



A review on the importance of genotyping and phenotyping in fluoropyrimidine treatment

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Abstract

Fluoropyrimidines, after more than 50 years from their discovery, are still the treatment of many types of cancer, and it is estimated that two million patients receive fluoropyrimidine therapy annually. The toxicity associated with fluoropyrimidines affects 30-40% of patients and some adverse effects can be lethal.

Dihydropyrimidine dehydrogenase is the main enzyme in the catabolism of 5-FU and DPD activity deficiency can cause important toxicity. This is an important reason to determine DPD activity in order to improve patient safety and to limit potential life-threatening toxicity.

At present multiple phenotypic and genotypic methods are available for the determination of DPD activity, some of these methods have proven their usefulness in practice, and yet they are not routinely recommended in clinical practice.

This review is another statement of the importance of the determination of DPD status, the phenotypic and genotypic methods that are available and can be used.

Keywords: chemotherapy, fluoropyrimidine treatment, toxicity

Introduction

Fluoropyrimidines still represent the foundation stone in the treatment of multiple cancers, such as colorectal cancer, gastric cancer, pancreatic cancer, esophageal cancer, head and neck cancer, breast cancer and every year it is estimated that two millions patients are treated with fluoropyrimidine [1,2]. The fluoropyrimidines used in general practice are 5-Fluorouracil (5-FU), Capecitabine and Tegafur, the last two are prodrugs of 5-Fluorouracil.

Capecitabine, the oral precursor of 5-Fluorouracil, is administered in advanced colorectal cancer, gastric cancer, and breast cancer, turning into 5-Fluorouracil at the tumor site [3,4,5]. Capecitabine passes unchanged through the intestinal wall and is converted in the liver to 5-deoxy-5-fluorocytidine (5'-dFCR) and then to 5-deoxy-fluorouridine (5'-dFUR), by carboxylesterase and cytidine-deaminase. 5'-dFUR turns into 5-FU by thymidine-phosphorylase or uridinephosphorylase [6,7].

Tegafur, another prodrug of

5-Fluorouracil, is converted by CYP2A6 to an intermediate metabolite, 5-hydroxy-tegafur, which spontaneously breaks down to 5-FU [6,7].

5-Fluorouracil: history, metabolic pathways and toxicity

5-Fluorouracil was patented in 1956 and become used in cancer treatment in 1962. In 1985, Tuchman et al. reported the first case of severe toxicity in a patient with familial pyridinemia and treatment with 5-FU. This was the first evidence that a genetic defect in pyrimidine catabolism can be associated with fluoropyrimidine-related toxicity. Now, we know that DPD activity is associated with 5-FU pharmacology and enzyme deficiency increases the risk of severe side effects, sometimes even lethal, in patients undergoing treatment with fluoropyrimidines [1-4].

In Japan sixteen deaths were reported after simultaneous administration of 5-FU and Sorivudine, which inhibits the activity of DPD. Sorivudine is catabolized in the intestinal wall to uracil, which inhibits dihydropyrimidine dehydrogenase

DOI: 10.15386/mpr-1564

Manuscript received: 04.01.2020

Received in revised form: 24.02.2020

Accepted: 20.03.2019

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(DPD), thus resulting into a prolonged systemic exposure to 5-FU [2,3].

After administration of 5-FU, only 1-5% of the amount is transformed at the intracellular level into cytotoxic metabolites such as fluorodeoxyuridine-monophosphate (FdUMP), fluorodeoxyuridine- diphosphate (FdUDP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). The main anti-tumoral mechanism of 5-FU is due to the cytotoxic metabolite FdUMP, which inhibits thymidylate-synthase (TS), a crucial enzyme in the DNA replication and repair. TS is an important enzyme for de novo synthesis of thymidylate required for DNA synthesis. FdUMP and FdUTP destroy the processes and function of DNA, by inhibition of DNA elongation and fragmentation, respectively FUTP influences the RNA processes of methylation, stability and synthesis [1-7].

About 80-85% of 5-FU is catabolized by dihydropyrimidine dehydrogenase (DPD) into the non-cytotoxic metabolite, 5-fluoro-5,6-dihydrouracil (DHFU) [7]. DHFU is then converted to fluoro-beta-ureidopropionate (FUPA) and fluoro-beta-alanine (FUBA), which are eliminated from the body through urine [7]. So

DPD is the main responsible enzyme for the catabolism of 5-FU. Because DPD is the first enzyme in the 5-FU catabolism, the amount of 5-FU which is converted into cytotoxic metabolites depends on the systemic activity of DPD [6,7] (Figure 1).

5-FU has a narrow therapeutic window so there is a small limitation between efficacy and the maximum tolerated dose. A decreased DPD activity means a decreased clearance and an increased half-life of 5- FU resulting an increased risk of dose-related toxicity [7,8].

Dihydropyrimidine dehydrogenase (DPD) is the most important enzyme for fluoropyrimidine catabolism pathway. Patients with a partial or complete DPD deficiency are at risk to develop severe toxicity due to the treatment with fluoropyrimidines [8,9]. DPD activity can be determined in many tissues, but the most intense activity is found in the liver, where catabolism occurs. Patients with decreased hepatic function are candidates for developing severe toxicity after treatment with fluoropyrimidines. DPD determination from the normal liver correlates with DPD activity from the peripheral blood mononuclear cells (PBM), so PBM level of DPD is equivalent to the total amount of DPD [8,9].

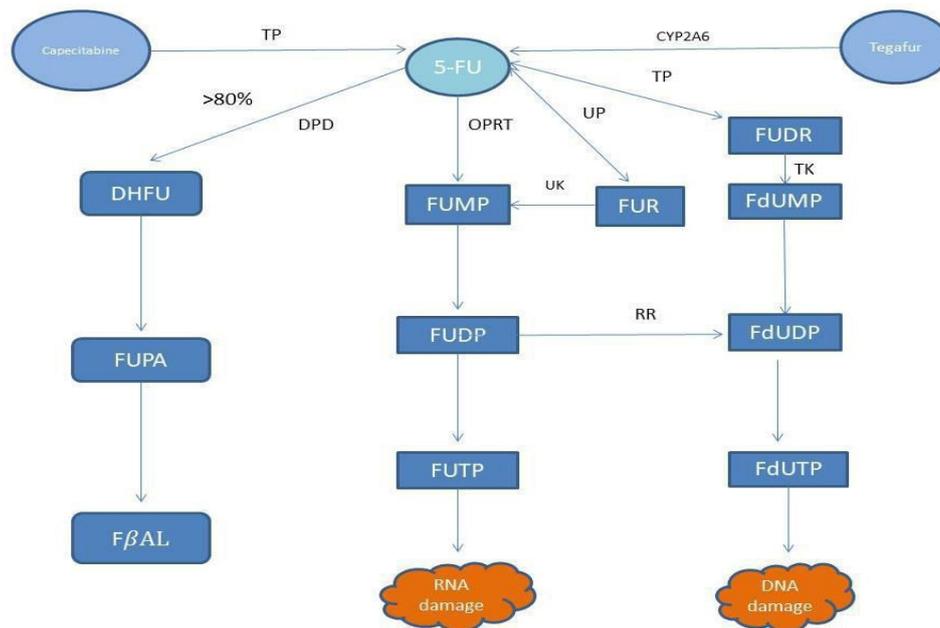


Figure 1. 5-FU metabolic pathways. Capecitabine and Tegafur are converted to 5-FU by thymidine-phosphorylase (TP) and CYP2A6. 1-5% of 5-FU is transformed into cytotoxic metabolites fluorodeoxyuridine-monophosphate (FdUMP), fluorodeoxyuridine-diphosphate (FdUDP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). 5-FU is directly transformed to fluoridine monophosphate (FUMP) by orotate phosphoribosyltransferase (OPRT), or indirectly through fluorouridine (FUR) by uridine phosphorylase (UP) and uridine kinase (UK). FUMP is then phosphorylated to fluorouridine diphosphate (FUDP), which is phosphorylated to fluorouridine triphosphate (FUTP). FUDP can also be converted to fluorodeoxyuridine diphosphate (FdUDP) by ribonucleotide reductase (RR). In an alternative activation pathway 5-FU is converted to fluorodeoxyuridine (FUDR) by thymidine phosphorylase. FUDR is converted through thymidine phosphate in fluorodeoxyuridine monophosphate (FdUMP), which is phosphorylated to fluorodeoxyuridine diphosphate (FdUDP), which is phosphorylated to fluorodeoxyuridine triphosphate (FdUTP). 80% of the 5-FU is catabolized by dihydropyrimidine dehydrogenase into the non-cytotoxic metabolites 5-fluoro-5,6-dihydrouracil (DHFU), which is then converted to fluoro-beta-ureidopropionate (FUPA) and fluoro-beta-alanine (FUBA), which are eliminated through urine.

Bosidron-Celle et al. compared the 5-FU clearance in two heterozygote patients with DPYD*2A and seven heterozygote patients with DPYD*9B with 163 patients with no deficient variant. In the heterozygote patients with the two deficient variants they observed that 5-FU clearance had decreased by 80% and 40-58% respectively, compared to the patients with no deficient variant [8]. The same group found in one patient three heterozygote variants: DPYD*2A, rs67376798 and 85 T>C resulting in a 5-FU plasma clearance close to zero. All these data indicate that heterozygote patients for deficient variants have significantly reduced fluorouracil clearance, by 40-80%, compared to patients with no deficient variants [6-10].

DPD activity turned out to be inversely proportional with plasma concentration of 5-FU in patients treated with continuous infusion [8-10]. A study showed a concentration area under the curve of 5-FU increased from 9.2 in group control to 24.7 in patients with DPD deficiency [10].

Fluoropyrimidines induce grade 3-4 toxicity in 30-40% of cases and lethal toxicity in 0.3-2% of cases (almost 5% of the elderly population). The level of DPD activity has an important inter-individual variability which is partly explained through different variants of DPYD gene which encodes DPD [11-13].

The complete deficit can be very rare, 0.1-0.5% in the general population and partial deficiency can be found in 3-10% in Caucasian population. DPD deficiency can be detected in 39-61% of patients with severe toxicity [14].

Strategies to determine the DPD deficiency are based on genotyping and phenotyping. The main phenotyping methods are measuring DPD enzyme activity in PBM, determination of the endogenous concentration of uracil and dihydrouracil in plasma, saliva and urine, 2-¹³C uracil breath test, uracil dose test [15,16].

Phenotyping methods – what to choose from so many alternatives?

Measuring DPD activity in PBM is the gold standard in phenotyping methods, the level of PBM activity correlates with the hepatic level. The clinical validity in measuring DPD enzyme activity in PBM has been demonstrated in many retrospective and prospective studies [15,16]. An important point of view is the DPD activity threshold value, which indicates whether there is a deficit or not. Milano et al. have proposed a cut-off under 70% of the population mean to classify patients with enzyme deficiency [14]. This value has been subsequently validated as a predictive value for fluoropyrimidine related toxicity in many retrospective studies.

An important phenotyping method to determine the interindividual variation in DPD activity is the 2-¹³C uracil breath test, an oral aqueous solution of 2-¹³C uracil which decomposes into ¹³CO₂ and can be detected from respiration by infrared spectroscopy [16,17]. Mattison et al. demonstrated in a study on 58 patients, 50 with normal

DPD activity, 7 with partial deficiency and one with total deficiency, who were submitted to this test at different time intervals up to 180 minutes, that measuring the level of 2-¹³C uracil at 50 minutes may detect the deficiency [16]. Another study showed that 2-¹³C uracil breath test has a moderate accuracy to differentiate between individuals with severe toxicity and moderate toxicity or not at all [17]. An important limitation of this test is the fact that the obtained results can be related to other enzymes from 5-FU metabolism, such as dihydropyrimidase and beta-ureidopropionase [18,19].

In the uracil dose test an oral solution of uracil is administered to the patient and then dihydrouracil and uracil pharmacokinetics are measured from plasma at different time intervals. van Staveren et al. compared the pharmacokinetics in two groups with comparable characteristics, except age, DPD status, and disease [20]. The group with normal DPD activity was established from young healthy individuals, and the group with DPD deficiency were patients with colorectal and breast cancer. Age is a factor that influences pharmacokinetics by functional modifications that come with age, therefore aging decreases hepatic perfusion and therefore we have a decreased clearance for some drugs through the first liver passage. Also, the presence of hepatic metastases and induced chemotherapy steatosis decrease the metabolism of drugs, which can lead to the alteration in the metabolism of uracil [20].

The pretreatment endogenous concentration of dihydrouracil and uracil from plasma has been investigated for determination of enzymatic activity of DPD. The association between UH2/Ura ratio, 5-FU clearance and treatment associated toxicity was demonstrated. In one study which enrolled 66 patients diagnosed with colorectal cancer, the mean value of UH2/Ura ratio in patients with adverse effects has statistically significantly lower (3.2 versus 7.1, p=0.009) than the reference population, healthy population. Twenty-one from twenty-four patients which presented adverse effects had a cut-off under 4, determined by ROC curve. UH2/Ura ratio has predictive for fluoropyrimidine related toxicity with a Se=87% and Sp=93% [16-19].

Carlsson et al. have determined pre-treatment dihydrouracil and uracil from the saliva of patients with colorectal cancer [23]. They took samples of saliva in fasting conditions from 73 patients before starting the adjuvant treatment, and dihydrouracil and uracil were measured by high-performance liquid chromatography (HPLC). The adverse effects were monitored before and after every cycle of chemotherapy and the most severe adverse reactions were noted. From the 73 patients, 56 patients needed a dose reduction due to side effects and 8 had to interrupt the treatment due to severe toxicity. The UH2/Ura ratio was low in these patients who experienced toxicity due to treatment with fluoropyrimidines [20-23].

The same group of authors determined the dihydrouracil and uracil from urine levels from 143 patients with colorectal cancer and compared them to the levels of control group [23]. The urine samples were taken from the first urine in the morning and measured with HPLC. According to this study, there was no statistically significant evidence between patient group and control group. In another study, Hayashi et al. established a threshold value for uracil and dihydrouracil in Japanese adult patients. The mean SD dihydrouracil and uracil levels were found to be 63.8-66.0 $\mu\text{mol/g}$ and 23.8-35.5 $\mu\text{mol/g}$ creatinine, and the mean SD UH2/Ura ratio was 0.36-0.1; these values are much lower compared to the values recorded by Carlsson et al [23-25].

Kristensen et al. determined the UH2/Ura ratio in plasma at 68 patients with colorectal cancer and 100 healthy individuals who formed the group control. Twenty-four patients with adverse effects were tested for DPYD*2A, and UH2/Ura ratio was low. Plasma concentration of 5-FU increased 130 times in 13 of the 24 patients [15].

Meulendijks et al. in a study published in 2017, show that pretreatment serum uracil was superior to dihydrouracil/uracil ratio as a predictor for severe toxicity associated with fluoropyrimidine treatment [26]. Serum uracil was strongly predictive of global toxicity ($p=0.004$), gastrointestinal toxicity ($p=0.0006$), toxicity-related hospitalization ($p=0.011$) and fatal toxicity ($p=0.007$), but not associated with hematological toxicity ($p=0.383$). The pretreatment uracil concentration ranged between 3.2-38.2 ngml^{-1} , and patients were classified in groups based on the distribution of concentration, $U >16 \text{ ngml}^{-1}$, 13.9-16 ngml^{-1} , 13-13.9 ngml^{-1} and $U <13 \text{ ngml}^{-1}$. The first two groups, Uracil $>16 \text{ ngml}^{-1}$ and 13.9-16 ngml^{-1} were at statistically significant increased risk of global toxicity compared with the group $<13 \text{ ngml}^{-1}$, with OR=5.3 and $p=0.0087$, respectively OR=8.2 and $p=0.0004$. Patients with pretreatment concentration of 13-13.8 ngml^{-1} , had no statistically significant risk [26-28].

Genotyping, yet another proof of clinical utility

The most important methods are those of genotyping for different genetic defects, such as the presence of a deleterious mutation in DPYD and mutation in miR-27A which influence the expression of DPD, but for which only a few pieces of information exist. The most studied is the DPYD gene that encodes DPD activity. The toxicity of 5-FU can be influenced by other genes, the most studied are ABCB1, MTHFR, and TYMS, but data are inconsistent and strategies to determine the activity of these genes are needed [29,30]. TYMS variants located in the regulatory regions seem to have an influence in the transcription rate. High intra-tumoral levels of TYMS

have been associated with 5-FU resistance [29,30].

In terms of MTHFR, two single nucleotide polymorphism (SNPs) located on exon 4 and exon 7 seem to lead to a decreased enzymatic activity according to some in vitro studies [30].

DPYD gene is located on chromosome 1p22 and contains 4399 nucleotides in 23 exons which encode 1025 amino acids such as uracil, flavine mononucleotide (FMN), NADPH and flavine adenine dinucleotide (FAD) binding sites, along with four Sulphur motifs [31-33].

The most frequent DPYD allelic variants are DPYD*5 (rs1801159 T>C), DPYD*6 (rs1801160 C>T), and DPYD*9A (rs1801265 A>G) at frequencies of 11.5–30, 0.7–9, and 2.9–13.7%, respectively, and data regarding their effects on DPD activity are contradictory. The Dutch pharmacogenetics working group have designated these variants as functional on the lack of an association between toxicity and these variants [33,34].

The most studied variant is DPYD*2A (IVS14 + 1G>A, c.1905+1G>A, or rs3918290) which is a single nucleotide polymorphism (SNP), at the intron boundary of exon 14 and result in a splicing defect, skipping of the entire exon and resulting in a non-functional protein. This allele is considered to be more common than others variants at a frequency range from 0.004-3.5% [33,34].

DPYD*2A was associated with multiple lethal events during the treatment with fluoropyrimidines. For example in a paper published in 2001 by van Kuilenburg et al., one female patient died because of adverse effects of the treatment, she presented stomatitis after the first administration of the drug, and after the second cycle of chemotherapy fever, severe stomatitis, leucopenia, thrombocytopenia, pancytopenia and died after the infection complications. Genotyping found that she had DPYD*2A, homozygous [29].

DPYD*2A was correlated statistically significantly with nausea and vomiting ($p<0.007$) and neutropenia ($p<0.001$), DPYD*9B with dehydration ($p=0.02$), diarrhea ($p=0.003$), leucopenia ($p=0.002$), neutropenia ($p<0.001$) and thrombocytopenia ($p<0.001$) [34].

In a meta-analysis, Terrazzino et al. showed that DPYD*2A variant is a risk factor of grade 3 or higher hematological toxicity and to a less grade 3 or higher mucositis or diarrhea [34].

DPYD*3 (rs72549303 C>del),*13 (rs55886062 A>C), and *9B (rs67376798) are relatively rare but result in low DPD activity and 36,37 seems to be related to 5-FU toxicity [33,34]. Recently in African-American patients, a novel DPYD variant, Y186C, was identified and it seems that it can be found in 26% of patients with reduced DPD activity. Individuals carrying this variant had a 46% reduction in enzyme activity as compared with noncarriers [33].

Table I. DPYD variants and dose reduction recommendations.

DPYD variant	NCBI SNP reference	Exon	Nucleotide	Protein	MAF	Dosing recommendation heterozygous/homozygous
DPYD*2A (IVS14+1G>A, c.1905+1G>A)	rs3918290	Intron 14	IVS14+1G>A	Exon 14 skipping	3.5%	50%/Contraindicate
DPYD*9B (c.2846A>T)	rs67376798	22	A2846T	D949V	0.1-1.1%	25%/50%
DPYD*13 (c.1679T>G)	rs55886062	13	T1679G	I560S	0-2%	25%-50%
HapB3 (c.1129-5923C>G)	rs75017182	1		E412E		25%/50%

Abbreviations: DPYD – the gene that encodes DPD, HapB3 – haplotype B3, MAF – minor allele frequency, NCBI – National Center for Biotechnology Information

Another well studied variant is DPYD*9B, c.2846A>T, described for the first time in 2000 by van Kuilenberg et al [34-36]. The c.2846A>T polymorphism leads to a structural change in the enzyme that interferes with a cofactor binding or electron transport. The frequency of this allele is between 0.1-1.1%. In vitro studies show that homozygous patients with c.2846A>T variant have a 60% activity of the enzyme compared with wild-type [35,36]. Therefore homozygous patients of this variant need a 50% reduction of the dose and heterozygous patients a reduction of 25% [36]. Rosmarin et al. in a study which enrolled 927 patients evaluated the toxicity induced by Capecitabine and found that DPYD*9B variant and DPYD*2A variant is the most frequent variants found in a relationship with Capecitabine toxicity, OR=5.51, p=0.0013 [30].

The B3 haplotype, a combination of various genetic changes, corresponds to an allele with several variations in linkage disequilibrium (c.1236G>A) in exon 1 a four intronic variations C.483+18G>A, c.680+139G>A, c.959-51T>G, and c.1129-5923C>G). The c.1129-5923C>G affects pre-mRNA splicing and probably represents the causal variation of Hap B3 [36]. Homozygous patients with variant c.1236G>A/Hap B3 have a 50% decrease in DPD activity. These data support a dose reduction of 25% in patients with heterozygous form and 50% in homozygous patients [36].

A meta-analysis which enrolled 7365 patients from 8 studies highlighted the fact that DPYD*13 and c1236G>A/Hap B3 are statistically significantly associated with high risk for fluoropyrimidines related- toxicity [36].

In a study including 2886 patients with stage III colorectal cancer, 2594 patients developed side effects, they were genotyped for DPYD*2A, DPYD*9B and DPYD*13. The incidence of grade 3 or greater adverse effects was 88% for DPYD*2A, 81.5% for DPYD*9B and 50% for DPYD*13. Statistically significant associations were demonstrated between grade 3 or greater 5-FU adverse effects for both DPYD*2A (OR=15.21, 95% CI= 4.54-

50.96, p<0.001) and DPYD*9B (OR=9.10, 95% CI=3.43-24.10, p<0.001) [37].

DPYD*13 has a frequency of 0-2% in Caucasian population [38]. Based on a meta-analysis the risk of hematological and gastrointestinal toxicities is increased up to almost 10, respectively 6 times in patients with this variant. A heterozygous genotype is expected to result in a 40-50% decrease in DPD activity [38] (Table I).

Discussion

The main benefit in determining a genetic deficiency would be to avoid toxicity and prevent the most severe and fatal effects of toxicity by using alternative drugs or reduce fluoropyrimidine doses. In patients who need treatment with fluoropyrimidine, DPD status should be determined before the start of the treatment to prevent the potential toxicity. At this moment, clinical validity is well known for four variants (DPYD*2A, DPYD*9B, DPYD*13, and c.1236G > A/Haplotype B3), but there are no official recommendations for genotyping before the treatment and also to adjust the dose based on these findings [40]. Also others characteristics such as age, gender, hepatic and renal function may increase the toxicity risk [38].

In clinical practice there are four phenotyping methods available, enzyme activity in PBM, endogenous uracil levels, 2-¹³C-uracil breath test and uracil dose test. Enzyme activity in PBM is gold-standard but is not easily implementable in clinical practice. Pretreatment uracil serum is a well feasible method in clinical practice and seems to be more predictive of fluoropyrimidine toxicity than dihydrouracil/uracil ratio. Based on the study of Meulendijks et al. it may be assumed that patients with a concentration of pretreatment serum uracil >16 ngml⁻¹ are at increased risk of developing severe and fatal toxicity related to the treatment with fluoropyrimidine [33]. Therefore before any DPD phenotype-guided dosing can be implemented in clinical practice, further prospective studies are required and established the appropriate cut-off values.

The most recommended strategies to increase the sensitivity of an upfront test is a DPYD genotyping and DPD phenotyping combination approach [33-34].

Even now when we know some of DPYD variant that can increase the risk of severe toxicity due to treatment with fluoropyrimidines, a negative test for these variants does not guarantee that a patient is DPD proficient. A screening only by genotyping has a low sensitivity to identify patients at risk for toxicity, and it is estimated that only a half of subjects with DPD deficiency can be identified by genotyping for the four variants (DPYD*2A, DPYD*9B, DPYD*13, and c.1236G >A/Haplotype B3) [34-38].

The French National Network of Pharmacogenetics recommend that if genotyping has found a partial deficiency for DPYD*2A, DPYD*9B or DPYD*13 variants, the dose should be reduced by 50% for the first cycle of chemotherapy [40]. If a complete deficiency is found by genotyping, fluoropyrimidines are contraindicated and another therapy should be administered. The Dutch Pharmacogenetics Working Group recommend using an alternative drug for homozygous patients and for heterozygous patients a reduced dose or alternative drug [39].

If the deficiency is demonstrated by phenotyping methods, the dose can be reduced in correlation with the degree of deficiency, by 25% to 75%. Also, if tolerance is observed after the first cycle of chemotherapy, the dose can be increased [40].

At present, despite all studies linking DPD deficiency to fluoropyrimidine related toxicity, pharmacogenetic testing for DPYD PNS is not described in the drug label. DPD deficiency is suggested in the summary of product characteristics of Capecitabine, in both the European Union and United States of America, but pre-testing is not recommended.

Even if the pre-testing is not recommended due to the small incidence of the DPD deficiency, in December 2015 the Food and Drug Administration (FDA) approved uridine triacetate (Vistogard) for use in emergency cases of overdose or life-threatening adverse reactions in adult and pediatric patients who undergo chemotherapy based on 5-fluorouracil or Capecitabine. Uridine triacetate is the first and the only chemotherapy antidote [41,42].

Conclusion

Fluoropyrimidine-based chemotherapy is still the first line treatment in many types of cancers even in our days when targeted therapy and immunotherapy is increasingly used. The importance of choosing the right patient who will benefit the most from treatment is an important public health issue with implications not only for the patient's life, but also for the health economy. By providing the right treatment for each patient according to

their own personal features we will be able to increase the effectiveness of our treatments, minimize the side effects and thus control the progression of the disease.

Despite all this data, more studies are needed, several new variants allelic to be searched, many other enzymes which can be investigated in order to diagnose those cases that are negative. Also, a phenotypic approach could be easier implemented in general practice and perhaps the time has come for one of these methods to become routine practice.

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