects with normal DPD status, 500 mg/m<sup>2</sup> uracil resulted in uracil  $C_{\max}$  levels of  $14.4 \pm 4.7$  mg/L at  $T_{\max} = 30.0 \pm 11.6$  min and in DPD-deficient subjects,  $20.0 \pm 4.5$  mg/L at  $31.5 \pm 1.1$ . The uracil  $AUC_{0-180}$  was  $31.2 \pm 5.1$  mg L/h in DPD-deficient subjects which was significantly higher ( $p < 0.05$ ) than in the subjects with normal DPD status ( $13.8 \pm 3.9$  mg L/h). Repeated uracil dosing showed reproducible uracil PK in subjects with normal DPD status and dose elevation of uracil suggested linear pharmacokinetics.

**Conclusion:** The PK of uracil differs significantly between subjects with a normal DPD activity and those with a deficient DPD status. The AUC and  $C_{\max}$  of uracil can be useful as a diagnostic tool to differentiate patients with regard to DPD status.

## INTRODUCTION

5-Fluorouracil and its prodrug capecitabine are commonly used chemotherapeutic drugs in the treatment of colorectal, breast and head and neck cancer. The intracellular metabolism of 5-FU is complex, requiring conversion into cytotoxic nucleotides. The main cytotoxic metabolite is 5-fluoro-2'-deoxyuridine 5'-monophosphate that inhibits thymidylate synthase [1]. However, only a small proportion of the administered 5-FU dose is converted to cytotoxic metabolites. Within a few hours after parenteral administration, 70–90% of the 5-FU dose is metabolized into inactive metabolites. DPD is the initial, and rate-determining enzyme in the catabolism of 5-FU [2-5]. Patients with a partial or complete DPD deficiency have a strongly reduced capacity to degrade 5-FU [6, 7] or its oral prodrug capecitabine [8, 9]. As a consequence, treatment with 5-FU and/or capecitabine in patients with reduced DPD activity can cause severe or life-threatening toxicity such as neutropenia, diarrhea and mucositis [10]. It was initially estimated that in 3–5% of Caucasians the activity of DPD is strongly reduced due to (epi)genetic variations in the gene encoding DPD. However, this percentage can be disputed because the incidence of DPD deficiency depends on the method that is used to detect it [11] and the cutoff level chosen or determined to define DPD deficiency [12]. A recent study found DPD deficiency in 40% of the patients included using the uracil/dihydrouracil ratio in plasma, an observation higher than the outcome expected with DPYD genetic polymorphism [13].

Several methods have been developed to detect patients with reduced DPD activity, such as genotyping [14], determination of DPD activity in peripheral blood mononuclear cells (PBMCs) [4, 10], phenotyping with a breath test using [2-<sup>13</sup>C] uracil [15-17], the administration of a 5-FU test dose [7, 18] and assessment of the endogenous uracil/DHU plasma ratio [19-27]. A major drawback of many of these methods is that they are costly and/or laborious or in the case of a 5-FU test dose potentially toxic, thus precluding the routine implementation in clinical practice for prospective screening of DPD deficiency. To achieve the most accurate and simple method to predict DPD deficiency prior to 5-FU- or capecitabine-based treatment, a combined testing strategy has been proposed [12, 21]. However, oral administration of an uracil test dose and subsequent measurement of uracil and its metabolite DHU in plasma might be a cheap, fast, and simple method for screening for DPD deficiency prior to 5-FU or capecitabine containing therapy, which can be used clinically. So far, only the pharmacokinetics of orally administered <sup>13</sup>C-uracil has been reported using low doses of 50, 100, and 200 mg uracil. The major drawback of using low doses of uracil

concerns the lower chance of reaching adequate plasma concentrations. The use of higher uracil doses is expected to result in a more adequate discrimination between normal and deficient individuals due to a prolonged DPD enzyme saturation in DPD-deficient subjects compared with lower doses of uracil. Moreover, this situation may better reflect the DPD enzyme dynamics in the clinical situation when 5-FU doses of 1,000–2,000 mg are being used. From previous work with 5-FU, we estimated that a uracil plasma level of at least 10 mg/l would be needed for proper discrimination between DPD-deficient and normal DPD subjects with high sensitivity and specificity [6].

The main objective of this study was to compare the pharmacokinetics of orally administered uracil between healthy volunteers with normal DPD activity and patients with DPD deficiency due to heterozygosity for a *DPYD* gene mutation. Secondary objectives involved the investigation of linearity of uracil pharmacokinetics at increased uracil dose and the intra- and interday variation in uracil pharmacokinetics.

## MATERIALS AND METHODS

### Study subjects

Eleven subjects with a normal DPD status and ten DPD-deficient subjects, aged 18 years and older, participated in this study. The eleven subjects were all healthy volunteers and the ten DPD-deficient subjects were colorectal and breast cancer patients who suffered CTC grade III or IV side effects following a 5-FU or capecitabine containing drug schedules and had DPD activity < 5 nmol/mg protein/hr. DPD activity was measured in PBMCs, and the DPD status was considered normal or deficient when the DPD activity in PBMCs was > 5 nmol/mg protein/h, or < 5 nmol/mg protein/h respectively [11]. In all patients, DPD deficiency was confirmed by sequence analysis of *DPYD* showing heterozygosity for a pathological mutation. Heterozygosity for the c.1905 + 1G>A (IVS14 + 1G>A), c.2846A>T, c.1129 - 5923C>G, and the novel c.2579delA mutation was detected in 5, 2, 2, and 2 patients, respectively. One of the patients was heterozygous for both the c.1905 + 1G>A mutation and the c.1129 - 5923C>G mutation.

Prior to uracil administration, blood samples were taken to measure creatinine, alanine transaminase (ALAT), and gamma-glutamyl transpeptidase (gamma-GT) as markers for renal and liver function.

The study was approved by the local Medical Ethics Committee of Diaconessen Hospital Meppel, the Netherlands. Informed consent was obtained from each subject.

### Uracil administration

Uracil (Pharmorgana GmbH, Raubling/Rosenheim, Germany) was administered orally at a test dose of 500 mg/m<sup>2</sup> body surface area, calculated by the DuBois and DuBois formula, after an overnight fast (last food intake > 8 h earlier). All subjects had to abstain food during 2 hours after ingesting the uracil. All the test doses were administered between 08:00 a.m. and 09:00 a.m. to avoid circadian effects. The uracil powder was mixed with 100–200 ml tap water, and immediately after preparation, the suspension was ingested within a few minutes. In addition, repeated administration on subsequent days (n = 2 in 3 subjects and n = 1 in one subject) with 500 mg/m<sup>2</sup> was performed in 4 volunteers to assess intra-individual variation and 1,000 mg uracil/m<sup>2</sup> was administered to 4 volunteers to assess linearity of pharmacokinetics, respectively.

### Collection of blood samples

A cannula was placed intravenously in one arm of each subject. Blood samples of 5 ml were collected in heparin-containing tubes. In an intensive sampling schedule, blood samples were collected just before and at t = 15, 30, 45, 60, 80, 100, 120, 150, 180, and 220 min (500 mg/m<sup>2</sup>) or 240 min (1,000 mg/m<sup>2</sup>) after uracil intake. Samples were immediately placed on ice and subsequently centrifuged at 2,500 x g for 10 min at 4°C and stored at -20°C until analysis.

For the repeated uracil administration in the 4 subjects, blood samples were collected according to a limited sampling schedule. This schedule is based on results from an interim analysis on intensive schedule results from both volunteers and patients, in which t = 60 min and t = 120 min were selected as optimal sampling points for the limited sampling strategy.

### DPD activity

The activity of DPD was determined in PBMCs using radiolabeled thymine followed by the separation of radiolabeled thymine from radiolabeled dihydrothymine using reversed-phase HPLC, as described before [28].

## Analytical method for uracil and dihydrouracil

Uracil and DHU plasma concentrations were measured by a validated HPLC method described by Maring et al. [29]. Calibration samples were prepared by spiking human heparinized plasma (Red Cross Blood Bank, Groningen, the Netherlands) with appropriate amounts of uracil and 5,6-DHU (Sigma Chemical Co, Zwijndrecht, the Netherlands). Uracil was quantified at 266 nm and DHU at 205 nm. The internal standard chlorouracil was quantified at both wavelengths. The limit of quantification in plasma was 0.004 mg/l for both uracil and DHU.

## Pharmacokinetic and statistical analysis

The pharmacokinetic parameters  $T_{\max}$ ,  $C_{\max}$ ,  $AUC_{0-180\text{min}}$  were calculated with Phoenix<sup>TM</sup> Winnonlin® Version 6 (Pharsight® Products, CA) using noncompartmental analysis. Mean plasma clearance was calculated with the formula Dose/AUC. Statistical analysis was performed by using SPSS version 16.0 (SPSS inc, Chicago, IL). Normality of data was tested by performing a Kolmogorov-Smirnov test.

To examine whether the uracil and DHU plasma concentrations and derived pharmacokinetic parameters differed between subjects with a normal DPD status and those with DPD deficiency, an unpaired Student's t test was performed on data obtained from the 500 mg/m<sup>2</sup> dose. To investigate whether the distribution of gender in both groups differs between the DPD-deficient and normal individuals, chi-square statistic was used. One-way ANOVA analysis was performed on the uracil and DHU values measured in plasma at t = 60 and 120 min in the four volunteers after repeated 500 mg/m<sup>2</sup> doses to study the inter- and intrasubject variability.

## RESULTS

The characteristics of the subjects included in this study are displayed in Table 4.1. No differences between the two groups were observed ( $p > 0.05$ ) except for age, DPD activity, and disease status.

The HPLC method that was used in this study revealed fully separated peaks for uracil and DHU in the chromatogram as is depicted in Figure 4.1.

**Table 4.1 Patient and volunteer characteristics**

|                           | Normal DPD activity<br>(n = 11) | DPD-deficient<br>(n = 10) | p value            |
|---------------------------|---------------------------------|---------------------------|--------------------|
| Age (years)               | 38 ± 9                          | 62 ± 12                   | < 0.001            |
| Sex (male/female)         | 5/6                             | 4/6                       | Chi square = 0.002 |
| Weight (kg)               | 74 ± 10                         | 76 ± 15                   | 0.636              |
| Height (cm)               | 177 ± 9                         | 173 ± 5                   | 0.154              |
| PBMC DPD (nmol/mg/l)      | 7.2 ± 1.3                       | 3.6 ± 0.8                 | < 0.001            |
| Serum creatinine (μmol/l) | 80 ± 10                         | 78 ± 18                   | 0.707              |
| Serum ALT (U/l)           | 20 ± 8                          | 25 ± 5                    | 0.104              |
| Serum gammaGT (U/l)       | 16 ± 6                          | 39 ± 40                   | 0.160              |

Values are displayed as mean ± SD.

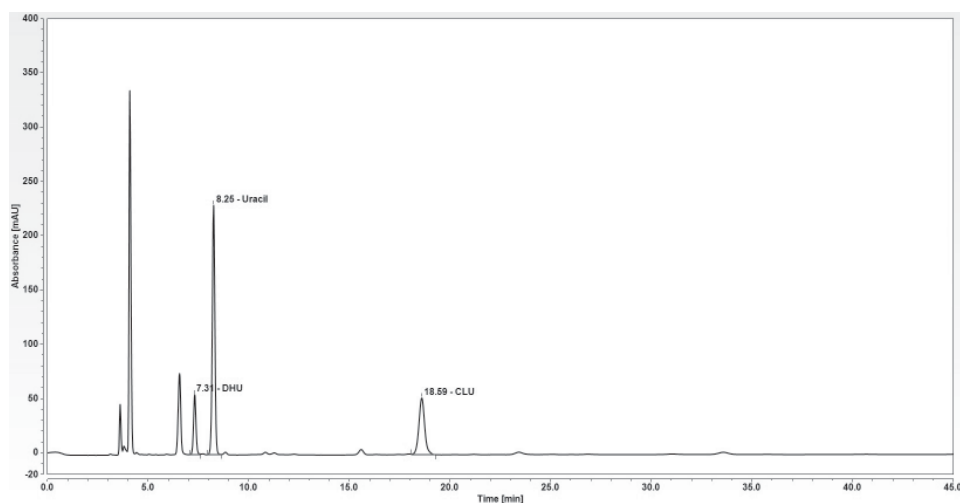


**Figure 4.1 Representative chromatogram obtained from a blood sample of a patient with normal DPD status at t = 60 min after oral intake of 1000 mg uracil.**

The chromatogram was recorded at 205 nm. The uracil and dihydrouracil concentrations were estimated as 9.5 mg/L and 3.4 mg/L, respectively.

The mean uracil and DHU plasma concentrations in the 11 subjects with normal DPD and in the 10 DPD deficient subjects following a uracil dose of 500 mg/m<sup>2</sup> are depicted in Figure 4.2 and the estimated pharmacokinetic parameters  $T_{max}$ ,  $C_{max}$ ,  $AUC_{0-180min}$  and the mean CI are displayed in Table 4.2.

Table 4.2 Pharmacokinetic parameters of 500 mg/m<sup>2</sup> and 1,000 mg/m<sup>2</sup> in volunteers and 500 mg/m<sup>2</sup> in patients

|                                  | Normal DPD activity                         |                                          | DPD deficient                               |                                          | p value (500 mg/m <sup>2</sup> ) | Normal DPD activity                          |                                           |
|----------------------------------|---------------------------------------------|------------------------------------------|---------------------------------------------|------------------------------------------|----------------------------------|----------------------------------------------|-------------------------------------------|
|                                  | Uracil 500<br>mg/m <sup>2</sup><br>(n = 11) | DHU 500<br>mg/m <sup>2</sup><br>(n = 11) | Uracil 500<br>mg/m <sup>2</sup><br>(n = 10) | DHU 500<br>mg/m <sup>2</sup><br>(n = 10) |                                  | Uracil 1,000<br>mg/m <sup>2</sup><br>(n = 4) | DHU 1,000<br>mg/m <sup>2</sup><br>(n = 4) |
| T <sub>max</sub> (min)           | 30.0 ± 11.6                                 | 104.5 ± 23.4                             | 31.5 ± 11.1                                 | 166.0 ± 55.6                             | 0.765                            | 33.8 ± 18.9                                  | 195.0 ± 57.4                              |
| C <sub>max</sub> (mg/l)          | 14.4 ± 4.7                                  | 3.0 ± 0.9                                | 20.0 ± 4.5                                  | 2.2 ± 0.9                                | 0.011                            | 24.1 ± 11.9                                  | 5.2 ± 1.1                                 |
| AUC <sub>0-180min</sub> (mg h/l) | 13.8 ± 3.9                                  | 5.9 ± 1.9                                | 31.2 ± 5.1                                  | 4.4 ± 1.7                                | < 0.001                          | 36.0 ± 14.4                                  | 8.0 ± 2.3                                 |
| Cl <sub>mean</sub> (l/min)       | 1.3 ± 0.5                                   | 3.1 ± 1.8                                | 0.5 ± 0.1                                   | 4.0 ± 1.6                                | < 0.001                          | 1.1 ± 0.6                                    | 4.5 ± 1.7                                 |

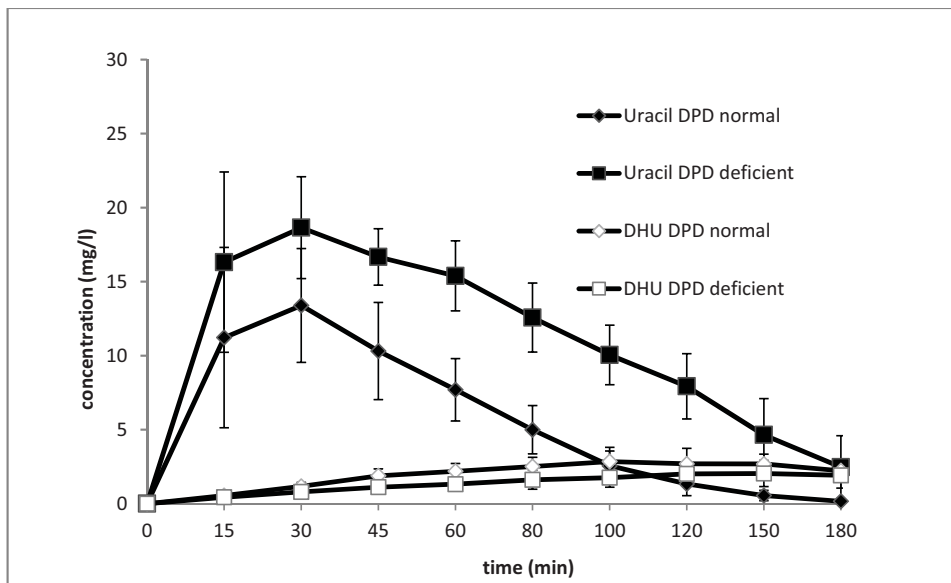


**Figure 4.2** Concentration-time profile of uracil and DHU in subjects with normal DPD ( $n = 11$ ) and DPD-deficient subjects ( $n = 10$ ) after oral intake of 500 mg/m<sup>2</sup> uracil suspension. The results shown are the mean  $\pm$  SD.

$T_{\max}$  of uracil,  $AUC_{0-180\text{min}}$  and Cl of DHU did not differ between the two groups ( $p > 0.05$ ). All the other displayed parameters differed significantly ( $p < 0.05$ ). After reaching  $T_{\max}$ , the decline of uracil concentration in both groups followed zero-order kinetics, which suggests that the DPD enzyme is fully saturated at the administered dose in both groups. In the subjects with normal DPD, after  $t = 100$  min, the elimination changed gradually from zero order to first order, which resulted in an exponential decline. The same phenomenon occurred in DPD-deficient subjects, although at a later stage (after  $t = 150$  min).

In subjects with normal DPD activity, uracil was completely eliminated within approximately 180 min, whereas in DPD-deficient subjects the uracil plasma concentration was still  $2.5 \pm 2.1$  mg/l indicating that in DPD-deficient individuals the clearance of uracil was decreased. In addition, due to the reduced DPD activity, the reaction rate of the formation and the absolute amount of DHU were reduced in the group of DPD-deficient subjects. The uracil and DHU plasma concentrations measured in DPD-deficient subjects differed significantly ( $p < 0.05$ ) as compared to the individuals with normal DPD except for uracil at  $t = 15$  min ( $p = 0.071$ ) and for DHU levels at  $t = 15$ , 120, 150 and 180 min ( $p = 0.149$ ,  $p = 0.111$ ,  $p = 0.087$ , and  $p = 0.363$ , respectively).

Comparable uracil concentrations at  $t = 60$  and  $120$  min were observed in subjects with normal DPD status who were tested multiple times. However, significant differences were observed for DHU levels at  $t = 60$  and  $120$  min ( $p = 0.012$  resp.  $p = 0.001$ ).

Figure 4.3 shows the plasma concentrations of uracil and DHU after administration of  $1,000$  mg uracil/ $m^2$  compared to  $500$  mg uracil/ $m^2$  in subjects with normal DPD status. The  $C_{max}$  of both curves is reached after approximately  $30$  min ( $T_{max}$ ). Only after  $45$  min, the uracil levels differed significantly between both dosages.

For DHU, the concentrations after ingestion of both doses differed not significantly the first  $100$  minutes, but after this point, the DHU concentrations measured in the group of  $1,000$  mg/ $m^2$  were significantly higher. The  $C_{max}$  values of uracil and DHU (Table 4.2) at  $1,000$  mg/ $m^2$  are, respectively  $1.7 \pm 0.3$  and  $1.5 \pm 0.4$  times higher compared with those after  $500$  mg/ $m^2$ .

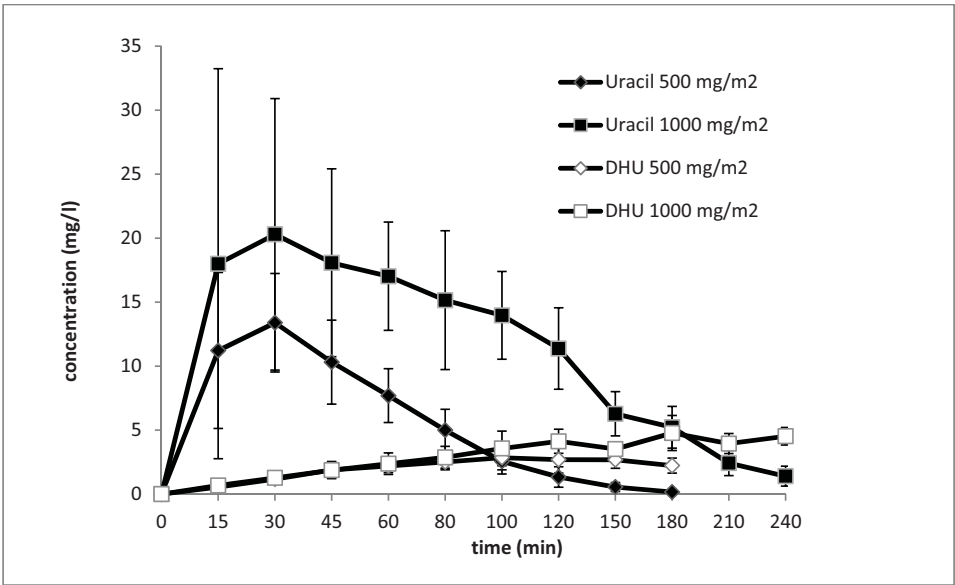


**Figure 4.3** Concentration-time profile of uracil and DHU in subjects with normal DPD activity after oral administration of a dose of  $500$  mg/ $m^2$  ( $n = 11$ ) and  $1,000$  mg/ $m^2$  ( $n = 4$ ) uracil suspension. The results shown are the mean  $\pm$  SD.

## DISCUSSION

In this study, it is shown that the pharmacokinetics of uracil after an oral uracil dose of 500 mg/m<sup>2</sup> was significantly different in subjects with DPD deficiency as compared to those with a normal DPD status suggesting that oral uracil administration may be useful as a test to determine patients with DPD deficiency.

The patient characteristics of the two groups in which we studied uracil and DHU pharmacokinetics were comparable except for DPD status, age, and disease state. The subjects with normal DPD consisted of young healthy individuals, in contrast to the subjects with DPD deficiency, who all were colorectal and breast cancer patients. Aging involves progressive impairments in the functional reserve of multiple organs, which might also affect drug metabolism and pharmacokinetics [30]. With age, the liver mass and its perfusion decreases causing a diminished first pass effect of highly cleared drugs, and renal clearance of drugs is reduced by the loss of kidney function [31]. The liver contains a high amount of DPD, so increasing age might lead to reduced first pass effect and metabolism of uracil in the liver causing higher plasma concentrations in elderly compared to younger individuals. In addition, in cancer patients, metastases of the liver and steatosis caused by systemic chemotherapy [32] can reduce liver drug metabolism, which may also lead to changes in uracil metabolism. However, Maring et al. [33] described that extensive hepatic replacement due to liver metastases had no effect on 5-fluorouracil pharmacokinetics, indicating that the amount of DPD is probably not influenced by reduction in liver function. In addition, in our study population of DPD-deficient patients receiving systemic chemotherapy, no significant differences were observed in liver function or serum creatine compared with the group of healthy individuals. As a result of these findings, we consider it unlikely that the 2.6-fold decrease in clearance of uracil can be ascribed to differences in age or disease state between the two groups. The differences between the two uracil curves representing both the groups with and without DPD deficiency at 500 mg/m<sup>2</sup> are caused by the amount of DPD available in deficient subjects, which is lower than in subjects with normal DPD activity causing reduces clearance of uracil. Mattison et al. [15] evaluated fixed doses of 2-<sup>13</sup>C-uracil of 100, 200, and 300 mg as well as doses adjusted to body weight (1, 3, 6 and 12 mg/kg). They demonstrated with their uracil breath test that an administered dose of 6 mg/kg 2-<sup>13</sup>C-uracil generated less variable  $C_{\max}$  and  $T_{\max}$  than single fixed doses of 100, 200, or 300 mg. The dose of 500 mg uracil/m<sup>2</sup> used in our study is in range with the 2-<sup>13</sup>C-uracil dose of 12 mg/kg that was also used by Mattison et al. and with the commonly applied

5-FU bolus doses of 400–600 mg/m<sup>2</sup> in the treatment of colorectal and breast cancer [34–37]. The gastrointestinal absorption of uracil is a pharmacokinetic first order process and the elimination follows a reversible and saturable Michaelis-Menten kinetics [17]. When DPD is saturated the elimination of uracil follows zero-order kinetics. The formation of DHU depends on the  $K_m$  of DPD and the amount of uracil and enzyme present. When all the present DPD enzyme is saturated, the metabolism of uracil will depend on the absolute amount of DPD present and not on the amount of uracil, i.e., if the same dose of uracil is administered to two individuals with different enzyme levels, the individual with the highest amount of enzyme will have the highest “zero-order” reaction rate and the lowest  $C_{max}$  of both individuals. So, in order to discriminate between individuals with a normal DPD activity and those with a DPD-deficiency the uracil dose used needs to be high enough to saturate the DPD enzyme both in individuals with and without DPD-deficiency in order to achieve significant different plasma levels. For oral uracil to be used as a diagnostic test, the pharmacokinetics of uracil between patients with and without DPD deficiency has to be clearly discriminating. Mattison et al. [15] describe that the AUC following a dose of 6 mg/kg 2-<sup>13</sup>C uracil is significantly different in subjects with normal DPD activity versus partial DPD-deficiency. This is in line with the results we found using a dose 500 mg/m<sup>2</sup> uracil. For a broad clinical use, a diagnostic test has to be simple, cheap, sensitive, and specific. The uracil breath test is expensive because of the use of 2-<sup>13</sup>C uracil and breath bags, and the technique of IR spectrophotometry for analysis of exhaled samples is not available in every hospital. Our test might be more cost effective and might lead to quick test results since the price of 1 gram uracil is about 1 US\$ and the HPLC equipment that is used for analysis is common in most hospitals for therapeutic drug monitoring purposes. If the HPLC equipment is not available at the testing site, the plasma samples have to be stable enough to be transported. Prior to this study, the stability of U and DHU in whole blood and plasma were determined. The results show that uracil in whole blood can be stored at 4°C for up to 4 hours. The degradation of U and DHU in plasma was less than 2% during 24 hour at room temperature. We therefore concluded that the stability in plasma is sufficient enough to perform the analytical extraction procedure without further precautions and that the plasma samples are suitable for transportation within 24 hours. As a result, our test can be incorporated broadly into common clinical practice. However, a disadvantage of the current setup of the test is the intensive blood sampling scheme that takes 4 hour to perform and makes it patient intense. The test has to be further optimized into a limited sampling strategy to be more patient friendly. Based on a limited sampling strategy, the parameters

AUC, clearance, and  $C_{\max}$  are less suitable for discriminating, but the uracil and/or DHU concentrations or U/DHU ratio at selected time points (e.g. 120 min after administration) might be. At this stage, it is unclear if monitoring DHU concentrations can be useful in a limited sampling strategy setting since we found only slightly different values in DHU pharmacokinetic parameters in both groups. This might be explained by the fact that the DHU levels are not only determined by the degradation of uracil by DPD but also by its volume of distribution and the subsequent hydrolysis of DHU into N-carbamyl- $\beta$ -alanine by dihydropyrimidinase. We conclude that uracil administration at a single dose of 500 mg/m<sup>2</sup> leads to significant and reproducible differences in pharmacokinetics of uracil and DHU between volunteers with a normal DPD activity and DPD-deficient patients.  $AUC_{0-180\text{min}}$  and  $C_{\max}$  might be useful to detect partial DPD deficiency. In addition, uracil doses above 500 mg/m<sup>2</sup> have no discriminating benefits but will only result in a right shift of the uracil and DHU concentration curve and unnecessary longer exposition to high uracil levels. The results presented here points toward a promising development of an oral uracil challenge as a diagnostic test for DPD deficiency. The sensitivity and specificity of this test are currently investigated in a larger population of cancer patients with and without DPD deficiency.

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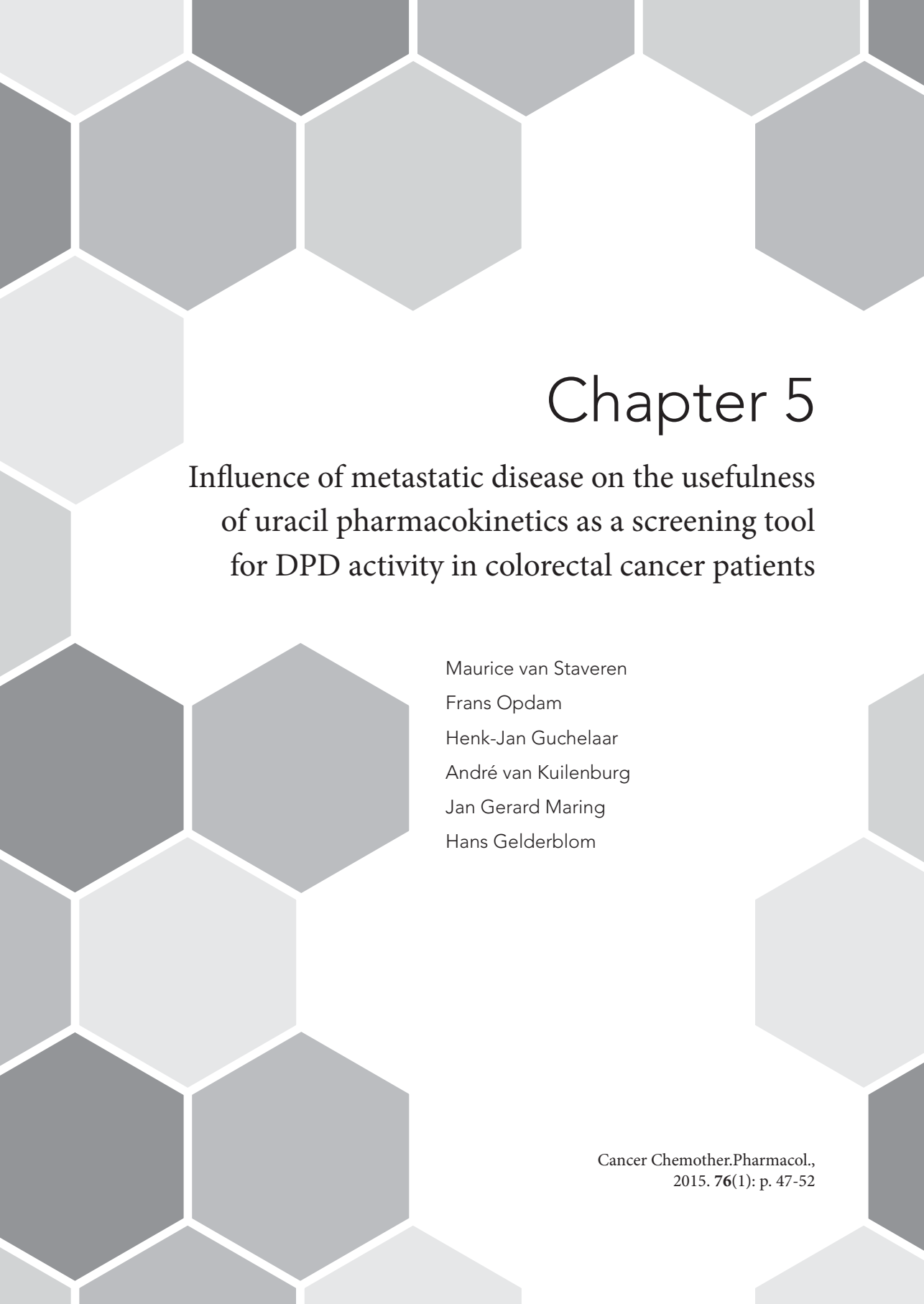
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# Chapter 5

## Influence of metastatic disease on the usefulness of uracil pharmacokinetics as a screening tool for DPD activity in colorectal cancer patients

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## ABSTRACT

**Purpose:** Dihydropyrimidine Dehydrogenase (DPD) deficiency can lead to severe toxicity in patients treated with a standard dose of a fluoropyrimidine such as 5-fluorouracil (5-FU) or capecitabine (CAP). Administration of oral uracil and subsequent measurement of uracil and dihydrouracil (DHU) plasma concentrations has been used to identify patients with DPD deficiency. Liver metastasis might influence systemic DPD activity. The aim of the study is to investigate the effect of metastatic disease on the pharmacokinetics of uracil and DHU after oral administration of uracil.

**Methods:** 500 mg/m<sup>2</sup> uracil was administered orally to 12 subjects with stage II-III colorectal cancer (CRC) who were treated in the adjuvant setting and to 12 subjects with stage IV metastasized CRC, all treated with capecitabine containing therapy. All subjects had a normal DPD activity defined as >6 nmol/mg/h determined in peripheral blood mononuclear cells (PBMCs).

**Results:** The mean uracil clearance [CL 51.7 (SD 6.4) versus 46.7 (SD 13.0) l/h], Area under the curve [ $AUC_{0-220\text{min}}$  20.6 (SD 6.4) versus 21.0 (SD 5.7) h\*mg/l], elimination half life [ $t_{1/2}$  21 (SD 7) vs 21 (SD 8) min], maximum concentration time [ $T_{\text{max}}$  27 (SD 9) vs 25 (SD 9) min], Volume of distribution [V 26.58 (SD 10.11) vs 21.10 (SD 8.48) l] and the elimination constant [ $k_{\text{el}}$  2.01 (SD 0.56) vs 2.41 (SD 0.72) h<sup>-1</sup>] did not differ significantly ( $p > 0.05$ ) non-metastatic CRC versus metastatic CRC.

**Conclusions:** Metastasis does not alter uracil pharmacokinetics and is similar in CRC patients with and without metastasis. Therefore, the uracil test dose could be used as a DPD phenotype test in both adjuvantly treated and metastatic CRC patients using similar cut off criteria to identify patients with DPD deficiency.

## INTRODUCTION

Capecitabine is an oral prodrug of 5-fluorouracil (5-FU). Both drugs are extensively used for the treatment of patients with colorectal, breast, gastric and head and neck cancer. Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme in the catabolism of capecitabine and 5-FU, converting > 80% of an administered dose of 5-FU to inactive metabolites, a process mainly occurring in the liver. Patients with a partial or complete DPD deficiency have a strongly reduced capacity to degrade 5-FU, which may thus result in severe toxicity [1-5]. Several methods have been proposed to identify patients with reduced DPD activity [6]. Since uracil is a non-toxic structural analogue of 5-FU, the metabolism of uracil is similar to that of 5-FU and can therefore be used as a phenotype probe for DPD activity. Like fluoropyrimidines, uracil is metabolized initially by DPD and subsequently degraded by other enzymes into eventually beta-alanine [7] (Figure 5.1).

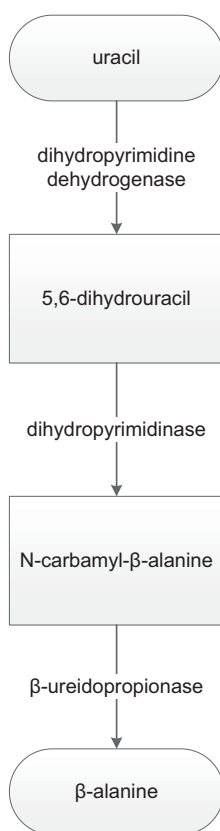


**Figure 5.1** Catabolic pathway of uracil.

In a previous study we described the use of an oral uracil loading dose to assess the DPD status in healthy volunteers and in DPD-deficient CRC patients [8]. The purpose of the current study was to investigate whether or not the presence of metastases might influence the pharmacokinetics of orally administered uracil. The catabolism of 5-FU by DPD occurs mainly in the liver and contributes substantially to the metabolism of 5-FU [9, 10]. Liver metastases might alter uracil pharmacokinetics since in cancer patients, metastases in the liver and steatosis, caused by systemic chemotherapy, have shown to reduce drug metabolism [11]. Secondly, concomitant inflammatory responses have been observed during initiation, invasion, and metastasis of tumours. Components of cancer inflammation like chemokines, prostaglandins, and cytokines have shown to down-regulate cytochrome P450 enzyme activity [12]. Indeed, for CYP2C19, a discordant slow metabolizer phenotype compared to the predictive genotype was found in patients with advanced metastatic cancer [13]. For this reason it might be possible that the presence of a significant metastasis burden might alter DPD activity and uracil pharmacokinetics as well. Therefore, to further validate the oral uracil loading test, we performed a study in colorectal cancer patients treated with capecitabine to compare uracil pharmacokinetics in patients with metastatic disease and patients who were treated in the adjuvant setting without metastatic disease.

## **MATERIALS AND METHODS**

### **Calculation of sample size**

Based on the pharmacokinetic analysis of the data from 11 healthy volunteers enrolled a previous study [8] we calculated a mean uracil clearance of 50.6 l/hour with a variance of 21%. We considered empirically that a difference in uracil clearance > 25% was clinically relevant. Based on this consideration, to achieve 80% power at a 0.05 significance level in order to detect a difference in uracil clearance in subjects with and without metastasizes, the calculated sample size is 24 (12 + 12).

### **Study subjects**

Twelve subjects with metastasized CRC and 12 subjects with CRC in the adjuvant setting were included in this study. All subjects were treated with capecitabine containing therapy and had a normal DPD activity > 6 nmol/mg/h measured in PBMCs to avoid an effect on

uracil pharmacokinetics caused by inactivating *DPYD* mutations. The value of  $> 6$  nmol/mg/h is a threshold level to distinguish individuals with and without DPD deficiency [14]. All subjects were aged  $> 18$  years and had adequate renal and liver function. Three hospitals in the Netherlands participated in this study that was approved by the Medical Ethics Committee BEBO in Assen, The Netherlands. Informed consent was obtained from all individual participants included in the study. Prior to uracil administration, blood samples were obtained to measure creatinine clearance, alanine transaminase (ALAT) and gamma-glutamyl transpeptidase (gamma-GT) as markers for renal function and liver damage.

### Uracil administration

Uracil (Pharma Waldhof GmbH, Düsseldorf, Germany) was administered orally at a test dose of 500 mg/m<sup>2</sup> body surface area, calculated by the Dubois and Dubois formula, after an overnight fast (last food intake  $> 8$  h earlier). All subjects had to abstain from food during 2 hours after oral administration of uracil. Administration took place at least 48 hours after the last administration of capecitabine. All the test doses were administered between 08:00 AM and 09:00 AM to avoid circadian effects. The uracil powder was mixed with 100–200 mL of tap water and immediately after preparation the suspension was ingested within a few minutes.

### Collection of blood samples

Blood samples were obtained at  $t = 0, 15, 30, 45, 60, 80, 100, 120, 150, 180$  and 220 min from an intravenous indwelling catheter.

Samples were immediately placed on ice and subsequently centrifuged at  $2,500 \times g$  for 10 min. The plasma was stored at  $-20^{\circ}\text{C}$  until analysis.

### DPD activity

The activity of DPD was determined in PBMCs using radiolabeled thymine followed by separation of radiolabeled thymine from radiolabeled dihydrothymine using reversed-phase HPLC and online detection of radioactivity, as described before [15].

## Analytical method for uracil and dihydrouracil

Uracil and DHU plasma concentrations were measured by a validated HPLC method described by Maring et al. [16]. Calibration samples were prepared by spiking human heparinised plasma obtained from volunteers with appropriate amounts of uracil and 5,6-DHU (Sigma Chemical Co, Zwijndrecht, The Netherlands). Uracil was quantified at 266 nm and DHU at 205 nm. The internal standard chlorouracil was quantified at both wavelengths.

## Pharmacokinetic and statistical analysis

The pharmacokinetic parameters area under the curve ( $AUC_{0-220min}$ ), uracil clearance (Cl), maximum uracil concentration ( $C_{max}$ ) and maximum concentration Time ( $T_{max}$ ) were calculated with 'KINFIT module' of MwPharm version 3.50 (Mediware, Groningen, the Netherlands)\*. KINFIT is a Bayesian curve fitting module in which we used a one compartment model. The AUC was calculated by a logarithmic trapezoidal rule. Statistical analysis was performed by using SPSS version 19.0 (SPSS inc, Chicago, IL). To examine whether the uracil and DHU plasma concentrations and derived pharmacokinetic parameters differed between the two study groups, an independent-samples Student's t-test was performed. Levene's test for equality was used to determine if the variance of each pharmacokinetic parameter was equal.

## RESULTS

Table 5.1 displays the characteristics of the patients included in this study. In the metastasized group all patients had liver metastasis. Length, weight, age, BSA, leukocyte count, renal- and liver function were inventoried in all patients and did not differ significantly ( $p > 0.05$ ) between the two study groups. DPD activity was equally distributed between the two groups [9.5 (SD 2.9) and 10.3 (SD 1.8) nmol/mg/h respectively]. The patients in the adjuvant and metastatic group were using capecitabine as monotherapy ( $n = 12$ ), combined with oxaliplatin ( $n = 7$ ) or combined with oxaliplatin and bevacizumab ( $n = 5$ ) for treatment of CRC. Capecitabine and bevacizumab was only used in the metastatic group. Mean age of subjects adjuvantly treated for CRC was 63 (SD 10 years) and 69 (SD 6 years) for those with metastatic disease. Mean Body Surface Area (BSA) did not differ between the patients in treated in the adjuvant setting ( $p = 0.601$ ) compared to patients with metastatic CRC.

**Table 5.1 Patient characteristics of the study population with standard deviation between brackets**

| Baseline characteristic          | Adjuvant    | Metastasis  |
|----------------------------------|-------------|-------------|
| DPD activity (nmol/mg/l)         | 9.5 (2.9)   | 10.3 (1.8)  |
| Creatinine ( $\mu\text{mol/l}$ ) | 76.6 (15.8) | 79.8 (18.1) |
| ALAT (U/l)                       | 27.4 (8.1)  | 22.7 (11.6) |
| GammaGT (U/l)                    | 39.8 (11.5) | 52.7 (39.3) |
| Leukocytes (mmol/l)              | 4.9 (1.6)   | 5.9 (1.8)   |
| Weight (kg)                      | 82 (13)     | 84 (11)     |
| Length (cm)                      | 174 (7)     | 177 (10)    |
| BSA ( $\text{m}^2$ )             | 1.97 (0.20) | 2.01 (0.19) |
| Age (y)                          | 63 (10)     | 69 (6)      |

Adjuvant, adjuvantly treated CRC patients; metastasis, study group with metastatic CRC; ALAT, Alanine Amino Transferase; GammaGT, Gamma-glutamyltransferase; BSA, Body Surface Area.

Table 5.2 displays the pharmacokinetic parameters of the two study groups. Clearance of uracil was lower in the group with metastatic disease [46.7 (SD 5.7) l/h] compared to clearance in adjuvantly treated patients [51.7 (SD 11.7) l/h], but the difference was not statistically significant ( $p = 0.327$ ). Figure 5.2 shows the concentration-time curves for uracil and DHU in both study groups. The mean exposure to uracil was not different ( $p = 0.889$ ) between the two groups [ $\text{AUC}_{0-220\text{min}}$  20.6 (SD 6.4)  $\text{h} \cdot \text{mg/l}$  for adjuvantly treated patients and for metastatic patients  $\text{AUC}_{0-220\text{min}}$  21.0 (SD 5.7)  $\text{h} \cdot \text{mg/l}$ ]. The time to reach  $T_{\text{max}}$  did not differ but the maximum concentration of uracil was significantly different between the two groups with 19.9 mg/l (SD 4.0) in the adjuvantly treated group and 25.8 mg/l (SD 5.7) in the

**Table 5.2 Pharmacokinetic parameters of the two study groups with standard deviation between brackets**

| Pharmacokinetic parameter               | Adjuvant      | Metastasis   | p value |
|-----------------------------------------|---------------|--------------|---------|
| $\text{AUC}_{0-220\text{min}}$ (h mg/l) | 20.6 (6.4)    | 21.0 (5.7)   | 0.889   |
| CL (l/h)                                | 51.7 (11.7)   | 46.7 (13.0)  | 0.327   |
| $T_{\text{half}}$ (min)                 | 21 (7)        | 21 (8)       | 0.927   |
| $T_{\text{max}}$ (min)                  | 27 (9)        | 25 (9)       | 0.600   |
| $C_{\text{max}}$ (mg/l)                 | 19.9 (4.0)    | 25.8 (5.7)   | 0.008   |
| V (l)                                   | 26.58 (10.11) | 21.10 (8.48) | 0.164   |
| $k_{\text{el}}$ ( $\text{h}^{-1}$ )     | 2.01 (0.56)   | 2.41 (0.72)  | 0.136   |

Adjuvant, adjuvantly treated CRC patients; metastasis, study group with metastatic CRC; AUC, Area Under the Curve;  $T_{\text{half}}$ , elimination half time;  $T_{\text{max}}$ , time point of maximum concentration;  $C_{\text{max}}$ , maximum concentration; V, volume of distribution;  $k_{\text{el}}$ , elimination constant.

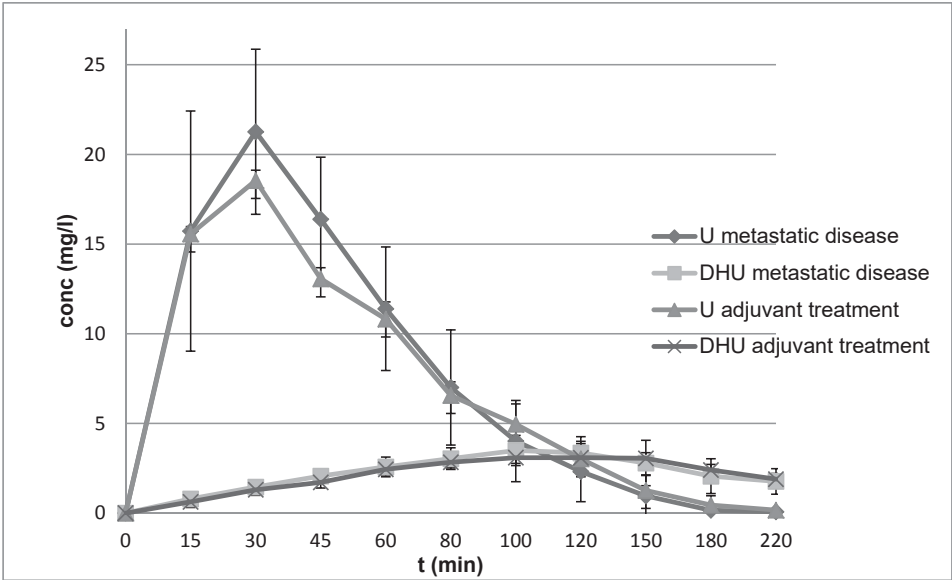


**Figure 5.2** Time versus concentration curve of the mean  $\pm$  SD values of U and DHU in both study groups.

group with metastatic disease ( $p = 0.008$ ). Also, the half-life of uracil did not differ and was 21 minutes for both groups, as was the case for the volume of distribution and elimination. No adverse events related to uracil administration of uracil were identified in the study.

DISCUSSION

This is the first study investigating the potential effect of metastatic disease on uracil pharmacokinetics in CRC patients with a normal DPD activity. Our results show that metastasis has no effect on uracil pharmacokinetics. Hypothetically, the extent of metastatic disease might influence DPD activity and uracil pharmacokinetics. In our study the patients with metastatic disease all had a good health performance. Liver enzymes were normal in both study groups and no patients with extreme cachexia have been identified. Therefore, we cannot fully exclude that the presence of cachexia, commonly seen in metastatic disease, might influence uracil PK.

This study did not reveal a difference in uracil pharmacokinetics between patients adjuvantly treated for CRC and those with metastatic disease with the exception of  $C_{max}$ . The  $C_{max}$

observed for the adjuvantly treated group is in line with  $C_{\max}$  observed in a previous study [9].  $C_{\max}$  is determined by dose, absorption/degradation rate of a drug and volume of distribution, which are considered to be equal between the two study groups. Based on the pharmacokinetic profile of capecitabine and the fact that both groups were treated with capecitabine, we find it not likely that capecitabine might tribute to this observation. In the metastatic group however, patients were treated with bevacizumab. The test was performed more than 14 days after bevacizumab administration, but since the long elimination half time of approximately 20 days of this drug, we cannot exclude the possibility that bevacizumab or the presence of metastatic disease might influence the gastrointestinal absorption and hence  $C_{\max}$ .  $C_{\max}$  however does not play a role of interest if the test is used to discriminate between DPD-deficient individuals and individuals with a normal DPD status. In this context we focused on uracil and DHU levels at  $t = 120$  min.

In this study, we did not perform a pharmacokinetic analysis of dihydrouracil. Dihydrouracil has its own unique elimination pathway (Figure 5.1). Dihydropyrimidinase deficiency is very rare and since its effect on the toxicity of fluoropyrimidines is not known, we did not investigate the pharmacokinetics off dihydrouracil.

We enrolled only patients with a normal DPD activity in the study. This study setup was chosen to exclude the effect of *DPYD* polymorphisms that have a large effect on uracil pharmacokinetics [17]. Such as large decrease of DPD enzyme activity caused by DPD polymorphisms would have excluded the detection of a smaller effect of metastatic disease on uracil PK. In this study, concomitant use of DPD-inhibiting medication such as cimetidine was not allowed and could therefore not have confounded the results.

The orally administered uracil did not result in any adverse events in our patients and can be used safely. Orally administered uracil  $500 \text{ mg/m}^2$  is considered to saturate DPD fully during the period that plasma concentration levels are above the Michaelis constant [8]. Because of this, differences in DPD activity between individuals will become more profoundly clear than the determination of physiological levels of uracil/DHU ratios, which show high variation between individuals [5, 18-21]. Our oral uracil loading test is useful to be introduced into clinical practice. However, the test can be further optimized with a limited sample strategy in combination with the dried blood spot method for sample selection and patient convenience. In conclusion, in patients with a normal DPD activity, with the exception of  $C_{\max}$  we found no evidence for different uracil pharmacokinetics in patients with metastatic CRC as compared to CRC patients treated adjuvantly. Since  $C_{\max}$  is not used

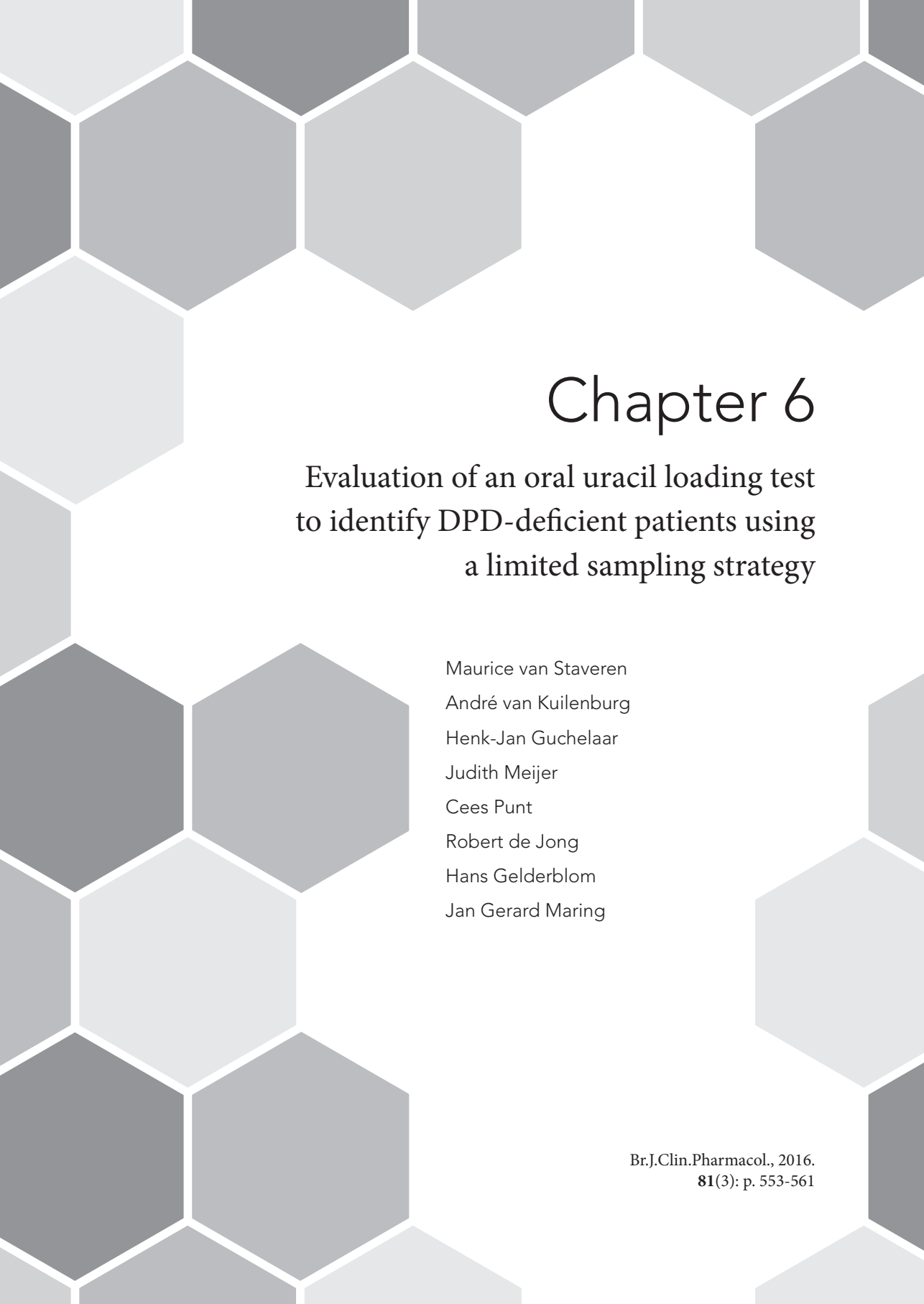
as a discriminating parameter, orally administered uracil could therefore be used as a DPD phenotype test in both adjuvantly treated and metastatic CRC patients using the same cut off criteria. The results of this study look very promising to use the oral uracil loading dose as a easy and robust test to evaluate DPD activity in a clinical setting.

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# Chapter 6

## Evaluation of an oral uracil loading test to identify DPD-deficient patients using a limited sampling strategy

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## ABSTRACT

**Aim(s):** Dihydropyrimidine dehydrogenase (DPD) deficiency can lead to severe toxicity following 5FU or capecitabine (CAP) treatment. Uracil (U) can be used as a probe to determine the systemic DPD activity. This study was performed to assess the sensitivity and specificity of an U loading dose for detecting DPD deficiency.

**Methods:** Cancer patients with Common Toxicity Score (CTC) grade III or IV toxicity after the first or second cycle of 5-FU or capecitabine treatment were asked to participate. Based on DPD activity in PBMCs, patients were divided in 2 groups: DPD activity in PBMCs  $< 5 \text{ nmol mg}^{-1}\text{h}^{-1}$  (deficient group) and  $\geq 5 \text{ nmol mg}^{-1}\text{h}^{-1}$ . U  $500 \text{ mg m}^{-2}$  was administered orally and plasma concentrations of U and dihydrouracil (DHU) in plasma were determined. In the deficient group, PCR amplification of all 23 coding exons and flanking intronic regions of *DPYD* was performed. An U pharmacokinetic model was developed and used to determine  $V_{\max}$  of the DPD enzyme of each patient. Sensitivity and specificity of  $V_{\max}$ , U concentration and the U/DHU concentration ratio were determined.

**Results:** 47 patients were included (19 DPD deficient, 28 DPD normal). Of the pharmacokinetic parameters investigated, a sensitivity and specificity of 80% and 98% respectively was obtained for the U/DHU ratio at  $t = 120 \text{ min}$ .

**Conclusions:** The high sensitivity of the U/DHU ratio at  $t = 120 \text{ min}$  for detecting DPD deficiency as defined by DPD activity in PBMCs, show that the oral U loading dose can effectively identify patients with reduced DPD activity.

## INTRODUCTION

5-Fluorouracil (5-FU) is extensively metabolized by dihydropyrimidine dehydrogenase (DPD) into fluorodihydrouracil (FDHU) [1-4]. Reduced activity of DPD will result in altered 5-FU pharmacokinetics (PK) [5-7] and the increased exposure to 5FU can cause severe side effects [8]. Pre-emptive testing followed by dose reduction for patients with DPD deficiency might prevent these severe side effects [7]. Despite the fact that several tests are available to screen for DPD deficiency, only a few are implemented on a broad scale in a pre-emptive setting [9]. Previously, we showed that administration of an oral loading dose of U allows the identification of patients with normal DPD activity and DPD deficient patients [10]. However, the intensive blood sampling scheme that was used previously is a major drawback for a pre-emptive setting [10]. Quantitative compartmental modeling has proven to be a sensitive tool in describing the mechanisms involved in PK [7]. The object of this study was to develop a limited sampling strategy, to detect decreased U elimination in patients with a DPD deficiency and to perform a more in-depth quantitative compartmental pharmacokinetic analysis of U plasma concentrations.

## MATERIALS AND METHODS

### Patients and study design

Patients were included in 10 hospitals in The Netherlands between August 2006 and December 2013. The study population consisted of cancer patients who suffered from CTC grade III or IV toxicity after the first or second cycle of treatment schedules containing 5-FU or capecitabine. Since the incidence of DPD deficiency is relatively (3–5%) low in the Caucasian population [2], only patients with toxicity were included in order to increase the likelihood to find DPD deficient patients. DPD activity in PBMCs [11] was determined and patients were divided in 2 groups: patients with DPD activity in PBMCs  $< 5 \text{ nmol mg}^{-1}\text{h}^{-1}$  were considered deficient (deficient group) while patients with activity  $\geq 5 \text{ nmol mg}^{-1}\text{h}^{-1}$  were classified as normal with respect to DPD status (normal group, mean  $\pm$  SD controls:  $9.9 \pm 2.8 \text{ nmol mg}^{-1}\text{h}^{-1}$ ). Coefficient of variation (CV) and limit of detection (LOD) of this assay were previously described [11]. No discrimination was made between tumour type or adjuvant treatment versus metastatic disease. The study flow diagram is displayed in Figure 6.1.

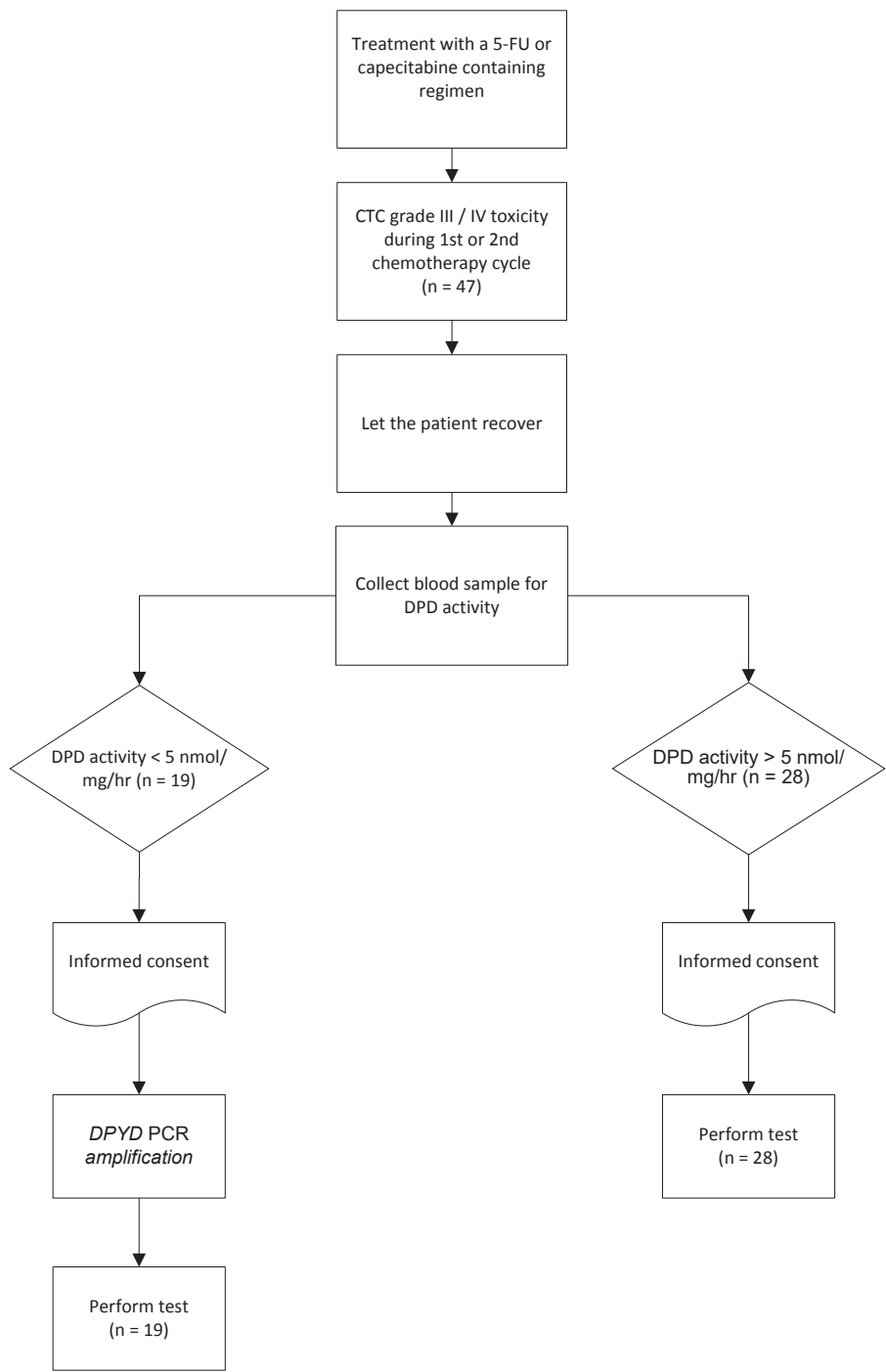


**Figure 6.1 Flowchart of the study design.**  
5-FU, 5-fluorouracil; DPD, dihydropyrimidine dehydrogenase; PCR, polymerase chain reaction.

In all participants, U 500 mg m<sup>-2</sup> as a loading dose was administered orally followed by blood sampling as described before by using 2 different sampling schemes [10]. The U test dose was administered in the morning around 08:00 after an overnight fast to minimize variance caused by possible circadian effects off DPD activity and food intake. Plasma concentrations following the loading dose of U and its metabolite dihydrouracil (DHU) were determined by high performance liquid chromatography (HPLC) [12]. CV of U were 4.8% (4 mg l<sup>-1</sup>) and 3.7% (18 mg l<sup>-1</sup>), and 8.8% (1 mg l<sup>-1</sup>) and 5.7% (3 mg l<sup>-1</sup>) for DHU. LOD is 0.060 mg l<sup>-1</sup> for both U and DHU. In the deficient group, genetic analysis of *DPYD* was performed by PCR amplification of all 23 coding exons and flanking intronic regions followed by sequence analysis, essentially as described before [8]. In all patients, prior before the U loading dose was administered, endogenous concentrations of U, thymine, DHU, dihydrothymine, N-carbamyl-β-alanine and N-carbamyl-β-aminoisobutyric acid in plasma were determined using reversed-phase HPLC combined with electrospray tandem mass spectrometry [13, 14]. CV and LOD of this method were described before [13]. Values of creatinine, alanine transaminase (ALAT), gamma-glutamyl transpeptidase (Gamma-GT) and white blood cell count measured before the occurrence of fluoropyrimidine related toxicity of all subjects were collected. Toxicity data was collected and scored by the local investigator of each participating hospital according to the Common Toxicity Score (CTC) version 3. The CTC was scored for the typical fluoropyrimidine side effects diarrhea, stomatitis, neutropenia and hand-foot syndrome. The cumulative CTC score of each individual was obtained by calculating the sum of all CTC ratings. The mean cumulative CTC score of both study groups was used to compare the toxicity burden between both groups. The study protocol was approved by the Ethics Review Committee of the Martini Hospital Groningen in the Netherlands and all patients gave written informed consent.

## Pharmacokinetic analysis

All patients received orally 500 mg m<sup>-2</sup> U after which blood was taken prior after intake. A full sampling scheme (FSS) in which blood samples were collected at 0, 15, 30, 45, 60, 80, 100, 120, 150, 180 and 240 minutes was applied in the first 10 patients of both study groups. A limited sampling scheme (LSS) in which blood was taken prior to the intake and at 60 and 120 minutes was applied to all other subjects in both study groups. The time points of the LSS were determined as the optimal LSS points based on results of an interim analysis as previously reported [10]. The pharmacokinetic parameters that were investigated were the U concentration at t = 120 minutes, the U/DHU ratio at t = 120 min and the V<sub>max</sub> value

derived from a pharmacokinetic U model. To calculate the  $V_{\max}$  of both study groups, the following approach was used: based on U and DHU concentrations measured in the blood samples of the FSS of both study groups, a population one-compartment model (EURMIX model) with Michaelis-Menten elimination PK was developed with the 'KINPOP module' of MwPharm version 3.50 (Mediware, Groningen, the Netherlands) [15]. The elimination parameters were: Michaelis-Menten constant ( $K_M$ ),  $V_{\max}$ , the apparent volume of the central or plasma compartment in a one compartment model (V1) and absorption constant ( $k_a$ ). Pharmacokinetic parameters were assumed to be distributed log-normally. Since no data is available with respect to the oral bioavailability of U, we assumed that 100% of the administered dose was absorbed from the gastrointestinal tract since the gastrointestinal absorption of U is a fast pharmacokinetic first order process [16].

The same principle was used to develop population models for the DPD deficient (EURDEF model) and DPD normal (EURNOR model) study groups. The EURMIX model was used to calculate the  $V_{\max}$  of all individual subjects based on the LSS, with the use of an iterative two stage Bayesian (ITBS) procedure [15, 17]. To establish the performance of the LSS, the FSS data were used for an explorative internal validation. Using the FSS data,  $V_{\max, \text{FSS}}$  values were calculated with the EURMIX model. Subsequently in the same dataset  $V_{\max, \text{LSS}}$  values were calculated with the EURMIX model using only U and DHU data of the  $t = 60$  and  $120$  minutes samples. Both  $V_{\max, \text{FSS}}$  and  $V_{\max, \text{LSS}}$  were compared by correlation analysis and the performance of the LSS was considered valid if the mean prediction error was  $< 5\%$  and the root mean square error (RMSE) was  $< 10\%$ .

## Statistical analysis

Normal distribution of data was tested by performing the Shapiro-Wilk test. Comparison of parameters between the two study groups was performed by using the two-sample Student's t-test and Chi square analysis. Receiver operating characteristic (ROC)-curves were used to determine the cut-off levels bases of sensitivity (true positive rate) and specificity (true negative rate). The level of significance was set at  $p \leq 0.05$ . Analysis was performed by using the Statistical Package for the Social Sciences (SPSS), version 19 (IBM SPSS Inc., Chicago, IL, USA).

## RESULTS

In total 47 cancer patients aged > 18 year were included, 19 in the DPD deficient group and 28 in the DPD normal group based upon DPD enzyme measurement in PBMCs. Because several patients were included simultaneously in different hospitals after starting the study, the FSS was applied to more than 10 patients in each study group. In the deficient and normal group the FSS was performed in 14 resp 12 patients. The patient characteristics are displayed in Table 6.1.

Of all characteristics, only the DPD activity in PBMCs differed significantly between the two groups ( $p < 0.05$ ). Weight, length, age, liver- and renal function did not differ significantly. From measurement of the endogenous pyrimidine metabolites, we found that none of the metabolite levels or the calculated metabolite ratios was significantly different between the two study groups ( $p > 0.05$ ). In the DPD deficient group all patients had at least one pathogenic *DPYD* variant: c.1129-5923C>G ( $n = 4$ ), c.2579delA ( $n = 2$ ), c.2846A>T ( $n = 3$ ), c.1905+1G>A ( $n = 10$ ), c.1679T>G ( $n = 1$ ). No difference in U PK was observed between the different variants.

### PK analysis of U

Table 6.2 displays the pharmacokinetic parameters of the EURDEF, EURNOR and EURMIX models. There are clear differences in the  $V_{\max}$  values of the different models and is lowest in the DPD deficient pharmacokinetic model (mean  $\pm$  SD  $494 \pm 133 \text{ mg} \cdot \text{h}^{-1} \cdot 1.85 \text{ m}^{-2}$ ) and highest in the population with normal DPD activity (mean  $\pm$  SD  $837 \pm 130 \text{ mg} \cdot \text{h}^{-1} \cdot 1.85 \text{ m}^{-2}$ ).

### LSS

Figure 6.2 displays the correlation between the  $V_{\max}$  calculated with the EURMIX model for the LSS and full sampling for the subjects who underwent the FSS. The root mean square error and the mean prediction error were  $< 7.3\%$  and  $< 1.8\%$ , respectively showing that  $V_{\max}$  values calculated from the data of the limited sampling schedule are comparable to those calculated from the intensive sampling schedule. The  $V_{\max}$  values calculated with the EURMIX model based on the LSS time points in all subjects differed significantly between the two study groups ( $p < 0.001$ ) as is shown in Figure 6.3.

Table 6.1 Patient characteristics

| Parameter                                                 | ID            | Mean | SD   | SEM    | p-value |
|-----------------------------------------------------------|---------------|------|------|--------|---------|
| Weight (kg)                                               | DPD normal    | 70   | 12   | 2.187  | 0.114   |
|                                                           | DPD deficient | 76   | 14   | 3.176  |         |
| Length (m)                                                | DPD normal    | 170  | 9    | 1.697  | 0.835   |
|                                                           | DPD deficient | 170  | 8    | 1.812  |         |
| Age (year)                                                | DPD normal    | 63   | 11   | 1.997  | 0.592   |
|                                                           | DPD deficient | 62   | 10   | 2.408  |         |
| Kreatinine (μmol l <sup>-1</sup> )                        | DPD normal    | 74   | 19   | 3.654  | 0.277   |
|                                                           | DPD deficient | 81   | 20   | 4.627  |         |
| ALAT (U l <sup>-1</sup> )                                 | DPD normal    | 28   | 20   | 4.268  | 0.22    |
|                                                           | DPD deficient | 22   | 6    | 1.572  |         |
| GammaGT (U l <sup>-1</sup> )                              | DPD normal    | 107  | 131  | 29.265 | 0.057   |
|                                                           | DPD deficient | 39   | 31   | 7.964  |         |
| WBC (10 <sup>6</sup> l <sup>-1</sup> )                    | DPD normal    | 6.0  | 1.8  | 0.3761 | 0.596   |
|                                                           | DPD deficient | 6.4  | 2.4  | 0.5878 |         |
| DPDact in PBMCs (nmol mg <sup>-1</sup> *h <sup>-1</sup> ) | DPD normal    | 9.9  | 3.5  | 0.6642 | < 0.001 |
|                                                           | DPD deficient | 3.8  | 1.5  | 0.3426 |         |
| Dhu (μmol l <sup>-1</sup> )                               | DPD normal    | 0.93 | 0.42 | 0.09   | 0.522   |
|                                                           | DPD deficient | 0.85 | 0.33 | 0.08   |         |
| Dht (μmol l <sup>-1</sup> )                               | DPD normal    | 0.93 | 0.55 | 0.11   | 0.250   |
|                                                           | DPD deficient | 0.77 | 0.21 | 0.05   |         |
| NCbala (μmol l <sup>-1</sup> )                            | DPD normal    | 0.19 | 0.10 | 0.02   | 0.284   |
|                                                           | DPD deficient | 0.15 | 0.10 | 0.02   |         |
| NCbaib (μmol l <sup>-1</sup> )                            | DPD normal    | 0.13 | 0.10 | 0.02   | 0.060   |
|                                                           | DPD deficient | 0.08 | 0.03 | 0.01   |         |
| Uracil (μmol l <sup>-1</sup> )                            | DPD normal    | 0.25 | 0.10 | 0.02   | 0.205   |
|                                                           | DPD deficient | 0.29 | 0.10 | 0.02   |         |
| Thy (μmol l <sup>-1</sup> )                               | DPD normal    | 0.05 | 0.02 | 0.00   | 0.995   |
|                                                           | DPD deficient | 0.05 | 0.03 | 0.01   |         |
| Uracil/DHU+NC-bala                                        | DPD normal    | 0.28 | 0.15 | 0.03   | 0.486   |
|                                                           | DPD deficient | 0.32 | 0.14 | 0.03   |         |
| Thy/DHT+NC-baib                                           | DPD normal    | 0.06 | 0.04 | 0.01   | 0.949   |
|                                                           | DPD deficient | 0.06 | 0.04 | 0.01   |         |
| U/NC-bala                                                 | DPD normal    | 1.66 | 1.24 | 0.2491 | 0.125   |
|                                                           | DPD deficient | 2.31 | 1.32 | 0.3314 |         |
| Thy/NC-baib                                               | DPD normal    | 0.56 | 0.39 | 0.0789 | 0.265   |
|                                                           | DPD deficient | 0.77 | 0.77 | 0.1921 |         |

WBC, white blood cells; Thy, thymine; DHU, dihydrouracil; U, uracil; Dht, dihydrothymine; NC-bala, N-carbamyl-β-alanine; NC-baib, N-carbamyl-β-aminoisobutyric; SD, standard deviation; SEM, standard error mean.

**Table 6.2** Population pharmacokinetic parameters  $\pm$  standard deviation estimated in dihydropyrimidine-dehydrogenase deficient subjects (EURDEF model). Subjects with normal DPD activity (EURNOR model), and all subjects (EURMIX).

|        | $K_m$ (mg l <sup>-1</sup> ) | $V_{max}$ (mg h <sup>-1</sup> 1.85 m <sup>-2</sup> ) | $k_a$ po (h <sup>-1</sup> ) | $V(l)$          |
|--------|-----------------------------|------------------------------------------------------|-----------------------------|-----------------|
| EURDEF | 5.99 $\pm$ 4.17             | 494 $\pm$ 133                                        | 5.61 $\pm$ 7.16             | 0.57 $\pm$ 0.09 |
| EURNOR | 7.69 $\pm$ 1.38             | 837 $\pm$ 130                                        | 4.65 $\pm$ 3.33             | 0.51 $\pm$ 0.14 |
| EURMIX | 7.10 $\pm$ 3.33             | 641 $\pm$ 178                                        | 5.13 $\pm$ 4.87             | 0.55 $\pm$ 0.12 |

$K_m$ , Michealis-Menten constant;  $V_{max}$ , maximum enzymatic conversion capacity;  $k_a$ , absorption constant;  $V$ , apparent volume of drug distribution; po, per oral.

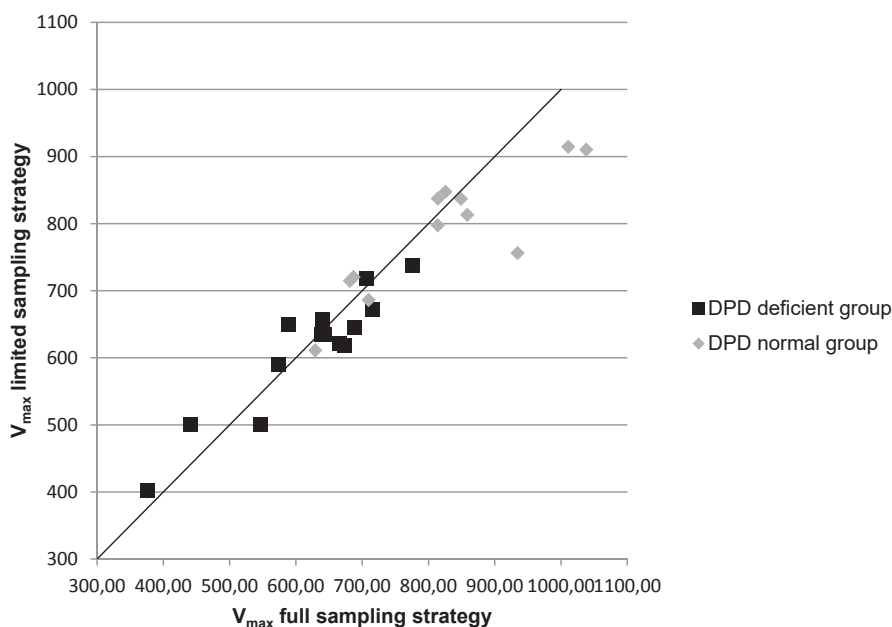


**Figure 6.2** Comparison of maximum enzymatic conversion capacity ( $V_{max}$  mg h<sup>-1</sup> 1.85 m<sup>-2</sup>) values calculated for the limited sampling and intensive sampling strategy with the EURMIX model.

The diagonal line represents  $y = x$ .

## Toxicity data

There was no statistical difference observed in fluoropyrimidine specific cumulative toxicity, i.e. diarrhea, stomatitis, neutropenia and hand-foot, between the DPD normal and DPD deficient group ( $p = 0.495$ ). Diarrhea was the most reported side effects in both study groups. Chi square analysis revealed no significant difference in reported numbers of CTC grade I, II, III or IV toxicity and toxic side effects diarrhea, stomatitis, neutropenia and hand-foot syndrome between the two study groups (all  $p$ -values  $> 0.05$ ).

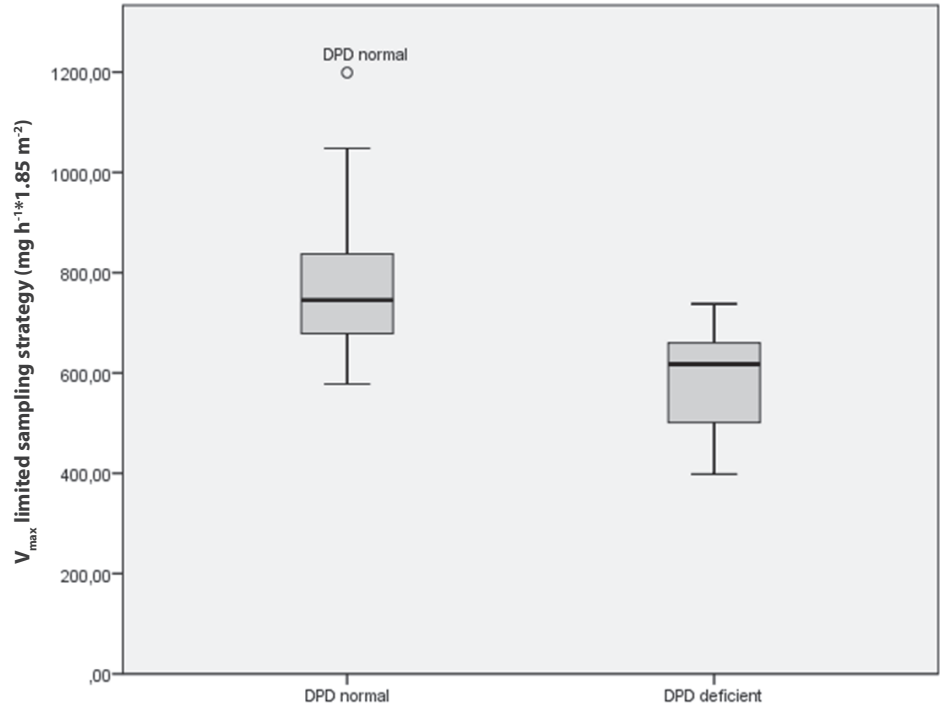


**Figure 6.3**  $V_{max,LSS}$  ( $\text{mg h}^{-1} \cdot 1.85 \text{ m}^{-2}$ ) values calculated using the limited sampling strategy model in normal dihydropyrimidine dehydrogenase (DPD) and DPD deficient patients. The top line, bottom line and middle line through the box correspond to the 75<sup>th</sup>, 50<sup>th</sup>, and 25<sup>th</sup> percentiles, respectively. The grey circle represents an outlier.

ROC curves

The cut-off levels for  $V_{max,LSS}$  value,  $U/DHU_{t=120\text{min}}$  ratio and  $U_{t=120\text{min}}$  concentrations were estimated by ROC analysis (Figure 6.4). The data are displayed in Table 6.3. For the  $U/DHU_{t=120\text{min}}$  ratio, a cutoff level of 2.4 will result in a sensitivity of 80% and specificity of 98% with a

**Table 6.3** Sensitivity, specificity and cut-off levels for maximum enzymatic conversion capacity for the limited sampling strategy ( $V_{max,LSS}$ ), uracil/dihydrouracil ratio at  $t = 120 \text{ min}$  ( $U/DHU_{t=120\text{min}}$ ) and  $U_{t=120\text{min}}$  concentration derived from ROC curves

| Test parameter                                              | Cutt-off level | Sensitivity (%) | Specificity (%) | npv% | ppv% |
|-------------------------------------------------------------|----------------|-----------------|-----------------|------|------|
| $V_{max}$ ( $\text{mg hr}^{-1} \cdot 1.85 \text{ m}^{-2}$ ) | 667            | 80              | 78              | 99   | 16   |
| Uracil $DHU^{-1}_{120\text{min}}$ ratio                     | 2.4            | 80              | 98              | 99   | 67   |
| Uracil $_{120 \text{ min}}$ ( $\text{mg l}^{-1}$ )          | 6.4            | 80              | 96              | 99   | 51   |

ppv, positive predictive value; npv, negative predictive value.

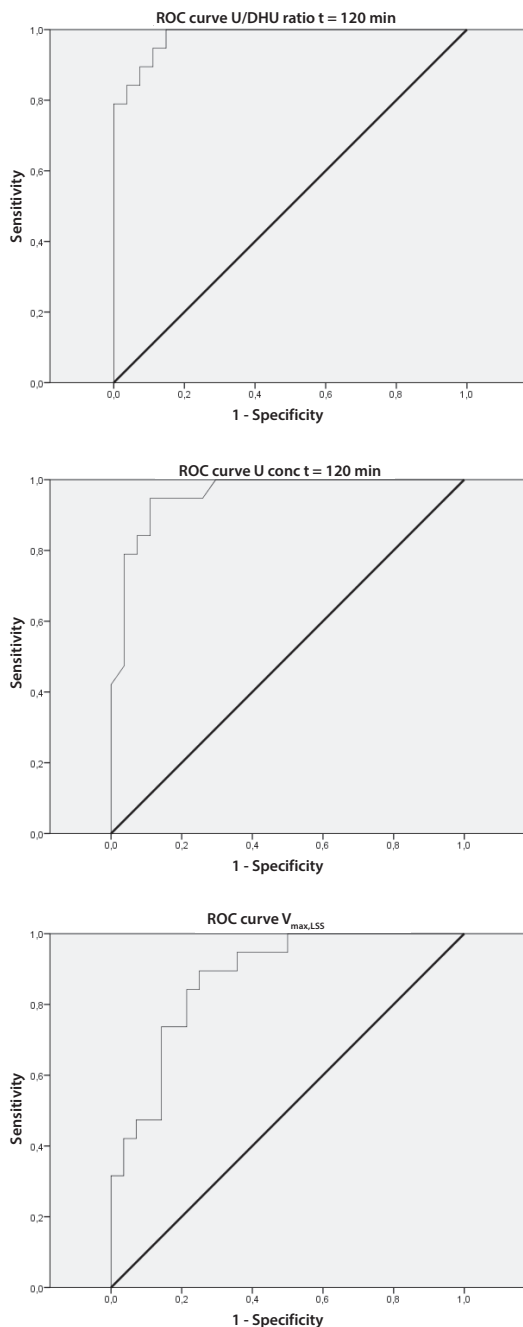


**Figure 6.4** Receiver operating characteristic (ROC) curves for the uracil/dihydrouracil (U/DHU) ratio at  $t = 120$  min, U concentration at  $t = 120$  min and maximum enzymatic conversion capacity for the limited sampling strategy ( $V_{\max,LSS}$ ).

The areas under the curves are 0.981; 0.957 and 0.874 for U/DHU at  $t = 120$  min, U concentration at  $t = 120$  min and  $V_{\max,LSS}$ , respectively. Reference line  $y = x$  is displayed bold in each graph

positive predictive value of 67% and a negative predictive value of 99% to discriminate between subjects with normal DPD activity and DPD deficient subjects. The cut-off levels of  $V_{\max, \text{LSS}}$  and  $U_{t=120\text{min}}$  showed lower specificity values when a sensitivity of 80% was chosen.

## DISCUSSION

In this study we developed a limited sampling model as a screening tool for DPD deficiency. Several parameters were evaluated with respect to sensitivity and specificity of DPD deficiency. Based on our experience on the field of 5-FU pharmacokinetic modeling [7] we investigated the potential value of  $V_{\max}$  to interpretate U pharmacokinetics. The PK models for a DPD deficient population and DPD normal population revealed significant differences in  $V_{\max}$  which is in the deficient population 42% lower compared to a normal population. Despite this significant difference, there is an overlap in  $V_{\max}$  because of the Gaussian distribution within the population. This result is in line with the results of pharmacokinetic analysis of 5-FU plasma levels performed by van Kuilenburg et al [7], and also with the fact that the mean DPD activity in patients heterozygous for a *DPYD* mutation is 48% of that observed in controls [7]. We validated the  $V_{\max, \text{LSS}}$  model by using LSS en FSS data employed in one population. This can be problematic because validation results and sensitivity/specificity values might be falsely influenced and elevated by this approach. Our validation involved only an explorative internal validation of the model in which each case is its own control. We realize that this should be followed by an external validation in which a different population should be used. There are two reasons that we did not perform this external validation. First, despite the fact that sensitivity and specificity results might be falsely influenced and elevated, the sensitivity and specificity are still lower compared to the U/DHU ratio. We consider it unlikely that external validation will result in higher sensitivity and specificity compared to U/DHU ratio. Secondly, to work with  $V_{\max}$  and PK modeling, highly sophisticated software and knowledge of PK are necessary and these are not present in every hospital. This latter issue means that  $V_{\max}$  is not a suitable candidate for use in a test performed in a prospective setting. The results of this study show that the  $U/DHU_{t=120\text{ min}}$  ratio is superior to  $V_{\max}$  as a discriminating parameter. A possible explanation for this is that the calculated  $V_{\max}$  value is based on U plasma data only. DHU plasma data are not included in the model. Compared to a previous developed 5FU iv PK model [7], the oral U PK model is more complex due to interindividual variability in bioavailability. Theoretically, the prediction of  $V_{\max}$  might improve when DHU data are included in a Michealis Menten PK model.

The arrangement of the two study groups was based on the DPD activity in PBMCs. This enzymatic assay is influenced by factors such as lymphocyte, granulocyte and monocyte composition in peripheral blood and protein concentration. In this study however, we took into account all the potential pitfalls of this assay as identified before [11]. The results of the analysis in the present study did not identify any outliers in the study population. The power of the DPD activity assay in this study was further demonstrated by the fact that in the deficient group, pathogenic *DPYD* variants were detected in all subjects.

The study population consisted of patients with a history of severe toxicity while treated with 5-FU or capecitabine in order to enrich for DPD deficiency. The results can therefore only be interpreted for this population. Theoretically it is highly possible that patients in the DPD normal group might have varying degrees of DPD deficiency. This makes it difficult to apply the results to the general cancer population that also consists of patients without toxicity. Further research is needed in order to determine if the results of this study also apply to the general population. In this study we defined patients with a DPD activity in PBMCs  $< 5 \text{ nmol mg}^{-1}\text{h}^{-1}$  as DPD deficient. This might lead to the discussion if a different value for DPD activity will result in different sensitivity and specificity. In order to investigate this, we performed a sensitivity analysis in which we calculated the sensitivity and specificity by ROC analysis based on a DPD activity cut off level of  $< 5.5$  and  $< 6.0 \text{ nmol mg}^{-1}\text{h}^{-1}$  which resulted in a specificity of 92.3% and 90.9% respectively when sensitivity is 80%. This shows that the cut-off chosen in the present study will have the smallest percentage of false positive test results.

The LSS strategy is an important improvement compared to the intensive sampling scheme that was used previously [9] and is more convenient for the patient. As the prevalence of a partial DPD deficiency in the general population is at least 3–5% [2], a prospective test in order to detect deficiency has to be applied to a large number of patients to find those few individuals with a low DPD activity. We decided to choose a cut-off level for our test whereby the number of individuals with true positive results favours the number of individuals with false positive results. In a population of 1000 persons and an incidence of 5%, a sensitivity of 80% and a specificity of 98%, there will be 40 individuals with a true positive test result and 19 with a false positive test result, leading to a positive predictive value of 67%. As positive test cases are at risk of developing severe toxicity when fully dosed, we recommend that a dose reduction of 50% should at least be considered in line with the recommendations of the Royal Dutch Association for the Advancement of Pharmacy for *DPYD* genotyping [18].

After an initial dose reduction and no toxicity, a dose escalation strategy could be followed in small steps based on clinical tolerability observed after each treatment cycle.

How do the results of this study compare with other strategies that are developed to detect DPD deficiency or prevent fluoropyrimidine related toxicity? There have been several genotyping studies in which sensitivity and specificity were established [19, 20]. It is difficult to compare our study results with these results, since these studies used different endpoints to the DPD activity that was used in our study. Only a head to head study would enable a fair comparison to be made between a genotyping and phenotyping strategy possible.

Compared to genotyping, an advantage of a phenotyping test strategies is that genetic variants outside the coding region of *DPYD* resulting in either a systemically altered DPD activity or altered 5-FU metabolism will be detected with these approaches. The incidence of these genetic variants however is very low as was proved by the fact that all subjects in the deficient group had variants inside the coding region of *DPYD*.

Among the included subjects, we identified one individual with a normal DPD activity in PBMCs of  $8.3 \text{ nmol mg}^{-1}\text{h}^{-1}$  who had highly elevated U and DHU levels comparable to the other DPD deficient subjects. Mutation analysis of *DPYD* in this individual revealed the presence of the pathogenic c.1905+1G>A variant. Heterozygosity for this mutation, in combination with a low but normal DPD activity has been previously described before by van Kuilenburg et al [21]. Another individual included in the present study had a very low DPD activity in PBMCs of  $1.0 \text{ nmol mg}^{-1}\text{h}^{-1}$  but the oral loading dose test results showed U and DHU considerably lower than the other DPD deficient subjects. Additional sequence analysis of *DPYD* in this case showed that there were no *DPYD* variants present. This observation was previously described by others investigators [8]. These two cases show that the results of the oral U loading test in these individuals correlated better with the results of genotyping than did the DPD activity in PBMCs which was used as the gold standard for inclusion in the present study.

It has been reported that homozygosity for mutant DPD alleles can lead to complete DPD deficiency resulting in elevated concentration of endogenous pyrimidine degradation metabolites [22] and neurologic disorders in children. In the present study, all patients were heterozygous for mutant DPD alleles and were only partial DPD deficient. Catabolism of the pyrimidine bases thymine and U consists of three consecutive steps [13]. The high concentrations of pyrimidine metabolites that accumulate in patients with a defect in

the enzyme responsible for pyrimidine degradation, compared with controls, could make identification of such patients feasible. DHU is not a metabolic end product but is further degraded into N-carbamyl- $\beta$ -alanine. In the present study, we found no statistical differences in the levels of any pyrimidine metabolites between the two study groups. Based on our results we see no beneficial effect of measuring these metabolites in heterozygous DPD deficient patients.

The U/DHU ratio at  $t = 2$  hr after U ingestion appears to be an easy-to-calculate biomarker for predicting the U to DHU conversion rate, and can be implemented in daily practice of most hospitals. The HPLC equipment needed for the U and DHU assay is present in most hospitals laboratories or pharmacies. Based on our experience, we estimate that the cost prize of the oral U loading dose currently stands at around 110 Euros. Additional improvement in the test principle and assay could decrease this price further. A thorough cost-effectiveness analysis should be performed to establish total cost when this test is used in a prospective setting in all patients with an indication for fluoropyrimidine containing therapy. Based on our study results we conclude that the U/DHU ratio at  $t = 120$  min following an oral U loading dose, is a suitable parameter for identifying patients at risk of developing severe toxicity as a result of DPD deficiency with a high sensitivity and specificity.

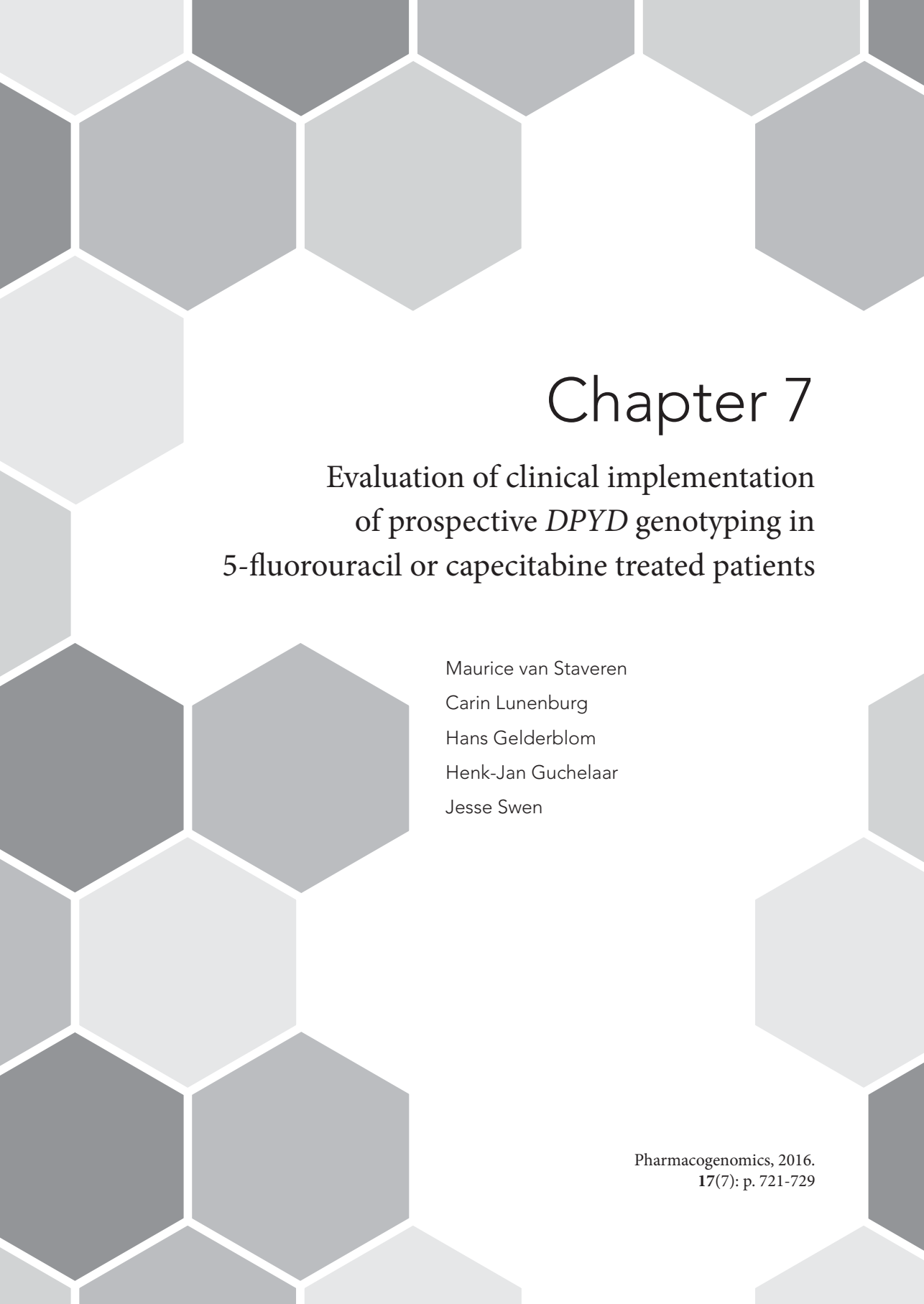
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# Chapter 7

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

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## ABSTRACT

**Introduction:** Prospective *DPYD* screening reduces severe fluoropyrimidine-induced toxicity. This study evaluated the routinely application of prospective *DPYD* screening at the Leiden University Medical Center.

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

**Results:** 86.9% of all patients with a first fluoropyrimidine prescription were screened. 14 out of 275 patients (5.1%) carried a *DPYD* variant and received a 25–50% dose reduction recommendation. None of the *DPYD* carriers treated with an initial dose reduction developed toxicities.

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

## INTRODUCTION

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

At Leiden University Medical Center (LUMC), a routine *DPYD* screening programme prior to prescribing 5FU or CAP was initiated in April 2013. In this retrospective study we evaluated

the physician's acceptance of prospective *DPYD* screening for patients who were prescribed 5FU or CAP in LUMC and the adherence of the recommended dose reduction.

## METHODS

### Setting

At LUMC all patients with an indication for a fluoropyrimidine containing therapy were routinely screened for *DPYD* variants by the laboratory of the department of Clinical Pharmacy and Toxicology (CPT) using two independent techniques (Taqman assay and pyrosequencing (PSQ), described previously) [17]. Within LUMC the Electronic Medication Record (EMR) system EZIS (version 5.2, Chipsoft) is used, which can be consulted by physicians, pharmacists and nurses. *DPYD* genotyping results are communicated electronically by the responsible pharmacist into the EMR and are visible for other users of the EMR.

The prospective screening programme was initiated on April 15<sup>th</sup> 2013. During a kick-off meeting attended by medical oncologists and fellows, the staff was informed and agreed on the prospective programme. New medical oncologists and fellows were informed about the prospective screening programme during the regular introduction programme for new staff members. Genotyping was performed 3 times per week (Monday, Wednesday, Friday) in order to minimize the lag time between sampling and test. This resulted in a turnaround time of 2 days, allowing rapid start of treatment if needed. Ethical approval by the Institutional Review Board of LUMC was not required for the current study as it evaluates standard care. Patient data from the EMR was handled following the Codes of Proper Use and Proper Conduct in the Self-Regulatory Codes of Conduct ([www.federa.org](http://www.federa.org)).

### Study endpoints

Three study endpoints were evaluated to determine the successfulness of the screening programme that was introduced at LUMC. We evaluated:

1. The 'implementation', i.e. requests of the *DPYD* tests as standard care in daily practice;
2. The proportion of test results with a dose recommendation provided by the pharmacist;

3. The follow up of the dose recommendations by oncologists, calculated as the number of follow-ups of dose recommendations by prescribers, excluding the patients in which a follow-up was not possible (e.g. no therapy).

## Study procedures

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

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

After completion of the study, an explorative analysis was executed in order to describe the course of toxicity in relation to the provided dose recommendations. In order to perform this analysis, toxicity information regarding the 5FU or CAP therapy was retrieved from the EMR for patients with a *DPYD* variant. Toxicity was scored by the oncologists using the Common Toxicity Criteria (CTC), version 4.

RESULTS

The implementation of the prospective screening programme for *DPYD*

The prospective *DPYD* screening programme was implemented on April 15<sup>th</sup> 2013 (start date study) at LUMC. From this date until December 31<sup>st</sup> 2014 (end date study) 540 patients were genotyped for *DPYD* variants at LUMC. Initially, patients were screened only for the presence of the *DPYD*\*2A variant. Later on *DPYD*\*13, c.2846A>T and c.1236G>A were added to the *DPYD* screening. An overview is shown in Table 7.1. After removal of duplicate or invalid records, 529 evaluable genotyped patients remained. Of these 529 patients, 275 patients were patients treated at the LUMC and 254 patients were treated at other hospitals, but genotyped as a service provided by the department of CPT of the LUMC. The dose reductions that were advised for each individual *DPYD* variant are displayed in Table 7.1.

Table 7.1 Recommended reductions of initial 5FU or CAP dose

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

Advice given by CPIC and DPWG guidelines at the time the variant was added to the routine screening.  
\* The dose reduction advice for c.2846A>T has been updated to 25% in February 2015.

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

Genotyped patients were compared with patients who were prescribed 5FU or CAP, resulting in 236 matching patients. 39 patients were genotyped for *DPYD*, but were not prescribed 5FU or CAP. Also, 101 patients were prescribed 5FU or CAP, but were not genotyped for *DPYD* variants (Figure 7.1).

Two patients, who received 5FU or CAP and were genotyped, were excluded because their medical records revealed they had received 5FU or CAP prior to April 15<sup>th</sup> 2013. Of

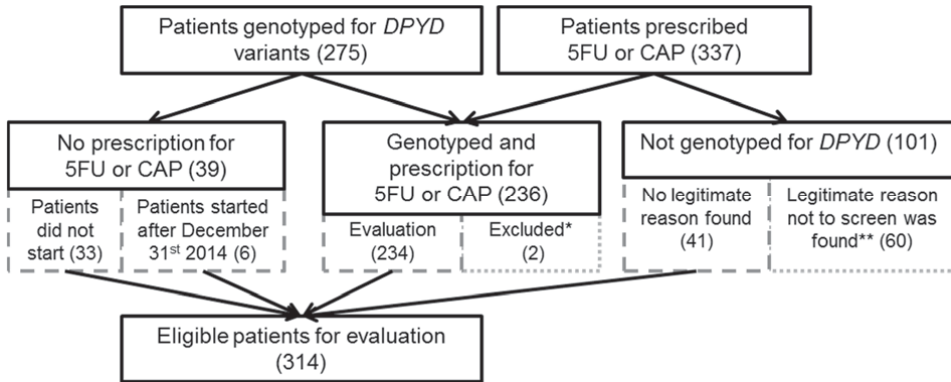


**Figure 7.1 Patient selection.**

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

\* These two patients were excluded because their medical records revealed they had received 5FU or CAP prior to April 15<sup>th</sup> 2013.

\*\* Legitimate reasons were: e.g. any notes on prior treatment with 5FU or CAP (e.g. outside LUMC) or invalid patient files (e.g. no medical dossier found for the oncology department).

the 39 patients who were genotyped without receiving 5FU or CAP therapy, 33 patients eventually did not start their therapy, although there was an intention to treat at the time of requesting the screening test. Six patients started their therapy after December 31<sup>st</sup> 2014 and were therefore not identified by the search. Of the 101 patients with a 5FU or CAP prescription and no *DPYD*-genotyping record, the medical records were screened resulting in a legitimate reason not to genotype in 60 cases (Table 7.2). Legitimate reasons included; any notes on prior treatment with 5FU or CAP (e.g. outside LUMC) or invalid patient files (e.g. no medical dossier found for the oncology department). For 41 patients who had a

**Table 7.2 Excluded patients**

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

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

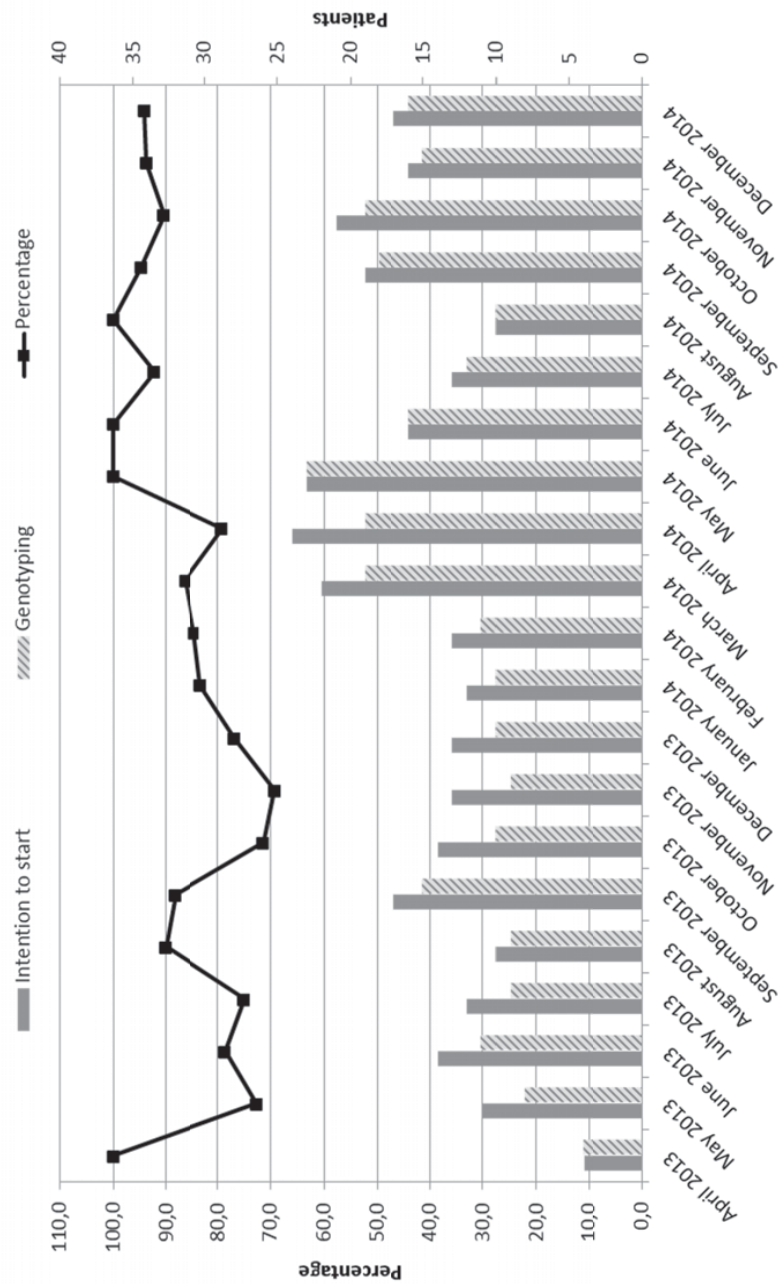


**Figure 7.2 Proportion of eligible patients that were genotyped.** The figure shows the eligible patients for evaluation per month in actual patient numbers. If the intention to treat with 5FU or CAP was present, patients were eligible. Also the actual patient numbers of the genotyped patients per month are shown and the calculated percentage which represents the clinical acceptance, or how well implemented the prospective DPYD screening is.

prescription for newly 5FU or CAP no reason was found to neglect genotyping. After data cleaning, 314 patients with a newly 5FU or CAP prescription remained in the dataset and 273 of these patients were genotyped as depicted in Figure 7.1. The clinical acceptance of the prospective *DPYD* screening programme is displayed as percentage per month in Figure 7.2. The average clinical acceptance was 86.9%.

The clinical acceptance of the prospective *DPYD* screening programme is displayed as percentage per month in Figure 7.2. The average clinical acceptance was 86.9%.

### Proportion of test results with a dose recommendation

During the study period 275 patients were screened for *DPYD* variants. Of these 275 patients, 14 patients (5.1%) were found to carry one or more variants. Shown in Table 7.3 are the variants that were screened for, and of each variant the frequency in comparison to the literature.

**Table 7.3** *DPYD* variants

| <i>DPYD</i> variant           | #                   | # of tested patients | % LUMC | % literature      |
|-------------------------------|---------------------|----------------------|--------|-------------------|
| <i>DPYD</i> *2A / c.1905+1G>A | 6                   | 275                  | 2.2    | ~1.0–1.8 [10, 18] |
| <i>DPYD</i> *13 / c.1679T>G   | 0                   | 214                  | 0      | ~0.1 [12]         |
| c.2846A>T                     | 1                   | 214                  | 0.5    | ~1.0–1.4 [10, 12] |
| c.1236G>A                     | 8                   | 109                  | 7.3    | ~2.6–4.9 [10, 19] |
| TOTAL                         | 15 ( <i>n</i> = 14) | 275                  | 5.1    | 4.7-8.2           |

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

For eight patients with a c.1236G>A variant a dose reduction of 25% was recommended. Five patients with a *DPYD*\*2A variant received a recommendation to reduce the dose by 50%. One patient carried both *DPYD*\*2A and c.2846A>T (Table 7.4, patient 9). For this patient no dose reduction was recommended. Instead it was advised to determine the DPD enzyme activity in PBMCs as applied Taqman and PSQ assays were not able to identify if the found mutations were in cis or trans configuration. Turnaround time of the DPD enzyme activity test is approximately 1–2 weeks, which could not be awaited for. The treating physician decided to treat this patient with a 50% dose reduction, taking into account the results of the genotyping and the fact that this patient had tolerated 5FU-containing regimens before.

Table 7.4 DPYD variant carrying patients

| Pt # | Cancer type  | Therapy   | DPYD variant | Prospective screening? | Initial dose adjustment?# | Toxicity (gr 3-4)? | Toxicity specifications                         | Hospital admissions? | Second dose adjustment?                | Toxicity (gr 3-4)? | Toxicity specifications   | Hospital admissions? |
|------|--------------|-----------|--------------|------------------------|---------------------------|--------------------|-------------------------------------------------|----------------------|----------------------------------------|--------------------|---------------------------|----------------------|
| 1    | Colorectal   | CAPOX     | c.1236G>A    | YES                    | YES                       | NO                 | N/A                                             | N/A                  | YES (to 100%)                          | NO                 | N/A                       | N/A                  |
| 2    | Mouth        | TPF + RT  | c.1236G>A    | NO <sup>11</sup>       | NO                        | YES                | Diarrhoea IV + Neutropenia/Thrombocytopenia III | YES (6 + 16 days)    | N/A (Quit after 2 <sup>nd</sup> cycle) | N/A                | N/A                       | N/A                  |
| 3    | Colon (met.) | OXACAPBEV | c.1236G>A    | YES                    | YES                       | NO                 | N/A                                             | N/A                  | NO                                     | N/A                | N/A                       | N/A                  |
| 4    | Anus         | 5FU + RT  | c.1236G>A    | YES                    | YES                       | NO                 | N/A                                             | N/A                  | NO                                     | N/A                | N/A                       | N/A                  |
| 5    | Colon        | N/A       | c.1236G>A    | YES                    | DNS <sup>13</sup>         | N/A                | N/A                                             | N/A                  | N/A                                    | N/A                | N/A                       | N/A                  |
| 6    | Pharynx      | 5FU + RT  | c.1236G>A    | YES                    | YES                       | NO                 | N/A                                             | N/A                  | NO                                     | N/A                | N/A                       | N/A                  |
| 7    | Pancreas     | CAP       | c.1236G>A    | YES                    | YES                       | NO                 | N/A                                             | N/A                  | N/A (Quit)                             | N/A                | N/A                       | N/A                  |
| 8    | Rectal       | CAP + RT  | DPYD*2A      | YES                    | YES                       | NO                 | N/A                                             | N/A                  | YES (to ± 80%)                         | YES                | Diarrhoea III + Enteritis | YES (31 days)        |

Table 7.4 Continued

| Pt # | Cancer type    | Therapy  | DPYD variant        | Prospective screening? | Initial dose adjustment? <sup>#</sup> | Toxicity (gr 3–4)? | Toxicity specifications               | Hospital admissions? | Second dose adjustment?          | Toxicity (gr 3–4)?        | Toxicity specifications | Hospital admissions?                       |
|------|----------------|----------|---------------------|------------------------|---------------------------------------|--------------------|---------------------------------------|----------------------|----------------------------------|---------------------------|-------------------------|--------------------------------------------|
| 9    | Mamma (met.)   | CAPOX    | DPYD*2A + c.2846A>T | YES                    | No dose recomm. <sup>*4</sup>         | NO                 | N/A                                   | N/A                  | N/A (Quit)                       | N/A                       | N/A                     | N/A                                        |
| 10   | Mamma (met.)   | CAPOX    | DPYD*2A             | YES                    | YES                                   | NO                 | N/A                                   | N/A                  | YES (to 100%)                    | YES (not in first cycles) | HFS II–III              | NO (switch to Paxclitaxel, after 8 cycles) |
| 11   | Rectal         | CAPOX    | DPYD*2A             | YES                    | YES                                   | NO                 | N/A                                   | N/A                  | YES (to 60%)                     | NO                        | N/A                     | N/A                                        |
| 12   | Gastric (met.) | EOX      | DPYD*2A             | NO <sup>*2</sup>       | NO                                    | YES                | Diarrhoea III                         | NO                   | YES (to 50%)                     | NO                        | N/A                     | N/A                                        |
| 13   | Rectal         | CAP + RT | DPYD*2A             | YES                    | NO                                    | YES                | Diarrhoea IV + Enteritis + Leukopenia | YES (18 days)        | N/A (Quit after TOX first cycle) | N/A                       | N/A                     | N/A                                        |
| 14   | Rectum         | N/A      | c.1236G>A           | YES                    | DNS <sup>*5</sup>                     | N/A                | N/A                                   | N/A                  | N/A                              | N/A                       | N/A                     | N/A                                        |

<sup>\*1</sup> Genotyping was performed on November 7th 2014, while therapy started on November 5th, 2014.

<sup>\*2</sup> Both genotyping and start of therapy where on January 24th, 2014. Therefore the result of the genotyping was not awaited.

<sup>\*3</sup> Patient did not start therapy on its own wish.

<sup>\*4</sup> For this patient no dose reduction advice was given because this patient was compound heterozygous (carrying two variants), and it was not possible to predict the remaining DPD enzyme activity with the current information. The advice given was to test the actual DPD enzyme activity with another method.

<sup>\*5</sup> Patient did not start therapy due to renal failure and presence of the DPYD variant.

<sup>#</sup> Initial dose adjustment is the dose adjustment made prior to the first dose of 5FU or CAP.

RT = radiotherapy, CAPOX = Capecitabine + Oxaliplatin, TPF = Docetaxel + Cisplatin + 5-fluorouracil, OXACAP8EV = Oxaliplatin + Capecitabine + Bevacizumab, 5FU = 5-fluorouracil, CAP = Capecitabine, EOX = Epirubicin + Oxaliplatin + Capecitabine, DNS = Did not start, dose recomm. = dose recommendation.

Fluoropyrimidine therapy was stopped in this patient after the first cycle due to toxicity ( $\leq$  grade 3).

**The follow up of the dose recommendations by oncologists**

Dose reduction was advised after the first administration of 5FU or CAP (post-dose) for 2 patients. The medical record of the first patient showed that the initial screening result became available after the start of therapy. Dose adjustments could not be applied, toxicity occurred and the advised dose reduction was applied in the second cycle (Table 7.4, patient 12). The other patient was screened after start of therapy, but stopped therapy completely due to toxicity, thus applying a dose reduction was not applicable. For this patient the reason not to screen prospectively was absent in the medical record (Table 7.4, patient 2).

For eleven patients a dose reduction was recommended prior to the start of therapy (prospective). This resulted in an initial dose reduction in 8 out of 11 patients. For one patient the recommend dose reduction was not applied and full dose was given (Table 7.4, patient 13). In two patients the recommended dose reduction could not be applied since they did not start therapy. One patient did not start therapy due to renal failure and the presence of a *DPYD* variant (Table 7.4, patient 14), and one patient refused to start therapy (Table 7.4, patient 5). Also one patient was genotyped prospectively, but received a recommendation for phenotyping due to compound heterozygosity (Table 7.4, patient 9). This patient started treatment with a 50% reduced dose at the oncologists discretion. An overview of the above mentioned data is displayed in Table 7.4. The adherence to the dose recommendations (pre- and post-dose) is 90% (9 out of 10).

**Analysis of results on clinical outcomes**

The explorative analysis showed that the prospective dose recommendations given, resulted in initial dose reductions in eight patients. None of these eight patients developed severe toxicity (grade  $\geq$  3) during the first cycle. After the first or second cycle it was possible to increase the dosages, guided by toxicity. Dosages were increased in four patients (from 50% up to 60, 80 and 100%, and from 75% to 100%, respectively, all receiving CAP). However, this led to the development of severe toxicity in two *DPYD*\*2A carrying patients (80% CAP led to diarrhoea grade 3 followed by 31 days of hospitalisation and 100% CAP led to hand-foot syndrome grade 3). Toxicity data can be found in Table 7.4.

In one patient with a *DPYD*\*2A variant who received CAP in combination with radiotherapy, the dose recommendation was not followed by the physician and this patient experienced diarrhoea (grade 4), enteritis and leukopenia, for which hospitalisation of 18 days was required and CAP therapy was permanently terminated (Table 7.4, patient 13).

## DISCUSSION

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

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

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

We believe patients do not need to be genotyped if previous 5FU or CAP usage without toxicity is known or if patients were genotyped (*DPYD*) or phenotyped (DPD) previously. However, within the 41 (13.1%) remaining patients legitimate reasons can still exist (e.g. well-tolerated treatment before 2013 with 5FU or CAP), but might not have been filed in the medical record. Therefore we can conclude the 90–100% ( $\leq 1$  patient not tested per

month) rate was an effective prospective *DPYD* screening implementation. Disputable is, if this clinical acceptance can become 100% continuously. In order to support the clinical implementation, the use of a clinical decision support (CDS) system might be suitable. In LUMC a CDS entitled adverse drug event alerting system (ADEAS) is used in daily practice in the hospital pharmacy of LUMC [20]. This system is used by hospital pharmacists to systematically select patients at risk of possible adverse drug events. It retrieves data from several information systems, and uses clinical rules to select the patient at risk of adverse drug events.

As mentioned before, sensitivity of genotyping is relatively low (< 14.5% for *DPYD*\*2A and c.2846A>T combined) [11]. Even if all patients with a *DPYD* variant are identified and treated with an appropriately reduced dose, not all fluoropyrimidine-related toxicity can be prevented. Adding a DPD phenotyping test may increase sensitivity, but is expensive and logistically challenging to implement in clinical practice [13]. SNPs located in other genes than *DPYD* (e.g. TYMS) have been associated with fluoropyrimidine-induced toxicity with conflicting results. However, testing for these SNPs holds the potential to increase sensitivity [21]. Even though *DPYD* screening cannot prevent all fluoropyrimidine-related toxicity, we feel that the available evidence strongly supports implementation in clinical practice and can prevent fluoropyrimidine-induced deaths [8, 11, 22].

The presence of one of the four *DPYD* variants that were pre-emptively tested resulted in a recommendation to the oncologist to reduce the initial dose of 5FU or CAP by 25–50% depending on the identified variant. In February 2015 the recommended dose reduction for c.2846A>T was changed from 50% to 25%, following the updated guidelines of the DPWG [23, 24].

One patient (Table 7.4, patient 13) received full CAP dose, since the treating oncologist argued that she was afraid of under dosing the patient as the dosage of CAP in chemoradiation schemes is already lower compared to other treatments and there is less opportunity to increase the dose in subsequent treatment cycles. The patient developed severe toxicity illustrating that the recommended dose reductions should also be applied to lower CAP doses used in chemoradiation, despite lack of published data about CAP toxicity during chemoradiation therapy.

In conclusion, this study for the first time shows that systematic prospective *DPYD* screening can be implemented successfully in real world daily clinical practice. The applied 25–50%

dose reduction for patients with a *DPYD* variant resulted in absence of toxicity. However, a more active follow-up of adherence to provided dose recommendations might improve patient safety even further.

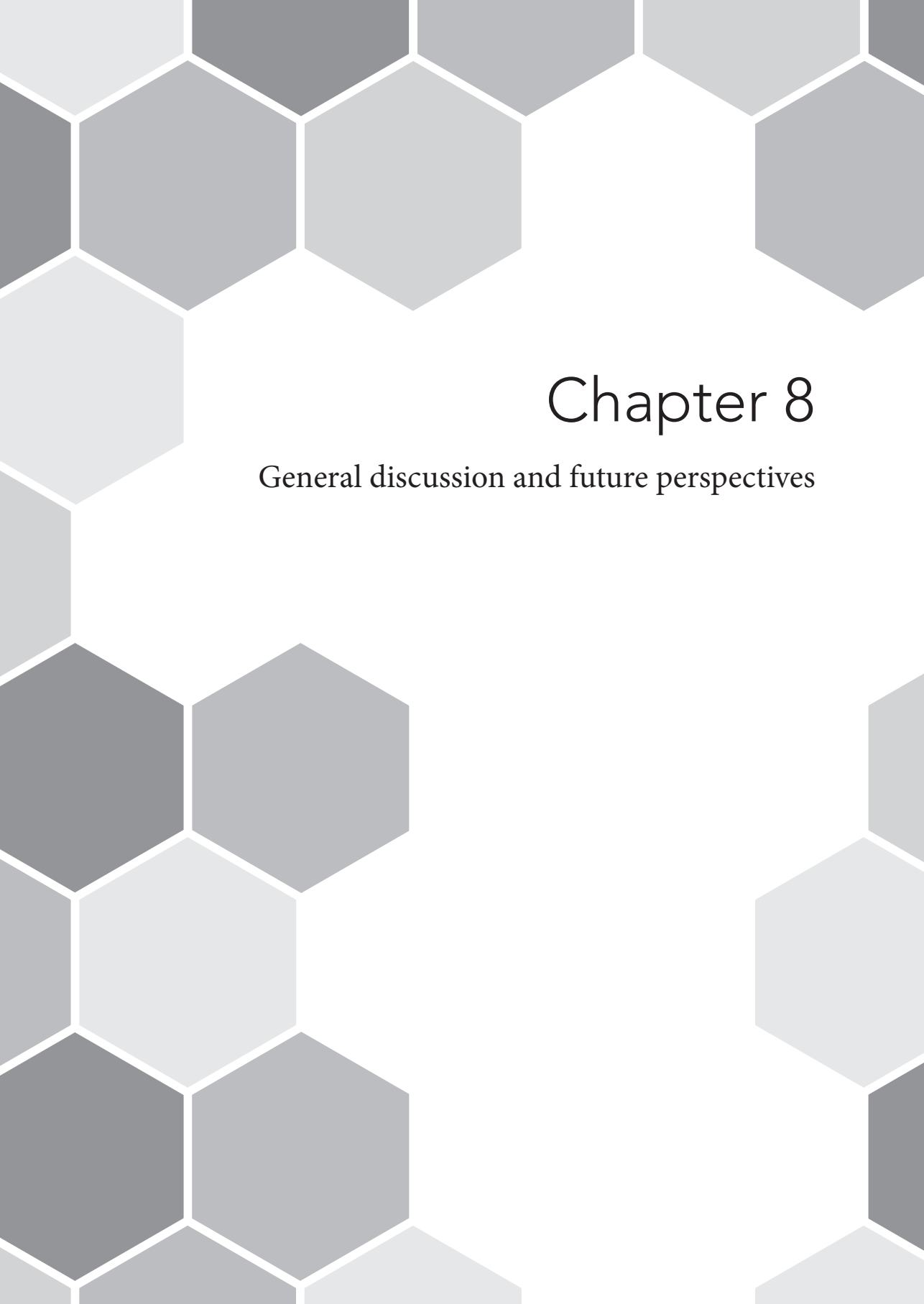
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# Chapter 8

General discussion and future perspectives

## Introduction

Since its approval in the 1960s, the fluoropyrimidine 5 fluorouracil (5-FU) has been extensively used, either as single agent or in combination with other drugs or radiotherapy in the treatment of many types of cancer such as breast, anal, vulvar, head and neck, and gastrointestinal cancer. In the last decades, 5-FU is increasingly used in an oral formulation, as the pro-drug capecitabine. The use of fluoropyrimidines often results, like other cytotoxic chemotherapeutic drugs, in undesired toxicity. The incorporation of the 5-FU metabolite fluorouridine triphosphate (FUTP) into RNA and inhibition of thymidylate synthase by FdUMP, underlying the antineoplastic effect in tumour cells, also interfere with the metabolism of normal, rapidly proliferating cells like the gastrointestinal mucosa, bone marrow, hair follicles and nail beds leading to leukopenia, alopecia, mucositis and stomatitis [1]. The first sign of 5-FU systemic toxicity is often stomatitis, varying from mild erythema to haemorrhagic ulceration of the oral cavity. Frequently, stomatitis is accompanied by diarrhoea. The effect of 5-FU on the brain stem can cause nausea and vomiting during administration and its effect on the skin can cause hyperpigmentation and hand-foot syndrome or when used in combination with radiotherapy skin inflammation and ulceration.

Besides these expected therapy related toxic effects, in a small proportion of fluoropyrimidine treated patients, extreme toxicity is observed. It has become clear that a large part of this severe toxicity is caused due to a partial or complete dihydropyrimidine dehydrogenase (DPD) deficiency and hence, a strongly reduced capacity to degrade 5-FU into metabolites [2-6]. DPD deficiency, however, can not explain the toxicity in all patients. There may be several reasons for this. Fluoropyrimidines are often used in combination treatments with other cytotoxic drugs or radiotherapy, and a contributory effect of non-fluoropyrimidines in the toxicity observed. Alternatively, polymorphisms involving genes other than *DPYD* (gene encoding for DPD) that play a role in fluoropyrimidine metabolism may cause non-DPD related toxicity. For example variants in the *TYMS* gene will lead to reduction of the enzyme thymidylate synthase. In breast cancer patients homozygous for the *TYMS* 3RG allele, have a significantly higher incidence of toxicity and a lower response was observed when compared to heterozygous patients, and patients in which the *TYMS* 3RG allele was not present [7]. In addition, the presence of the *TYMS* 2R/2R variant was also associated with higher toxicity [8]. Variants in the dihydropyrimidinase coding gene, *DPYS*, were likely to cause structural destabilization and protein misfolding [9] and patients with a partial deficiency of dihydropyrimidinase are at risk to develop severe 5-FU related toxicity

[10]. For the  $\beta$ -ureidopropionase enzyme which catalyzes the last step in the 5-FU and uracil-degradation pathway, it is suggested that variants of the *UPB1* gene coding for  $\beta$ -ureidopropionase are likely to be associated with increased toxicity although the role of these variants are less significant than alterations in *DPYD* [11-13].

Besides its role in 5-FU pharmacokinetics and toxicity, the level of DPD present in tumour cells is likely to be correlated to 5-FU resistance and treatment response. Indeed, prospective studies in colorectal cancer patients showed that overexpression of thymidylate synthase and DPD in certain types of tumours may explain the resistance to 5-FU therapy [14-16]. A similar correlation was found for bladder cancer [17], and for lung cancer where a high DPD expression in NSCLC tumour cells is correlated with EGFR mutations [18].

The examples given in the previous paragraphs show that the individual's genotype greatly influences the behaviour of a drug. Pharmacogenetics, the heritability of drug response, may help to explain some of the variability in drug response between individuals [19]. However, besides the variation in drug response caused by the genotype of an individual, non-genetic factors such as age, organ function, food, smoking status, concomitant therapy, drug interactions and nature of the disease may also influence the drug's effect in an individual [20].

DPD is encoded by the gene *DPYD* for which 567 coding genetic variants are currently known [21], some of them being pathogenic since they reduce enzyme function or stability [22, 23]. We hypothesized that variability in DPD activity and its effect on DPD related toxicity could be best examined by a phenotyping approach, since current *DPYD* genetic testing only explains part of the fluoropyrimidine-related toxicity. Tests based on phenotyping approaches are already known and used in pharmacokinetics research, such as probes for phase I and phase II metabolic enzymes and drug transporters [24]. In this thesis, uracil being used as an oral loading dose is studied as a probe for DPD deficiency in cancer patients treated with fluoropyrimidines and our aim was to develop a test procedure that is suitable to be incorporated broadly into daily practice in hospital care.

## Status of DPD testing

Several tests have been developed aiming to predict or explain DPD related fluoropyrimidine toxicity [25] (**Chapter 2**). These tests include both genotyping and phenotyping-based assays. To date, in daily practice, DPD testing is often used as counselling for family members of

DPD deficient subjects or in a retrospective setting to provide an explanation for severe fluoropyrimidine related toxicity. In our view this is not useful, as the toxicity then already has taken place and the aim is to avoid toxicity. The prospective screening of DPD deficiency is only used sparsely internationally, but was recently successfully implemented in a number of hospitals in the Netherlands (**Chapter 7**). The severity of DPD related toxicity is illustrated by prolonged hospitalization and by its rare, but potential lethal outcome [26, 27]. This observation raises the question why prospective DPD testing is not yet adopted as standard care in the field of oncology.

### **The successfulness of a diagnostic test or screening method**

There are numerous guidelines and criteria for appraising diagnostic test studies and diseases [28-30]. It is interesting to investigate to what extent these criteria for diagnostic tests are met for routinely DPD screening in patients with an indication for fluoropyrimidine containing therapy. One of the criteria for a diagnostic test state that there should be a suitable test or examination to detect DPD related toxicity. The term *suitable* can be interpreted in multiple ways, and makes it unclear to what extent a test has to be validated or studied to be considered as suitable. In recent history, there are several well examples of diagnostic tests in the field of oncology that have been accepted by oncologists and were implemented broadly within a short period. Interestingly, for these tests, no prospective clinical trial was performed. This is illustrated by the discovery of Kirsten rat sarcoma viral oncogene (KRAS) mutations and its role in the treatment of colorectal cancer with cetuximab and panitumumab. It was first discovered that cetuximab had little or no effect in colorectal tumours harbouring KRAS mutation [31, 32]. Patient selection by analysis of KRAS mutations has been a fundamental event to increase efficiency and reduce cost after multiple retrospective studies all showed the same results [33]. Recently this was extended to RAS wild type tumours. Apparently a strong biological rationale and effect (shown by multiple retrospective studies all showing the same direction of effect) doesn't need a prospective randomised study to change clinical practice.

It is remarkable that KRAS testing was successfully implemented while DPD testing is still under discussion. With KRAS testing, there is a very clear correlation between the test result and the treatment outcome. This is not fully the case with prospective DPD testing. It is obvious that DPD testing can prevent 5-FU related toxicity, however, not all 5-FU toxicity can be explained by reduced DPD activity. This is illustrated by the fact that approximately 30–50% of all patients suffering from 5-FU related toxicity have no decreased DPD enzyme

activity and [34, 35] and it is thus important to realize that prospective testing for DPD deficiency will not exclude all 5-FU related toxicity.

An important criterion for a diagnostic test is that the cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole. Prospective screening for DPD deficiency can reduce treatment costs by preventing toxicity initiated by low DPD activity. Severe Common Toxicity Criteria (CTC) grade III or IV toxicity might lead to hospitalization of patients for several days at high care units or even death (grade V). Ideally to be cost effective, the costs of prospective testing must be in favour to the costs of treatment of toxicity due to DPD deficiency. The prevalence of partial DPD deficiency in Caucasian population is approximately at least 3% [36–38]. As a result, many patients with an indication for fluoropyrimidine-containing therapy need to be screened prospectively in order to diagnose the minority with DPD deficiency. Nevertheless, it was described that the average total treatment cost per patient was lower with a prospective DPD screening strategy compared to nonscreening [39].

Importantly, the test result of a diagnostic test should lead to an actionable, clinical recommendation, or in the case of DPD deficiency, to a fluoropyrimidine dose adjustment or advice for alternative therapy. The test result of DPD deficiency measured in PBMCs can present as a complete or partial malfunction of the enzyme and there is a linear correlation between DPD activity and 5-FU clearance [40]. For the genotyping approach in which *DPYD* variants are determined, there are clear dosing advices that are related to the presence of *DPYD* variants, though there is data available that the toxicity risk of certain *DPYD* variants is also influenced by factors such as gender, mode of administration and co-treatment with folinic acid [41]. **Chapter 7** shows, that it is possible to implement prospective *DPYD* screening effectively in a hospital setting, with the result that 90–100% of all patients with an indication for a fluoropyrimidine containing therapy are being screened.

## Evaluation of the oral uracil loading dose

Uracil is not registered for human use, but the quality of commercially available uracil is very high without impurities as is described in **Chapter 3** and can be used safely in patients. The safety of oral uracil is further demonstrated by the fact that there were no side effects of any form observed in all subjects following the administration of the uracil loading dose. Uracil in plasma can be determined by High Pressure Liquid Chromatography (HPLC) of

with HPLC-mass spectrometry. This equipment is present in clinical or pharmaceutical laboratories in hospitals. The uracil dose that was used in this thesis is 500 mg/m<sup>2</sup>. There is a disadvantage of a uracil dose that is based on the body surface area (BSA) of patients since this requires individual doses of uracil have to be on stock or prepared by the pharmacy. In **Chapter 4 and 5** an intensive blood-sampling scheme was used in order to establish comprehensive plasma-concentration curves of uracil following oral intake. Because a full sampling scheme and the associated test length of 4 hours can be considered as patient unfriendly, it was replaced by a limited sampling schedule in which blood is taken after 60 and 120 minutes following uracil ingestion. Is this feasible in daily clinical practice and patient-friendly enough? There are other diagnostic tests based on the same blood sampling principle that are accepted and implemented broadly, and used successfully. As an example, the oral glucose tolerance test is used to diagnose diabetes and gestational diabetes mellitus defined as glucose intolerance identified during pregnancy [42, 43]. Diabetes may be diagnosed based on HbA1C criteria or plasma glucose criteria, either the fasting plasma glucose or the 2-hour plasma glucose value after a 75-g oral glucose tolerance test. The use of this glucose test shows that it is indeed possible that the oral uracil loading in its present form is suitable to be incorporated into daily practice in hospitals.

### Patient selection and uracil pharmacokinetics

In this thesis, uracil was orally administrated to healthy volunteers and cancer patients with, and without DPD deficiency. All study subjects who were tested, were Caucasian which is interesting, since differences are reported in DPD activity among different populations. The prevalence of DPD deficiency is 3–5% in a European/Caucasian population, but is estimated at approximately 8% for the African-American population [37, 44]. This difference in prevalence will not influence the test result of the oral uracil loading dose. Genetic variations in *DPYD* might be unique depending on race and were investigated for different ethnical groups in which several *DPYD* variants showed different distributions [45–48]. In addition, differences in pyrimidine catabolism have been reported between men and women. In general women suffer more from side effects during fluoropyrimidine-containing therapy [49–52]. A possible explanation may be the lower clearance of 5-FU in women compared to men [53]. Moreover, differences have been observed in endogenous uracil and dihydrouracil levels between men and women, but the mean uracil/dihydrouracil ratio was comparable [54]. For the oral uracil loading dose, it was not investigated if uracil pharmacokinetics and the uracil/dihydrouracil ratio differed between men and women.

In **Chapter 5** we showed that the presence of metastatic disease in colorectal cancer patients has no effect on uracil pharmacokinetics. This is in line with the observation that extensive hepatic replacement due to liver metastases had no effect on 5-FU pharmacokinetics indicating that the amount of DPD is probably not influenced by moderate reduction in liver function [55]. The gastrointestinal absorption of uracil is a pharmacokinetic first order process and the elimination follows a saturable Michaelis-Menten kinetics [56]. Of note, all patients that were included in the studies described in this thesis had an intact gastrointestinal (GI) tract. Surgical alteration of the structure of the GI tract such as gastric or bowel resection, may alter its function which can have impact on drug absorption [57]. For this reason, the oral uracil loading dose is not suitable for patients with such GI alterations and alternative testing has to be performed.

In this thesis, the oral uracil loading dose was not used in a prospective setting. All patients already received one or multiple dose of 5-FU or capecitabine before they received the oral uracil test dose. They were included based on the DPD activity that was assessed by measuring the DPD activity in peripheral blood mononuclear cells (PBMCs) [58] and in **Chapter 6** severe toxicity occurred before DPD activity was measured. Despite the fact the uracil loading was not investigated in a true prospective setting, the test was applied to a variable group of patients with and without toxicity, variable DPD activity and with and without metastatic disease.

## Future perspectives

The results in this thesis indicate that the oral uracil loading dose is suitable as a phenotyping probe for DPD deficiency and can be incorporated into daily practice in hospital care. As mentioned before the equipment for analysing the plasma samples is already present in most hospitals, so that the turnaround-time of the test can be short with 1 or 2 days. The local pharmacy can order or prepare the uracil that is needed for the test as long as there is no commercially uracil solution available. Most hospitals in the Netherlands have special wards that are used for short stay of patients or the clinical laboratories can handle the patient logistic that is needed to perform the test.

Nevertheless, the test still might be optimised to simplify the test procedure in order to further increase acceptance and applicability. Since the uracil/dihydrouracil ratio after uracil ingestion is determined by DPD enzyme activity and not so much by the dose administered, a fixed dose uracil will not influence the test result and is for practical reasons preferred

over the BSA determined dose of a subject that was used in this thesis. Subsequently, a dried blood spot sampling might be candidate to replace the venous sampling method that is currently used.

Pharmacokinetics can be used as a tool to optimise 5-FU therapy. With PK guided 5-FU dosing, the start dose of 5-FU is based on BSA and titrated during following administration based on 5-FU plasma concentration and AUC [59, 60]. Besides its role in diagnosing DPD deficiency, the oral uracil loading dose might be useful to establish a more specific 5-FU starting dose when therapy is started close to the desired plasma level and Area Under the Curve if a clear relation exists between uracil and 5-FU PK.

Although not studied in this thesis, it may be interesting to investigate if the oral uracil loading dose can play a role in individual pharmacokinetically (PK) guided 5-FU dosing. This 5-FU dosing approach leads to higher efficacy and tolerability compared to 5-FU dosing based on BSA. It has already been suggested that the use of 5-FU Michaelis-Menten pharmacokinetic models might be suitable to predict a-priori 5-FU plasma concentrations [61].

The genotyping strategy that was evaluated in **Chapter 7** can be further improved. There are more *DPYD* variants than the four tested that alter DPD enzyme activity, and novel variants are still discovered. The presence of one of these non-tested variants potentially will lead to a false negative test result with the risk to develop toxicity. Since the costs for genotyping continues to decrease [62, 63], the number of *DPYD* variants tested could be expanded to improve sensitivity. The variants that are tested in the current test strategies are mostly coding variants, but there have been pathogenic variants described in the noncoding *DPYD* gene regions that are not routinely tested [64]. This can be resolved by sequencing the entire *DPYD* gene, but although more informative, this is far more expensive than the strategy that was used in Chapter 7.

This thesis does not answer the question which DPD test strategy is the most efficient one to prevent and predict DPD related toxicity. This requires the multiple test strategies being compared to each other in a prospective, head to head study in which the outcome should be the prevention of fluoropyrimidine related toxicity. At this moment a large prospective, multicentre study is performed in which different strategies to prevent DPD related toxicity are evaluated (EudraCT registration number: 2014-005064-15).

Despite the fact that the cost effectiveness of DPD screening should be investigated more thoroughly and not all fluoropyrimidine related toxicity can be prevented, pre-treatment

DPD testing, irrespective of what specific test is used, should be standard care and incorporated in oncology guidelines. We advocate that all patients who are first time treated with a fluoropyrimidine containing therapy should be screened for DPD deficiency.

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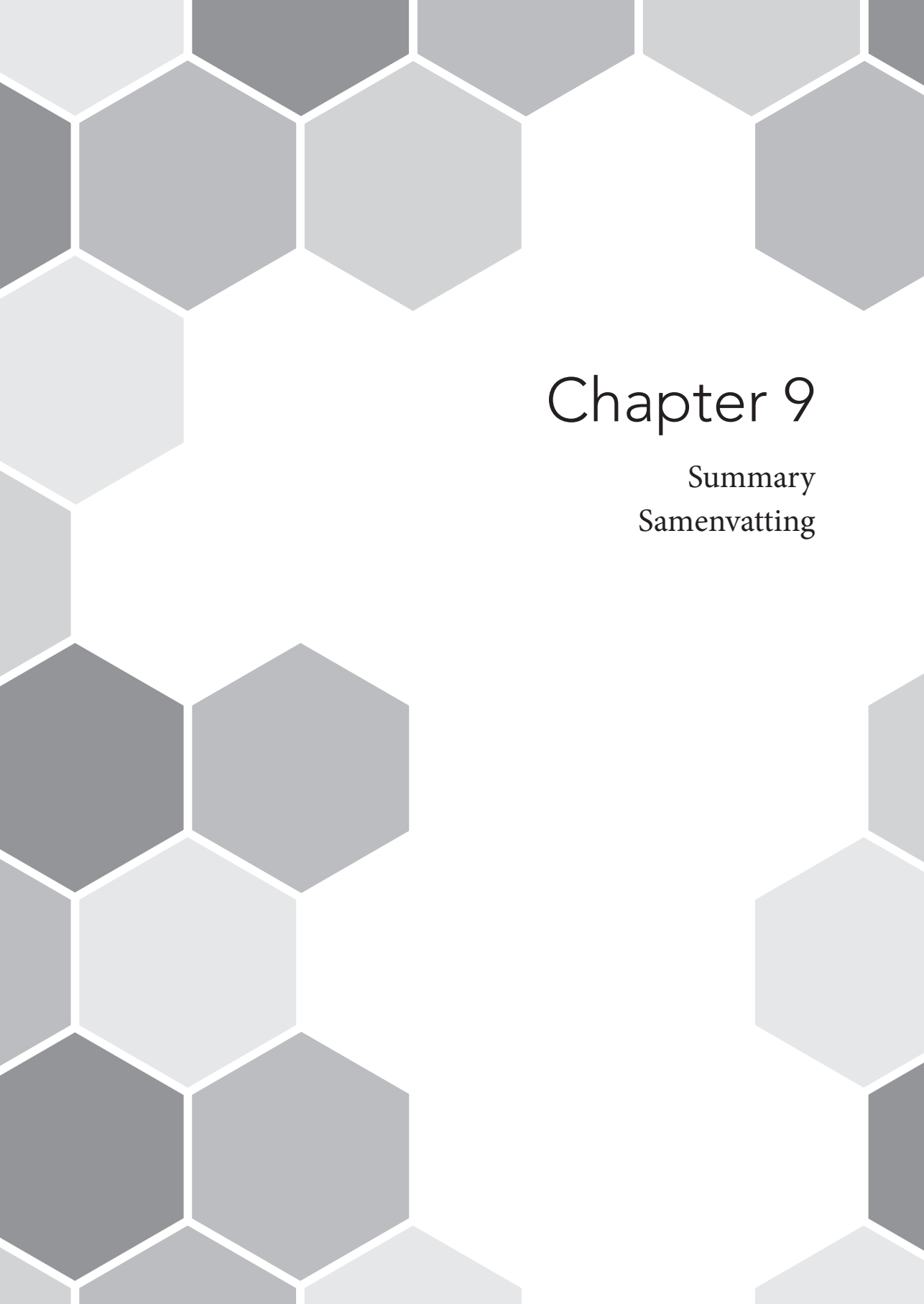
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# Chapter 9

Summary  
Samenvatting

## 9.1 SUMMARY

Fluoropyrimidines have been used in the treatment of different types of cancer for many decades. The most common fluoropyrimidine is 5-fluorouracil (5-FU), which is administered intravenously as a bolus or as prolonged infusion. 5-FU itself is not available in an oral presentation form. It is, however, converted by thymidine phosphorylase in the metabolic pathway of the oral 5-FU prodrug capecitabine. Many tissues throughout the body express thymidine phosphorylase. Some human carcinomas express this enzyme in higher concentrations than surrounding normal tissues. Dihydropyrimidine Dehydrogenase (DPD) is involved in the degradation of endogenous pyrimidine bases uracil and thymine, but also in the degradation of 5-FU. More than 80% of the amount of 5-FU administered is catabolized primarily in the liver where DPD is abundantly expressed. DPD is encoded by the *DPYD* gene for which 567 coding variants are known to date, some of them being pathogenic by reducing enzyme capacity. Interindividual variability in the activity of DPD influences 5-FU pharmacokinetics and a reduced DPD activity can lead to severe toxicity and even death following administration of 5-FU or capecitabine. Knowledge regarding the clinical impact of reduced DPD activity on the pharmacokinetics and pharmacodynamics of fluoropyrimidines may be useful to dose-individualized therapy of 5-FU and capecitabine. In this thesis, an in depth overview is provided of methods and their potential to optimize fluoropyrimidine dosing based on individual DPD enzyme activity. Furthermore an oral uracil loading dose as probe for DPD deficiency in cancer patients treated with fluoropyrimidines for this purpose is studied.

In **Chapter 2** an overview of the literature is presented on predictive tests that have been described until 2016 for screening for dihydropyrimidine dehydrogenase deficiency. Tests based on assessing DPD enzyme activity, genetic variants in *DPYD* and mRNA variants have been studied for screening for DPD deficiency, but only few are sparsely implemented into clinical practice in a prospective setting, or are only used in a retrospective setting. A problem with many tests is that they require expensive equipment that is not available in every hospital or that analytical procedures are laborious, and for that reason not applicable to screen large numbers of patients. Beside these practical drawbacks, there is a no consensus about the definition of DPD deficiency and definition of selectivity and specificity of the tests. If determined, they are not related to the same endpoint, which makes it even harder to compare the efficacy of the various tests. Future studies should focus more on cost-effectiveness and sensitivity and specificity in order to determine which test or strategy

is most suitable to predict patients at risk of developing severe fluoropyrimidine related toxicity caused by DPD deficiency.

Like 5-FU, uracil is metabolised by DPD. The measurement of plasma uracil levels following administration of an oral uracil loading might be indicative for the DPD activity of an individual patient and might thus be able to prevent severe fluoropyrimidine related toxicity. In **Chapter 3** the Investigational Medicinal Product Dossier (IMPD) for uracil is presented. The IMPD is the basis for approval of clinical trials by the competent authorities in the EU. It was used for the medical ethical approval of the studies that were performed with uracil powder since uracil is not registered for human use. All paragraphs of the IMPD were written for uracil and all tests considering impurities, assays and quality were performed by an analytical monograph with the use of liquid chromatography with UV photo diode array detection. Acceptance criteria were derived from the European Pharmacopeia General Monograph. The manufacturing of the uracil study medication was performed under Good Manufacturing Practice and Good Clinical Practice conditions.

The pharmacokinetics of uracil should only depend on DPD activity and should not be influenced by disease status or differ between healthy volunteers and cancer patients. In order to exclude this last aspect, in Chapter 4 the results of a pharmacokinetic study are described that investigates the differences in uracil pharmacokinetics in healthy volunteers and DPD deficient patients following administration of an oral uracil loading dose in two doses.

The use of higher uracil doses is expected to result in a more adequate discrimination between normal and deficient individuals due to a prolonged DPD enzyme saturation in DPD-deficient subjects compared with lower doses of uracil. Secondary objective of the study performed in **Chapter 4** was to investigate linearity of uracil pharmacokinetics at increased uracil dose and the intra- and interday variation in uracil pharmacokinetics. The conclusion of this study was that uracil administration at a single dose of 500 mg/m<sup>2</sup> Body Surface Area (BSA) leads to significant and reproducible differences in pharmacokinetics of uracil and dihydrouracil between volunteers with a normal DPD activity and DPD-deficient patients. Uracil doses above 500 mg/m<sup>2</sup> have no higher discriminating potential to distinguish between subjects with and without DPD deficiency. Uracil dose elevation will only result in a shift to the right of the uracil and dihydrouracil concentration curve and unnecessary longer exposition to high uracil levels.

Uracil metabolism by DPD takes mainly place in the liver. Hence, the presence of liver metastasis might influence uracil pharmacokinetics following administration of oral uracil. In **Chapter 5** the uracil dose of 500 mg/m<sup>2</sup> BSA was further studied and compared in adjuvantly treated colon cancer patients, and colorectal (CRC) patients with metastatic disease. The results of this study showed that in patients with a normal DPD activity, with the exception of  $C_{\max}$ , there is no difference in uracil pharmacokinetics in patients with metastatic CRC compared to colon cancer patients that are treated adjuvantly.

The pharmacokinetic profile of uracil in the former two studies were established with the use of an intensive blood sampling scheme that is not ideal for use in a clinical setting. Therefore in **Chapter 6** the oral uracil loading dose was further evaluated (i) to develop a limited sampling strategy, (ii) to detect decreased uracil elimination in patients with a DPD deficiency and (iii) to perform a more in-depth quantitative compartmental pharmacokinetic analysis of uracil plasma concentrations. In this study, several pharmacokinetic parameters were determined and compared to each other, leading to the conclusion that the uracil/dihydrouracil ratio at  $t = 120$  min following the oral uracil loading dose is the most suitable parameter for identifying patients at risk of developing severe DPD related toxicity with a high sensitivity and specificity.

The Investigator Brochure of capecitabine states that its use is contraindicated in patients with a known DPD deficiency, that is defined as rare, unexpected and severe toxicity. For 5-FU, the investigator brochure indicates that when applicable, DPD enzyme activity should be measured prior 5-FU treatment. Until now, prospective DPD screening is not yet considered standard care in oncology practice and are not advocated in ESMO or Dutch guidelines. This may be a result of the fact that there is no consensus internationally about the optimal strategy to identify patient at risk of developing DPD based toxicity. Despite this, there are however well organised initiatives involving a prospective DPD test setting. **Chapter 7** describes a study that evaluates a routine *DPYD* screening programme in a Dutch academic center prior to prescription of 5-FU or capecitabine. In this study, the physician's acceptance of prospective *DPYD* screening for patients who were prescribed 5-FU or capecitabine and the adherence of the recommended dose reduction were evaluated. The number of patients in which screening was performed was relatively high with 86.9% of all eligible patients and follow-up of dose recommendations given by the pharmacist was possible in most cases. This study showed that implementation of prospective *DPYD* screening is feasible and can be implemented successfully in clinical practice.

In **Chapter 8** the results of the studies performed in this thesis are discussed in a broader setting and future perspectives are mentioned regarding improving the oral uracil loading dose and prospective DPD screening in general. Despite further improvements of the test procedure, the oral uracil loading dose in its present form has potential as a phenotyping probe for DPD deficiency in a prospective setting.

## 9.2 SAMENVATTING

Fluoropyrimidines worden al decennia lang gebruikt ter behandeling van verschillende soorten kanker. De meest bekende fluoropyrimidine is 5-fluorouracil (5-FU) dat intraveneus als bolus of langlopend infuus kan worden toegediend. 5-FU als stof zelf is niet beschikbaar in een orale toedieningsvorm, maar wordt gevormd door thymidine fosforylase uit de metabolieten van de orale 5-FU prodrug capecitabine. Veel weefselstructuren in het lichaam bevatten thymidine fosforylase. Sommige humane carcinomen bevatten dit enzym echter in hogere concentratie dan omliggend weefsel. Dihydropyrimidine dehydrogenase (DPD) is betrokken bij de afbraak van de endogene pyrimidine basen uracil en thymine, maar ook bij de afbraak van fluoropyrimidines. Meer dan 80% van de hoeveelheid 5-FU toegediend, wordt voornamelijk afgebroken in de lever waar DPD voornamelijk aanwezig is. DPD wordt gevormd uit het *DPYD* gen waarvoor op dit moment 567 coderende varianten beschreven zijn en waarvan sommige pathogeen zijn doordat ze de enzymactiviteit verminderen.

Interindividuele variatie in DPD-activiteit beïnvloedt 5-FU farmacokinetiek en verminderde DPD-activiteit kan leiden tot ernstige toxiciteit en zelfs overlijden ten gevolge van 5-FU of capecitabinegebruik. Kennis over de klinische gevolgen van verminderde DPD-activiteit en de farmacodynamiek van fluoropyrimidinen kan behulpzaam zijn bij individueel doseren van 5-FU en capecitabine. In dit proefschrift wordt een dieper overzicht gegeven van methoden en het potentieel daarvan om het doseren van fluoropyrimidinen te optimaliseren op basis van de individuele DPD enzymactiviteit. Aanvullend wordt een orale uracil belastingtest bestudeerd die geschikt zou kunnen zijn om DPD-deficiëntie op te sporen in kankerpatiënten die behandeld worden met fluoropyrimidinen.

In **hoofdstuk 2** wordt een overzicht gegeven van beschikbare literatuur over voorspellende testen voor DPD-deficiëntie die beschreven zijn tot 2016. Er zijn testen bestudeerd die gebaseerd zijn op het schatten van de enzymactiviteit en genetische variaties in *DPYD* en mRNA, maar er zijn maar enkele testen spaarzaam geïmplementeerd in de kliniek in een prospectieve setting. Een probleem met veel testen is dat deze dure apparatuur vereisen die niet in elk ziekenhuis beschikbaar is, of dat de testmethode zeer bewerkelijk is en om die reden niet geschikt om grote aantallen patiënten te testen. Naast deze praktische nadelen is er ook geen consensus over de definitie van DPD-deficiëntie en zijn selectiviteit en specificiteit, indien bepaald, niet gecorreleerd aan dezelfde eindpunten waardoor het lastig is om testen qua effectiviteit onderling te vergelijken. Toekomstig onderzoek zou zich meer moeten richten op kosteneffectiviteit, sensitiviteit en specificiteit om te bepalen welke strategie het

meest geschikt is om te voorspellen welke patiënten het risico lopen om fluoropyrimidine-gerelateerde toxiciteit te ontwikkelen veroorzaakt door DPD-deficiëntie.

Uracil wordt net als 5-FU gemetaboliseerd door DPD. Het meten van uracil plasmaspiegels na toediening kan indicatief zijn voor de DPD-activiteit in een individuele patiënt en kan ernstige fluoropyrimidine-gerelateerde toxiciteit voorkomen. In **hoofdstuk 3** wordt het Investigational Medicinal Product dossier (IMPD) gepresenteerd dat de basis is voor het goedkeuren van klinisch onderzoek door bevoegde instanties in de Europese Unie. Het IMPD is gebruikt voor de medisch-ethische toetsing van de studies die met uracilpoeder zijn uitgevoerd, aangezien uracil niet geregistreerd is voor humaan gebruik. Alle paragrafen van het IMPD zijn toegespitst op uracil en alle testen met betrekking op onzuiverheden, gehalte-analyses en kwaliteit zijn uitgevoerd aan de hand van een analytische monografie met behulp van vloeistofchromatografie in combinatie met UV-foto diode array detectie. Acceptatiecriteria zijn afgeleid van de Europese farmacopee General Monograph. Het bereiden van de uracil studiemedicatie werd uitgevoerd onder Goede Manier van Produceren (GMP) en Good Clinical Practice (GCP) omstandigheden.

De farmacokinetiek van uracil zou alleen af moeten hangen van DPD-activiteit en niet worden beïnvloed door ziektebeeld of verschillen tussen gezonde vrijwilligers en patiënten. Om dit laatste uit te sluiten zijn in **hoofdstuk 4** de resultaten beschreven van een farmacokinetische studie waarin het verschil in uracil farmacokinetiek tussen gezonde vrijwilligers en DPD-deficiënte patiënten is onderzocht na toediening van een orale uracil belastingtest in twee doseringen.

De verwachting was dat het gebruik van een hogere dosis uracil tot een duidelijker onderscheid leidt tussen normale en deficiënte individuen als gevolg van verlengde DPD enzymverzadiging in DPD-deficiënte personen ten opzichte van de lagere uracildosis. Het tweede doel van de studie die in **hoofdstuk 4** is uitgevoerd was het onderzoeken van de lineariteit van uracil farmacokinetiek bij een verhoogde dosering uracil en de intra- en interdagvariatie van uracil farmacokinetiek.

De conclusie van deze studie is dat uraciltoediening op basis van een dosering van 500 mg/m<sup>2</sup> lichaamsoppervlak leidt tot significante en reproduceerbare verschillen in uracil en dihydrouracil farmacokinetiek tussen vrijwilligers met een normale DPD-activiteit en DPD-deficiënte patiënten. Uracildoseringen groter dan 500 mg/m<sup>2</sup> hebben geen beter onderscheidend vermogen om personen met en zonder DPD-deficiëntie te onderscheiden.

Verhoging van de uracildosering leidt alleen tot een rechtsverschuiving van de uracil- en dihydrouracil-concentratiecurve in de tijd en daardoor onnodige lange blootstelling aan hoge uracilconcentraties.

Uracil wordt voornamelijk in de lever gemetaboliseerd en om deze reden zou de aanwezigheid van metastasen in de lever de kinetiek van uracil na toediening van de orale uracil belastingtest kunnen beïnvloeden. In **hoofdstuk 5** wordt de uracildosering van 500 mg/m<sup>2</sup> lichaamsoppervlak verder bestudeerd en vergeleken in adjuvant behandelde patiënten met colonkanker en patiënten met colon/rectumkanker (CRC) met metastasen. De resultaten van deze studie laten zien dat in patiënten met normale DPD-activiteit, met de uitzondering van C<sub>max</sub>, er geen verschil is in uracil farmacokinetiek tussen patiënten met gemetastaseerd CRC ten opzichte van adjuvant behandelde colonkankerpatiënten.

Het farmacokinetische profiel van uracil in deze twee studies is tot stand gekomen met behulp van een intensief bloedafnameschema dat niet ideaal is voor gebruik in een klinische setting. Om deze reden is in **hoofdstuk 6** de orale uracil belastingtest verder onderzocht (i) om een vereenvoudigd bloedafnameschema te ontwikkelen, (ii) om verminderde uracileliminatie aan te tonen in patiënten met DPD-deficiëntie, (iii) om een verdiepende, kwantitatieve compartimentele farmacokinetiekanalyse uit te voeren op basis van uracil plasmaconcentraties. In dit onderzoek zijn verscheidene farmacokinetische parameters bepaald en met elkaar vergeleken. Dit heeft tot de conclusie geleid dat de uracil/dihydrouracilratio op t = 120 min na de orale uracilinnname de meest geschikte parameter is om patiënten die het risico lopen ernstige DPD-gerelateerde toxiciteit te ontwikkelen, met een hoge sensitiviteit en specificiteit te identificeren.

De samenvatting van de productkenmerken van capecitabine vermeldt dat het gebruik gecontraïndiceerd is in patiënten met een bekende DPD-deficiëntie, gedefinieerd als zeldzame en onverwachte ernstige toxiciteit. Bij 5-FU geeft de samenvatting van de productkenmerken aan dat wanneer dat mogelijk is, de DPD enzymactiviteit gemeten zou moeten worden voor behandeling met 5-FU. Tot op heden wordt het vooraf screenen van DPD niet als standaardzorg beschouwd binnen de oncologie en wordt het niet beschreven in ESMO of Nederlandse richtlijnen. Dit kan een gevolg zijn van het feit dat er internationaal geen consensus is over de optimale strategie om patiënten te identificeren die het risico lopen ernstige DPD-gerelateerde toxiciteit te ontwikkelen. Desondanks zijn er goed georganiseerde initiatieven in een prospectieve setting waarbij DPD getest wordt. **Hoofdstuk 7** beschrijft een studie waarin een routinematig *DPYD* testprogramma werd

geëvalueerd in een Nederlands academisch centrum vooraf aan het voorschrijven van 5-FU of capecitabine. In deze studie werd de acceptatie van de voorschrijvers geëvalueerd om bij patiënten die 5-FU of capecitabine voorgeschreven kregen, prospectief *DPYD* te laten screenen en werd het opvolgen van de dosisadviezen onderzocht. Het aantal patiënten dat daadwerkelijk werd gescreend was met 86,9% relatief hoog en de dosisadviezen die door de apotheker werden afgegeven werden in de meeste gevallen opgevolgd. Deze studie toont aan dat het implementeren van *DPYD*-screenen in een prospectieve setting mogelijk is en dat implementatie in de dagelijkse praktijk succesvol kan plaatsvinden.

In **hoofdstuk 8** worden de resultaten van de studies in dit proefschrift bediscussieerd in een breed perspectief en worden toekomstige ontwikkelingen genoemd betreffende het verbeteren van de orale uracil belastingtest en het prospectief screenen van DPD in het algemeen. Los van de verdere ontwikkeling van de testprocedure, is de orale uracil belastingtest in de huidige vorm potentieel geschikt als een fenotyperende test om DPD-deficiëntie te voorspellen in een prospectieve setting.





Dankwoord

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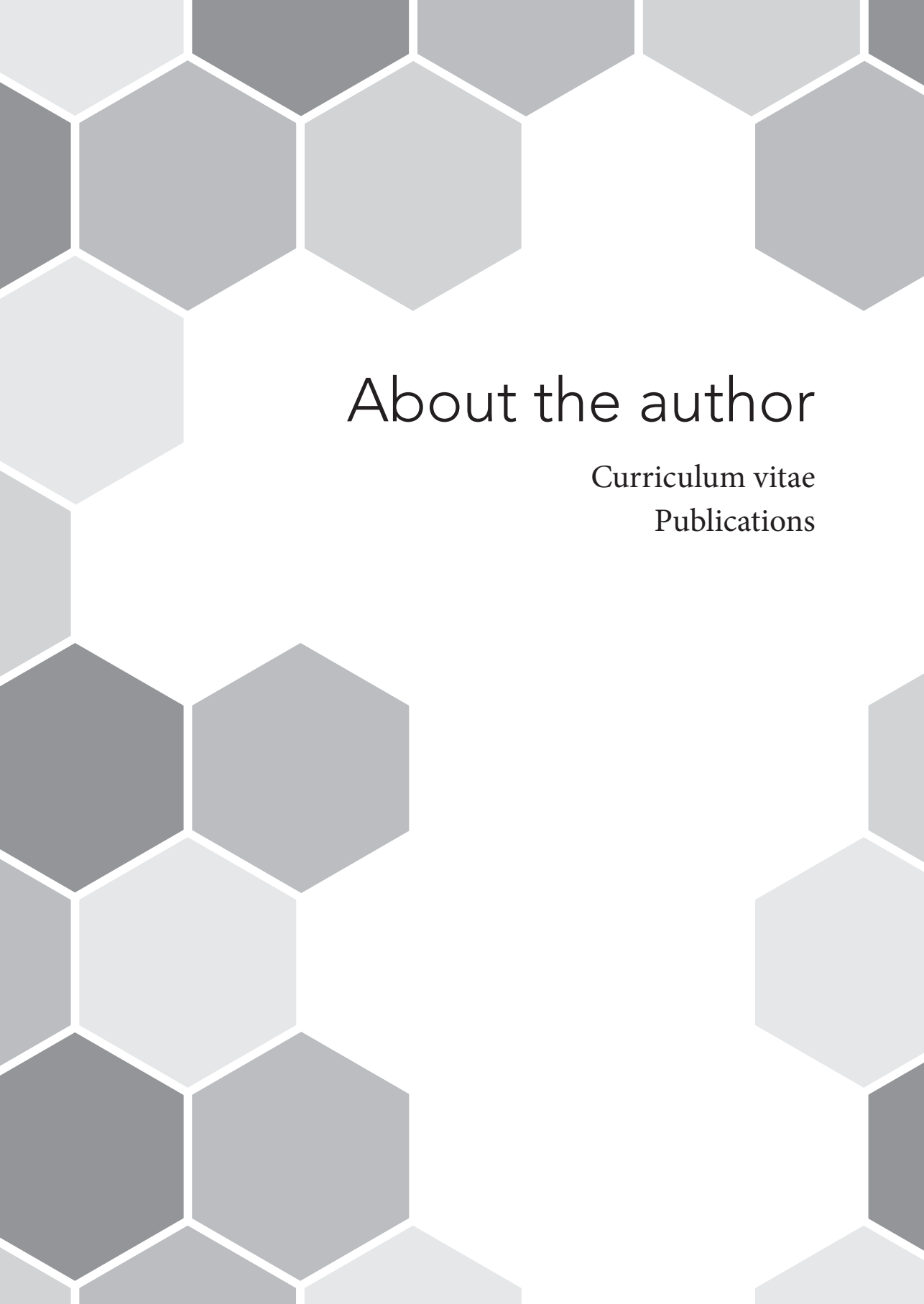
Paranimfen Roelof en Sander, het was voor mij een logische keuze om jullie te vragen om dit met mij te delen. Sander, wij kennen elkaar echt al ons leven lang, ik had je hierbij niet willen missen. Roelof, wij hebben elkaar goed leren kennen tijdens de oncologiedagen. Wij bleken het niet alleen professioneel maar ook privé goed met elkaar te kunnen vinden, net als onze gezinnen. Leuk dat we beide in 2016 ons promotietraject hebben afgerond.

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# About the author

Curriculum vitae  
Publications

## **CURRICULUM VITAE**

Maurice Charles van Staveren werd op 19 mei 1975 geboren in Den Haag. Zijn middelbare schooltijd doorliep hij op het Katholiek Drents College in Emmen, waar hij in 1994 zijn eindexamen behaalde. In dat jaar is hij ook begonnen met de studie Farmacie aan de Rijksuniversiteit Groningen. Zijn afstudeeronderzoek voor het doctoraalexamen deed hij in het Tjongerschans ziekenhuis in Heerenveen en het Medisch Centrum Leeuwarden (begeleider: prof. dr. J.R.B.J. Brouwers). In 2001 behaalde hij zijn apothekersexamen waarna hij aansluitend ging werken als projectapotheker in het Gemini ziekenhuis in Den Helder. Na een jaar werd hij aangenomen als projectapotheker in het Scheper ziekenhuis in Emmen waar hij als voornaamste taak had het ondersteunen van de dagelijkse klinisch farmaceutische zorg en het begeleiden van klinisch geneesmiddelen onderzoek. In 2005 is hij gestart met de opleiding tot ziekenhuisapotheker in het Martini Ziekenhuis in Groningen. In 2008 werd de opleiding tot ziekenhuisapotheker succesvol afgerond en is hij gaan werken als ziekenhuisapotheker klinisch farmaceutische zorg en laboratorium in het Scheper Ziekenhuis Emmen. In 2009 is hij begonnen met zijn promotieonderzoek bij de afdeling Klinische Farmacie en Toxicologie van het LUMC. Het onderwerp van zijn promotieonderzoek is een vervolg van het onderzoek dat hij verrichte in het kader van de opleiding tot ziekenhuisapotheker.

Hij is momenteel werkzaam in de ziekenhuisapotheek van de Treant Zorggroep waarvan het Scheper Ziekenhuis onderdeel is geworden.

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