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# Endogenous plasma and salivary uracil to dihydrouracil ratios and *DPYD* genotyping as predictors of severe fluoropyrimidine toxicity in patients with gastrointestinal malignancies

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ABSTRACT

**Objective:** The aim of this study was to evaluate the use of plasma and saliva uracil (U) to dihydrouracil ($UH_2$) metabolic ratio and *DPYD* genotyping, as a means to identify patients with dihydropyrimidine dehydrogenase (DPD) deficiency and fluoropyrimidine toxicity.

**Methods:** Paired plasma and saliva samples were obtained from 60 patients with gastrointestinal cancer, before fluoropyrimidine treatment. U and $UH_2$ concentrations were measured by LC–MS/MS. *DPYD* was genotyped for alleles **7*, **2A*, **13* and *Y186C*. Data on toxicity included grade 1 to 4 neutropenia, mucositis, diarrhea, nausea/vomiting and cutaneous rash.

**Results:** 35% of the patients had severe toxicity. There was no variant allele carrier for *DPYD*. The $[UH_2]/[U]$ metabolic ratios were 0.09–26.73 in plasma and 0.08–24.0 in saliva, with higher correlation with toxicity grade in saliva compared to plasma ($r_s = -0.515$ *vs* $r_s = -0.282$). Median metabolic ratios were lower in patients with severe toxicity as compared to those with absence of toxicity (0.59 *vs* 2.83 saliva; 1.62 *vs* 6.75 plasma, $P < 0.01$). A cut-off of 1.16 for salivary ratio was set (AUC 0.842), with 86% sensitivity and 77% specificity for the identification of patients with severe toxicity. Similarly, a plasma cut-off of 4.0 (AUC 0.746), revealed a 71% sensitivity and 76% specificity.

**Conclusions:** *DPYD* genotyping for alleles *7*, **2A*, **13* and *Y186C* was not helpful in the identification of patients with severe DPD deficiency in this series of patients. The $[UH_2]/[U]$ metabolic ratios, however, proved to be a promising functional test to identify the majority of cases of severe DPD activity, with saliva performing better than plasma.



## 1. Introduction

Since its introduction >50 years ago, 5-fluorouracil (5-FU) has become a component of the standard therapy for a variety of solid tumors, particularly gastrointestinal cancers [1]. The standard approach for calculating 5-FU dosage is based on patient's body surface area (BSA). Unfortunately, there is no rigorous scientific basis for this strategy, which has been associated with considerable variability in plasma 5-FU levels [2]. Considering an optimal target exposure of 20–30 $mg \cdot h \cdot L^{-1}$, only 20% to 30% of patients have 5-FU levels that are in the appropriate therapeutic range, while approximately 40% to 60% of patients are under-dosed and 10% to 20% over-dosed. Such inter-patient as well as intra-patient pharmacokinetic variability is a major contributor to patient toxicity and treatment failure [2]. Previous studies revealed that up to 30% of patients experience severe toxicities following 5-FU administration, with 0.5% to 3% toxic deaths [3–6]. The most common adverse reactions after 5-FU administration include neutropenia, fever, mucositis, nausea, vomiting, diarrhea and hand-foot syndrome [7].

As dihydropyrimidine dehydrogenase (DPD) accounts for about 80% of the metabolic inactivation of 5-FU, DPD deficiency has been recognized as an important risk factor predisposing patients to the development of severe toxicity. In a retrospective study, Ciccolini et al. demonstrated that 71% of severe toxicities and 80% of toxic deaths

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could be related to functional DPD deficiency [8]. Thus, the assessment of individual DPD activity has been proposed as a means to individualize 5-FU administration before the first dose [4,8–12].

DPD is encoded by the gene *DPYD* and variants in *DPYD* may cause a decreased DPD activity. Although prospective *DPYD* genotyping was suggested as a valuable tool to identify patients with DPD deficiency, and thus at risk for severe and potential life-threatening toxicity, results of genotyping studies have not yet been fully implemented in daily clinical care. Genotypic studies focusing on the identification of *DPYD* polymorphisms have produced so far controversial results. Some studies reported that variant allele carriers are at increased risk of developing severe toxicity [9,13–15], while other reports fail to confirm such association [8,16,17]. Furthermore, using current genotypic methods, *DPYD* gene abnormalities has been identified in about 5% of cases [16].

Alongside *DPYD* genotyping, several phenotypic methods have been proposed for establishing, indirectly, the presence or absence of a DPD deficiency status. These approaches have manly focused on the measurement of endogenous plasma uracil (U) to dihydrouracil ($UH_2$) ratio [8–10,18,19] or, alternatively, the ratio at defined time points after administration of a loading dose of U [20,21]. Monitoring the [$UH_2$]/[U] ratio and comparing it with a toxicity cut-off value, determined from a reference population, should allow the detection of patients at risk and subsequently lead to a possible dose adjustment [4]. Recently, Carlsson et al. described the evaluation of endogenous [$UH_2$]/[U] ratios in saliva of patients under fluoropyrimidine chemotherapy, describing significant differences between patients with or without severe toxicity [22]. However, the authors had not evaluated the diagnostic performance of the test.

In the presence study, our objective was to evaluate and compare two DPD phenotyping strategies, namely using endogenous [$UH_2$]/[U] ratios in plasma and saliva, as well as *DPYD* genotyping for most common inactive variants **2A*; **13* [23] and *Y186C* [24], as predictive tests for fluoropyrimidine toxicity in patients with gastrointestinal malignances.

## 2. Methods

### 2.1. Study population and data collection

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee of Hospital de Clínicas de Porto Alegre (approval number 402.139). After informed consent, 60 adult patients diagnosed with digestive cancer (colorectal, stomach or pancreas) were enrolled in the study between July 2014 and July 2015. All patients were scheduled for adjuvant, neoadjuvant or palliative chemotherapy the fluoropyrimidine-containing regimens FOLFOX (folinic acid + 5-FU + oxaliplatin), FOLFIRI (folinic acid + 5-FU + irinotecan), FOLFIRINOX (folinic acid + 5-FU + irinotecan + oxaliplatin), 5-FU-cisplatin, LV-5-FU (leucovorin + 5-FU), CapeOx (capecitabine + oxaliplatin) and capecitabine. No patient had received a fluoropyrimidine before. Patients with kidney, liver or heart dysfunctions and prolonged use of corticosteroids were excluded. Demographic data were recorded.

Blood and saliva samples were taken before the first chemotherapy cycle for the evaluation of DPD functional status and genotyping analysis. Venous blood samples were drawn into tubes containing EDTA as anticoagulant, with patients being fasten for 8 h. The saliva sample was collected at the same time by chewing on for 2 min the cotton wool swabs of a commercial saliva collecting device (Salivette®, Sarstedt, Germany). Immediately after, the collecting device was centrifuged and the saliva transferred to a 2 mL polypropylene tube. Sample collection was performed between 8:00 and 9:00 a.m. Plasma, saliva and whole blood samples were stored at −70 °C until analysis. [$UH_2$]/[U] metabolic ratios were calculated after the quantification of U and $UH_2$ in plasma and saliva samples by liquid chromatography–tandem mass spectrometry (LC–MS/MS).

Toxicity was monitored using the standard Common Terminology Criteria for Adverse Events (CTCAE) v. 4.0, including mild (grade 1), moderate (grade 2) and severe (grade 3 and 4) neutropenia, mucositis, diarrhea, nausea/vomiting, cutaneous rash or other 5-FU related adverse event. Blood cell counts were performed two days prior to the chemotherapy cycle. Toxicities were recorded when observed between first and third chemotherapy cycles, within 3 weeks of the infusion.

### 2.2. Determination of U and $UH_2$ concentrations in plasma and saliva

Plasma and saliva concentrations of U and $UH_2$ were measured by LC–MS/MS after a simple liquid-liquid extraction, according to Hahn et al. [25]. Briefly, a 500 μL aliquot of plasma or saliva was transferred to 5 mL polypropylene tube added with 100 μL of internal standard solution 5-fluorouracil (5-FU) 0.2 μg $mL^{-1}$, followed by 30 s of vortex mixing. After, proteins were precipitated with 500 mg of ammonium sulfate, followed for 1 min of vortex mixing. The resulting mixture was added with 3.5 mL of a mixture of ethyl acetate and isopropanol (85:15, *v*/*v*) and mixed for 10 min in a rotatory mixer at 50 rpm. After 10 min centrifugation at 3000*g*, 3 mL of the supernatant was transferred to an evaporation tube and dried at 60 °C under vacuum. The dried extract was reconstituted with 100 μL of ultrapurified water and centrifuged for 10 min at 10,000*g*. An aliquot of 25 μL of supernatant was injected into an Ultimate 3000 XRS UHPLC system (Thermo Scientific, San Jose, USA). Separation was performed in an Acquity C18 column (150 × 2.6 mm, p.d. 1.7 μm) from Waters (Milford, USA), maintained at 10 °C and eluted at a mobile phase flow rate of 0.2 mL $min^{-1}$. Column chilling was necessary to increase retention of U and $UH_2$, as previously described [26]. The mobile phase consisted of acetic acid 0.5% (eluent A) and acetonitrile plus 0.1% formic acid (eluent B). Initial eluent composition was 96% A, maintained for 2.5 min, and followed by a linear 1.0 min ramp to 50%, which was maintained for 2.0 min. The mobile composition returned to 96% A at 6.5 min. Equilibration time was 4.5 min. Detection was performed in a TSQ Quantum Access triple quadrupole mass spectrometer (Thermo Scientific, San Jose, USA). The MS conditions were as follows: electrospray ionization (ESI), positive mode, capillary voltage of 4 kV; sheath gas, nitrogen at a flow rate of 60 arbitrary units; auxiliary gas, nitrogen at flow rate of 50 arbitrary units; collision gas, argon; vaporizer temperature, 202 °C; and ion transfer capillary temperature, 250 °C. The scan time was set at 0.1 s per transition. The following transitions were used for MRM acquisition: U *m*/*z* 113 → 70 (quantitation), 113 → 40 and 113 → 96 (qualification); $UH_2$ *m*/*z* 115 → 70 + 115 → 30 (quantitation) and *m*/*z* 115 → 55 (qualification); 5-FU *m*/*z* 131 → 114 (quantitation), 131 → 58 and 131 → 67 (qualification collision energies were 13, 33 and 14 eV for U; 15, 17 and 19 eV for $UH_2$; 13, 26 and 14 eV for 5-FU). The method was linear from 5 to 1000 ng $mL^{-1}$ with LLOQ of 5 ng $mL^{-1}$ for U and 10 ng $mL^{-1}$ for $UH_2$, in both matrices. Accuracy was in the range of 91.3–103.4% and 89.9–103.1%, and within and between assay coefficients of variation were in the range of 3.8–10.4% and 2.9–8.7% for plasma (N = 45) and saliva (N = 45), respectively. Daily calibration curves were processed in all analytical batches. Internal quality control samples (QCL and QCH) were processed every 10 samples. It is also important to point out that the assay was developed to evaluate patients before initiation of therapy, and no 5-FU would be present. Otherwise, a washout period must be observed.

### 2.3. DPYD genotyping

Genomic DNA was extracted and purified from 200 μL of whole blood using PureLink® Genomic DNA Mini Kit (Invitrogen) according to manufacturer's directions. The DNA concentration was measured using Qubit® 2.0 Fluorometer (Cat. no. Q32866). Patients were tested for four *DPYD* polymorphisms *DPYD *2A* (IVS14 + 1G>A, rs3918290); **13* (1679T>G, I560S, rs55886062), *Y186C* (rs115232898), and **7* (rs72549309). Genotyping was performed using a TaqMan® Genotyping