Personalised medicine of fluoropyrimidines using DPYD pharmacogenetics

Lunenburg, C.A.T.C.

CHAPTER 3

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

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Linda M. Henricks*, Carin A.T.C. Lunenburg*, Didier Meulendijks, Hans Gelderblom, Annemieke Cats, Jesse J. Swen, Jan H.M. Schellens, Henk-Jan Guchelaar
**Contributed equally*

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

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Introduction

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

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

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

Previous guidelines and recommendations

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

Known *DPYD* alleles and their effect on DPD enzyme activity

DPYD*2A (rs3918290)

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

in a meta-analysis by Terrazzino *et al*. a strong correlation between the *DPYD**2A allele and overall grade >3 toxicity was found (odds ratio [OR] 5.42, p<0.001).[33] Deenen *et al.* described a mean capecitabine dose reduction of 50%, guided by toxicity, in patients carrying *DPYD**2A, compared with a mean dose reduction of 10% in wild-type patients.[42] Also, an initial dose reduction of capecitabine or 5-FU of 50% of standard dose has proven to decrease the risk of severe toxicity in *DPYD**2A carriers.[7,8] The above mentioned *in vitro, ex vivo* and *in vivo* studies provide solid evidence for the nonfunctionality of *DPYD**2A and a 50% reduced function in patients heterozygous for *DPYD**2A.

c.2846A>T (rs67376798)

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

DPYD*13 (rs55886062)

*DPYD**13 was first described by Collie-Duguid *et al*. as "T1679G".[48] The allele frequency was found to vary from 0.07 to 0.1% in Caucasians.[19,24] The precise functional consequences of the *DPYD**13 variant have not yet been unraveled, but are thought to be related to destabilization of a sensitive region of the protein.[16] *DPYD**13 has been found in patients with decreased enzyme activity, not in patients showing normal DPD enzyme activity.[29] Homozygous expression of this variant resulted in a 75% reduction of DPD enzyme activity compared with wild-type, as reported in an *in vitro* study by Offer *et al.*[27] This suggests that this variant almost completely inactivates the protein. Decreased DPD enzyme activity in patients with the *DPYD**13 variant was determined only in a limited number of *ex vivo*

studies using PBMCs.[16,29,30,48] A major variation of enzyme activity was found, ranging from 1.7 times to 500 times decreased as compared with the normal enzyme activity and once the enzyme activity was undetectable,[30] although it must be mentioned that these results could be influenced by other copresent *DPYD* variants. Patients with *DPYD**13 showed severe toxic side effects in several studies.[4,24,29,44,48,49] Also dose adjustments were described by two groups.[4,24] Morel *et al.* described a heterozygous patient that experienced severe grade 4 toxicity. After a 6-week treatment interruption, 5-FU was safely reintroduced with individual pharmacokinetic adjustment, based on 5-FU plasma levels.[4] The above mentioned studies show that *DPYD**13 results in an almost nonfunctional enzyme and consequently low enzyme activity levels. Without a dose reduction toxicities are likely to develop, however safe use of 5-FU is still possible with a dose adjustment. We suggest a starting dose of 50% for patients carrying *DPYD**13 to ensure safe and effective use of fluoropyrimidines.

c.1236G>A/HapB3 (rs56038477)

The c.1236G>A variant was first described by Seck *et al*., as a silent mutation that displays normal DPD enzyme activity.[46] The c.1236G>A polymorphism occurs in exon 11 and is a synonymous variant that is in complete linkage with c.483+18G>A, c.680+139G>A, c.959-51T>G and c.1129-5923C>G;[14] these variants in linkage have been termed haplotype B3.[14,15] The c.1129-5923C>G intronic polymorphism (rs75017182) results in aberrant splicing and is likely to be the responsible variant for the effect on DPD enzyme activity.[3,14] The frequency of heterozygous patients in Caucasian populations was reported to vary between 2.6 and 6.3%.[14,15,42,49,50] DPD enzyme activity for c.1236G>A carriers was measured in PBMCs in two studies.[14,46] Enzyme activities were reported to be 2.9, 4.2, 6.2 and 1.6 nmol/(mg*h) (normal value=9.6±2.6 nmol/[mg*h]) for one homozygous and three heterozygous carriers of c.1236G>A, respectively.[14] In addition, a heterozygous patient in another study was found to have an enzyme activity of 10.2 nmol/(mg*h), which was reported as 'normal activity', since the enzyme activity of the population ranged from 4.8—15 nmol/(mg*h).[46] Unfortunately data on c.1236G>A and enzyme activity are limited and not consistent. The homozygous patient still had 30% DPD activity remaining.[18] Furthermore we observed two homozygous patients with this variant in our own institute with a relevant DPD enzyme activity left of around 50%, showing that this variant does not result in a completely nonfunctional enzyme (author's unpublished data). In the study of Sistonen *et al.* the ratio between endogenous dihydrouracil (DHU) and uracil (U) was measured in patients carrying the c.1129-5923C>G variant.[50] This ratio can be used as a phenotyping marker for DPD enzyme activity, as described in several studies.[51-55] Sistonen *et al.* found a statistically significant decrease in DHU/U-ratio compared with wild-type patients (p=0.044). However, no significant effect for the other *DPYD* risk variants (*DPYD**2A, *DPYD**13 and c.2846A>T) was observed, which might be caused by the small sample size of patients with those variants. The c.1236G>A/HapB3 variant has been associated with severe and lethal toxicity.[14,15,42,49,56] For example, Froehlich *et al.* found a relative risk of 3.74 ($p=2x10^{-5}$) in c.1236G>A/HapB3 carriers for severe toxicity (grade 3—5).[49] In contrast, no significant effect of the c.1236G>A/HapB3 variant was found in two other studies.[44,47] A dose reduction to prevent toxicity may be advantageous since multiple studies found a correlation with severe toxicity; however the

degree of dose reduction cannot easily be determined with the enzyme activity from only two published studies and conflicting results in clinical studies. In heterozygous patients, a dose reduction of 50% would be too large since c.1236G>A/HapB3 does not result in a completely nonfunctional enzyme. No dose reduction at all would be in contradiction to the correlation found between this variant and toxicity. Therefore a more cautious dose reduction of 25% seems appropriate, to avoid both increased risk of toxicity and prevent underdosing.

Also our own experimental data support the differentiation between various SNPs in *DPYD*. We determined the endogenous pretreatment ratio between DHU and U in a large cohort of patients (*N*=539) treated with capecitabine or 5-FU.[57] This cohort is a subset of patients participating in a prospective multicenter trial of *DPYD**2A-guided dosing of fluoropyrimidines (clinicaltrials.gov identifier: NCT00838370).[7,8] The DHU and U levels were measured in pretreatment serum samples using a validated LC-MS/MS method,[58] chromatographic separation was performed on an Acquity UPLC® HSS T3 column (150 x 2.1 mm ID, particle size 1.8 µm), and a triple quadruple mass spectrometer (API5500, AB Sciex, USA) was used for quantification of U and DHU. The method was validated over a concentration range of 1 to 100 ng/ml for U and 10 to 1000 ng/ml for DHU. Genotyping of *DPYD* variants was performed using standard PCR methods. A distinction was made between patients heterozygous for *DPYD**2A, c.2846A>T, *DPYD**13 or c.1236G>A and wild-type patients (Figure 1). For patients heterozygous for *DPYD**2A, c.2846A>T, *DPYD**13 and c.1236G>A the median relative DHU/U ratio compared with wild-type is 52, 68, 50 and 101% respectively. These results confirm that DPD enzyme activity differs between carriers of certain *DPYD* polymorphisms and points toward a differentiated dose reduction for each individual SNP.

Gene activity score

The gene activity score method is based on the principle that variant alleles can differ in the extent to which they influence enzyme activity. Such a method was first described by Steimer *et al.* where a 'quantitative functional gene dose' is assigned to alleles of the gene *CYP2D6*, a highly polymorphic gene that is involved in the metabolism of various clinically used drugs, including antidepressants, antipsychotics and opioids.[59] Thereafter Gaedigk *et al.* introduced the 'activity score' and divided *CYP2D6* alleles in three categories, consisting of fully functional alleles (value of 1), reduced activity alleles (value of 0.5) and nonfunctional alleles (value of 0).[60] The values for both alleles of a patient are summed, leading to an individual gene activity score that represents the enzymatic phenotype of the patient. This method results in a uniform way of describing phenotypes and can be used for adjusting the dose of a drug. For *CYP2D6* it has been demonstrated that the gene activity score is valid and easy-to-use for translating genotype and predicted phenotype.[60] The gene activity score may also be useful to properly interpret different DPD enzyme activities, translate these into a phenotype and thus personalize fluoropyrimidine treatment according to *DPYD* genotype. With this tool a more precise distinction between nonactive and reduced activity alleles can be made and it also provides the possibility to include novel SNPs which may be identified in the near future using whole exome and whole genome sequencing. The activity score as

proposed by Gaedigk *et al.* has proven beneficial for *CYP2D6*, for which a large number of polymorphisms are known.

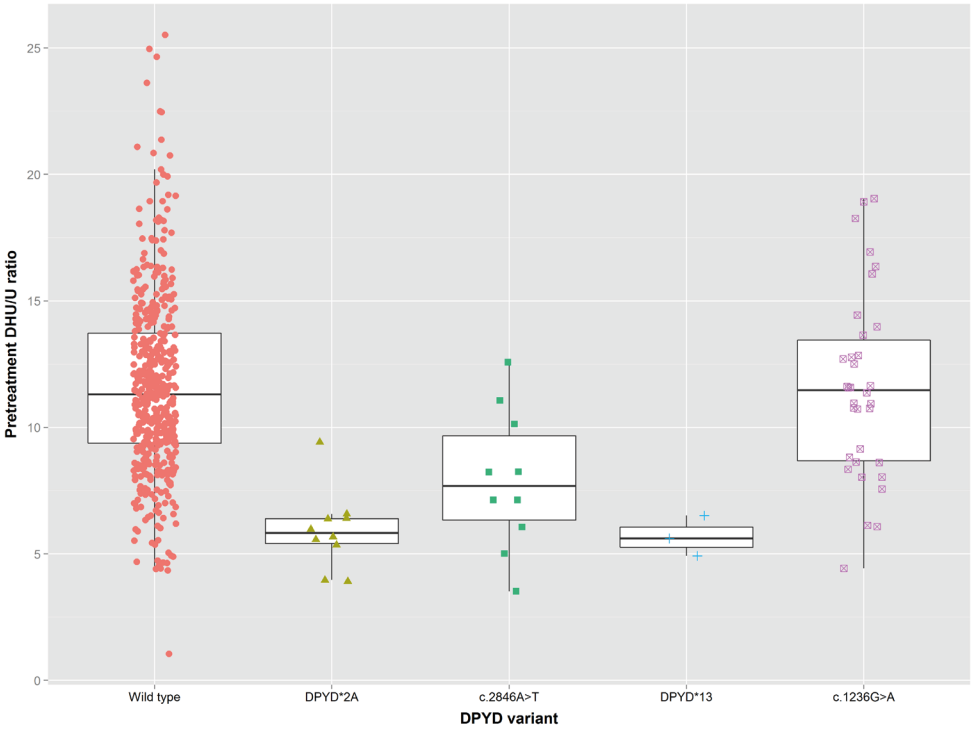

Figure 1. DHU/U ratio according to *DPYD* genotype

Shown are individual values and a box plot with the median of the DHU/U ratio for patients with a *DPYD* polymorphism or *DPYD* wild-type patients.

Abbreviations: DHU: Dihydrouracil, U: Uracil

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

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

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

Activity Value	Alleles	Ref.
0	*DPYD**2A (rs3918290)	4,8,10,11,19,27,29-46
	*DPYD**13 (rs55886062)	4,19,30,32,33,46,49,50
0.5	c.2846A>T (rs67376798)	4,13,24,33,36,41,42,44,47
	c.1236G>A/HapB3 (rs556038477)	14,15,42,44,46,47,49,50,56
1	*DPYD* *1 (wild-type)	

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

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

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

Discussion and conclusion

There is ample evidence that shows that DPD-deficient patients develop severe toxicities when treated with a normal dose of fluoropyrimidines. Even though this relation is widely known, there is no global systematic approach to prevent severe toxic side effects using *DPYD* polymorphisms as predictive markers. Upfront *DPYD**2A screening has been implemented in a limited number of institutions and other SNPs are increasingly added to the standard genetic screening. Testing for an increasing number of SNPs that result in different DPD enzyme activities makes it harder to derive a dosing advice. The gene activity score is a new method for translating *DPYD* genotype into DPD phenotype. It can be used to standardize the process of describing DPD enzyme activity, which stimulates uniformity. In the CPIC guideline a dose recommendation of 50% is advised for *DPYD**2A, *DPYD**13 and c.2846A>T.[19] In the gene activity score as proposed in this manuscript we adopt these recommendations

for *DPYD**2A and *DPYD**13, but deviate in the dose advice for c.2846A>T and include a dose advice for c.1236G>A/HapB3. We have summarized *in vitro, ex vivo* and *in vivo* studies to determine the appropriate dose recommendation for these SNPs. In addition, we have shown our own experimental data. Our data are in agreement with previous data and show a 50% reduced DPD enzyme activity in patients heterozygous for *DPYD**2A and *DPYD**13 and an ~25% decreased activity for heterozygous patients with c.2846A>T. Unfortunately, our data on c.1236G>A do not correspond and additional data containing DPD enzyme activity measurements in patients with c.1236G>A/HapB3 are scarce and not in agreement. Including our study, three out of four studies suggest that c.1236G>A results in an enzyme activity close to normal levels. However, Sistonen *et al.* showed a significant reduction in DHU/U ratio in patients carrying this variant[50] and associations with the development of severe toxic side effects have also been described. The toxicity data point out that a dose reduction for c.1236G>A/HapB3 is required, but a dose reduction of 50% would be too large considering the measured enzyme activities. Therefore a dose reduction to 75% of the normal dose for heterozygous patients seems appropriate in order to prevent toxicity as well as to prevent underdosing. After the initial dose reduction the patient should be closely monitored and the dose can be adjusted according to occurring toxicity.

Currently only four SNPs in *DPYD* are allocated a gene activity score, since we consider these variants are the most relevant polymorphisms. It has been described before that 13 to 19 variants are expected to result in DPD deficiency[61,62]. However, more research is necessary on the effect of these other SNPs on DPD enzyme activity before they can be included in the gene activity score. With the gene activity score approach it is possible to continuously keep adding variant alleles or updating the values of the gene activity score that are assigned to variant alleles. When new information on effects on enzyme activity is published, this can be included, while the currently proposed gene activity score can already be used in clinical practice. In addition, more research is needed with regard to compound heterozygous patients (patients who carry two different SNPs) and homozygous patients. These patients would benefit from an additional phenotyping test to measure the DPD enzyme activity as to determine the optimal dose adjustment or decide to treat with an alternative drug.

Both genotyping and phenotypic biomarkers have been proposed in order to predict and reduce toxicity in patients. However, the gold standard of phenotyping (measuring DPD enzyme activity in PBMCs) is not easy to implement as a routine test and other phenotyping methods, such as uracil test dose, endogenous DHU/U ratio and 2-^{13}C-uracil breath test, have not yet been fully validated or standardized.[63] Compared with phenotyping methods, genotyping methods are faster, easier and less expensive, so it is expected that it will be implemented more often as standard of care for patients undergoing fluoropyrimidine treatment.

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

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

Future perspective

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

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