UNIVERSITÀ DEGLI STUDI DI PADOVA

# UNIVERSITA' DEGLI STUDI DI PADOVA

## DIPARTIMENTO DI SCIENZE DEL FARMACO

**CORSO DI LAUREA MAGISTRALE IN CHIMICA E TECNOLOGIA FARMACEUTICHE**

TESI DI LAUREA

# Personalized therapy for oral anticancer drugs: an integrated approach based on therapeutic drug monitoring and pharmacogenetics for sunitinib and imatinib

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ANNO ACCADEMICO 2021/2022

*Questo lavoro di tesi è stato svolto presso la Struttura Operativa Complessa di Farmacologia Sperimentale e Clinica del Centro di Riferimento Oncologico di Aviano (Istituto di Ricovero e Cura a Carattere Scientifico).*

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# 1 INTRODUCTION

## 1.1 Oral anticancer drugs

Oral anticancer drugs which have been approved in the last two decades play a key role in the treatment of multiple type of cancer. These orally administered anticancer drugs have contributed to improve treatment outcomes and provide considerable advantages for patients, because of the more convenient way of application compared with intravenously administered drugs (1). Nevertheless, there are multiple factors that have been recognized as limit the benefit of these anticancer agents.

As of April 2020, 71 oral antineoplastic drugs (OADs) targeting a large set of molecular targets have been approved by the European Medicines Agency (EMA) and/or the US Food and Drug Administration (FDA). With more OADs available, both the route of administration and the treatment typology are changing. OADs allow outpatient care with both its advantages and disadvantages: strong advantages are the level of independence and reduction of health care costs; at the same time, the responsibility for adhering to treatment schedules and to take the correct dose is moved to the patient. Given the often-complex treatment regimens, patients must be well trained and motivated to take their medication. For this reason, with oral anticancer drugs patient compliance became of primary importance in the treatment (2).

Many oral targeted therapies are characterized by complex pharmacological profiles, due to their oral formulation which involves a complex pharmacokinetics. This leads to a high interindividual variability in OADs exposure. Consequently, fixed dosing results in a wide range of plasma drug concentrations between patients, and most importantly, many of these new oral targeted therapies have a narrow therapeutic window: this means that the area between toxicity curve and efficacy curve is very small and, therefore, there is a small range of therapeutic concentrations.

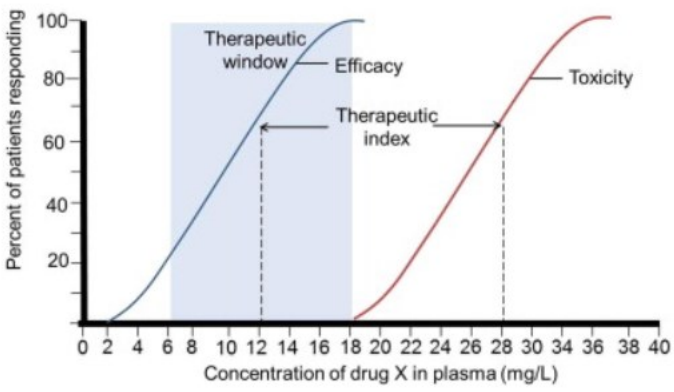


Figure 1: representation of therapeutic window of drug, correlating plasma concentrations and efficacy.

### 1.1.1 Small therapeutic index of OADs

As a result, the problem with this OADs and the currently used fixed dose, is that ± 30% of patients with cancer are being underdosed (drug concentrations are under the efficacy curve) and hence drug may not be fully effective; whereas another ± 15% of patients are currently being overdosed (above the curve of toxicity) potentially resulting in excess of drug, which can develop several types of toxicities. For this reason, precision/personalized medicine would not only include selecting the right drug, but also selecting the right dose (3). The correct dose can be obtained by selecting the right starting dose for each individual patient, "right-dose-first-time" paradigm, after which this dose can be further optimized by PK-guided dosing (adjusting the dose based on measured drug concentrations, taking count of the pharmacogenetic profile, and evaluating drug-drug interactions). This point makes important and useful a monitoring of the drug's PK during therapy, that is called TDM (therapeutic drug monitoring).

The significant inter-individual variability of the therapeutic response, together with other distinctive features of anticancer agents, makes this class of drugs really good candidate for TDM practice.

## 1.2 Tyrosine kinase inhibitors (TKIs)

Imatinib and sunitinib are the two OADs analysed in this work. As reported in the last paragraph, these drugs are OADs administered at a fixed dose, this entails high inter-patient variability in exposure for patients treated with imatinib and sunitinib.

These two drugs belong to the class of Tyrosine kinase inhibitors (TKIs), anti-cancer drugs that target tyrosine kinases. TKI are enzymes involved in multiple cellular processes which catalyses the transfer of a phosphate group from ATP to a specified molecule. This functionality may cause other molecules to become either active or inactive. Kinases are a part of many cellular processes; some cancer treatments target certain kinases that are linked to that specific type of cancer.

Receptor tyrosine kinases (RTKs), a cell-surface receptors family, transduce signals to polypeptide and protein hormones, cytokines, and growth factors;

these are key regulators of critical cellular processes such as proliferation and differentiation, cell survival and metabolism, cell migration and cell cycle control. In the human genome, RTKs have been identified, which fall into 20 families.

The parent TKI is imatinib, which targets the BCR-ABL fusion kinase that is responsible of chronic myelogenous leukaemia (CML). Constitutive BCR-ABL signalling activates well-characterized oncogenic pathways including Ras/ERK, Jak Stat, PI3K, and c-MYC.

Although, the normal function of the BCR protein is still unclear/unknown, ABL activation is overexpressed in several tumours and is heavily implicated in cancer cells growth and survival (4).

Imatinib inhibits the BCR-ABL protein by binding himself to the ATP pocket in the active site: it binds with high affinity to the inactive form of the kinase domain, blocks ATP binding, and thereby inhibits the kinase activity of the enzyme by interrupting the transfer of a phosphate from ATP to tyrosine residues on substrate proteins, thus preventing downstream phosphorylation of the target protein. The development of imatinib to target the BCR-ABL tyrosine kinase has been one of the greatest advancements of cancer therapy.

The aberrant chromosome which results in expression of the BCR-ABL as well as activate a number of signalling pathways, affects integrin function: this causes an impair of cell-cell contact resulting in decreased sensitivity of CML progenitor cells to the influence of bone narrow stromal cells.

The stem cell receptor tyrosine kinase KIT in normal cells promotes physiological functions, including haematopoiesis, melanogenesis and gametogenesis, as well as growth and differentiation of mast cells and interstitial cells of Cajal. KIT also plays an important role in development human cancers: activating mutations of KIT occur up to 90% of gastrointestinal stromal tumours (GISTs), underlie GIST transformation (5). Approximately 35% of GISTs lacking KIT mutations have instead activating mutations of platelet-derived growth factor receptor alfa (PDGFRa); other dysregulated tyrosine kinases may serve as substrates for imatinib such as FIP1L1-PDGFRα associated hyper eosinophilic syndrome and COL1A1/PDGFRβ associated with dermatofibrosarcoma protuberans.

For this pathway, imatinib is indicated, firstly, for the treatment of adult and pediatric chronic myeloid leukemia with Philadelphia chromosome mutation (Ph+) in blast crisis, accelerated phase, or chronic phase after IFN-alpha therapy

failure(6). Additionally, imatinib is also approved for the treatment of:

- adult and pediatric Ph+ acute lymphoblastic leukemia
- adult myelodysplastic/myeloproliferative diseases
- adult aggressive systemic mastocytosis
- adult hypereosinophilic syndrome and/or chronic eosinophilic leukemia (CEL)
- adult dermatofibrosarcoma protuberans
- malignant GIST.

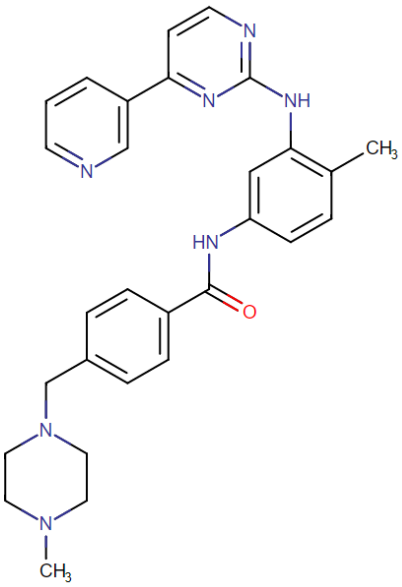


Figure 2: imatinib chemical structure

Sunitinib, the other drug analysed in this work, is also a TKI inhibitors, which has more target receptors compared to imatinib.

Sunitinib was firstly approved by the U.S. FDA in January 2006 for advanced renal cell cancer (RCC) (7). Since then, it has been approved globally for this indication and for patients with imatinib-resistant or -intolerant GIST and advanced pancreatic neuroendocrine tumours. Historically, before the introduction of targeted therapies, cytokine-based therapy with interferon-*a*(IFN) and/or interleukin-2 (IL-2) was considered standard first-line treatment for patients with metastatic RCC (mRCC). However, the response rates were low (only approximately 15%), survival was limited, and treatment-related toxicities restricted their usage. In addition, previous studies of other therapies for cytokine-refractory patients with RCC were unable to show benefits (7).

Sunitinib is used for its inhibitory activity against a variety of kinases (>80 kinases) and is identified as an inhibitor of platelet-derived growth factor

receptors (PDGFRa and PDGFRb), vascular endothelial growth factor receptors (VEGFR1, VEGFR2 and VEGFR3), stem cell factor receptor KIT (CD117), the RTK that drives the majority of GIST; Fms-like tyrosine kinase-3 (FLT3), colony stimulating factor receptor Type 1 (CSF-1R), and the glial cell-line derived neurotrophic factor receptor (RET).

The formation of new blood vessels is a highly regulated process in which proangiogenic factors work in concert with anti-angiogenesis factors. Two of the key mediators, both from a mechanistic and from a therapeutic perspective, are the vascular endothelial growth factor (VEGF) and the platelet derived growth factor (PDGF). The binding of these ligands to their receptors located in endothelial cells results in the activation of the RTK that will trigger the activation cascade of several intracytoplasmic signal transduction mediators. This will lead to the proliferation of new endothelial cells and pericytes, recruitment of endothelial cell precursors, and the generation of new capillaries (8). For this reason, targeting these receptors is a winning strategy for an anticancer therapy.

Sunitinib inhibition of the activity of these RTKs has been demonstrated in biochemical and cellular assays, and inhibition of function has been demonstrated in cell proliferation assays (9).

For this mechanism of action, sunitinib is indicated for:

- Treatment of adult patients with GIST following disease progression (or intolerance) on imatinib, as a second line treatment.
- Treatment of adult patients with advanced RCC.
- Adjuvant treatment of adult patients at high risk of recurrent RCC following nephrectomy.
- Treatment of progressive, well-differentiated pancreatic neuroendocrine tumors (pNET) in adult patients with unresectable, locally advanced or metastatic disease.

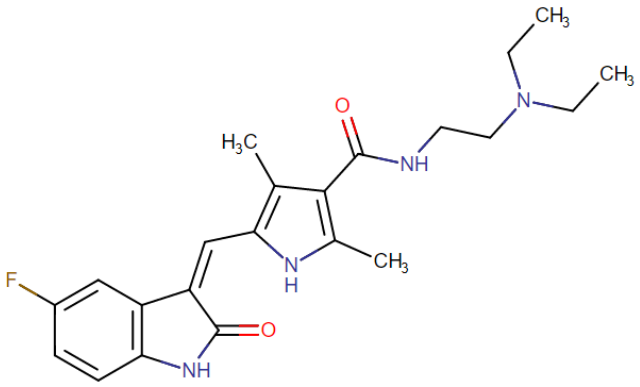


Figure 3: sunitinib chemical structure

Currently, multiple oral TKIs have been introduced in the treatment of solid tumours: all are administered at a fixed dose, although large interpatient pharmacokinetic (PK) variability is described. For both imatinib and sunitinib exposure-treatment outcome (efficacy and toxicity) relationships have been established and therapeutic windows have been defined (2); therefore, dose optimization based on the measured blood concentration (PK data), called therapeutic drug monitoring (TDM), can be valuable in increasing efficacy and reducing the toxicity of these anticancer drugs.

### 1.2.1 Metabolism and pharmacokinetics of imatinib

Pharmacokinetic studies done in healthy patients, CML patients, GIST and other cancers showed that orally administered imatinib is well absorbed, it has an absolute bioavailability of 98% irrespective of oral dosage form (tablet, capsule) nor dosage setting (100 mg, 400 mg) and the food has no relevant impact on the bioavailability of the drug. The terminal elimination half-life is approximately 18 hours; imatinib plasma concentrations increase by 2- to 3-fold when reach the steady state with daily administration, to 2,6 ± 0,8 μg/mL at peak and 1,2 ± 0,8 ug/mL at trough, exceeding the 0,5 ug/mL (1 μmol/L) concentrations needed for tyrosine kinase inhibition in vitro. Imatinib is

approximately 95% bound to human plasma proteins, mainly albumin and α1-acid glycoprotein and it is eliminated predominantly via the bile in the form of metabolites.

Imatinib is primarily metabolized by CYP3A4 and CYP3A5 to its active metabolite, N-desmethylimatinib (NOR-IMA)(10); also CYP2C8 can metabolize this conversion, and in patients with liver disease and compromise CYP3A4, CYP2C8 is able to maintain imatinib clearance.

In vitro studies showed capability of CYP3A4, CYP1A1, CYP4F2 to form NOR-IMA (11): it has a similar potency to that of the parent drug; the elimination half-lives are approximately 13 hours for imatinib and 20 hours for the metabolite. Other CYPs, such as CYP1A2, CYP2D6, CYP2C9 and CYP2C19 are listed in the drug label and FDA documentation because they have a minor role but have not been verified in peer-reviewed studies. Two n-oxide metabolites of imatinib, imatinib pyridine n-oxide (CGP 72383) and imatinib piperdine n-oxide (CGP 71422) have also been identified in patient urine 2 hours post-dose but were not observed at 24 hours after dosing (5).

Imatinib is transported by P-glycoprotein (ABCB1) and Breast Cancer Resistance Protein (BCRP/ABCG2). With respect to ABCB1, among the patients homozygous for allele 1236C>T 85% achieve a BCR-ABL level 3 log reduction when treated with imatinib, versus 47.7% for the other genotypes. The mechanism of resistance to imatinib appears to be due to a variety of factors, including BCR-ABL gene amplification, mutations in the protein that could alter binding or over-expression of transporters.

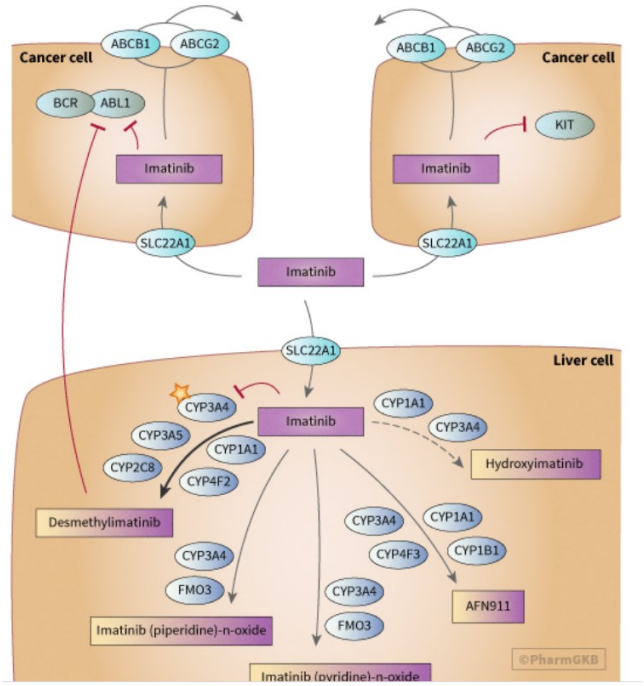


Figure 4: imatinib first metabolism and transporter.

The metabolism of imatinib may be decreased and increased when it is co administered with drugs that inhibit, induce or are major substrate of CYP3A4 and CYP3A5. Major inducers of CYP3A4 and CYP3A5 activity may increase metabolism and decrease the exposure to imatinib: these drugs are for example carbamazepine, rifampicin, hypericum, barbiturates, phenytoin, dexamethasone, phenobarbital. Other interactions are found with the inhibitors: protease inhibitor as indinavir, lopinavir/ritonavir, saquinavir, telaprevir, nelfinavir, boceprevir; or antifugal as ketoconazole, itraconazole, voriconazole; macrolides as erytromicin, clarithromycin e telytromicin; they can reduce imatinib metabolism and can increase plasma concentrations. Drugs which are substrates of CYP3A4 and CYP3A5 (as ciclosporin, simvastatin, tacrolimus, pimozide, ergotamine) are influenced by imatinib coadministration and their plasma concentration can be reduced.

### 1.2.2 Metabolism and pharmacokinetics of sunitinib

Pharmacokinetics studies of sunitinib denote no differences between healthy volunteers and tumour patients, including patients with gastrointestinal stromal tumour (GIST) and metastatic renal cell carcinoma (MRCC).

Maximum plasma concentrations (Cmax) of sunitinib are generally observed between 6 and 12 hours (Tmax) following oral administration. Food has no effect on the bioavailability of sunitinib, for this reason it can be taken with or without food.

Sunitinib is metabolized mainly by the cytochrome P450 family, particularly CYP3A4, to produce its primary active metabolite N-desethyl sunitinib (N-DES SUN), which is then also metabolized by CYP3A4. N-DES SUN includes 23 to 37% of the total exposure to sunitinib.

The binding of sunitinib and N-DES SUN to human plasma protein in vitro is 95% and 90%, respectively, with no concentration dependence in the range of 100 – 4000 ng/mL. The apparent volume of distribution Vd for sunitinib is 2230 L. In the dosing range of 25 – 100 mg, the area under the plasma concentration-time curve (AUC) and Cmax increase proportionately with dose(12).

Following administration of a single oral dose in healthy volunteers, the terminal half-lives of sunitinib and N-DES SUN are approximately 40 to 60 hours and 80 to 110 hours, respectively. With repeated daily administration, sunitinib accumulates 3- to 4-fold while N-DES SUN accumulates 7- to 10-fold. Steady-state concentrations of sunitinib and its primary active metabolite are achieved from the 11 to 14 day of therapy. In Day 14, combined plasma concentrations of sunitinib and N-DES SUN ranged from 62.9 – 101 ng/mL (therapeutic window). No significant changes in the pharmacokinetics of sunitinib or N-DES SUN are observed with repeated daily administration or with repeated cycles in the dosing regimens tested(13).

Elimination occurs via feces (61%), and by renal elimination (16% of the administered dose). Sunitinib and its active metabolite were the major drug-related compounds identified in plasma, urine, and feces, representing 91.5%, 86.4% and 73.8% of radioactivity in pooled samples, respectively. Minor metabolites were identified in urine and feces but generally not found in plasma. Sunitinib is neither an inhibitor nor an inducer of CYP-enzymes, therefore the drug is considered not prone to drug-drug and drug-food interactions, while other TKIs (e.g., imatinib, erlotinib, gefitinib) appear to be substrates and/or inhibitors of several CYP-enzymes in vivo and in vitro.
Nevertheless, it is not recommended the administration of sunitinib with the strong CYP3A4 inhibitor (ketoconazole, voriconazole, indinavir, bocepevir) resulted in 49% and 51% increases in the combined (sunitinib + primary active

metabolite) Cmax and AUC values, respectively, after a single dose of sunitinib in healthy volunteers. A dose reduction for sunitinib should be considered when it must be co-administered with strong CYP3A4 inhibitors.
On the other hand, administration of sunitinib with the strong CYP3A4 inducer (rifampin, rifabutin, phenytoin, hypericum, carbamazepine) resulted in a 23% and 46% reduction in the combined (sunitinib + primary active metabolite) Cmax and AUC values, respectively, after a single dose in healthy volunteers. A dose increment for sunitinib should be considered when it must be co-administered with CYP3A4 inducers (13).

## 1.3 Therapeutic drug monitoring

The science of TDM introduced a new aspect of clinical practice in the 1960s with the publication of initial pharmacokinetic studies linking mathematical theories to patient clinical outcomes (14). Since that studies, clinical pharmacokinetics emerged as a discipline in the late 1960s and early 1970s.
TDM is the clinical practice of measuring specific drug concentrations in a designated interval to have a tracking of patient's concentrations during a defined period of therapy. It is used mainly for monitoring drugs with narrow therapeutic windows, drugs with marked pharmacokinetic variability, medications for which target concentrations are difficult to monitor, and drugs known to cause adverse effects (14).
TDM approach is used with the assumption that there is a relationship between concentration (drug exposure) and therapeutic effects and/or toxicity. With this instrument, it's possible to collect patient samples during his/her treatment period and then verify if the drug concentration is within the therapeutic window or not (15).
TDM has been used in the clinical treatment with the aim of simultaneously maximizing the therapeutic effect and minimizing the occurrence of toxicity. Nowadays, this approach is applied in the pharmacological treatment with antibiotics, cardiovascular drugs, antiepileptics, antidepressants, anti-inflammatory agents, airway smooth muscle relaxants, immunosuppressants and chemotherapeutic agents.

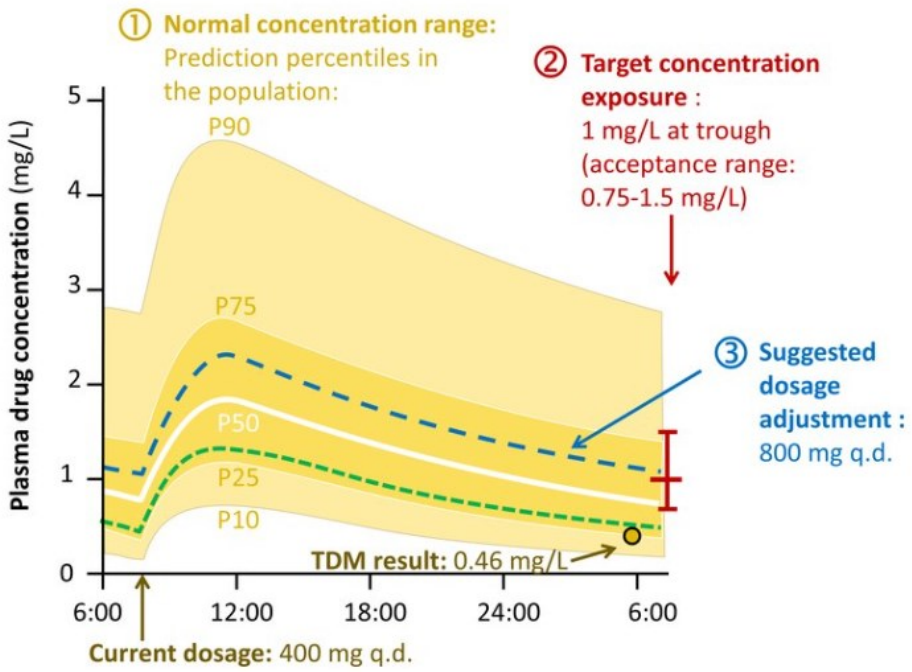


Figure 5: TDM results in plasma concentration among a population.

### 1.3.1 TDM as multidisciplinary approach

TDM applications required a multidisciplinary approach: accurate drug concentrations useful for a correct drug monitoring can be obtained only by the collaboration of a TDM team.

This team includes different professional figures according to the different steps involved in TDM practice: collection of blood samples, usually performed by a nurse who can also be responsible of correct timing of sampling for Cmin evaluation; samples analysis (the gold standard technique in this field is liquid chromatography tandem mass spectrometry LC-MS/MS) that is carried out by an analyst to measure drug concentrations in the biological fluid. Once this data is obtained, the pharmacokinetic parameter can be calculated and/or interpreted according to information reported in literature: this task is the responsibility of a pharmacologist/pharmacist in order to provide the correct interpretation of the value to be useful to the physician to monitor the therapy and, ideally, to adjust the drug dosage accordingly.

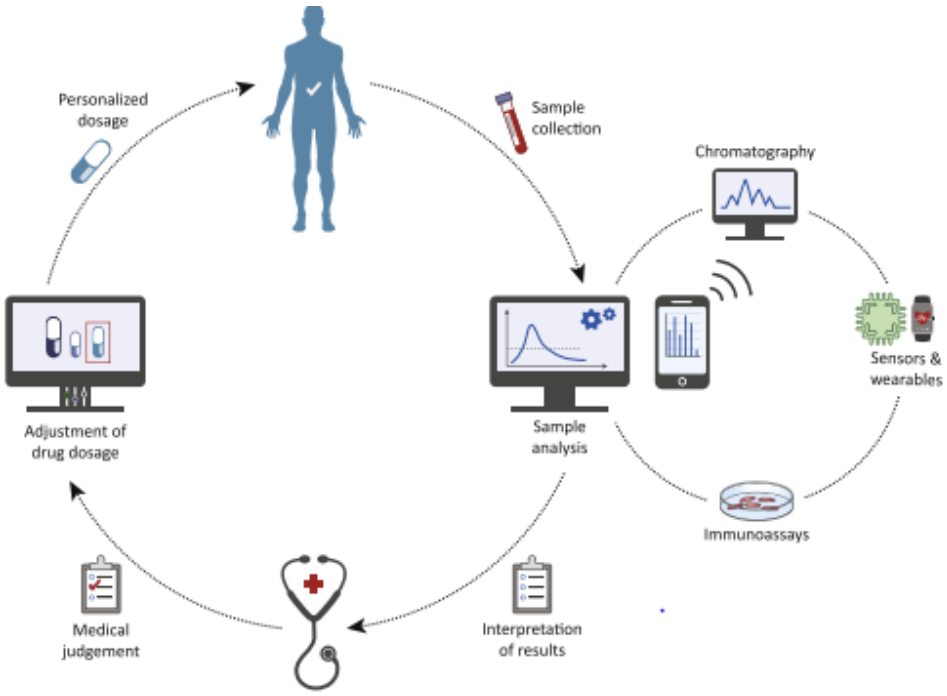


Figure 6: TDM procedure

## 1.4 LC-MS/MS in TDM approach

Nowadays, the reference analytical method for the determination of drug concentration in biological samples is the liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS)(16). In fact, LC enables the components of a complex matrix (for example whole blood, serum, or plasma) to be separated with high reproducibility and precision, while a mass spectrometer in tandem configuration provides information regarding both the identity and the exact amount of the analyte present in the biological matrix.

The LC-MS/MS methods can simultaneously quantify different molecules and, fort this reason, has become a diffuse technique applied in many laboratories.

The chromatographic process that allows the separation of compounds contained in a mixture, is based on different physicochemical properties characterizing each compound which need to be separated, such as mass, charge, or polarity. The principle of the chromatography is the separation of the analytes under the same conditions and their different distribution between the two phases: one fixed in the column, named stationary phase (SP), and the eluent that flows through it, called mobile phase (MP).

The technique adopted in this project is the reverse phase (RP) separation, it is used for the separation of lipophilic molecules, like most of drugs, because the

prevailing retention mechanism is due to hydrophobic interactions. The selectivity is therefore based on the differences in hydrophobicity between the molecules to be separated. During the chromatographic process, lipophilic molecules carried by the MP are retained on the SP which fills the chromatographic column: SP presents similar characteristics with the analytes, in contrast with the MP. The SP is usually constituted by a matrix (composed, depending on the uses, by silica polymer or a silica-polymer hybrid) derivatized with apolar chains of various length (e.g., octilsilyl, octadecilsilyl, phenyl, etc.).
Connected to HPLC there is the mass spectrometer: MS is a technique that allows the detection of substances based on the ratio between the molecular mass and the charge ($m/z$), with an accuracy that reach 0.01%.
The first step in MS is the ionisation of the neutral compound of interest, mainly through electron ejection, electron capture, protonation, deprotonation or adduct formation. This process takes place into the source, and it is essential because only charged molecules can be detected by the spectrometer. A variety of ionisation technique can be used for MS: electron ionisation (EI), chemical ionisation (CI), atmospheric-pressure chemical ionisation (APCI), Matrix assisted laser desorption/ionisation and electrospray ionisation (ESI) which is the source of the spectrometer used for this study. From the source, the produced ions are transferred to the analyser, where they are discriminated according to their $m/z$ values. In order to separate ions according to their $m/z$ ratio, several types of mass analysers have been developed: some separate ions in space, others by time. Among these types of analysers, coupled to an ESI-type source, a triple quadrupole can generally be found to perform tandem MS analysis for quantitative purposes.

### 1.4.1 TDM in the clinical practice

TDM refers to the individualization of drug dosage by maintaining plasma or blood drug concentrations within a targeted therapeutic range or window (14). The goal of this process is to individualize therapeutic regimens for optimal patient benefits.

In pharmacotherapy, many medications are generally used without monitoring blood levels, as their dosage can usually be varied according only to the clinical response that a patient gets to that substance. For certain drugs, this is impracticable, because insufficient levels will lead to undertreatment (be underdosed) which can affects the efficacy, and excessive levels can lead to develop toxicities and tissue damage (17).

Indications in favor of therapeutic drug monitoring include:

- Consistent and clinically established pharmacodynamic relationships between plasma drug concentrations and pharmacological efficacy and/or toxicity.
- Significant pharmacokinetic variability between patients: the standard dosage achieve different concentration levels among patients (while the drug disposition remains relatively stable in a given patient).
- Narrow therapeutic window of the drug, which prohibits giving high doses in all patients to ensure the efficacy.
- drug dosage optimization that cannot be achieved only based on clinical observation alone.
- potential patient compliance problems that might be remedied through concentration monitoring.

TDM determinations are also used to detect and diagnose poisoning with drugs if the suspicion arises.

Examples of drugs widely analyzed by therapeutic drug monitoring:

- Aminoglycoside antibiotics (gentamicin)
- Antiepileptics (such as carbamazepine, phenytoin and valproic acid)
- Mood stabilisers, especially lithium citrate
- Antipsychotics (such as pimozide and clozapine)
- Digoxin

- Ciclosporin, tacrolimus in organ transplant recipients

For some agents, TDM has already proven feasible (2). Strong evidence exists for imatinib in CML and GIST. Additional compounds, for which TDM resulted feasible in prospective studies are sunitinib, pazopanib, tamoxifen and abiraterone.

In Mueller-Schoell et al. (2) (Table 1) the current evidence for TDM-guided dosing of OADs are reported. In this review, each drug was classified according to the level of evidence available for TDM. If there is an established exposure-response relationship and a PK target, TDM is considered potentially useful. If additionally, a feasibility study has been performed, TDM is recommended. If randomized, prospective studies demonstrated a positive effect of TDM, it is strongly recommended. If there is no evidence for an exposure-response relationship, TDM is considered exploratory. If there are minimal data on the PK of a drug, there are more useful targets than plasma concentration or there is evidence that TDM is not useful, it is not recommended (2).

For both imatinib and sunitinib TDM is classified as recommended.

| Evidence level | Recommendation | Description |
|---|---|---|
| 1 | Strongly recommended | Randomised, prospective studies demonstrated positive effect of routine TDM with regards to efficacy and/or safety. |
| 2 | Recommended | There is an established exposure-response relationship using standard dosage from retrospective studies, a target is established **and** a feasibility study has been performed. |
| 3 | Potentially useful | An exposure-response or exposure-safety relationship using standard dosage has been identified and a potential target has been reported. |
| 4 | Exploratory | An exposure-response or exposure-safety relationship using standard dosage has been identified but no target has been reported. |
| 5 | Not recommended | No exposure-response or exposure-safety relationship using standard dosage has been identified **and/or**<br>- there is very few data on pharmacokinetics of the drug;<br>- there are more useful targets than plasma concentration (PD);<br>- there is evidence that TDM is not useful |

Table 1: Evidence for TDM for OADs.

### 1.4.2 Application of TDM for imatinib and sunitinib- available guidelines

TDM targets means concentrations to which refer to define patient's drug exposure, an established range of concentrations where the exposition is within the therapeutic window.

For most drugs, TDM target is based on exposure–efficacy analyses. If these analyses were not available (yet), the mean or median exposure of the drug was taken as a reference (15). In fact, for the compounds with TDM targets based on exposure–efficacy analyses, the PK targets amounted to 81%–85% of the average population exposure. Therefore, targeting the mean or median concentration will generally leads to an efficacious exposure. In the meantime, thorough exposure–efficacy analyses will be awaited, which can provide a definitive target for TDM.

Imatinib and sunitinib have consistent and clinically established relationships between plasma drug concentrations and pharmacological efficacy and/or toxicity, therefore they are suitable candidates for TDM clinical practice.

As related to imatinib, in a retrospective analysis of a randomized phase II study conducted by Demetri et al. (18) patients with GIST and treated with imatinib showed a better overall survival when the imatinib trough level was ≥1100 ng/mL. Since then, several other groups have established the same target exposure for imatinib associated with better treatment outcome (19), (20). These evidences lead to the publication of consensus guidelines for TDM of imatinib by the *International Association of Therapeutic Drug Monitoring and Clinical Toxicology* (IATDMCT) (21). Moreover, while consensus suggests that $C_{min}$ of ≥1100 ng/mL is needed for imatinib efficacy, trough concentrations >3000 ng/mL have been correlated with adverse effects, resulting in treatment interruption. In fact, several relationships between imatinib concentrations and toxicity, including Cmin with thrombocytopenia (22) and AUC with absolute neutrophil count decrease, have been established. A trend towards higher incidences of hematological Grade 3/4 adverse events (Aes) for patients with very high Cmin (>3000 ng/mL) was reported.

TDM for sunitinib is generally performed using the sum of concentrations (total Cmin) of both sunitinib and N-DES-SUN. The target total trough plasma concentrations of SUN plus active metabolite N-DES-SUN have been deduced from preclinical data and are in the range of 50–100 ng/mL (23). Subsequently, it was shown that total trough levels >50 ng/mL was associated with an objective response in a small cohort of patients with advanced solid tumor treated with sunitinib 50 mg once daily for 4 weeks followed by a 2-week drug holidays (24). In patients treated with sunitinib 37.5 mg continuously daily dosing, the minimal total trough levels of 37.5 ng/mL have been recommended, based on dose proportionality of plasma exposure at therapeutic doses. However, target trough concentrations for sunitinib plus active metabolite have not been confirmed in retrospective or prospective studies. Faivre et al. (24) showed that dose limiting toxicities were often most seen in patients with sunitinib doses >75 mg/day and sunitinib + N-DES-SUN trough concentrations >90 ng/mL, respectively. Dose limiting and Grade 3 toxicities of sunitinib have been associated with total Cmin 100 ng/mL. Grade 2 mucositis and altered taste have also been related to higher total Cmin. A relationship was also found between sunitinib AUC and Grade 3 toxicity (P 5 0.0005). Based on the above, an upper Cmin cut-off of 100 ng/mL could be considered (24).

### 1.4.3 Pharmacogenetics and other factors influencing drug exposure

Several factors may influence the systemic exposure: variability in oral drug absorption and metabolism, as reported before, drug–drug interactions (DDI), food–drug interactions, patient non adherence, and decrease of plasma exposure over time during the first months of treatment (25).

The number of potential DDI increases exponentially with the number of taken drugs, making it hard to consider all drug interactions in polypharmacy patients, as patient with cancer (26). A retrospective study in 898 patients treated with oral antitumor agents identified potential drug-drug interactions in 46% of the patients (1); in 16% of the patients, these were considered to be of

major clinical relevance. Medication errors can also occur due to lack of required screening.
The time interval between drug intake and meals is also a critical factor for approximately 50% of the oral anticancer drugs since absorption can be increased or decreased by concomitant food intake and drug exposure may be influenced.

Imatinib and sunitinib are both metabolized by cytochromeP450 enzyme (CYP) 3A4 in the liver, which is involved in metabolism of approximately 70% of the oral anticancer drugs. Therefore, combination with agents that inhibit, induce or are major substrate of CYP3A4 may lead to increased or decreased plasma exposure of the TKIs. Moreover, imatinib is substrate of CYP2C8, CYP2D6 and of the transporter enzyme P-glycoprotein, which may add to the interpatient variability observed due to drug–drug interactions, drug-gene-interactions, and polymorphisms.

These latter in the field of pharmacogenetics, which detects genetic information such as single nucleotide polymorphisms (SNP) of metabolism enzymes, haplotypes, microsatellites or simple sequence repeats, insertion and/or deletion, copy number variations and tumour tissues, with the aim of discover patient's genotype and explain some metabolic behaviours.

Guiding the dose by using pharmacogenetics offers an additional strategy for treatment individualization, as the initial dose can be adjusted based on polymorphisms in the genes encoding for metabolizing enzymes and drug efflux transporters. Nevertheless, variability in exposure will remain, as genotype is only one of the many factors affecting exposure. In fact, measured drug concentrations are the translation of all these factors, allowing for better precision dosing. As this work seeks to implement, these two approaches should be combined by first selecting the right starting dose based on pharmacogenetics, which could then be further optimized by PK-guided dosing. However, if one of the two approaches should be preferred over the other, PK-guided dosing takes into account all factors that introduce variability, including pharmacogenetics.

Nowadays, do not exist pharmacogenetics guidelines for imatinib and sunitinib, but only explorative data. Is nevertheless plausible that SNPs impacting on gene encoding for metabolizing enzymes or transporter play a crucial role in pharmacogenetics analysis.

Since many factors may influence the plasma exposure of imatinib and sunitinib, it is not possible to predict whether an individual patient will reach an adequate plasma exposure using a standard fixed dose of the drug, hence exposure is necessarily influenced by the fixed dose, as reported in the first paragraph.

## 1.5 Pharmacogenetics as a tool to predict plasma exposure

Pharmacogenetics (PG) has been defined as "the study of variability in drug response due to heredity" and should help in reducing morbidity and mortality caused by drugs. This field also emphasizes the development of novel drugs based on newly discovered genes, as the entire human genome becomes sequenced.

The term "pharmacogenetics", which denote the study of genetic factors that influence response to drugs and chemicals, was first coined in 1959 (27). Since the beginning of this millennium, large-scale human genetic studies have been transformed pharmacogenetics or pharmacogenomics into potential clinical disciplines. Many effective drugs, including anticancer drugs, have been studied in different patients and different tumour categories.

Patient's genetic information has been used to forecast disease risk, choosing treatment agents and drug dosage. Pharmacogenetics or pharmacogenomics detects genetic information such as single nucleotide polymorphisms (SNP) of human metabolism enzymes, haplotypes, microsatellites or simple sequence repeats, insertion and/or deletion, copy number variations of human metabolism enzymes and tumour tissues (26).

As it was said at beginning of the introduction, plasma concentrations, drug

responses and toxicities of anticancer drugs can vary considerably among different patients, tumour tissues which are given the same dosages of anticancer drugs; this happens because of genetic variations or genetic polymorphisms in drug metabolism enzymes or in the cancer biomarkers.

The purpose of PG is to predict or to investigate the fraction of active or inactive metabolites, the required dosage of a drug, and the possible sensitivity of tumours to anticancer drugs. This purpose is completed by analysing the genetic status of the drug metabolism enzymes or targeted cancer molecules, or by targeting the biomarker status of the cancer tissues. There are several different metabolites of anticancer drugs in human blood or plasma, and they are created by many human metabolizing enzymes.

Overall, pharmacogenetics or pharmacogenomics are an effort to maximize efficacy and minimize toxicities of drugs among patients.

PG represents the study of human genetic factors affecting drug toxicities or responses in treated patients. One human metabolizing enzyme can be affected by genetic polymorphism, as a result some anticancer drugs cannot produce enough active drug metabolites, hence tumour inhibition of these drugs will be reduced. On the other hand, active anticancer drugs may be more quickly detoxicated or excreted by human metabolizing enzymes: if these human metabolizing enzymes are inactivated by the genetic polymorphism of enzyme genes, the active anticancer drugs will be more intensively accumulated in blood and plasma in human bodies. These patients will show the strong toxicity, some of them even life-threatening(28). For this reason, it is extremely useful to investigate patient's polymorphisms or variations which can affect metabolism and transport of this drugs, firstly understanding which SNPs relate to the specific drug, which gene are implicated in the drug's metabolism and which co-administered drugs can modify anticancer plasma concentrations using some cytochrome to be metabolise.

For many cancer therapy PG protocols or applications are available, for other OADs need to be improved in future (28).

### 1.5.1 SNPs

A single nucleotide polymorphism, or SNP (pronounced "snip"), is a variation at a single position in a DNA sequence among the population.

Remembering basic information about DNA, it is known that the DNA sequence is formed from a chain of four nucleotide bases: A, C, G, and T, every DNA sequence has its complementary in the strand (DNA duplex). If more than 1% of a population does not carry the same nucleotide at a specific position in the DNA sequence, this variation can be classified as a SNP (29).

SNPs represent the most common type of polymorphisms in human population and may be useful in association studies (as they may be functionally relevant) and/or might be in linkage disequilibrium with other variants, which may have different functionally effect.

The number of discovered SNPs has increased tremendously during the past few years. Public SNP databases (dbSNPs) are a highly valuable resource of information about polymorphisms in the candidate genes. At present, several dbSNPs exist in public domain, their SNP content significantly overlaps but also sometimes complements. The dbSNP of National Centre for Biotechnology Information is one of the central repositories for newly discovered genomic and cDNA sequence variations, both single base changes and short deletions and insertions (30).

If a SNP occurs within a gene, then this gene is described as having more than one allele; in these cases, SNPs may lead to variations in the amino acid sequence and thus in the protein code. SNPs, however, are not just associated with genes; they can also occur in noncoding regions of DNA (intron zone).

Although a particular SNP may not cause a particular disorder, some SNPs are associated with certain diseases: these associations allow scientists to look for SNPs in order to evaluate an individual's genetic predisposition to develop a disease. In addition, if certain SNPs are known to be associated with a trait, then scientists may examine stretches of DNA near these SNPs to identify the gene or genes responsible for the trait.

SNP which may not cause a disorder can caused variations in protein transcription, modifying the expression and the functionality of the resulting protein/enzyme. If this protein is involved in drug ADME (adsorption, distribution, metabolism, and eliminations), pharmacokinetics will change, and different metabolic phenotype can be identified. If the protein it is involved in the drug's target the efficacy can be compromise, but during my thesis period my work was focused on SNP involved in drug metabolism.

When the SNP belongs to hepatic cytochrome it can affect the functionality of this enzyme; nowadays 4 types of metabolic phenotypes has been identified:

- poor metabolizer, the patient whose metabolism is lower/less functional than the other.
- Intermediate metabolizer when the cytochrome's activity is between the poor and normal metabolizer.
- normal metabolizer when the patient's enzyme works normally and the drug are metabolized correctly, with the common alleles on the gene involved.
- rapid metabolizer who metabolizes the drug quicker than the population and in elevate quantities, plasma concentration of drug is lower; for the last.
- ultrarapid metabolizer whose cytochrome works very fast instead of the population, entailing the lowest plasma concentration of the drug.

# 2 AIMS OF THE PROJECT

Oral anticancer drugs are highly potent drugs, which have substantially changed treatment options for patients with cancer. In the treatment of several cancers, OADs have moved away from the use of cytotoxic drugs and non-specific chemotherapy to a chronic oral treatment with targeted molecular therapies. These treatments are characterised by unique mechanisms of action and are highly specific for single or multiple key cellular biological pathways, implicated in the cancer process.

OADs show a high interpatient variability in pharmacokinetics, leading to large differences in drug exposure. For many of these drugs, exposure has been linked to efficacy and toxicity. Despite this knowledge, these drugs are still administered in a one-size-fits-all approach with a long-term chronic administration of a fixed drug dose, without requirement of a pharmacological monitoring. Consequently, individual patients have a high probability to be either chronically underexposed to the drug, which can lead to decreased antitumor efficacy, or overexposed, which could potentially result in increased risk of toxicity (2).

Another remarkable aspect is that this variability in drug exposure for almost all OADs is at least in part related to monooxygenases of the Cytochrome P450 (CYP) family metabolism (31), which belong to first pass metabolism, and trans-cellular transport mediated by ATP-binding cassette (ABC) transporter family. Both those gene classes are characterized by highly polymorphic isoenzymes as in example *CYP3A4, CYP3A5*, and *ABCB1* contributing to the interindividual variability in drug exposure. The expression/activity of CYP and ABC enzymes may be additionally influenced by concomitant administration of inducers/inhibitors agents, environmental factors, smoking and food intake.

This existing multi-causal inter-individual variability in OADs exposure needs to be addressed: the clinical study of “Farmacovigilanza Attiva”, ongoing at the Experimental and Clinical Pharmacology of CRO-Aviano, in which I have taken part, proposes to apply a combined pharmacological approach including a pharmacogenetic frame joined with a therapeutic drug monitoring and check of

drug-drug interaction with the aim of improving the clinical outcomes of a list of selected OADs.

Specifically, my work of thesis was focused on two drugs in the list (imatinib and sunitinib), presenting robust data and clinical guidelines supporting a therapeutic drug monitoring approach to reduce underdosing and overdosing (21). For those drugs, data on the role of CYPs and ABCs pharmacogenetics in determining a differential drug exposure are also available.

The primary aim of the thesis was to verify the feasibility of therapeutic drug monitoring of imatinib and sunitinib in the clinical practice to drive patients with cancer clinical management.

Secondary aim was an exploratory analysis of the potential association between pharmacogenetics variants in ADME related genes and the plasmatic drug level of imatinib and sunitinib.

To reach the thesis aims the following activities were carried on:

1. Participate in patients' enrolment, interviews for clinical, demographic, and quality of life data collection (including co-administered drugs), and support for biological sampling for pharmacological analyses.
2. Collaborate in TDM data generation and interpretation. Specifically, I observed the revalidation of an LC-MS method of quantifications and samples analysis for sunitinib and its active metabolite to obtain plasma concentration of the drug.
3. Contribution to the selection, method set-up and analysis of a set of genetic polymorphisms, involved in sunitinib and imatinib metabolism; work in the SNP line assaying cytochrome polymorphisms in order to understand the metabolic profile of the patients, always with the perspective of personalise therapy.
4. Exploratory correlation between pharmacogenetics and drugs plasmatic exposure.

# 3 MATERIALS AND METHODS

In the Clinical Pharmacology Unit of CRO Aviano a pharmacological counselling service for patients undergoing treatment with TKIs was started.
The patient samples analysed in this work of thesis derive from two prospective clinical studies carried on at the Clinical Pharmacology Unit of CRO Aviano. Patients treated with sunitinib were enrolled in "Farmacovigilanza attiva" a study started in June 2022, still ongoing. For imatinib samples collection started in 2015 within a previous clinical study entitled "*Pilot study to evaluate the feasibility of an innovative approach to monitor patients with gastrointestinal stromal tumour treated with imatinib*" and continued with the "Farmacovigilanza attiva" study, together with sunitinib patients.
Patients were requested to sign an informed consent for the use of the diagnostic data for research purposes.
Clinical data concerning the disease stage, the molecular profiling, the treatment regimen and setting, drug dose, adverse drug reactions, co-administered treatments, biographical information, and quality of life data are recorded from the patient and from electronic medical record in the clinical software of CRO Aviano.
Ten millilitres of blood were collected as protocols methodology in K2-EDTA containing tubes at the time of regular medical check-ups, every 3–6 months. Plasma was separated by centrifugation and stored at -80 °C until LC-MS analysis. Genomic DNA was extracted from the harvested buffycoat by means of the GeneJET Whole Blood Genomic DNA Purification Mini kit (Thermo Fisher Scientific, Wilmington, DE, USA) and quantified by Quantus Fluorometer (Promega, Madison, WI, USA). Genomic DNA was stored at 4C.

## 3.1 Sample's collection for TDM

Blood samples collection has been done in respecting important kinetic rules.
To obtain solid PK data, samples should be collected with high accuracy: if the PK value that I want to product will be reliable, attention must be paid to the timing of blood sampling, the type of blood sample, the measurement technique,

and the results interpretation. Several PK parameters can be used for TDM purpose, such as AUC (area under the concentration vs time curve), Cmax (maximum concentration), and, specifically for metronomic oral drugs, Cmin, parameter used in this work of thesis.

For an adequate evaluation of drug Cmin, a prerogative is to obtain the blood sample for measuring the drug concentration at the correct time after dosing: errors in the timing of sampling are likely responsible for most of errors in interpreting the results and can also produce an incorrect data.

)(14).

The correct phase of distribution in which drug has been collected is when drug reach the steady state, which is a concept of fundamental importance in pharmacology. Following repeated administration of a drug, a steady state is reached when the quantity of drug eliminated in the unit of time equals the quantity of the drug that reaches the systemic circulation in the unit of time. The steady state describes a dynamic equilibrium in which drug concentrations stay within therapeutic limits for long, potentially indefinite, periods. The concentration around which the drug concentration consistently stays is known as the *steady-state concentration*, and this is the concentration used in the TDM approach(32).

Reaching a steady-state concentration is generally necessary for effective pharmacological action on the diseases. An example would be antibiotics for the treatment of infectious diseases (33).

For these reasons, blood samples collection for the evaluation of Cmin at the steady state has been done in respecting the following indications:

- Since imatinib is taken every day without interruption, steady state is reached after 4 days from the therapy start. Imatinib blood samples has been preferably collected 24 hours from the last assumption, to ensure the correct quantification of imatinib and NOR-IMACmin. If the blood collection was not performed with this timing, the following formula, previously validated by Wang et al. (34), was used to calculate imatinib and nor-imatinib Cmin:

$$C_{\mathrm{min,SS}} = C_{\mathrm{measured}} * 0.5^{\frac{\text{Dosing Interval [h]} - \text{Time after last dose[h]}}{\text{Half-life [h]}}} \quad [2]$$

where $C_{measured}$ is the actual measured drug concentration; dosing Interval

is, for once daily administration, 24h; half-life correspond to 19 h for imatinib and 40 h for nor-imatinib. The samples collected up to 5 h or after 35 h from the last imatinib administration were excluded from the analysis as they were outside the algorithm's range of linearity.

The cadence of imatinib withdrawals was about 5-6 months, because oncological examinations have this schedule.

- In the case of sunitinib, the proper samples collection at the steady-state resulted more challenging, because sunitinib and N-DES-SUN reach steady-state 10-14 days after the first intake, and the drug schedule used at CRO is 2 weeks on and 1 weeks off: for this reason, the blood collection must be precisely done in the last 4 days of therapy (a narrow range of suitable days for Cmin evaluation). Sunitinib blood samples were also preferably collected 24 hours form the last assumption, for Cmin proper evaluation. Anyway, also in this case, if this timing for blood collection was not respected, the previous formula [2] was used.

  The cadence for sunitinib withdrawals was 1 month, because oncological examinations have this tighter schedule.

## 3.2 LC–MS/MS quantification of IMATINIB and NOR-IMA plasma concentrations

Quantification of imatinib and NOR-IMA was performed using a liquid chromatography with tandem mass spectrometry (LC-MS/MS) instrument consisting of a Prominence LC-20 AD UFLC XR(Shimadzu, Tokyo, Japan) and an API 4000 QTrap mass spectrometer (SCIEX, Framingham, MA, USA).

Imatinib and NOR-IMA has been quantified after a sample preparation: simple protein precipitation using acetonitrile (ACN) as an extraction method and the analytes are separated on a Synergi Fusion RPC18 chromatography column 4µm, 50 x 2.0 mm coupled to a C18precolumn (Phenomenex, Torrence, CA, USA). Elution has been performed in gradient mode chromatography. The mass spectrometer is equipped with an electrospray ionization (ESI) source interface and operated in positive ion mode. The biological samples have been analysed in selected reaction monitoring mode. Quantifications were performed using the

following transitions: m/z 494.4 > 394.2 for imatinib, m/z 480.4 > 394.2 for nor-imatinib and m/z 502.4 > 394.2for imatinib-D8, employed as an internal standard. The developed method was validated according to FDA and European Medicines Agency (EMA) guidelines for validation of bio-analytical methods, evaluating linearity, recovery, limit of detection, limit of quantification, matrix effect, inter and intra-day precision and accuracy, selectivity, stability and reproducibility (35).

## 3.3 LC-MS/MS quantification of Sunitinib and N-DES-SUN plasma concentrations

From an analytical point of view, the most challenging peculiarity of SUN is represented by its photo-isomerization in solution. In fact, SUN and N-DES SUN are 5-fluoro-2-oxindoles linked to a dimethyl pyrrole carboxamide by an exocyclic double bond. Z-isomer is the thermodynamically stable form due to the presence of an intramolecular hydrogen bond between the C2 carbonyl group of the oxindole and the NH group of the pyrrole ring. Conversely, the E-isomer is characterized by an extensive steric hindrance due to 3D spatial contacts of the substituted benzene and pyrrole rings leading to a non-planar molecule. The pharmacological active substance of Sutent© (capsules for oral assumption) is Z-SUN, which is photostable in solid form. The analysis of the content of Sutent capsules conducted in protection from light confirmed that the E-SUN accounted for less than 2% (1.90 ± 0.66%), in our condition (36).
The light induced isomerization reaction of both SUN and N-DES-SUN only occurs ex vivo in plasma samples and other solutions: thus, during collection, handling, and analysis of biological samples containing these compounds particular precautions are necessary. A comprehensive study of Z- to E-SUN photo-transformation kinetics in solvent was firstly conducted by Maafy and Lee (37). On the basis of these results, many published methods overcame the isomerization problem by conducting the blood sampling and the whole handling procedures under light protection in order to avoid the formation of E-isomer: e.g., use of opaque tubes, sample manipulations conducted quickly and with minimal light exposure, which, surely does not represent a suitable option in the perspective of an analytical assay applicable to clinical practice.

In this work, following the procedure reports by B. Posocco at al. (36), the quantitative reconversion of the E-isomer to the Z-form was exploited, a procedure applicable to analytical methods for the quantification of SUN and its metabolite in human plasma samples.

The quantification in this work of thesis was performed with the high-performance liquid chromatography–tandem mass spectrometry method for the determination of sunitinib and N-DES SUN in human plasma, published by E. Marangon et al. (38), it was used applying some modifications:

- Eight working solutions, instead of seven, has been done for the preparation of calibrators (from H to A).
- The calibration range has been modified: from 5 to 500 ng/mL for sunitinib (instead of 0,1-500 ng/mL) and from 1 to 100 ng/mL for N-DES SUN (instead of 0,1-250 ng/mL). The Lower limit of quantification (LLOQ) was increased for the current analysis.
- During sample preparation a step of dilution has been added to the preparation.

The addition of the dilution step determined a change of the matrix sample; for this reason, a qualitative re-evaluation of the matrix effect was conducted by means of the post column infusion. In addition, a intra-day precision and accuracy test of the method was performed

### 3.3.1 Standards and chemicals

Analytical reference standards of SUN malate (N-[2-(Diethylamino)ethyl]-5-[(Z)-(5-fluoro-1,2-dihydro-2-oxo-3H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrole-3-carboxamide (2S)-2-hydroxybutanedioic acid (1:1) salt, batch 030M4706V, chemical purity ≥98%) was purchased from Sigma-Aldrich Co. (Milan, Italy) while N-DES SUN free base (N-[2-(Ethylamino)ethyl]-5-[(Z)-(5-fluoro-1,2-dihydro-2-oxo-3H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrole-3-carboxamide, batch S820002, chemical purity 96%) and the stable isotope labelled internal standard (IS) SUN d-10 (N-[2-(Diethyl-d10)aminoethyl]-5-[(Z)-(5-fluoro-1,2-dihydro-2-oxo-3H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-

pyrrole-3-carboxamide; batch D289650, chemical and isotopic purity 96 and 99,4% respectively) were purchased from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). Dimethyl sulfoxide (DMSO), LC-MS grade acetonitrile (MeCN) and formic acid (FA) were purchased from Sigma-Aldrich Co.; LC-MS grade methanol (MeOH) was purchased from VWR (Radnor, Pennsylvania, USA); type 1 ultrapure water ($H_2O$) was obtained from a Milli-Q Advantage A10 system (Millipore, Billerica, MA, USA). Control human plasma/$K_2$-EDTA, used to prepare daily calibration curve and quality control samples (QCs) for the validation study was prepared by mixing plasma samples from 16 healthy volunteers (8 males and 8 females) to reduce the bias of the variability between matrices. Each plasma sample was provided by the transfusion unit of the National Cancer Institute (Aviano, Italy).

### 3.3.2 Preparation of calibrators and quality control samples

Two different sets of stock solutions for both SUN and N-DES SUN were prepared in DMSO at a concentration of 0,05 mg/mL. By mixing and diluting the first set with ACN, eight working solutions for the preparation of calibrators were obtained with the following concentrations: 100, 200, 500, 1500, 4000, 6000, 8000, 10000 ng/mL and 20, 40, 100, 300,800, 1200, 1600, 2000 ng/mL (from H to A) for SUN and N-DES SUN respectively. The stock solutions belonging to the second set were mixed and diluted with ACN obtaining three working solutions for the preparation of quality controls (QCs) with the following concentrations: 300, 5000, 8500 ng/mL and 60, 1000, 1700 ng/mL (QCL (Low), QCM (Medium) and QCH (High) respectively) for SUN and N-DES SUN, respectively. The stock solution for IS was prepared in DMSO at a concentration of 0,05 mg/mL and was diluted in ACN to 100 ng/mL obtaining the IS working solution. All the solutions were kept in polypropylene tubes and stored at -80 °C.

An eight-point plasma calibration curve was prepared freshly every day during the validation study. For each calibrator, 95 µL of pooled human plasma were spiked with 5 µL of the respective working solution (from H to A to obtain the final concentrations reported in Table 2)

Each calibration curve included a blank (no analytes, no IS) and a zero-

calibrator (blank plus IS). QCs were processed at least in triplicate for each concentration level and to prepare them the same procedure of calibrators (reported above) was followed (obtaining the final concentrations reported in Table 2).

| | sunitinib | | N-desetil |
|---|---|---|---|
| | final conc. (ng/mL) | | final conc. (ng/mL) |
| **h** | 5 | **h** | 1 |
| **g** | 10 | **g** | 2 |
| **f** | 25 | **f** | 5 |
| **e** | 75 | **e** | 15 |
| **d** | 200 | **d** | 40 |
| **c** | 300 | **c** | 60 |
| **b** | 400 | **b** | 80 |
| **a** | 500 | **a** | 100 |
| **QCL** | 15 | **QCL** | 3 |
| **QCM** | 250 | **QCM** | 50 |
| **QCH** | 425 | **QCH** | 85 |

Table 2: final plasma concentrations of calibrators and QCs for both SUN and N-desethyl-SUN

### 3.1.1 Sample preparation

Each plasma sample was thawed at room temperature, vortex-mixed for 10 seconds and centrifuged at 2'450 g for 10 min at nominally 4°C. Calibrators and QC (prepared as reported above) or a 100 µL-aliquot of the patient sample, were transferred to a 1.5 mL Eppendorf polypropylene tube (Eppendorf, Amburg, Germany), spiked with 5 µL of the IS working solution (100 ng/mL) and the resulting mixture was vortex-mixed for 5 seconds. Then, each sample underwent a protein precipitation by adding 500 µL of pure MeOH and, after having vortex-mixed it for 10 seconds, a 10 min centrifugation at 16'200 g was performed to allow the separation of the protein residue from the supernatant. From each tube, 200 µL of the obtained clear supernatant were transferred to a second 1.5 mL Eppendorf polypropylene tube and added with 200 µL of MilliQ water. Finally, the mixture was transferred to a Waters borosilicate glass vial with a pre-slit PTFE cap (Waters, Milford, USA).
Since all the sample handling steps described above occur without any light-protection, the additional step was introduced to revert the isomerisation and thus to obtain only the active Z-isomer (36): the vials containing the supernatant were heated at 70 °C for 5 min with stirring at 100 rpm in a Vortemp 56 (Illumina, San Diego, USA). Then, the transfer from the bath to the autosampler represents the only step, in the entire sample handling procedure, that needs to be performed in the dark. To address the carry-over effect, after the injection of the ULOQ, three mobile phase samples were injected. This procedure guaranteed that no peak higher than 20% of LLOQ was detected. For the same reason, the same number of mobile phase samples were injected between successive real samples.

## 3.3.3 Chromatographic conditions

The HPLC system consisted of a Prominence UFLC XR composed by a SIL-20AC XR auto-sampler, two LC-20AD XR pumping modules, two FCV-11AL solenoid valve units, a DGU-20A3 degasser, a CBM-20A system controller and a CTO-20AC column oven (Shimadzu Corporation, Tokyo, Japan). Samples were separated on a Synergi Fusion-RP 4 µm (80 Å, 50 x 2 mm) chromatographic column coupled with a 4 x 2 mm Fusion-RP (Phenomenex, California, USA) pre-column and thermostatically controlled at 50°C. As far as the chromatographic

method is concerned, the flow rate was kept constant at 0.3 mL/min following a multi-step gradient elution:
1) 90% MPA (initial condition) kept constant for 0.5 min.
2) from the initial condition to 30% MPA over 1 min and kept constant for 1.2 min.
3) from 30% MPA to 60% over 0.3 min and from 60% MPA to initial condition over 0.5 min.
4) reconditioning for 4 min. The mobile phases (MPs) used for chromatographic separation were 0.1% HCOOH in $H_2O$ (A) and 0.1% HCOOH in MeCN (B).
The total run time was 7.5 min.

### 3.3.4 Mass spectrometric conditions

The HPLC system was coupled to an API 4000 QTrap, a triple quadrupole mass spectrometer (SCIEX, Massachusetts, USA). The instrument was equipped with a Turbo Ion Spray source operating in positive ion mode The source temperature was set at 625°C, and the ion spray voltage at 5500 V. Zero air was employed as nebulizer gas (30 psi) and as heater gas (70 psi) while nitrogen as curtain gas (20 psi) and collision gas (CAD) (at medium intensity).
Each quantitative analysis was performed in multiple reaction monitoring (MRM) by following three MRM transitions for each analyte: one qualifier transition was exploited for the quantification while two qualifier transitions were monitored in order to increase the specificity. All data were processed with Analyst 1.6.3 while peaks integrations were performed with MultiQuant 2.1 software package (AB SCIEX).

### 3.3.5 Post column infusion

During the partial validation study, matrix effects on the quantification of the two analytes, SUN and N-DES SUN, were tested.
These phenomena arise due to effects of plasma matrix endogenous components on the ionization of the analytes to quantify and IS. These phenomena related to plasma matrix endogenous components were investigated by means of the post-column infusion: a constant flow of standard solutions of SUN, N-DES SUN and IS, each prepared in 0.1% HCOOH acetonitrile/water 1:1 (50 ng/mL), were

infused by a syringe pump during the chromatographic run of an extracted pooled blank human plasma sample. The extracted plasma sample eluted from the LC column and the flow from the infusion pump were combined by means of a zero-dead-volume 'T' union and inserted into the mass spectrometer source. A variation in the signal response of the infused analyte, caused by the coeluted interfering compounds, indicates ionization enhancement or suppression.

### 3.3.6 Accuracy and precision

The precision and accuracy of the presented method were evaluated by analysing 5 replicates of QC samples and LLOQ within a single-run analysis for intra-day assessment. The method precision, at each concentration, was reported as the coefficient of variation (CV%).0

CV% was defined expressing the standard deviation as a percentage of the mean calculated concentration. The accuracy of the method was determined by expressing the mean calculated concentration as a percentage of the nominal concentration.

The measured concentration had to be within 15% of the nominal value (20% for LLOQ).

## 3.4 Pharmacogenetic analysis

### 3.4.1 SNPs selection

SNPs tested for the pharmacogenetic analysis consists of 33 variants for 7 pharmacogenes selected according to different criteria:

- genes that are implicated in the pharmacokinetics of TKIs inhibitors, as reported in the "imatinib/sunitinib metabolism" paragraph, differentiating for some pharmacogenes which are implicated only in the pathway of imatinib and not in sunitinib's one.
- polymorphisms for which there are guidelines drawn up by the CPIC including treatment recommendations for a specific genotype.
- the literature search showed that the genetic variant has a significant functional impact in the protein/enzyme encoded by one of the genes that could be involved in pharmacokinetic profile of index drug.
- the minor allele frequency (MAF) in the Caucasian population is ≥1% referring to an archive of simple genetic polymorphisms called Single Nucleotide Polymorphism database (dbSNP).

Pharmacogenes selected for imatinib are: CYP3A4, CYP3A5, CYP2D6, CYP2C8, CYP1A2, ABCB1, ABCG2, SLC22A1, SLC1B3, considering its ADME pathway including transporter.

Pharmacogenes selected for sunitinib are: CYP3A4, CYP3A5, ABCB1, ABCG2, CYP1A2, considering the different pathway in respect of imatinib and its different metabolism.

### 3.4.2 Collection, extraction, and quantification of DNA

Two 7.5 mL blood's tubes are drawn from each patient; these tubes contain EDTA, used as an anticoagulant, necessary to prevent clotting processes inside the tubes. This can happen because of the presence of calcium ions interacting with the negatively charged walls of the tube, which can induce a conformational change that activates the clotting factors: this phenomenon is called "contact activation". Therefore, anticoagulants such as EDTA are very

important, it can act as an irreversible calcium chelator preventing the clotting process.

Every sample is processed at room temperature; using the Eppendorf 5810R bench-top centrifuge, a centrifuge is performed at 1855 RCF (Relative Centrifugal Force: "xG") for 10 minutes with brake. The separation of the plasma is achieved with this first step, then will be possible to see plasma with the corpuscular part stratified on the bottom of the tube: above the ring of white blood cells and platelets (buffy coat) and below the red blood cells.

The supernatant (plasma) is collected under a biological hood, taking care to aspire only plasma, no buffy coat, and then transferred to three 1,5 tubes for storage. The buffy coat is also gathered, and 1 mL is aliquoted into two 1,5 mL tubes for storage too.

Centrifugate the sample is essential to separate the white blood cells (which contain DNA) from the plasma and erythrocytes since they lack a cell nucleus, so they don't have DNA inside.

The tubes containing buffy coat and plasma are stored at -80 °C according to the storage protocol.

### 3.4.3 . DNA extraction

This is the essential step for all the genetic analyses: to obtain the DNA.

Obtaining high-quality and high-concentrated, intact genomic DNA is the first and most critical step in molecular applications; generally, this extraction/purification process is characterized by the following four phases:

- *Cell lysis:* performed by various techniques such as osmotic shock, repeated freeze-thaw cycles, enzymatic or detergent-mediated lysis and by mechanical methods such as blade homogenizer, Ultra-Turrax or ultrasonication and others.
- *Inactivation of cellular nucleases:* (DNase and RNase), necessary to prevent DNA degradation. Lowering the temperature can be exploited but are mainly used a mixture of chelating agents such as EDTA and EGTA, and protease inhibitors such as PMSF.

- *Separation of genomic DNA:* this step can be exploited by using organic solvents such as phenol or chloroform, or by using the adsorption of DNA on a silica gel matrix thanks to chaotropic agents or by magnetic particles.
- *Recovery of purified DNA:* by ethanol, isopropanol, depurated water, or TE buffer.

These different steps can be performed by manual extraction or with an automated extraction: in this work it has been used only manual extraction.

Manual DNA extraction is performed by using the Thermo Scientific GeneJETTM Whole Blood Genomic DNA Purification Mini Kit K0871 50prep (Thermo Fisher Scientific, US).

The procedure has been designed to exploit silica-based membrane technology insert in a little spin column, and the procedure takes about 20 minutes.

Whether starting from whole blood or buffy coat, first step is always enzymatic cell lysis by the proteinase K, an enzyme capable of cleave carboxyl ends of hydrophobic, aliphatic and aromatic amino acids, allowing a broad spectrum of activity. Proteinase K is essential as it purifies the nucleic acids from the contaminating proteins which are contained in the sample, and inactivates the DNases and RNases released during the subsequent addition of the lysis solution. Additionally, it must be remembered enzymes are stable in the pH range of 4-12,5 (8 is the optimal value) and their catalysing action works at the temperature range from 25 to 65 °C; so that's why the next step is to incubate the sample solution at 56 °C for 10 minutes. This step is also important because of the activation of the proteinase K in the presence of denaturant reagents like guanidine hydrochloride, contained in the lysis solution. Ethanol (96-100%) is then added to cause DNA precipitation.

These initial steps are the key steps: they enable to trigger the mechanism of DNA extraction.

The mixture obtained is then transferred to the spin column; here is where guanidine hydrochloride starts its activity as a chaotropic agent, it destabilizes the ordered structure of the silanols of the silica, which become dissociated at a pH of 8 and are dissolved out of the water, leaving the precipitated DNA bound to the silica membrane.

Under normal conditions, DNA has a negative polyelectrolyte, so it cannot interact with the silica due to electrostatic repulsion; as a result, sodium ions come into play to shield the negative charges of phosphate groups of DNA and the dissociated silanols, bridging these two groups together. In this way, only

the DNA remains bound to the silica, since it has an ordered and periodic structure that allows, with strong affinity, formation of stable interactions instead of RNA, proteins and lipids which form non-specific interactions with the silica. The whole process is favoured by a little entropy which is loss when the DNA interacts with the silica. Subsequently, centrifugation techniques and various washes are used to elute all impurities from the spin column, leaving only the DNA attached to the silica membrane. In the final step, the DNA is eluted from the silica-based membrane with galenic water and stored at +4 °C for short-term use or at -80 °C for long-term storage.

### 3.4.4 Sample quantification and normalization

DNA quantification is performed using Thermo Scientific NanoDropTM 2000c spectrophotometer (Thermo Fisher Scientific, US). This UV-Vis full-spectrum (190-840 nm) spectrophotometer can measure DNA, RNA, or proteins samples with high accuracy and reproducibility and features a patented sample retention technology which analyse micro-volume samples up to 0,5 µL.

To be highlighted also the ability to analyse concentration over a wide range of values, measurement time is less than 20 seconds, a user-friendly software, and the possibility to pipette a sample directly onto the optical measurement surface.

The measurement is made at three different wavelengths: 230 nm, 260 nm, 280 nm.

According to the Lamber-Beer law, absorbance is directly proportional to sample concentration: using this law DNA concentration of the sample can be quantified. The wavelength at 260 nm is characteristic of nucleic acid, a peak at about 260 nm is found in all four absorption spectra of deoxyribonucleotides Analysis procedures needs a DNA concentration of 20 ng/µL, therefore an aliquot of 1,5 µL in each well, will yield 30 ng of DNA. The protocol also indicates that the optimal amount of DNA for the success of the analysis is 15 ng for each assay, except for CYP2D6 assays, that require an approximately amount than 60 ng. This procedure allows to understand if samples need to be diluted to achieve the optimal concentration of 20 ng/µL, or not.

The standardized samples at the desired concentration are stored in aliquots of 200uL in the freezer at -80 °C for long term storage and -20° C for short term storage.

### 3.4.5 SNPline Genotyping platform

Once the sample is ready at the desired concentration, it is analysed for a panel of genetic variants customized for this study. These genotyping analyses are carried out through SNPline platform.


Figure 7: SNPline genotyping platform

### 3.4.6 KASP genotyping technology

SNPline platform is based on the Kompetitive Allele Specific PCR genotyping system (KASP), a genotyping technology which exploit competitive allele-specific polymerase chain reaction (PCR) for high-precision bi-allelic evaluation of single nucleotide polymorphisms (SNPs), insertions and deletions (InDels), at specific loci. This technology works with the fluorescence generated by two allele-specific forward primers that enable bi-allelic discrimination through their competitive

binding; it works also by using labelled probes associate with polymerase chain reaction.

The required components are:

- genomic DNA samples from the patients to be genotyped;
- Standard/Low Rox Master Mix (2x).
- KASP Assay Mix (72x).

The KASP Master mix contains: KASP TaqTM DNA polymerase, free nucleotides, the universal FRET (fluorescence resonance energy transfer) cassettes: one labelled with FAMTM dye and the other with HEXTM dye, ROXTM passive reference dye, MgCl2; the whole in an optimized buffer solution stored in the freezer.

Taq DNA Polymerase is a highly thermostable recombinant enzyme capable of catalysing the 5'-to-3' synthesis of DNA or amplification.

The FRET cassettes (*figure8*) are a double-strand moiety linked to a fluorophore and a quencher molecule: a molecule that dampens the fluorescence of the nearby fluorophore. There are two different fluorophores represented by FAM and HEX, for which different excitation and emission wavelength allow their discrimination, and this happens when they are not linked with the quencher. The FAM fluorophore is excited at 485 nm and emits at 520nm, while the HEX fluorophore is excited at 535 nm and emits at 556 nm.


Figure 8: KASP Master mix component FRET cassette

The KASP Assay mix contains three assay-specific unlabelled oligos: two forward primers and one common reverse primer (*figure 9*). Each primer has a 5'-terminal tail complementary to the filaments on which the fluorophores FAM and HEX are linked.

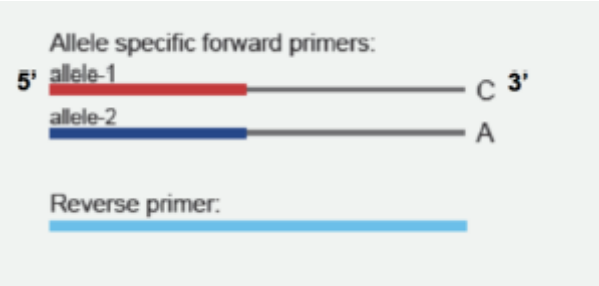


Figure 9: KASP Master mix component primers

The two allele-specific forward primers are composed of the same nucleotide base sequence and differ only by the SNP being analysed; they are complementary to the 3'-5' filament, one for each allele, while reverse primer is complementary to the 5'-3' filament for both alleles. If the complementary filament has the specific SNP, one of the two forward primers will anneal to the complementary filament during PCR.
This technique can be performed with different plates: 96 or 384 wells and 9 to 380 DNA samples can be analysed at the same time.

### 3.4.7 KRAKEN software

The entire genotyping process realized by the KASP technology take place through several specific instruments, and it uses the KRAKEN: a workflow manager software for the processing of biological samples, allowing the development of the samples and the analysis obtaining a fast and digital final elaboration.
Instruments that make up SNPline are:

- Meridian
- Kube
- Hydrocycler
- FLUORstar Omega.

The software KRAKEN was developed by LGC Group and rules every step of the genotyping process:

- Creation of the work project (file).
- Creation of working plates,
- Elaboration of the analytical data.

*Workflow*

A standardized workflow was developed for the analysis of all the polymorphisms chosen for the customized panel except for those concerning the *CYP2D6* gene.

This workflow allows to genotype 9 samples at once in about two days.

Through the software KRAKEN, the first 96-well *Master plate*, containing the 9 samples (or more, it depends on the sample number that needs to be analysed) is designed.

Then, starting from the *Master plate*, the working plates of 384 wells are assembled: analysis are performed in the working plate. Each working plate, defined *Daughter plate*, is identified by a barcode; this is a great advantage because the plates are automatically recognized from the designing, during the assay delivery, during the data analysis and in the moment of importing the analytical data and converting into clinical data.

According to KRAKEN, each well is identified with the sample's number which will be distributed in it. About 1,5 µL of DNA sample is manually aliquoted into each well and each sample is loaded in duplicate. Plate is then allowed to dry overnight and once the DNA is dried, plate is loaded into Meridian.

Figure 10: MeridianTM system (LGC Biosearch technologies, UK)

The MeridianTM system (LGC Biosearch Technologies, UK) is a single or multi-channel dispensing robot: it can dispense reagents ultra-fast, non-contact, automatically and continuously with ultra-low volume precision. It aspirates and dispenses up to 16 different assays in a very little time. The correct volume of calculated Assy mix and Master mix is dispensed into a special holder from which the Meridian's robotic arm retracts and then put into each well of the daughter plate thanks to the automatic detection. This tool speeds up the

operator's work and, above all, makes it more reproducible by reducing the analytical errors.


Figure 11: LGC Kube Thermal Plate Sealer (LGC Biosearch Technologies, UK)

Then the *daughter plate* is loaded into the LGC Kube Thermal Plate Sealer (LGC Biosearch Technologies, UK). It seals the plate in just 6-8 seconds working at 172 °C with a propylene film which provides an high temperatures resistant seal. This step is essential before PCR as it protects the material in the well from the water.

Plate is now centrifuged for 1 minute at 1500 RPM with brake. In this way, the mixture formed in the well is perfectly homogenized and ready for the PCR reaction. Hence, steps with the KASP technique are the following:

1. In the first PCR cycle, after the initial denaturation, one of the two allele-specific primer binds the target SNP and, amplifies the target region while the common reverse primer is linked to the complementary filament.

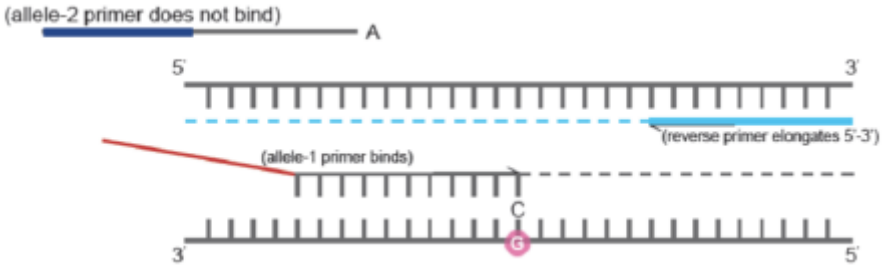


Figure 12: First step of PCR

2. In the second cycle, complex DNA strand and the elongated primer separate himself from the complex and the reverse primer binds to this last neosynthesized strand, elongates and forms a complementary filament.

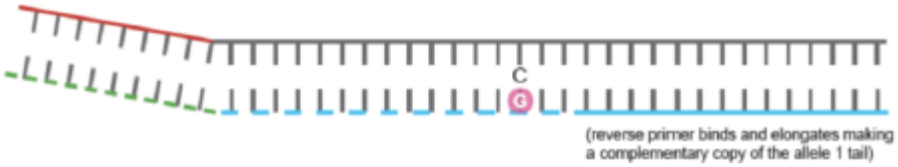


Figure 13: Second step of PCR

3. From the next cycle, the reaction starts to become productive, and the number of allele-specific tails increases. The fluorophore labelled portion of the FRET cassette (FAM or HEX) hybridized the new complementary tails sequences and separates itself from the quencher, generating a fluorescent signal.

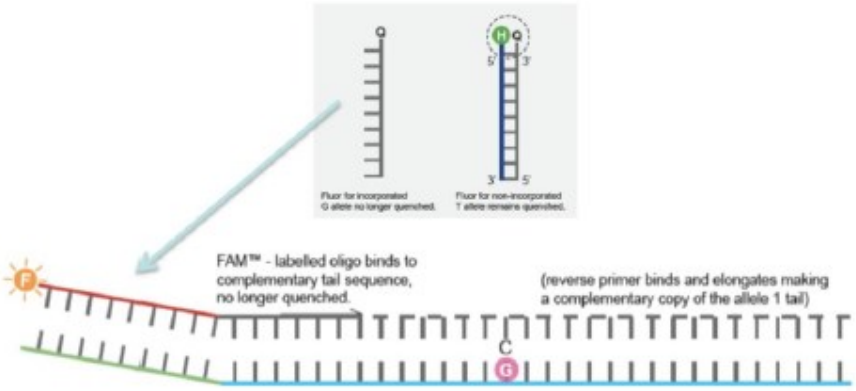


Figure 14: Third step of PCR 1

The double-strand moiety of the FRET cassette is usually connected to a fluorophore (FAM or HEX) and a quencher before PCR, so that no signal is generated. Nevertheless, oligos linked to the fluorophore can pair with the amplified DNA sequences and generate a signal that is distinctive according to the different allele combination present in the analysed DNA. The instrument used for the PCR reaction is the LGC Genomics Hydrocycler2 (LGC Biosearch Technologies, UK). The hydrocycler is a water bath thermal cycler suitable for performing the PCR and cycle sequencing reaction.

Figure 15: LGC Genomics Hydrocycler (LGC Biosearch Technologies, UK)

To perform PCR, it moves the plates between four baths to achieve the reaction temperature according to this program:

| Protocol Stage | Temperature | Duration | Number of cycles for each stage |
|---|---|---|---|
| Stage 1<br>Hot-start *Taq* activation | 94°C | 15 minutes | x 1 cycle |
| Stage 2<br>Touchdown | 94°C | 20 seconds | x 10 cycles |
| | 65°C<br>(65°C decreasing 0.8°C per cycle to achieve a final annealing / extension temperature of 57°C) | 60 seconds | |
| Stage 3<br>Amplification | 94°C | 20 seconds | x 26 cycles |
| | 57°C | 60 seconds | |
| Optional Stage 4<br>(read stage for qPCR instruments only) | 30°C<br>(any temperature below 40°C is suitable for the read stage) | 60 seconds | x 1 cycle |

61-55° touchdown protocol

Figure 16: PCR program

After the PCR reaction, a double centrifuge is performed at 1500 RPM for 1 minute: the first with the plate down and the second with the plate in the correct position.

The fist centrifuge is used to properly mix the reaction mixture in the wells, the second one is essential to deposit the reaction mixture on the bottom of the wells so that plate reading can be performed efficiently.

At the end, plates containing samples are analysed using FLUOstar® Omega (BMG LABTECH, Germany), a multimode microplate reader which works with the BMG LABTECH's Tandem Technology. FLUOstar® Omega is a combination of an ultra-fast, absorbance-sensitive full-spectrum spectrometer and an advanced, sensitive filter-based detector with an optics and photomultiplier tube. Usually, it analyses one single plate at a time.

FLUOstar Omega has the advantage of being a versatile, automated microplate reader with six detection modes, including UV/vis absorbance spectra, fluorescence intensity, time-resolved FRET and luminescence. The mode used for the project analysis is fluorescence detection.


Figure 17: FLUOstar Omega (BMG LABTECH, Germany)

The spectroscopic phenomenon of fluorescence is founded on the decay of absorbed electromagnetic energy, so it is a radiant decay process with the advantage of being more sensitive in respect of absorption, because of the background noise is almost zero, since there are few fluorescent molecules.
This phenomenon occurs when a molecule called chromophore absorbs energy from UV/Vis radiation: it transits from the electronic ground state to one of the vibrational levels of excited electronic states.
Then, if chromophore is also a fluorophore, it undergoes an internal conversion phenomenon passing from one of the vibrational levels, of the excited electronic state, to the lower vibrational level of the lower energy excited electronic state. During this process also energy dissipation occurs, which is not through radiant form but through kinetic energy. This only occurs in fluorophore molecules because of their greater delocalization of π electronic density, and because of their rigidity and planar structure.
When the excitation ceased, the fluorophore decays to arbitrary vibrational levels of the electronic ground state, releasing the remaining part of the absorbed energy in the form of electromagnetic energy, in the form of radiation emission, giving rise to the fluorescence phenomenon.

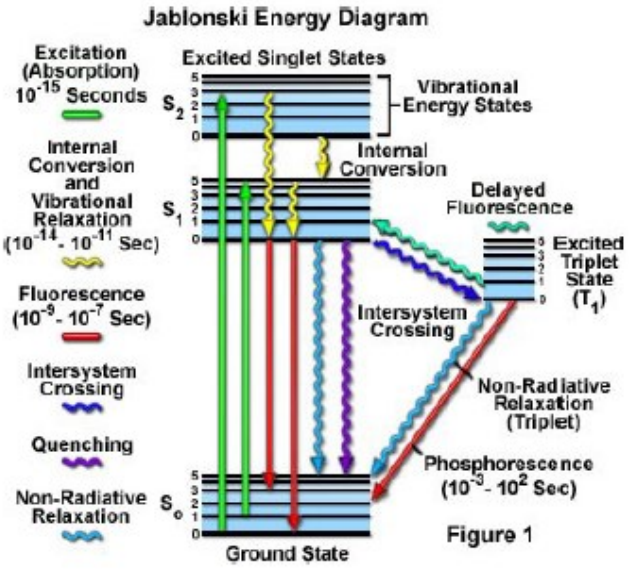


Figure 18: Jablonski energy Diagram

*(from https://www.olympus-lifescience.com/)*

Looking at the Jablonski energy diagram you may notice an energy gap between the electronic ground state and the excited electronic state: the principle of energy quantization must be respected. Then, the emitted radiation's frequency will be smaller than the frequency of the absorbed radiation, because the energy difference is smaller due to the internal conversion phenomenon. This entails two radiations with two different wavelengths: the wavelength of the emitted radiation is larger than the wavelength of the absorbed ones, so it is possible to measure the fluorescence.

Fluorophore Excitation (nm) Emission (nm) FAM 485 520 HEX 535 556 ROX 575 610.

FLUOstar Omega's excitation system uses a monochromator or optical filter to select a specific wavelength able to cause fluorophores excitation, while the radiation emitted by the fluorophores is detected by an optical system.

Allele discrimination is achieved by the difference in fluorescence of the two fluorophores FAM and HEX, which allows the determination of the patient's genotype:

- If the patient is homozygous, only one of two signals is detected: the wavelength can be used to distinguish between homozygous mutated and homozygous wild type.

- If the patient is heterozygous, there is a mix of the two fluorescence signals.

The various signals emitted by the fluorophores are converted into an identifiable value, which KRAKEN elaborates.

| Fluorophore | Excitation (nm) | Emission (nm) |
|---|---|---|
| **FAM** | 485 | 520 |
| **HEX** | 535 | 556 |
| **ROX** | 575 | 610 |

Table 4: Wavelengths of excitation and emission of fluorophores

The software provides a graphically view of the results, i.e. a Cartesian plot is generated with the fluorescence values FAM and HEX (Fam is plotted on the X-axis; HEX is plotted on the Y-axis) and the results are reported as three different genotyping clusters formed by little points representing each DNA sample.

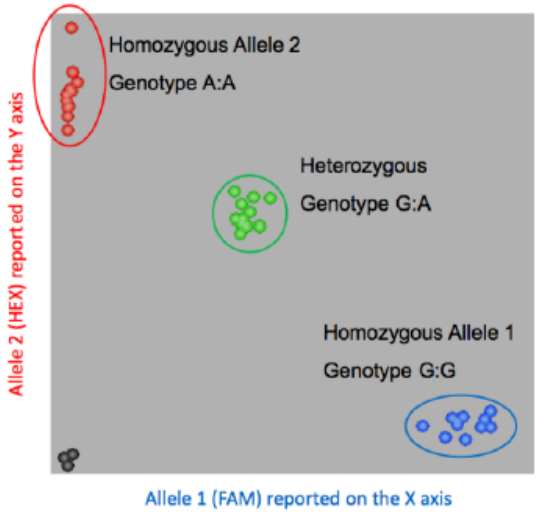


Figure 19: Example of an ideal result throughout FLUOstar Omega

## 3.4.8 CYP2D6 analysis

Analyses concerning the enzyme cytochrome P450 (CYP) 2D6, encoded by CYP2D6, did not follow the standard workflow just explained. This is because *CYP2D6* locus is characterized by large genomic aberration such as gene deletion or amplification. It contains the pseudogenes CYP2D8 and CYP2D7, which are highly homologous to CYP2D6 and may also contain some CYP2D6-CYP2D7 hybrid genes (singly or as a tandem hybrid). For these reasons, when analysing CYP2D6 polymorphisms, a nested PCR is performed to reduce

nonspecific amplification of the DNA template: nested PCR consists of a Long-Range polymerase chain reaction (LR-PCR) of the DNA sample performed in an Eppendorf Mastercycler proS gradient (Eppendorf AG, Hamburg, Germany) before preparing one of the *daughter plates*. Long Range PCR (LR PCR) is a classical PCR in which larger fragments are amplified.

*SNPline analysis protocol: LR PCR*

Three LR-PCR are prepared with specific primers and is performed with TaqGold DNA Polymerase (AB Applied Biosystem, Foster City, CA, USA):

The protocol includes the use of three specific primers:

1. Amplification for assays related to CYP2D6 except xN and * 5;
2. Duplication (xN), to investigate the presence of multiple copies of the gene.
3. Deletion (* 5), to investigate the absence of the gene.

The reaction mix is the following:

- 12 µL primer and 12 µL reverse primer, specific for each assay;
- 18 µL of Enhancer;
- 42 µL of Long Range Mix.

The LR -PCR is prepared according to protocol in a 96-well plate placed on a cooler. 7 µL of the reaction mix is dispensed into each well followed by the DNA. Starting from the master plate, 4 µL of DNA is dispensed for amplification and 3 µL of DNA is dispensed for both duplication and deletion. The plate is then loaded onto a Eppendorf Mastercycler Gradient Thermal Cycler for amplification under the following reaction conditions:

| Reaction condition | N° of cycles |
|---|---|
| 2’ at 95°C | X 1 |
| 30” at 95°C | X 38 |
| 30” at 62°C | |
| 6’ at 68°C | |
| 5’ at 72°C | X 1 |

Table 5: PCR steps and conditions of LRPCR

*Agarose gel electrophoresis*

Agarose gel electrophoresis is used to verify that the LR-PCR was successful and to perform a double check in cases of duplication and deletion with respect to the SNPline; it is a check, before working in the SNPline is practice to check if samples have been properly amplified by LR PCR.

The principle on which electrophoresis is based is essentially the displacement of negatively charged molecules towards the positive pole of the chamber under the influence of an applied electric field. DNA does indeed have negative charges given by the presence of glucosidic residues that lie outside the double helix. This allows the electric field applied to the buffer, in which the gel is immersed, to migrate the DNA fragments towards the anode, favoring their separation as a function of molecular weight (MW).

Fragments with lower PM have a higher migration speed through the gel mesh; molecules with high (MW) migrate slowly because they are hindered by the polymer network. However, this inverse proportionality is determined not only by the size of the molecule, but also by the concentration of the gel. The size of the networks is determined by the concentration of agarose. Small DNA fragments can be easily separated with high polymer concentrations. Generally, the LR PCR control is performed with a 0.8% gel in TAE (buffer consisting of tris-acetate-EDTA). This is done by dissolving the agarose in the buffer and adding 100 µl of GelRed® containing ethidium bromide, an intercalator for double-stranded DNA, which causes the complex to absorb in the UV. The mixture is then poured into a mold with special combs that define the wells once the gel has solidified. Before loading the sample (2 µl), a small amount of Loading Buffer (3 µl) containing SDS (anionic detergent), EDTA (to adjust pH), glycerol and bromophenol blue is added to the solution containing the DNA fragments to be tested. Glycerol promotes precipitation of the DNA so that the solution does not leave the wells after loading; bromophenol blue mixed with the DNA allows you to visibly follow the electrophoretic run as it too migrates to the positive pole.

The first well of the gel is filled with 0.5 µL of ladder (molecular weight marker), 3 µL of loading buffer and 1.5 µL of TAE. After loading the samples into the wells, a voltage of about 100V is applied to the chamber (Figure 13). The electrophoretic bands resulting from the migration, thanks to the presence of the intercalated ethidium bromide, are visualized by the transilluminator emitting into the UV.

The image is acquired using the tool Gel DOC EZ Imager by BIO -RAD. Ethidium bromide is a molecule that fluoresces at 620 nm and has 2 wavelengths of maximum absorption, 300 nm and 520 nm. The wavelength of 300 nm is preferred because the molar extinction coefficient is larger, consequently the number of molecules decaying from the excited state to the ground state by emission of radiation is larger.

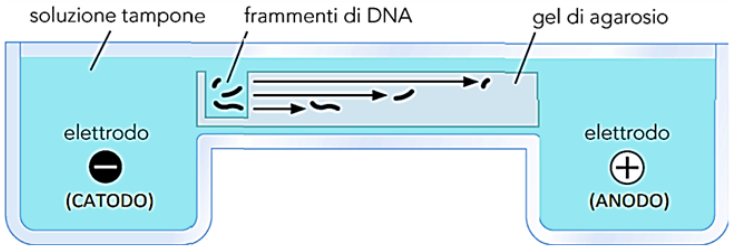


Figure 20 Functioning of the electrophoresis chamber

The electrophoretic bands are interpreted as follows (Figure 14):

1. Amplification: the bands must all be at the same level, at the level of the third band of the ladder, corresponding to a fragment of 6557 bp.
2. Duplication: in general, this mutation occurs under heterozygous conditions. Therefore, the gel shows a band referring to the wild-type allele (below the third band of the ladder, 6557 bp) and a band referring to the duplicated allele (below the fourth band of the ladder, 4361 bp), corresponding to the hybrid CYP2D6- CYP2D7.
3. Deletion: this mutation occurs under heterozygous conditions. Therefore, in the gel, one band is visible for the deleted allele (under the third band of the ladder, 6557 bp) corresponding to the CYP2D6-CYP2D7 construct and one for the wild-type allele (under the fourth band of the ladder, 4361 bp).

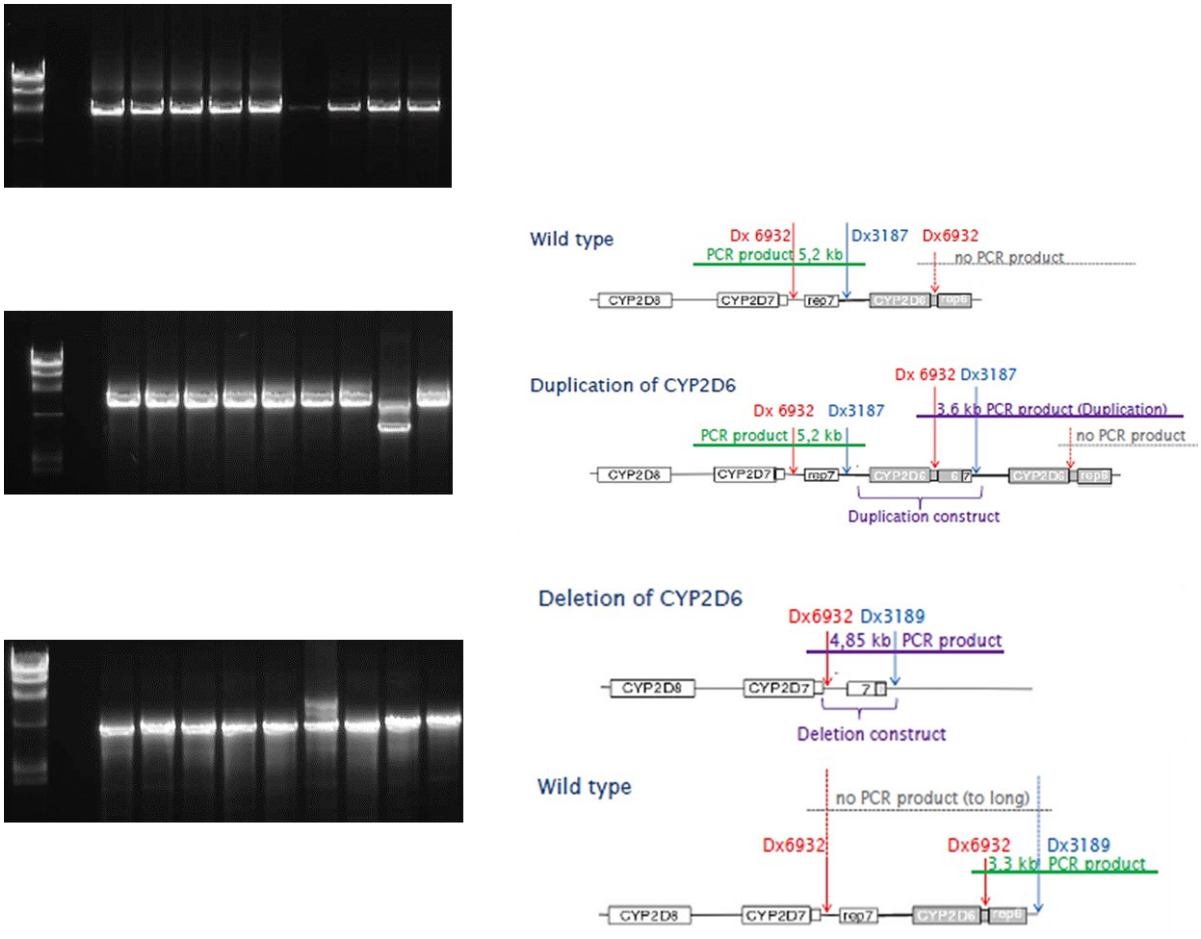


Figure 21 Electrophoresis bands of amplification (up), duplication (middle) and deletion (below)

### 3.4.9 Real Time PCR (RT PCR)

Allelic discrimination is the determination of the two variants of a single nucleic acid sequence using the "5'-nuclease fluorogenic assay". The basis of TaqMan® allele discrimination can be the use of a Real Time PCR (RT PCR), i.e. an amplification of fragments that closely follows the different phases of the PCR in real time. Moreover, thanks to the use of 96-well plates, this method allows the genotyping of a large number of samples using a sequence of PCR analysis without having to resort to other methods.

In RT-PCR, in addition to the two primers, an oligonucleotide called probe is used to pair with the template, which occupies an intermediate position between the sense primer and the antisense primer. The probe is labeled at both ends: on one side there is a quencher (TAMRA fluorochrome) that acts as a

fluorescence silencer, and on the other side a reporter (FAM or VIC fluorochrome) is connected. The silencing action of the quencher occurs by transferring energy from one fluorochrome to another when they are close to each other. The presence of this oligo in an intermediate position to the primers, hence, makes it possible to obtain fluorescence emission proportional to the amount of PCR product. In addition, the amplification of the SNP-bearing fragment as soon as the quencher separates from the reporter.

The operating principle of the method combines the three basic phases of PCR with fluorescence emission from the specific reporter associated with the sequence leading to the polymorphism. Specifically, the probe complementarily bound to the mold fragment is detached and digested by Taq polymerase at the time of elongation. The distance of the quencher from the reporter determines the fluorescence emission of the fluorochrome present in that specific probe. Thus, if the probe is complementary to the sequence WT, one fluorochrome is released, while Taq polymerase determines the release of the other fluorochrome if it perfectly matches the mutated sequence.

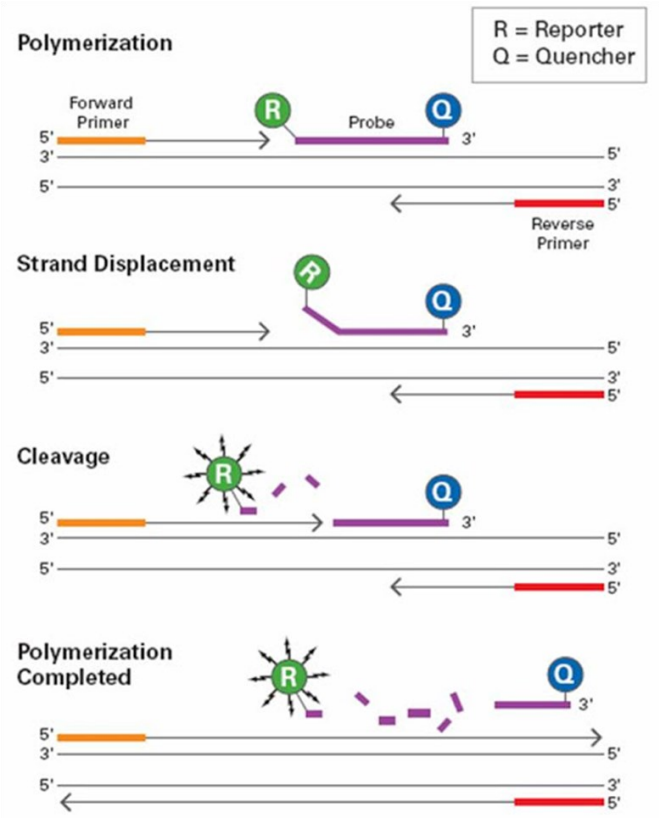


Figure 22: TaqMan assay working principle

Using an optimal annealing temperature ($T_a$) for the probe in the elongation phase of RT PCR, you will get less fluorescence from the uncoupled probes because they have a lower melting temperature ($T_m$) than the paired probes. The

probe is selected based on already defined assays that take into account some specifics:

- The $T_m$ must be at least 5°C higher than the $T_m$ of the two primers, since they must bind to the nucleotide sequence when the complementary strand is synthesized.
- The oligonucleotide must have a length of about 20-30 bp and a G and C content of 50%.
- The extension phase must take place at a temperature lower than the usual temperature of 72°C used in PCR, so that the probe does not detach from the template (high concentrations of MgCl2 are used for this purpose).
- The probe must not form dimers or even pair with itself.

The spectrophotometric data are collected at a wavelength between 500 and 600 nm. Then, using specific algorithms, software converts the raw data, expressed as fluorescence signal versus λ, into clean color signals. The data provided by the Taq Man® assay are in fact presented in the form of dots grouped and arranged in a two-dimensional diagram (Figure 20). In this diagram, three main groups can be distinguished: the first, along the ordinate axis, represents the homozygous samples for the Y allele; the second, along the abscissa, determines the homozygous genotypes for the X allele and, finally, the central group, arranged along the diagonal, it is given by heterozygous samples. In addition, in the diagram of Taq Man® analysis, there is a point called NTC (No Template Control) which corresponds to a control that is not allowed to develop a fluorescent signal ( mix ). There may be some points on the graph where it is not possible to accurately determine the group to which they belong. These samples, defined as outliers, may arise from:

- rare sequences;
- lack of amplification of the RT PCR;
- errors in sample distribution;
- contamination.

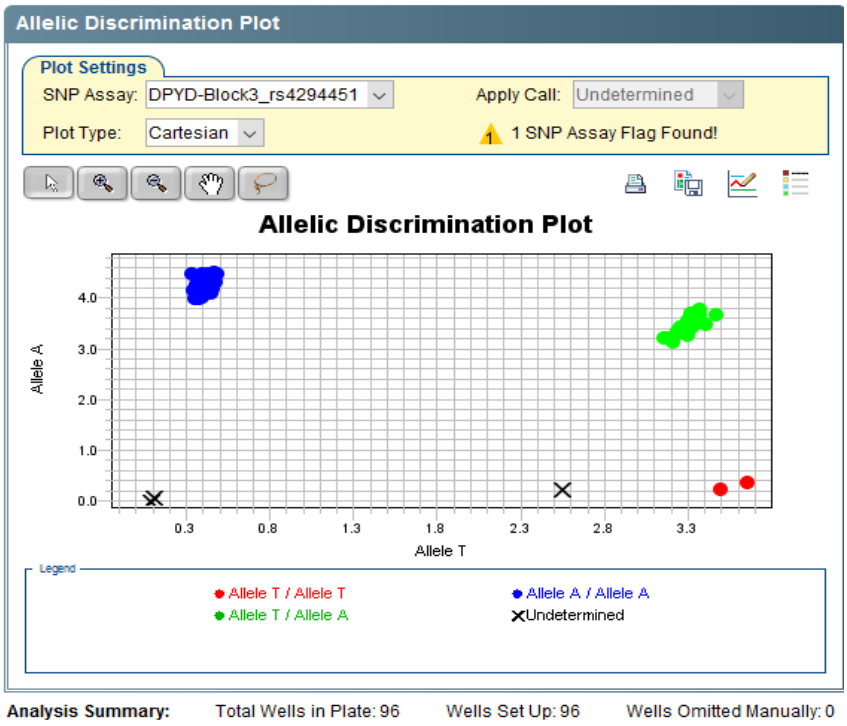


Figure 23: TaqMan Allelic Discrimination Plot

Ultimately, the Taq Man® method allows rapid analysis of the genotype using only one universal mix (master mix) and one marker. 96-well plates are used in the sample preparation phase. The mix is prepared by combining the mix containing the primers (forward and reverse) and the labeled probes with the master mix. The solution is distributed in the wells and finally the genomic DNA samples are added. In the next step, the plate is covered with an adhesive sheet and then centrifuged for a few minutes to remove air bubbles at the bottom of the wells. The plate is then placed in the device. At this stage, the conditions of the RT PCR are established (temperatures, times and cycles), the analysis volumes (10µl) and, most importantly, the markers (VIC and FAM) are assigned to the alleles of the SNP. The characteristics of the amplification of the genomic fragment are as follows:

| Reaction condition | N° of cycles |
|---|---|
| 5' at 50°C | X 1 |
| 10' at 95°C | X 1 |
| 15" at 92°C | X 50 |
| 1' at 60°C | |

Table 6: PCR steps and conditions of RT PCR

The analysis of the results is performed after PCR using a TaqMan® analysis and readout program.

## 3.5 Statistical associations

In this work of thesis, patients' PK and genetic data are described through descriptive statistical tests. For the Cmin of imatinib the mean value among patient's samples was reported, and to show the variability, standard deviation has been specified.

Imatinib Cmin data were then analyzed for their association with patients' sex, genotype, and metabolic phenotype. For the association between Cmin and genotype/phenotype parametric analysis were selected; in the association between two groups the U-Mann-Whitney test has been selected, for the association among more than two groups the Kruskal-Wallis test was used instead.

For every association, a plot representing the mean, the median and the quartile values was obtained; additionally, a p-value for every association was calculated.

P-value is the probability of seeing results as supportive of a genetic or other association (39); if the p-value is under 0,05, results are considered statistically significant and if it's below 0,005 they are considered highly statistically significant; variables for which the association has a p-value >0,5 are not statistically significant.

A commercial software (STATISTICA) was used to perform a statistical analysis of the data.

It is possible to achieve statistical associations only with a homogeneous and large patient population, for this reason, only for imatinib data statistical association has been tested, considering only the 400 mg/day population.

For sunitinib a too small population of samples was collected, and furthermore within this population three different dose were administered to the patients. Thus, no statistical evaluation was performed.

# 4 RESULTS

## 4.1 Case study description

A case study of 56 Caucasian male and female patients of Centro di Riferimento Oncologico CRO di Aviano has been analyzed in this work.
Patients were included in two distinct clinical protocols, as specified in the methods section. Patients were treated either with imatinib (54), or sunitinib (10).
The first 34 patients treated with imatinib were initially enrolled in the clinical trial (2017-002437-369), active in the Pharmacology Unit of CRO di Aviano (35); they are now being re-enlisted in the 'Farmacovigilanza attiva' protocol as hand.
All the other patients were directly enrolled in 'Farmacovigilanza attiva' protocol.
An informed consent for research purposes was collected from all the patients.
All the 47 imatinib treated patients were affected with GIST (gastrointestinal stromal tumour), some of them are not still on treatment.
Sunitinib treated patients were affected either with RCC or with GIST (as a second line treatment, after failure of imatinib therapy).
Patients of case study are all over 41 years old, the average age was around 70 years old.
Some descriptive information about this case study can be found in Table below.

| | | ALL | IMATINIB | SUNITINIB |
|---|---|---|---|---|
| Number of patients | | 57 | 47 | 10 |
| age, median (range) | | 67,6 (41-88) | 68 (88-41) | 66 (46-79) |
| Sex | male | 33* | 25 | 7 |
| | female | 24 | 21 | 3 |
| therapy setting at the enrolment | first line | / | 30 | 5 |
| | second line | / | 4 | 5 |
| | adjuvant | / | 13 | 0 |
| drug dosage at enrolment (continued schedule) | 400 mg/die | / | 44 | / |
| | 300 mg/die | / | 3 | / |
| | 800 mg/die | / | 1 | / |
| drug dosage (interrupted schedule, 2 week on 1 off) | 50 mg/die | / | / | 2** |
| | 37,5 mg/die | / | | 6** |
| | 25 mg/die | / | / | 2 |
| BMI, median (range) | | 25 (34,9-18,3) | 24,5 (34,9-18,3) | 25,26(33,4-18,8) |

Table 7: case study clinical description

*Among the male subgroup a trans gender patient was included
**one of these patients does the original sunitinib schedule reported in data sheet: 4 weeks on, 2 weeks off.

## 4.2 Therapeutic drug monitoring in the case study

The quantification of plasma samples was performed using the analytical methods reported in the previous section for imatinib and sunitinib.

### 4.2.1 Imatinib results

Plasma concentrations of the drug are related to the single sample: imatinib's samples are in greater quantity, because the collection started in the 2017. Every sample is identified by a progressive number assigned to the patient (given to the patient according to his entrance in the study) followed by the number of the sample: i.e., the patient named 2002 at the first sample was 2002.1, the second sample, which happens at least 1 month later, sample was named

2002.2.

At each patient a progressive number was assigned, data related to drug dosage at the time of sampling and hours spent from last administration (in order to understand if it is at the Cmin or not) were collected. Then, concentrations of imatinib and nor-imatinib are reported respectively.

230 imatinib samples has been successfully quantified, Cmin obtained were recalculated, when necessary, by the Formula [2] and the following distribution was obtained (figure 24).

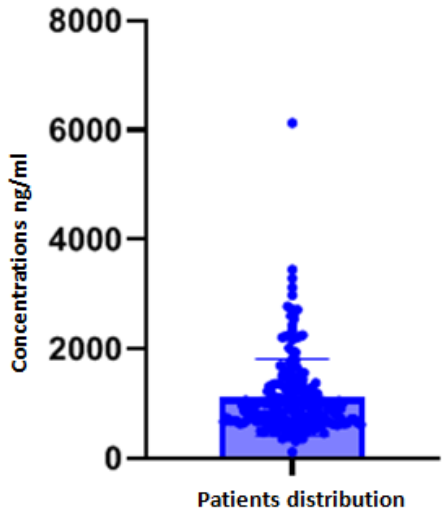


Figure 24: distribution of imatinib samples concentrations

It may be noted that most of samples are distributed within the range 800-1000 ng/mL; the average value is 1085 ng/mL and there are some samples outside the TDM target. The higher Cmin value measured was 6132 ng/mL, while the lowest Cmin was 116 ng/mL.

Published guidelines (21) have shown a correlation between trough concentrations of imatinib and clinical response, leading to the suggestion that maintaining levels above 1100 ng/mL for GIST may improve clinical outcomes. There is evidence that Cmin > 3000 ng/mL can lead to toxicity in patients treated with 50 mg/die. While consensus suggests that Cmin of >1100 ng/mL is needed for imatinib efficacy, trough concentrations >3000 ng/mL have been correlated with adverse effects, resulting in treatment interruption. Kang et al. (40), successfully used TDM to reduce imatinib drug dosing in patients experiencing excessive toxicity, without an interruption of treatment. In this work Cmin data ≥ 1100 ng/mL and < 3000 ng/mL are considered on target.

Our analysis reported that 74 samples were within the Cmin target range, 4 samples had a concentration higher than 3000 ng/mL, 152 samples had a concentration below the selected target.

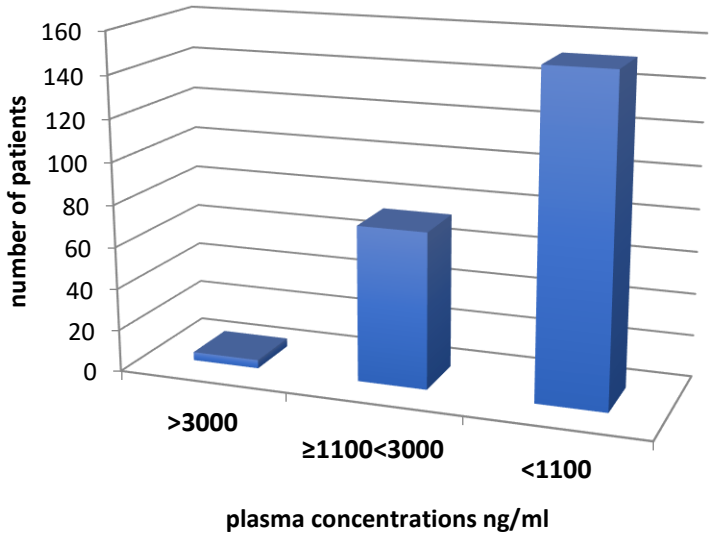


Figure 25: sample distribution into 3 selected range of concentration

These first results support the thesis on which this work was based: inter-patient variability is relatively wide, imatinib Cmin levels ranged from a minimum of 116 ng/mL to a maximum of 6177 ng/mL; the 63% of these concentrations are <1100 ng/mL, so under the TDM target. As a result, it is necessary to investigate plasma concentration and to monitor therapies with TDM approach.

Applying TDM approach, another interesting information was pointed out: there were 4 samples which concentrations were below the lower limit of quantification (LLOQ) thus, they were not quantifiable. All of these samples belonged to the same patient (203-0020): according to what the patient reported, each sample was collected at the steady-state and 24 hours from the last drug intake. Hence, this could be hypothesised as a case of non-compliance. The identification of this case highlights how TDM approach can be a useful and important tool to monitor therapies and how it may contribute to improve clinical outcome also by the identification of non-compliant patients.

In Table 8 the TDM results related to case 203-0020 are reported, including date of sampling, hours from last administration and the Cmin values.

| patient_code | samples_name | DOSE (mg/die) | Date of last intake | hour of last intake | DATA cof sample | hours from last intake | Cmin (ng/mL) | Cmin (ng/mL) METABOLITE |
|---|---|---|---|---|---|---|---|---|
| **203-0020** | 20_3 | 400 | 21/02/2018 | 23:00 | 22/02/2018 | 15:00:00 | (<LLoQ) | <LLoQ |
| **203-0020** | 20_4 | 400 | 13/11/2018 | 12:00 | 14/11/2018 | 23:00:00 | <LLoQ | <LLoQ |
| **203-0020** | 20_5 | 400 | 04/06/2019 | 12:00 | 05/06/2019 | 23:50:00 | <LLoQ | <LLoQ |
| **203-0020** | 20_8 | 800 | 30/07/2020 | 20:30 | 31/07/2020 | 16:15:00 | <LLoQ | <LLoQ |

Table 8: case report 203-020, parameter reported for kinetic analysis

Another parameter which is taken into account in TDM analysis is the ratio between imatinib and its metabolite (NOR-IMA): based on the literature data, the metabolite formed, NOR-IMA, has similar biologic activity to imatinib and represents approximately 20% to 25% of the parent drug level at steady-state (SS) in patients with GIST (18) (41).

The ratio between NOR-IMA and imatinib concentration that has been obtained in this work has an average value of 23% ± 9% (SD), thus inside the range prompted by literature.

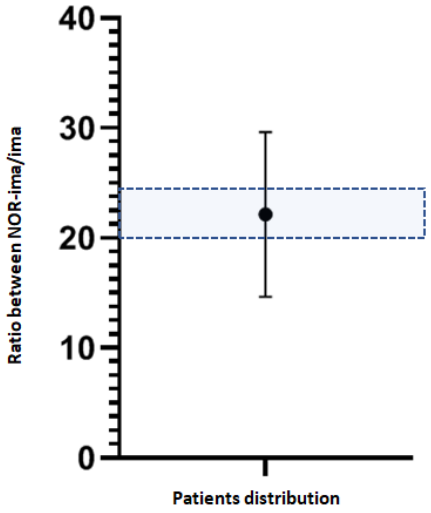


Figure 26: ratio between NOR-IMA and imatinib for all samples. The mean value of 23% was highlighted (dark circle).

As explained in the case-study table, patients received different drug dosage at the time of enrolment, and also during the period of monitoring patients often have changed their dose, usually decreasing it due to toxicities. Overall, patients were treated with six different dosages. In Table 9, for each dose-level, the mean Cmin was calculated and reported. The objective was to understand whether the dose could influence the exposure.

In the Table 9 the 6 different dosages are reported, number of samples, number

of patients treated at that dosage, concentration's average and standard deviation (SD).

| Dose (mg/die) | number of patients | number of samples | mean concentrations ± SD ng/mL |
|---|---|---|---|
| **100** | 1 | 5 | 306,2±97,3 |
| **200** | 1 | 2 | 706,7±40,1 |
| **300** | 4 | 7 | 1150,1±631,7 |
| **400** | 43 | 203 | 1114,4±700,1 |
| **600** | 1 | 2 | 816,0±310,5 |
| **800** | 3 | 11 | 699,1±808,5 |

Table 9: dose-concentration relationship

On average, the highest exposition (Cmin) was observed at the standard dose of 400 ng/mL. As evidenced by the standard deviation, a large interindividual variability among exposure was observed.

A dose-exposure comparison cannot be made between these data, due to the high value of the standard deviation and the large number of patients populating the 400 mg/day dose. For this reason, the comparison between dose-exposure has been reported considering all the samples' values. In the Figure 27 the sample distribution among the six different dosages is represented.

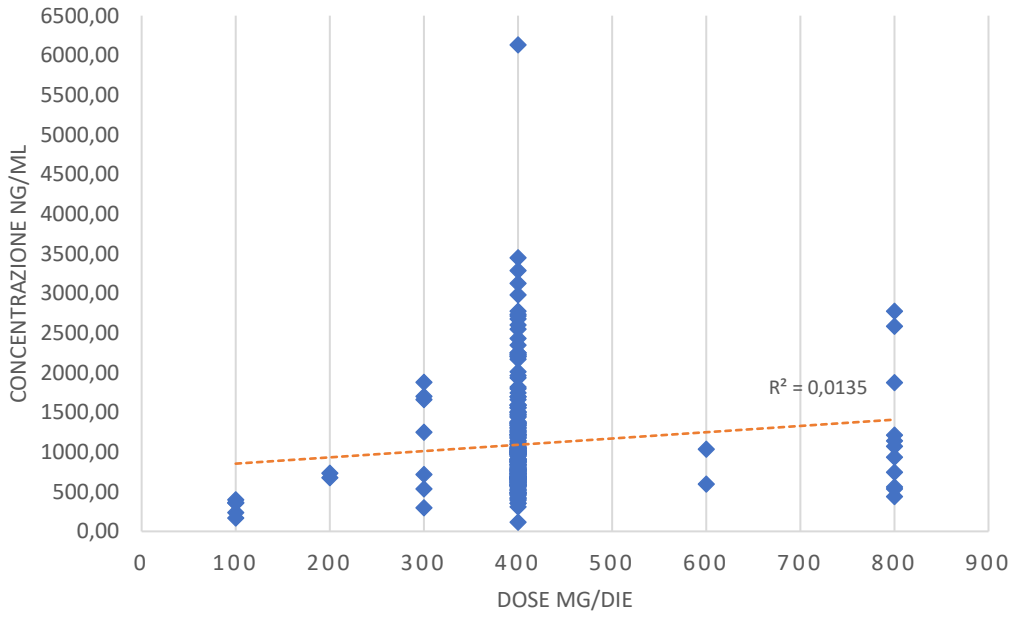


Figure 27: dose vs concentration graph, samples distribution among the six dosage levels, the orange line represents the tendency line whit $R^2$ value.

As the Figure 27 shows, 400 mg/die represents the most populated dose. The tendency line shows the hypothetical linear dependency between dose and exposure: from this graph no linear correlation between the dose increasing and drug plasma concentration has been found. No linear trend is detectable, as shown in the Figure 27 (R =0,0135). However, as reported before, some bias about this correlation came from the non-homogeneous distribution of patients among the six dose levels.

Considering the expected effect of patient's sex on imatinib Cmin, a statistical analysis was performed to compare median Cmin values between male and female patients. To exclude the bias represented by drug dosage inhomogeneity among patients, only samples corresponding to 400 mg/day administration were included in the analysis. The comparison was not statistically significant but a trend for a higher exposure among females as compared to males was observed (p-value: 0,0358 by U-Mann Withney test). For sunitinib there was high heterogeneity among the case study population, and no statistical analysis could be done.

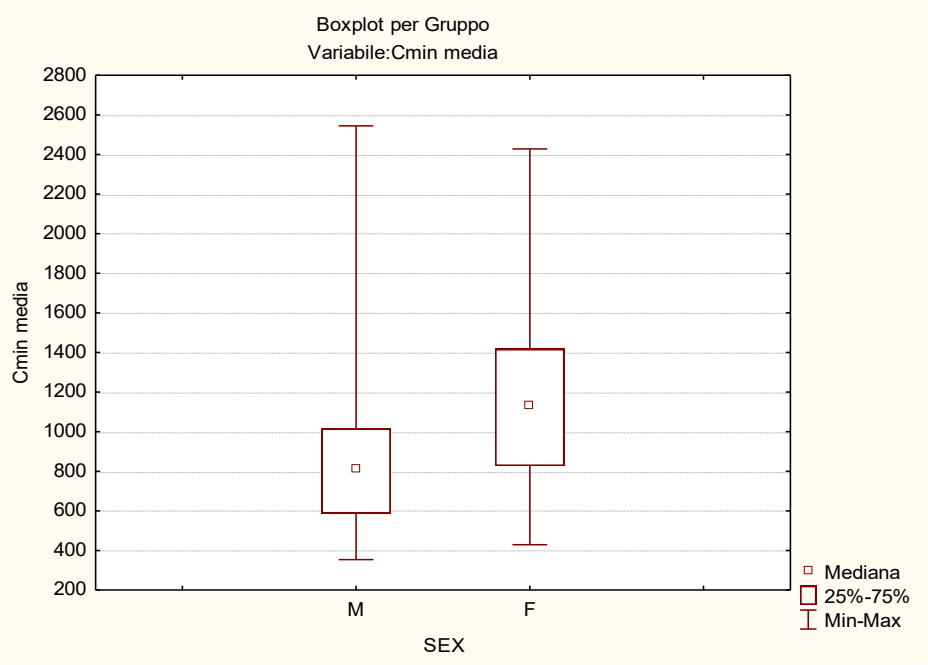


Figure 28: association between mean Cmin and sex, a trend between the variable is showed, female patients have highest exposure in respect of male.

## 4.2.2 Sunitinib results

### 4.2.2.1 Method partial-validation

Due to the addition of a dilution step before LC-MS analysis, the matrix sample was modified, and a re-evaluation of the matrix effect was necessary. Thus, the post column infusion test was conducted showing no major matrix effects on sunitinib and N-DES SUN quantification. In Figure 29, the signal of both compound during the chromatographic run of a blank sample (plasma extracted without analytes nor IS) was recorded. the intensity of both sunitinib and N-des sun seemed not to be affected by enhancement or decreasing phenomena at their retention times (2.64 min for sunitinib and 2.59 min for N-DES SUN) thus indicating the absence of matrix effect and, in particular, that the additional step of dilution did not affect the quantification.

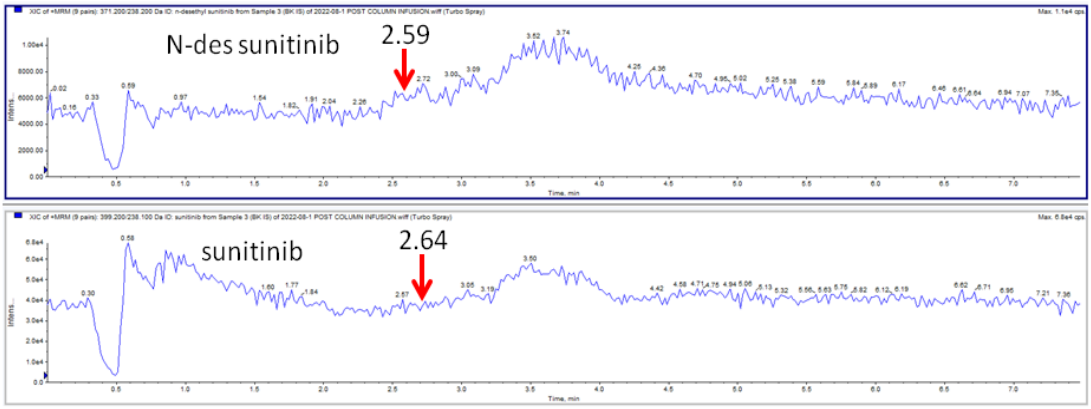


Figure 29. Post column infusion test.

The assessment of intra-day accuracy and precision was also performed to ensure that the modification introduced during sample preparation does not affect the precision and accuracy of the method. In Table 10 and 11, data related on this test were reported. For both sunitinib and its metabolite, the method resulted very accurate, being the obtained values ranging from 95,9 to 106,4 %, and very precise, being the CV% always ≤ 5,1%.

| sunitinib | LLOQ | QCL | QCM | QCH |
|---|---|---|---|---|
| actual value (ng/mL) | 5,0 | 15,0 | 250,0 | 425,0 |
| mean (found value, ng/mL) | 4,8 | 16,0 | 259,7 | 412,6 |
| Acc | 95,9 | 106,4 | 103,9 | 97,1 |
| CV% | 2,6 | 5,1 | 4,0 | 3,7 |

Table 10: intra-day accuracy and precision data of SUN.

| N-DES SUN | LLOQ | QCL | QCM | QCH |
|---|---|---|---|---|
| actual value (ng/mL) | 1,0 | 3,0 | 50,0 | 85,0 |
| mean (found value, ng/mL) | 1,0 | 3,0 | 50,8 | 84,4 |
| Acc | 100,8 | 101,0 | 101,5 | 99,3 |
| CV% | 3,5 | 4,8 | 3,8 | 4,2 |

Table 11: intra-day accuracy and precision data of N-DES SUN.

#### 4.2.2.2 Results of Cmin measured in patients

For sunitinib 16 samples were collected from 10 patients of CRO di Aviano, the patient population was smaller because of the low tolerability of the drug and thus few patients are currently under sunitinib treatment.

As already reported for imatinib, every sample is identified by a progressive number assigned to the patient (given to the patient according to his entrance in the study), followed by the number of the samples (first, second).

In the results the following data are reported: identification number, pathology, dosage, hours form last intake, date of sampling, concentration data of SUN and DES-SUN and their SUM, percentile of metabolite in respect of sunitinib.

In the Table 12 the quantification data are reported. All these samples were collected on the 14$^{th}$ day of therapy (i.e., at the steady-state).

| Patient N. | patology | Dose mg/die | sampling date | hours from last administration | SUN [ng/ml] | N-DES SUN [ng/ml] | SUM [ng/ml] | % metabolite with respect to sum (%) |
|---|---|---|---|---|---|---|---|---|
| 1991,1 | RCC | 37,5 mg/die | 25/07/2022 | 43:54:00 | 42,5* | 10,8* | 53,3 | 22 |
| 1752,2 | GIST | 50 mg/die | 03/06/2022 | 24:00:00 | 86,2 | 18,3 | **104,5** | 17 |
| 1752,3 | GIST | 50 mg/die | 26/07/2022 | 23:00:00 | 70,2 | 19,7 | 89,9 | 22 |
| 1914,1 | RCC | 50 mg/die | 08/06/2022 | 22:30:00 | 46.7 | 11,4 | 58.1 | 19 |
| 1914,2 | RCC | 50 mg/die | 20/07/2022 | 23:15:00 | 47,8 | 13,5 | 61,2 | 22 |
| 1974,1 | RCC | 37,5 mg/die | 13/07/2022 | 23:30:00 | 62,9 | 19,7 | 82,6 | 24 |
| 2002,1 | RCC | 25 mg/die | 03/08/2022 | 23:30:00 | 39 | 17,5 | 56,5 | 31 |
| 2002,2 | RCC | 25 mg/die | 21/09/2022 | 24:27:00 | 68,6 | 22,9 | 91,5 | 25 |
| 16_4 | GIST | 37,5 mg/die | 24/06/2022 | 24:30:00 | 43,7 | 14,3 | 58,1 | 25 |
| 16_5 | GIST | 37,5 mg/die | 14/09/2022 | 27:30:00 | 43.0 | 13.7 | 56.7 | 25 |
| 1897,1 | RCC | 25 mg/die | 30/05/2022 | 25:15:00 | 28,1 | 10,1 | 38,2 | 26 |
| 1946,1 | RCC | 37,5 mg/die | 23/06/2022 | 24:10:00 | 45,3 | 8,7 | 54 | 16 |
| 1946,2 | RCC | 37,5 mg/die | 04/08/2022 | 24:00:00 | 44,6 | 8,3 | 52,9 | 16 |
| 1952,1 | RCC | 37,5 mg/die | 27/06/2022 | 24:10:00 | 35,9 | 12,9 | 48,8 | 26 |
| 1929,1 | RCC | 37,5 mg/die | 15/06/2022 | 23:45:00 | 53,4 | 11,6 | 64,9 | 18 |
| 1929,2 | RCC | 37,5 mg/die | 27/07/2022 | 23:00:00 | 48,9 | 13,1 | 62 | 21 |

Table 12: : data related to sunitinib quantification. Values of sunitinib and N-des-sunitinib concentration and their sum were reported along with the percentage of the metabolite with respect to the sum.

As reported in the table 12, the first sample was collected 44 hours after last intake, so it was necessary to reconvert the concentration using the Formula [2]: for sunitinib $t_{1/2}$ is 50 hours (15) (Table 13).

| Patient N. | patology | Dosage mg/die | date of sample | hours form last administration | SUN [ng/ml] | N-DES SUN [ng/ml] | SUM [ng/ml] | Reconverted concentration |
|---|---|---|---|---|---|---|---|---|
| 1991,1 | RCC | 37,5 mg/die | 25/07/2022 | 43:54:00 | 42,5* | 10,8* | 53,3 | **70,2** |

Table 13: sample 1991,1 whit reconverted concentration

The TDM target for sunitinib (expressed as the sum of sunitinib and its metabolite) according to the dose are as follow: for 50 mg/die dose, the TDM target is 50-100 ng/mL (24), above 100 ng/mL patients may develop toxicities (42) and under 100 up to 50 ng/mL is considered within the therapeutic window.

This target was extrapolated to Cmin ≥ 37.5 ng/mL for 37,5 mg/die (continuous dosing) exploiting dose linearity. Under this level, patients may become under-exposed.

In figure 30 the distribution of sunitinib concentrations in the case study is displayed.

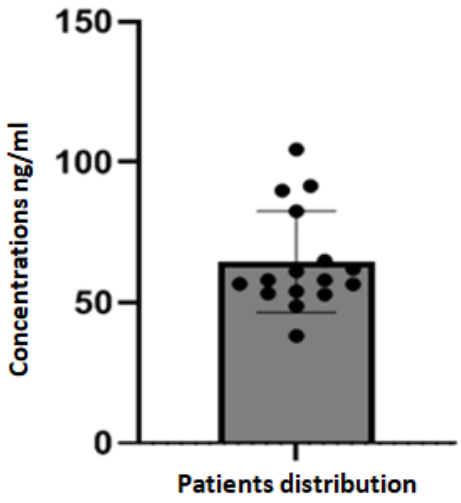


Figure 30: sunitinib samples distribution

Regardless the dose level, the mean Cmin value was 63.6 ± 18.2 ng/mL, with a maximum Cmin equal to 104,5 ng/mL and a minimum of 38,2 ng/mL. Despite most of the patients were treated with a reduced dose (both 37,5 and 25 mg/die),

except for 2 samples, the measured Cmin were always within the TDM target of 50-100 ng/mL, as shown in Figure 29. Taking into account patients treated with 50 mg/die, only one sample slightly exceeded the TDM target, being equal to 104.5 ng/mL. Considering the dose level of 37.5 mg/die, all patients resulted over the target of 37.5 ng/mL (and even over the 50 ng/mL target) but always < 100 ng/mL. As related to patients treated at the dose of 25 mg/die, no indication of TDM target has been reported in literature. Anyway, we observed that all samples showed a Cmin > than 37.5 ng/mL.

Regarding one of the aims of this work namely evaluating the TDM relevance, patients interindividual variability among drug exposure has been observed, making useful and interested to monitor sunitinib treatment.

A tentative correlation between dose and exposure was performed (Figure 31), as previously done for imatinib.

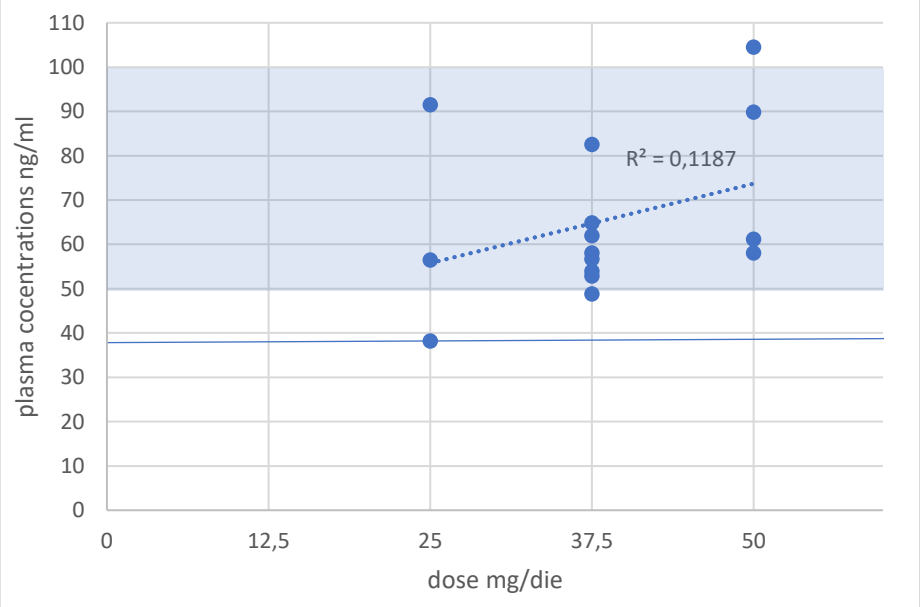


Figure 31: correlation graph between drug dose and plasma concentration (Cmin). In azure is highlighted the TDM target 50-100 ng/mL; the blue line indicates the 37,5 ng/mL target, and the dotted line is the trend line which represent the linear dependence.

Differently from imatinib, in this case exposure seems to depend on the dose, as the concentrations in the plot grow according with the dose level.

As the Figure 31 shows, there is a wide variety of dosage in this small population. In Table 14 patients' distribution between the three different doses is represented, and the mean Cmin related to the dose level has been reported:

again, mean Cmin seemed to grow with the dose. This is a very preliminary observation due to the paucity of patients and samples.

| Dose (mg/die) | number of patients | number of samples | mean concentrations ± SD ng/mL |
|---|---|---|---|
| **25** | 2 | 3 | 61,7±19,2 |
| **37,5** | 6 | 9 | 65,6±18,3 |
| **50** | 2 | 4 | 85,2±17,3 |

Table 14: dosage-concentration relationship.

Another interested parameter to evaluate is the percentage of active metabolite N-DES SUN with respect to sunitinib.

Sunitinib is subject to microsomal oxidative dealkylation through CYP3A4 to N-DES SUN, which shows a similar inhibitory action on tyrosine kinases as the parent drug. It has been estimated that N-DES-SUN represents approximately 30% of the overall exposure (sunitinib + N-DES-SUN). N-DES-SUN is further metabolized through CYP3A4, and its elimination half-life is even longer, up to 80–100 hours, while its $V_d$ is similar or greater than that of sunitinib.

Biotransformation of sunitinib is influenced by sex: in fact, female-patients have up to 30% lower apparent clearance (Cl/F), but even this difference does not imply different dosing for women (15). Additionally, the population pharmacokinetic analysis identified female gender and low body weight as covariates that significantly increase exposure to sunitinib (43)

In this case study, the mean percentage of N-DES-SUN concentration respect to the overall exposure was 22±4,20%, which is lower than the literature data (30%).

In the table 15 progressive number, sex, and percentage of metabolite with respect to overall concentration are reported.

| N. progressive number | SEX | % Metabolite with respect to sum | exposure | Dose mg/die |
|---|---|---|---|---|
| **1991,1** | M | 22 | **53,3** | 37,5 mg/die |
| **1752,2** | F | 17 | **104,5** | 50 mg/die |
| **1752,3** | F | 22 | 89,9 | 50 mg/die |
| **1914,1** | M | 19 | 58.1 | 50 mg/die |
| **1914,2** | M | 22 | 61,2 | 50 mg/die |
| **1974,1** | M | 24 | **82,6** | 37,5 mg/die |
| **2002,1** | F | 31 | **56,5** | 25 mg/die |
| **2002,2** | F | 25 | **91,5** | 25 mg/die |
| **16_4** | F | 25 | **58,1** | 37,5 mg/die |
| **16_5** | F | 25 | **56.7** | 37,5 mg/die |
| **1897,1** | M | 26 | **38,2** | 25 mg/die |
| **1946,1** | M | 16 | **54** | 37,5 mg/die |
| **1946,2** | M | 16 | **52,9** | 37,5 mg/die |
| **1952,1** | M | 26 | **48,8** | 37,5 mg/die |
| **1929,1** | M | 18 | **64,9** | 37,5 mg/die |
| **1929,2** | M | 21 | **62** | 37,5 mg/die |

Table 15: samples of sunitinib reported with gender, in pink female, and % of metabolite with respect to the sum.

Taking into account only the female patients, the mean percentage of metabolite with respect to the sum resulted 24±4% instead, for male patients this percentage was 21±3,5%. This result seems to confirm that biotransformation is influenced by female-patients. However, this data should be considered preliminary, due to the paucity of patient's population.

Thus, is not even possible to compare female/male exposure, because of the inhomogeneity of the dose and the patient's paucity (3 female vs 7 male patients). Although, for the same reason no statistical analysis has been done yet.

## 4.3 Polymorphisms distribution in the case study

Pharmacogenetic analyses focused on the investigation of polymorphisms in *CYP3A4, CYP3A5, ABCB1*and *ABCG2* genes for both drugs. CYP2C8, SLC22A1, SLCO1B3, CYP2D6 were selected only for imatinib relying on its metabolism. These are genes encoding for enzymes involved in phase I and phase II metabolism or in the transport of intermediates of oral anticancer drugs. The SNps selection was based on the before mentioned criteria and led to the identification of the list reported in Table below.

| Gene | SNP ID | Genetic variation |
|---|---|---|
| **CYP3A5** | rs776746 | c.6981A>G |
| | rs10264272 | c.14685G>A |
| | rs41303343 | c.27126_27127insT |
| **CYP3A4** | rs2242480 | c.313C>T |
| | rs35599367 | c.15389T>C |
| **CYP2D6** | rs35742686 | c.2550delA |
| | rs3892097 | c.1847G>A |
| | rs5030655 | c.1708delT |
| | rs5030865 | c.1759G>A,T |
| | rs5030656 | c.2616delAAG 100C>T |
| | rs1065852 | c.100C>T |
| | rs28371706 | c.1022C>A |
| | rs28371725 | c.2989G>A |
| | CYP2D6_5 | |
| | CYP2D6_xN | |
| **CYP2C8** | rs10509681 | c.35506A>G |
| | rs11572080 | c.7225G>T |
| | rs11572103 | c.16149A>T |

Table 16: selected polymorphisms on cytochrome involved in drug metabolism, in green are shown SNPs selected only for imatinib.

| Gene | SNP | Mutation |
|---|---|---|
| **ABCB1** | rs1128503 | c.1236C>T |
| | rs1045642 | c.3435CT |
| | rs2032582 | c.2677G>A,T |
| **ABCG2** | rs2231142 | c.412C>A |
| **SLCO1B3** | rs4149117 | c.52843T>G |
| **SLC22A1** | rs628031 | c.408G>A |
| | rs683369 | c.160G>C |

Table 17: selected polymorphisms on the transporter of drug or intermediates, in green are shown the ones implicated only in imatinib transport.

Pharmacogenetic data regarding this list of SNPs were collected for all 57 patients under study using the Real Time PCR and via the SNPline PCR Genotyping System platform as previously described.

The data obtained for the genotyping with the polymorphisms selected are presented in the Table 18 and 19, which show the genotype frequency within the case study, along with the minor allele frequency.

| Gene | SNP | Star allele | Wild type | Heterozygote | Mutated | Wild type | | Heterozygote | | Mutated | | MAF GnomAD* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | n % | | n % | | n % | |
| **CYP3A5** | rs776746 | *3 | AA | AG | GG | 0 | 0,000 | 6 | 10,526 | 51 | 89,474 | 0.276806 |
| | rs10264272 | *6 | CC | CT | TT | 57 | 100,000 | 0 | 0,000 | 0 | 0,000 | 0.0399 |
| | rs41303343 | *7 | -:- | A:- | AA | 57 | 100,000 | 0 | 0,000 | 0 | 0,000 | 0.0315 |
| **CYP3A4** | rs4986910 | *3 | TT | TC | CC | 57 | 100,000 | 0 | 0,000 | 0 | 0,000 | 0.0052 |
| | rs67666821 | *20 | -:- | TT | :T | 57 | 100,000 | 0 | 0,000 | 0 | 0,000 | N.A. |
| | rs35599367 | *22 | C:C | C:T | T:T | 53 | 92,982 | 4 | 7,018 | 0 | 0,000 | 0.0326 |
| **ABCB1** | rs1128503 | | C:C | C:T | T:T | 19 | 33,333 | 23 | 40,351 | 15 | 26,316 | 0.3684 |
| | rs1045642 | | C:C | T:C | T:T | 13 | 22,807 | 23 | 40,351 | 21 | 36,842 | 0.421 |
| | rs2032582 | | GG | G:T | T:T | 18 | 31,579 | 25 | 43,860 | 14 | 24,561 | N.A. |
| **ABCG2** | rs2231142 | | C:C | A:C | A:A | 41 | 71,930 | 14 | 24,561 | 2 | 3,509 | N.A. |

* GnomAD obtained from dbSNP https://www.ncbi.nlm.nih.gov/snp/;

Table 18: Frequency of selected polymorphisms of phase I and II enzymes and transporter of imatinib and sunitinib metabolism.

| Gene | SNP | Star allele | Wild type | Heterozygote | Mutated | Wild type | | Heterozygote | | Mutated | | MAF GnomAD* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | n | % | n | % | n | % | |
| **CYP2D6** | rs35742686 | *3 | A:A | -:A | -:- | 46 | 97,872 | 0 | 0,000 | 1 | 2,128 | 0.0128 |
| | rs3892097 | *4 | G:G | A:G | A:A | 31 | 65,957 | 15 | 31,915 | 1 | 2,128 | 0.1434 |
| | rs5030655 | *6 | T:T | -:T | -:- | 47 | 100,000 | 0 | 0,000 | 0 | 0,000 | 0.0088 |
| | rs5030865_1 | *8 | G:G | T:G | T:T | 47 | 100,000 | 0 | 0,000 | 0 | 0,000 | N.A. |
| | rs5030656 | *9 | AAG:AAG | -:AAG | -:- | 44 | 93,617 | 3 | 6,383 | 0 | 0,000 | 0.0183 |
| | rs1065852 | *10 | C:C | T:C | T:T | 31 | 65,957 | 16 | 34,043 | 0 | 0,000 | 0.1867 |
| | rs5030865_2 | *14A/B | G:G | A:G | A:A | 47 | 100,000 | 0 | 0,000 | 0 | 0,000 | N.A. |
| | rs28371706 | *17 | C:C | T:C | T:T | 47 | 100,000 | 0 | 0,000 | 0 | 0,000 | N.A. |
| | rs28371725 | *41 | G:G | G:A | A:A | 38 | 80,851 | 9 | 19,149 | 0 | 0,000 | 0.0674 |
| | CYP2D6_5 | *5 | AA:AA | AG:AC | GG:CC | 46 | 97,872 | 0 | 0,000 | 1 | 2,128 | N.A. |
| | CYP2D6_xN | xN | GG:CC | AG:AC | AA:AA | 45 | 95,745 | 0 | 0,000 | 2 | 4,255 | N.A. |
| **SLCO1B3** | rs4149117 | | G:G | G:T | T:T | 35 | 74,468 | 12 | 25,532 | 0 | 0,000 | N.A. |
| **SLC22A1** | rs628031 | *1 | G:G | G:A | A:A | 23 | 48,936 | 21 | 44,681 | 3 | 6,383 | N.A. |
| | rs683369 | | C:C | C:G | G:G | 33 | 70,213 | 13 | 27,660 | 1 | 2,128 | 0.1499 |
| **CYP2C8** | rs10509681 | | A:A | A:G | G:G | 36 | 76,596 | 10 | 21,277 | 1 | 2,128 | 0,083 |
| | rs11572080 | | G:G | G:A | A:A | 36 | 76,596 | 10 | 21,277 | 1 | 2,128 | 0,081 |
| | rs11572103 | | T:T | T:A | A:A | 47 | 100,000 | 0 | 0,000 | 0 | 0,000 | 0,049 |

* GnomAD obtained from dbSNP https://www.ncbi.nlm.nih.gov/snp/;

Table 19: Frequency of selected polymorphisms of transporter and phase I and II enzymes of imatinib metabolism.

### 4.3.1 Diplotype classification of patients genotype for cytochrome encoding genes

Cytochrome encoding genes own an intrinsic genetic complexity, hence, the identification of the genotype related to a single SNPs is not enough to address the overall predicted activity of a specific isoform.

A series of SNPs within the same gene it is necessary to define a genotype, so in this work more SNPs for every cytochrome were selected and genotyped (for example in the case of CYP2D6 the SNPs analyzed are 11).

Subsequently, a haplotype deriving from the various combinations of these SNPs is elaborated and will be identified through a specific *allele denomination (such as: * 1, * 4, * 8, etc).

Each haplotype identifies the single allele to which a CPIC committee has assigned a score called "*allele activity score*", based on in vitro or in vivo evidence, and on available clinical studies. This "*allele activity score*" identifies the predicted residual activity of a protein encoded by each polymorphic allele.

The activity score available on the CPIC website corresponding to each allele (only for CYP2D6), were included in table 20.

This parameter was found to be extremely relevant because it provides clear prediction of the functionality of the protein encoded by that specific haplotype.

| Gene | SNP | Allele | Allele activity score* | Allele functional status* |
|---|---|---|---|---|
| CYP3A5 | | *1 | N.A. | Normal function |
| | rs776746 | *3 | N.A. | No function |
| | rs10264272 | *6 | N.A. | No function |
| | rs41303343 | *7 | N.A. | No function |
| CYP3A4** | rs4986910 | *3 | N.A. | N.A. |
| | rs67666821 | *20 | N.A. | No function |
| | rs35599367 | *22 | N.A. | Decreased function |
| CYP2D6 | | *1 | 1 | Normal function |
| | rs35742686 | *3 | 0 | No function |
| | rs3892097 | *4 | 0 | No function |
| | rs5030655 | *6 | 0 | No function |
| | rs5030865_1 | *8 | 0 | No function |
| | rs5030656 | *9 | 0.5 | Decreased function |
| | rs1065852 | *10 | 0.25 | Decreased function |
| | rs5030865_2 | *14A/B | 0.5 | Decreased function |
| | rs28371706 | *17 | 0.5 | Decreased function |
| | rs28371725 | *41 | 0.5 | Decreased function |
| | CYP2D6_5 | *5 | 0 | No function |
| | CYP2D6_xN | xN | 2 | Increased function |

Table 20: Allele functional status of selected polymorphisms of phase I and II enzymes drug metabolism

Obviously, this Table refers to the single allele and since each gene is present in the human genome as two-allelic, then the patient will be identified by a diplotype (combination of two alleles).

Given that, the two alleles of the diplotype may have a different allele activity score, and, whit the aim of making a prediction of the overall diplotype functionality, the CPIC has developed a system: by adding the allele activity score of each allele, the overall activity score of each patient will be obtained ("*gene activity score*", GAS). Finally, each GAS was assigned a genetically predicted enzyme phenotype.

For other genes (ex. CYP3A5, ABCG2, …) the single allele activity score was not available on the CPIC website, consequently a GAS cannot be calculated according to this approach. However, a prediction of the overall phenotype is

provided for each genetic diplotype on the CPIC website and was used for this thesis.

Genotype-predicted phenotypes are distinguished into:

- poor metabolizer (PM): carry two inactive alleles.
- intermediate metabolizer (IM): can result from a combination of normal allele and one non-functional allele or one with reduced functionality; they may also result from both alleles with reduced or no function, or by the combination of one allele with reduced function and the other with no function.
- normal metabolizer (NM): generally, have two alleles with normal function. In some cases, genes with one normal allele and one with reduced activity can also be defined as normal metabolizers.
- rapid metabolizer (RM): simply have one normal allele and one allele with increased activity
- ultra-rapid metabolizer (UM): are the result of a combination of two alleles with increased activity or, in the specific case of cytochrome 2D6, the presence of duplications even in alleles with normal functionality.

Therefore, the table below shows diplotypes found in the genetic analysis of the case study (in black) and other examples (in grey), subdivided according withtheir respective metabolic status. In addition, their frequency in relation to thetotal number of patients has been calculated and the frequency of each cytochrome in the European population has been provided as a reference, again based on the CPIC guidelines.

| Gene | Phenotype (metabolic status) | Combined activity score* | Diplotype | Study popolation phenotype frequency | European phenotype frequency** |
|---|---|---|---|---|---|
| **CYP3A5** | PM | N.A. | * 3/* 3, * 6/* 6, * 7/* 7; * 3/* 6; * 3/* 7; * 6/* 7 | 0,9123 | 0.8573 |
| | IM | N.A. | * 1/* 3, * 1/* 6, * 1/* 7 | 0,0887 | 0.1372 |
| | NM | N.A. | * 1/* 1 | 0 | 0.0055 |
| **CYP2D6** | PM | 0 | * 3/* 3, * 3/* 3x2, * 3/* 4, * 3/* 4≥2, * 3/* 5, * 3/* 6, * 3/* 8 | 0,0425 | 0.0647 |
| | IM | 0.25 - 0.5 -0.75 - 1 | * 1/* 3, * 1/4, * 1/* 5, * 1/* 6, * 3/* 10,* 10/* 10, * 10/* 41, * 4/* 10, * 4/* 10xN, * 5/* 10, * 6/* 10, * 9/* 10 | 0,3617 | 0.3895 |
| | NM | 1.25 - 1.5 -2 - 2.25 | * 1/* 1, * 1/* 9, * 1/* 10, * 1/* 17, * 1/* 41, * 1/* 41xN, | 0,5957 | 0.5105 |
| | UM | ≥ 2.5 | * 1/xN | 0 | 0.0307 |

Table 21: Metabolic status of phase I and II drug metabolism enzymes selected polymorphism

*"diplotype-phenotype table" from CPIC guidelines accessed by 23.11.2021,
**"allele frequency table" from CPIC guidelines accessed by 23.11.2021 https://cpicpgx.org/guidelines/

Under each gene encoding the cytochrome, the number of patients out of a total of 57 in whom the metabolic status of the enzyme could be assigned is indicated. For CYP2D6 are considered only imatinib patients, a total of 47.

The graph in Figure 32 and 33 gives an overview of the distribution between metabolic phenotypes of this case study, taking count of CYP2D6 and CYP3A5 phenotypic frequencies.

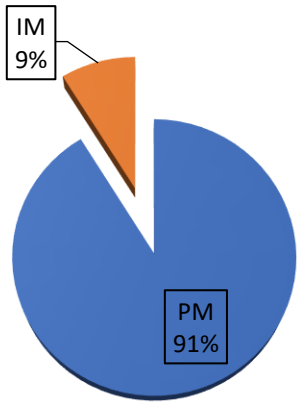


Figure 32: metabolic phenotypes distribution of CYP3A5, PM is poor metabolizer, IM intermediate metabolizer.

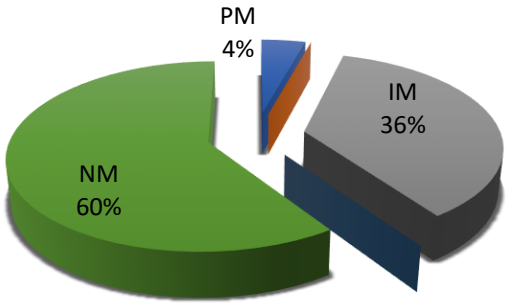


Figure 33: metabolic phenotypes distribution of CYP2D6. PM is poor metabolizer, IM intermediate metabolizer.

### 4.3.2 Pharmacogenetics as a predictive tool for imatinib exposure

*Association of Cmin with CYP genotypes/ phenotypes*

With the aim of finding a genetic factor that could be predictive for imatinib exposure, a statistical analysis was performed by associating mean Cmin of patients with the metabolic phenotypes of the cytochromes tested (CYP2D6, CYP3A5, CYP2C8) and single polymorphisms in transporters encoding genes (ABCB1, ABCG2, SLC22A1).

Firstly, the association between the mean Cmin and the genotype/ phenotype of the cytochrome involved in drug metabolism has been evaluated.

The association between mean Cmin and CYP3A5 metabolic phenotype shows a trend for a higher Cmin in intermediate in respect to poor metabolizer. It should be noted that in this case series there are only 3 intermediate metabolizers in respect of 44 poor metabolizers, so the trend will be influenced from this bias; p-value in this association is not significant.

Then, mean imatinib Cmin was analysed for association with CYP3A4 in a single polymorphism CYP3A4*22 (rs35599367): in this case the heterozygosity is represented only by 2 patients. The correlation it is not significant, and because of the too small number of heterozygous neither a trend is visible.

Thereafter, the association between CYP2C8 polymorphisms and mean Cmin was tested. Polymorphisms rs10509681 and rs11572080 were associates with mean Cmin, for both polymorphisms only AG and AA genotypes was identified. The association whit mean Cmin did not turned out significant, nor a well-defined trend was turned out.

Then, Cmin was associated with metabolic phenotypes of CYP2D6, following Dalle Fratte C. et al(44) where is suggest that the GAS and the predicted metabolic phenotype of CYP2D6 may help refine the prediction of imatinib plasma Cmin in GIST patients.

The association was not found significant, but, as reported in literature, PM and IM were turned out to be more exposed to imatinib with respect to NM. Even in this case, the bias was found in the paucity of the poor metaboliser phenotype

patients.

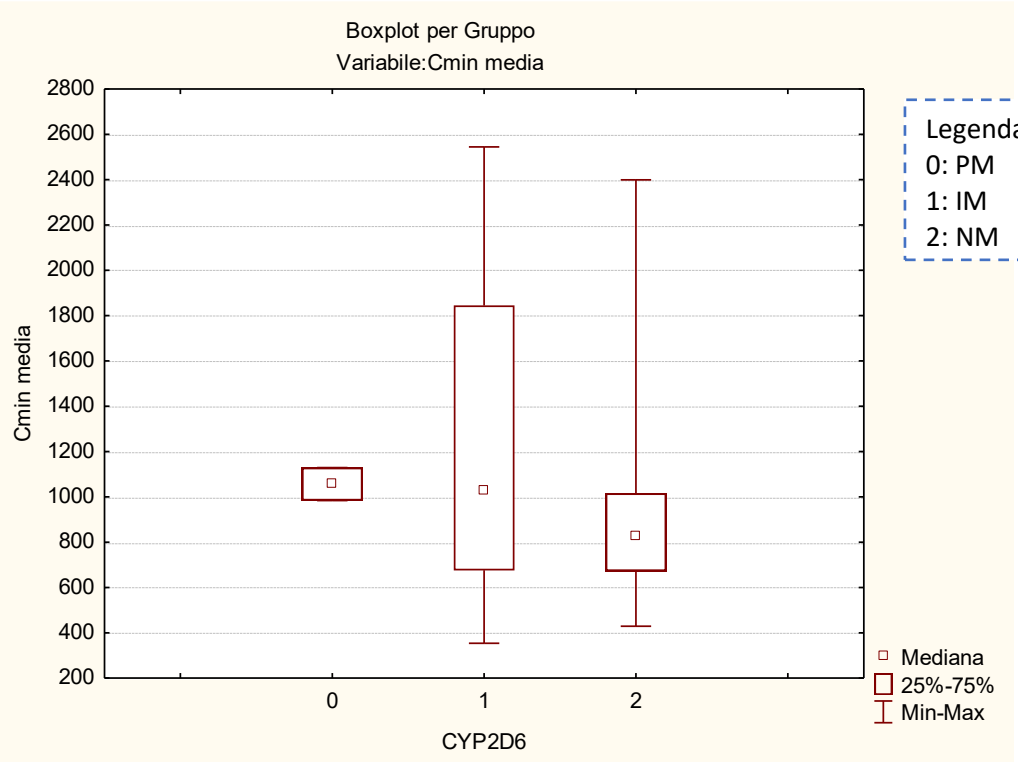


Figure 34: association between mean Cmin and CYP2D6 metabolic phenotypes.

### *Association of Cmin with transporters encoding genes genotypes*

The following association study was carried out between mean imatinib Cmin and transporter's polymorphisms.

The first analysis gave a significant association between mean Cmin and the *ABCB1* transporter genotypes. Three different polymorphisms c.3435C>T (rs1045642), c.2677G>T/A (rs2032582) and c.1236C>T (rs1128503) were tested; these polymorphisms lead to *ABCB1* mRNA instability, thus leading to a reduced protein expression and to a reduced functionality of P-gP *in vivo.* These 3 polymorphisms are in linkage disequilibrium.

Association between c.2677G>T/A (rs2032582) and c.1236C>T with the mean Cmin was not significant (in terms of p-value). The association with 3435 C>T genotype was significant with a p-value of 0,013. The trend describes the CC genotypes (wild type) more exposed to the drug in respect of TT genotypes.

The same trend is highlighted in the other 2 polymorphisms tested: the CC genotype for 1236 C>T and the GG genotype for 2677 G>T/A are more exposed to the drug in respect of the other genotypes.

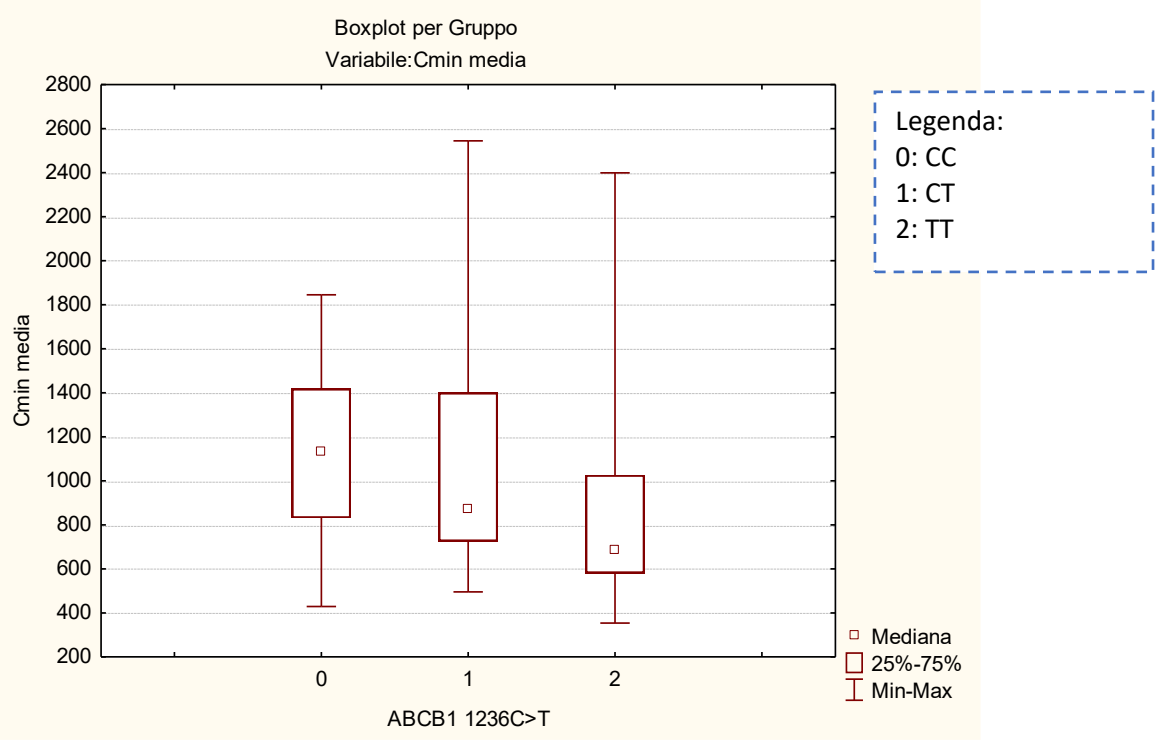


Figure 35: association between mean Cmin and ABCB1 1236 C>T polymorphism.

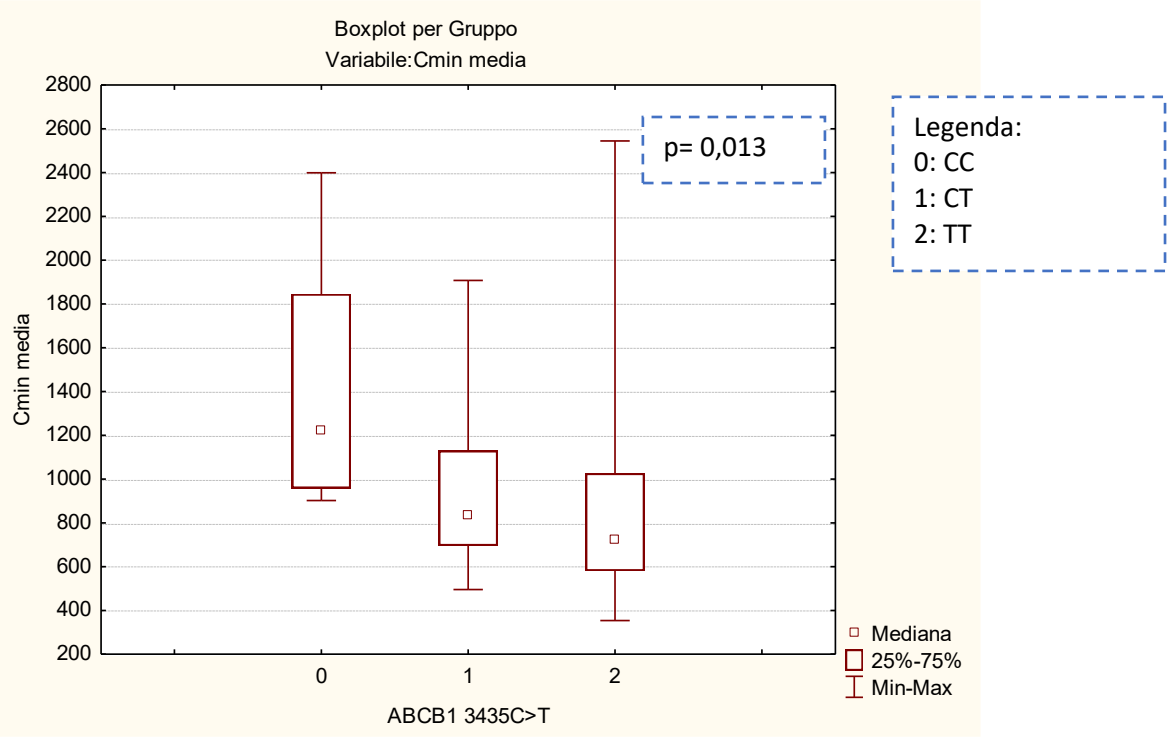


Figure 36: association between mean Cmin and ABCB1 3435C>T polymorphism.

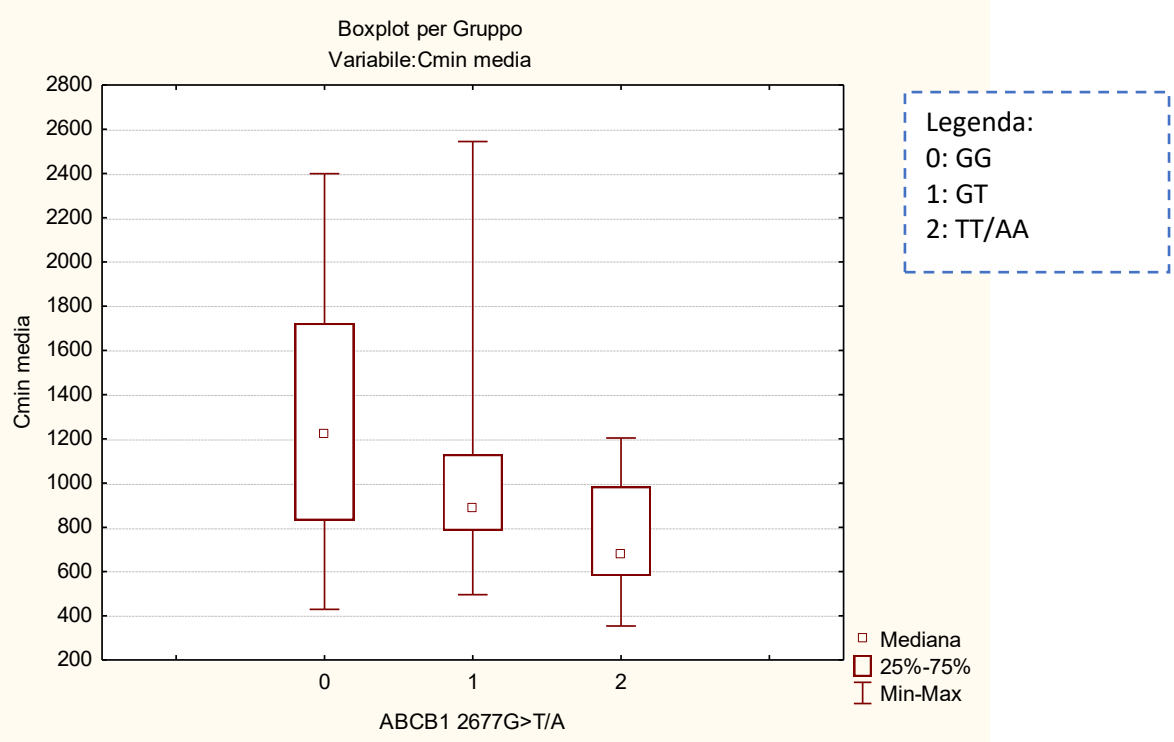


Figure 37: association between mean Cmin and ABCB1 2677g>T/A polymorphism.

Thereafter, the BCRP gene encoded by *ABCG2* has been tested. Association between the mean Cmin and *ABCG2* 421 C>A polymorphism proved to be not significant, but a clear trend has been evidenced: AA genotype was shown to have a high exposure to the drug with respect to wild CC genotype. In this plot, CA genotype was put together with CC genotype, in order to better show the trend between WT and homozygous for the variant. P-value is not significant.

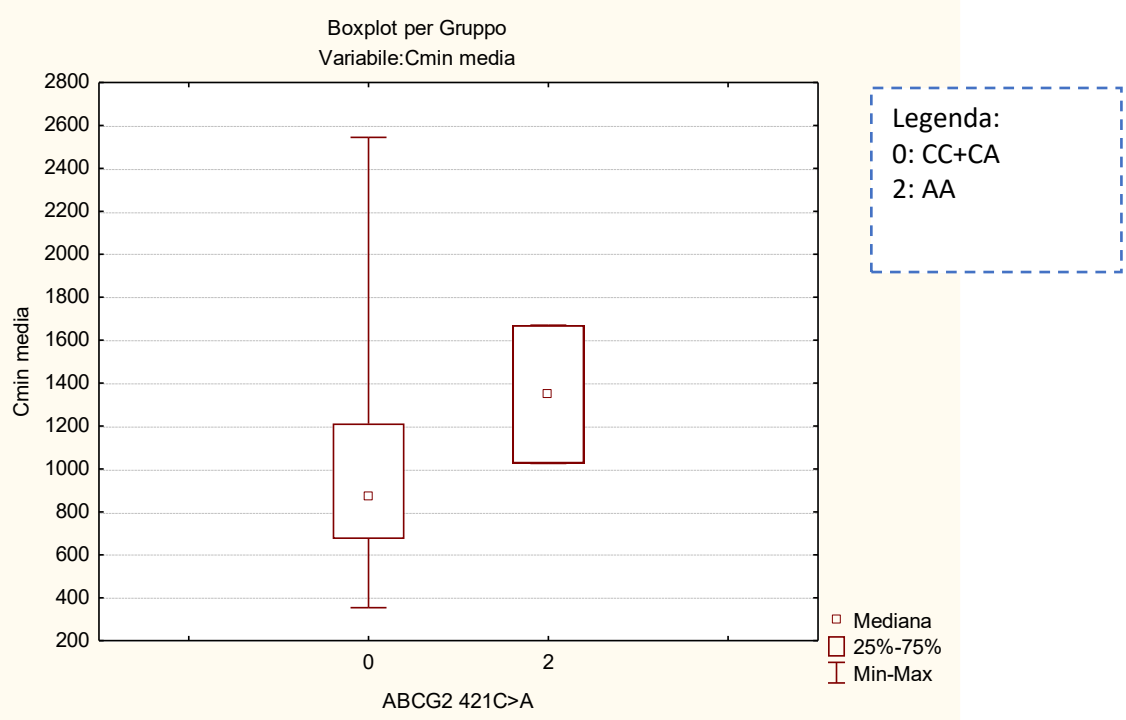


Figure 38: association between mean Cmin and ABCG2 421C>A polymorphism.

Then the SLC class of transporters has been analysed.

During the study of imatinib metabolism and transportation, SLCA221 and SLCO1B3 were selected. Association between mean Cmin and SLCA221 c.1222A>G (rs628031) and SLCA221 c.480G>C (rs683369) are shown in the Figure 33 and 34.

As for ABCG2, also for SLCA221 c.1222A>G, the GG genotype and the GA genotype are considered in the same group: as a result, a well-defined trend is shown in Figure 33. Patients with AA genotype for c.1222A>G polymorphism become more expose to imatinib in respect to GG genotype; indeed, the p-value is 0,0138 thus the association is significant.

In Figure 34 is showed the association between mean Cmin and the other SLCA221 polymorphism c.480G>C (rs683369), in this case the 3 genotype are distinguished. Also in this case a trend can be identified, the same of the before mentioned polymorphism. Nevertheless, in c.480G>C the CC genotype is represented by only 1 patient; thus, the association may not be reliable. P-value is not significant.

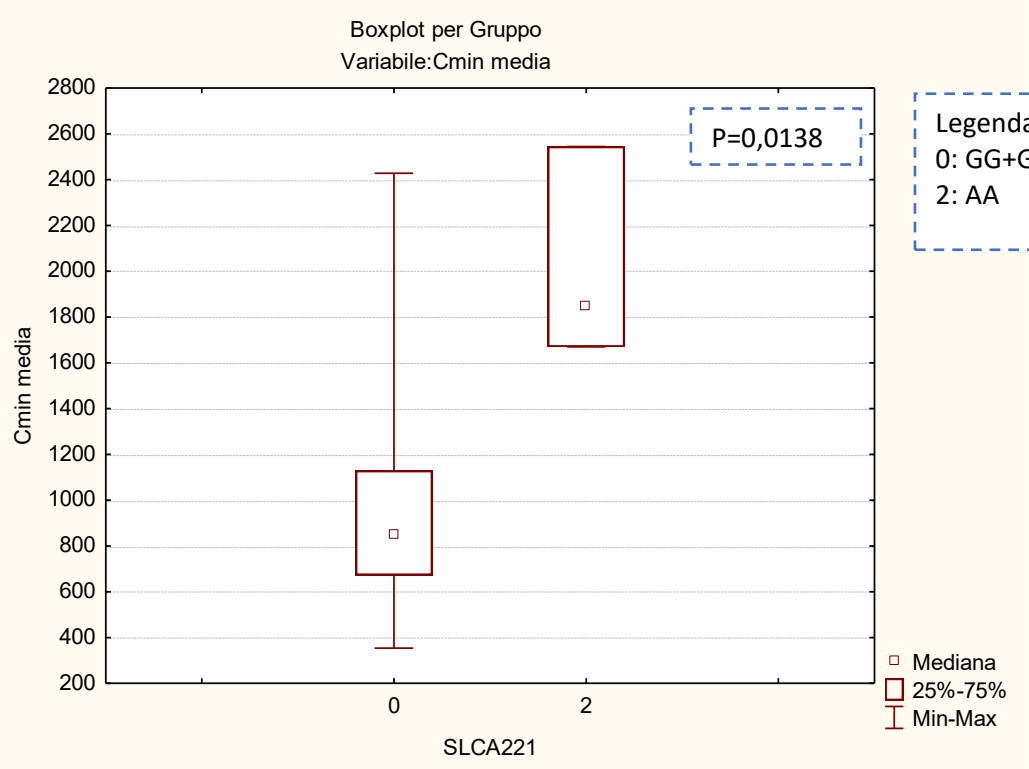


Figure 39: association between mean Cmin and SLCA221 rs628031 polymorphism.

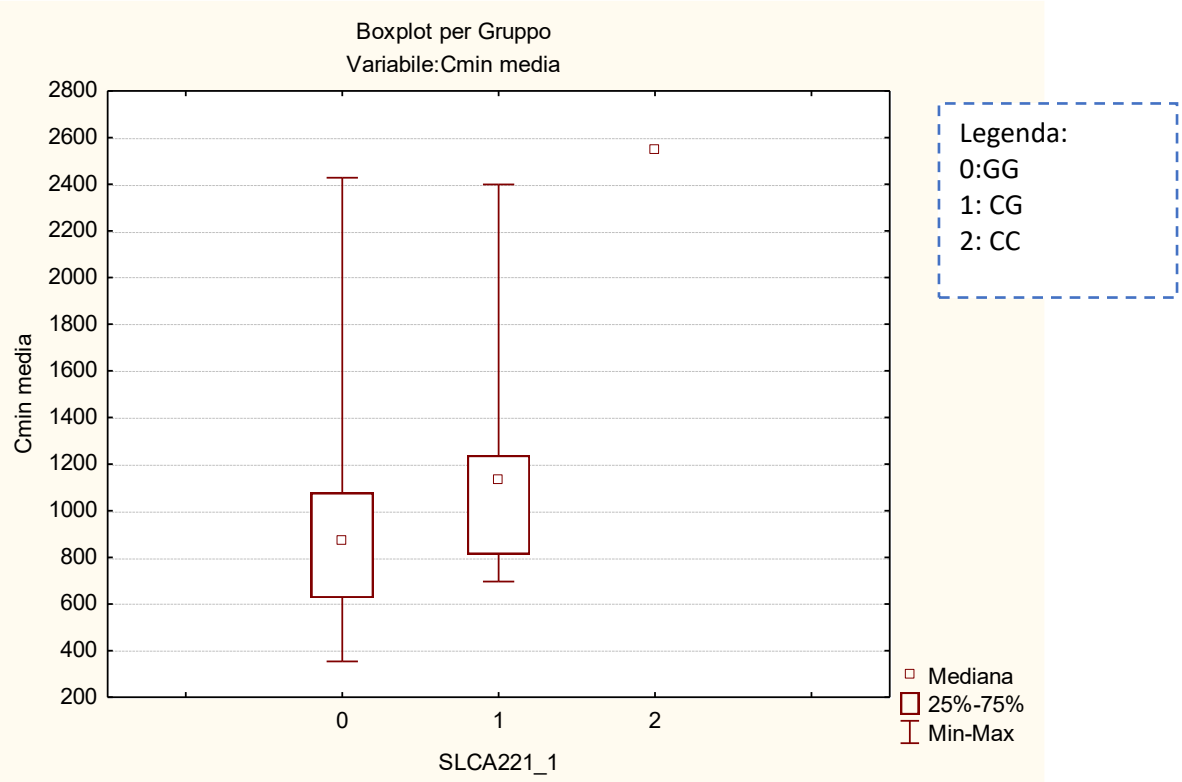


Figure 40: correlation between mean Cmin and SLCA221 rs683369 polymorphism.

# 5 DISCUSSION

Interindividual variability in drug exposure to oral anticancerdrugs depends on both exogenous factors, including the genetic variability of the tumor and the host, such as environmental factors or pathophysiological factors. In this work it is evaluated the combined approach between therapeutic drug monitoring and pharmacogenetics, namely analysing polymorphisms in drug metabolism and transport, in order to highlight and monitor interindividual variability.

High interindividual variability in exposure has been reported for imatinib and sunitinib (3): variability was confirmed, as Figure 25 and Figure 30 prove, showing a large distribution of concentrations values both for imatinib samples and sunitinib's ones.

Additionally, some samples were find to be outside of the TDM targets, where the efficacy was proven (2). Thus, hypothetically, can lead patients' progression in the disease or development grade≥3 toxicities. Sample distribution between TDM target is represented in Figure 25 for imatinib and Figure 30 for sunitinib, in which are highlighted the targets areas.

Correlation between dose and exposure has been studied both for imatinib and sunitinib, yielding opposite results: imatinib exposure seems not depending on the dose, Figure 26 represents the non-linear trend of the sample distribution between the six dosages regarded in the study; for sunitinib, on the other hand, there seems to be a correlation between dose and exposure, as represented in Figure 31, samples distribution among different doses follows a linear trend.

The clinical relevance of TDM has been established, by referring to Table 1, where the current evidence for TDM-guided dosing of OADs was reported, and to Groenland et al (15) where a large TDM study in OADs has been reported.

Then, the feasibility of TDM approach in OADs has been evaluated. The monitoring of drug exposure was performed as a rutinary test: patients came to the collection center of Centro di Riferimento Oncologico and did the collection together with the quality-of-life questionnaire, with the aid of our TDM team. This collection method has been functional in this work of thesis, nevertheless with the goal of applying TDM to the clinical practice, the necessity to get physically the patient to the collection center may prove to be an obstacle.

In order to solve this issue, the Pharmacology Unit of CRO Aviano is evaluating the possibility to substitute plasma sample, which need the patient to go to the hospital, with the Dried Blood Spot (DBS), which is a form of bio-sampling where

blood samples are blotted and dried on filter paper. This dried samples can easily be shipped to an analytical laboratory and analysed using various methods such as HPLC-MS/MS. Pharmacology Unit of CRO is developing chromatographic method (45) to analyse DBS and is now studying the correlation between plasma samples and dried samples, trying to find out whether plasma sample can be replaced with the dried ones. For this replacement a cross validation study is required with the application of proper statistical tests (46).

Another objective of this thesis was to investigate factors that affect exposure: data about co-medications and food intake were collected even though not systematically analysed for the purpose of this thesis. More attention has been paid on the co-medications; basic drug-drug interactions were analysed by referring to "Le interazioni tra farmaci" of the Mario Negri institute of research, where the major pharmacological interactions are reported and explained. In this thesis, the role of drug-drug interaction in drug exposure has not been fully developed.

Patients with cancer usually are polypharmacy patients, as well aged patients usually take several types of drug, and it is very likely that at least two of these drug are inducer/inhibitor of the same cytochrome (1).

Hence, the assessment of drug-drug interactions resulted to be of great importance in the application of this combined approach between TDM and pharmacogenetics in OAD, indeed, the Pharmacology Unit of CRO di Aviano has already reported a case study regarding drug-drug interaction and drug exposure correlation. A 63-year-old male treated with imatinib for which a TDM evaluation was performed, highlighting an imatinib Cmin of 406 ng/mL and NOR-imatinib Cmin of 213 ng/mL. Potential causes of such poor exposure were assessed by investigating patient's compliance, pharmacogenetics and DDIs, applying the integrated approach reported in this work.

No significant pharmacogenetic markers were identified, compliance to therapy was considered adequate but by analysing potential DDIs a significant interaction was identified between imatinib and carbamazepine, known to be a potent inducer of CYP3A4 and P-gp(47). Analysing also NOR-ima/imatinib ratio it was confirmed the metabolism induction. After reporting these data to the clinician through pharmacological counselling the dose was increased: in the further drug quantifications Cmin got up to 892 ng/mL, double the previous

quantification. Despite the imatinib concentration was still lower than plasma levels associated with efficacy, the TDM approach together with the DDIs examine was decisive for the therapy's outcome.
This case study underlines how the combined approach are of great significance in clinical practice. Without the monitoring of plasma concentration, it would not have been possible to discover this poor exposure and to investigate the possible causes. TDM is a necessary tool to ensure that the therapy is carried out correctly.

Supporting the utility of TDM in clinical practice, a case of imatinib non-compliance was highlighted through the application of the TDM approach. A male-patients treated with imatinib, for whom TDM was requested, was discovered to have imatinib plasma level under the limit of quantification of the chromatographic method, means too low to be quantified. Patients always refers to follow the therapy correctly. This suggests a low compliance to the therapy, and as well the possibility of disease progression despite treatment.
TDM approach was crucial in discovering this case of non-compliance, to evaluate drug exposition can also be a useful strategy for compliance monitoring.

Another interesting aspect reported in this work concerns the influence of sex in drug exposure, which can be evaluated using the TDM approach.
For imatinib, a correlation between mean Cmin and sex has been tested and reported in the Figure 32. A trend was obtained, which denotes female patients to be more exposed to imatinib with respect to male patients. Nevertheless, the correlation did not have a significant p-value.
For suntinib no statistical evaluation could be done due to the non-homogeneity of the group of patients and its low numerosity. As a result, further analyses will be needed to provide more precise considerations of the impact of gender in drug exposure.

Among all these reported factors, pharmacogenetics results to be one of the most causative factors of the observed inter-individual variability in term of plasmatic drug exposure. Genetic variability within genes encoding for enzymes involved in drug metabolism and transport may cause many differences in drug

exposure; identifying the patient's metabolic phenotype is of great importance to understand the variability in exposure.
Pharmacogenetic analysis of the enzyme implied in drug metabolism and transporter has already entered in the clinical practice and proved to be extremely useful for therapies outcomes. Nowadays in the Pharmacology Unit of CRO polymorphisms analysis are carried out as diagnostics analysis, directly prescribed by the clinicians. The important role of pharmacogenetics in imatinib exposure has already been tested in Dalle Fratte C et Al. (35), reporting several interesting information: *CYP2D6* poor and intermediate metabolizers were predicted to have a lower nor-imatinib/imatinib metabolic ratio than normal metabolizers (0.197 and 0.193 *vs*. 0.247, *P* = .0205), whereas *CYP2C8**3 carriers had a higher ratio than *CYP2C8*1/*1* patients (0.263 *vs*. 0.201, *P* = .0220). These findings underline that *CYP2D6* plays a major role in imatinib pharmacokinetics, and the other players (i.e., *CYP2C8*) may influence imatinib exposure. Based on these findings, with another research phase, these cytochromes were selected in this work to evaluate pharmacogenetic polymorphisms for imatinib.

Accordingly, in my thesis an association trend was observed between CYP2D6 metabolic phenotype and mean imatinib Cmin, where NM were found to be more exposed to the drug as compared to PM and IM phenotypes. Regarding the association between ima/Cmin and the other cytochromes genotypes/phenotypes in the study, there were not enough patients to represent the different metabolic phenotypes/genotypes groups, thus the results were not significant and neither a trend was detectable.

The activity of the efflux transporters such as P-gP, BCRP and SLC plays a pivotal role modulating the quantity of drug reaching the systemic circulation, and hence the tumor cell, as well as the amount of drug that is delivered to the intracellular molecular.
Hence, the correlation between ABCB1 transporter and mean Cmin has been tested: proved to be well-defined: seven studies including 649 patients administered with imatinib 400 mg per day were available for the association between *ABCB1* c.3435C>T and imatinib plasma Cmin levels (48),(49).
Polymorphisms c.3435C>T, c.2677G>T/A and c.1236C>T were selected: they are in strong *linkage disequilibrium* with each other, forming and haplotype, and

have been associated with a reduced P-gP expression and functionality. The presence of the minor allele in each genotype for the three *ABCB1* polymorphisms was previously associated to a lower P-gP activity with respect to the wild-type *ABCB1* and to a lower imatinib export capacity(50). In this case study a trend has been identified among ABCB1 polymorphisms, which seems to be in agreement with the literature: the CC genotype as GG genotype and CC genotype (wild type) resulted to be more exposed to the drug in respect of the genotypes containing the minor allele.
For c.3435 C>T the association whit the mean Cmin has turned out to be significant, with a p-value of 0,013.

Then, the mean Cmin has been associated with ABCG2 genotype, testing the c.421 C>A non-synonymous variant at the coding position 421 at exon 5, which entail a C>A nucleotide transition, and it is one of the most studied polymorphisms of *ABCG2* (rs2231142).
A trend was highlighted between *ABCG2* c.421A allele, and higher imatinib Cmin with respect to the C allele has been reported, which is concordant to the expected effect of the SNP on the protein.
This behaviour finds an explanation in literature (51), were the ABCG2 421C/A genotype and A/A genotype are associated with a higher imatinib exposure (Cmin ), as this case study reports. Intriguingly, Gardner *et al.* showed that cells expressing the BCRP protein with 421AA genotype had a significantly higher imatinib accumulation with respect to the wild-type protein(52), sustaining a defective extrusion of the drug in presence of the variant allele,
Therefore, this study and previous reports suggest that the *ABCG2* c.421C>A genotype might play a key role in modulating imatinib disposition in GIST pathology.

Subsequently, SLC family has been correlated with mean Cmin. The human organic cation transporter 1 (hOCT1), also known as solute carrier family 22 member 1 (SLC22A1), has been proposed as the major uptake transporter for IM. Several evidences suggest that variability in hOCT1 expression and its activity can play a role on IM treatment response (53). The two non-synonymous polymorphisms in SLC22A1, rs628031 (A1222G,) and rs683369 (C480G) were tested: among them, the correlation between A1222G (rs628031) and the mean Cmin turned out to be significant, with a p-value of

0,014. Patients with 12222AA genotype seems to be more expose to the drug instead of 1222GG genotype (wild type) and the 1222 GA genotype.

As a result, pharmacogenetics could be a predictive tool for drug exposure, and, for this purpose, pharmacogenetic analysis must be implemented and incorporated in the clinical practice.

All these result supports the rationale of this work, for which using an integrated approach between TDM, and pharmacokinetics can provide important additional information about the ongoing therapy.

# 6 CONCLUSION

In conclusion, in this work we have demonstrated the relevance and the feasibility of the integrated approach between TDM and pharmacogenetics.
Interindividual variability in exposure among patients treated with imatinib and sunitinib has been highlighted, firstly analysing plasma samples and then performing the quantifications with the HPLC-MS/MS.
Mean Cmin has been evaluated following the published guidelines, where TDM targets for imatinib and sunitinib were identified. Groups of patients above or below the target were found, the impact of the different dosage were analysed for both imatinib and sunitinib. Sex as a variable in drug exposure was evaluated, achieving results only for imatinib.
Concurrently, SNPs correlated with imatinib and sunitinib metabolism and transport were selected and then tested on the collected blood samples. Pharmacogenetic results were obtained using SNPline Genotyping Platform and RealTimePCR.
For imatinib, a statistical association between mean Cmin and metabolic phenotype of the cytochromes selected could be done. CYP2D6 metabolic phenotypes appeared to be related to imatinib exposure, and some significant correlation was obtained between mean Cmin and ABCB1 or SLCA221, where a higher exposition to the drug could be assigned to a specific genotype.
As shown below, the integrated approach between TDM and pharmacogenetics proved to be essential in order to make personalised therapies, by considering patient's physiological and genetic characteristics. In addition, this integrated approach has turned out to be feasible in the clinical practice.
At Centro di Riferimento Oncologico Cro di Aviano, in the Pharmacology Unit, an integrated pharmacological consults service is already available: by using this integrated approach, pharmacological consults are being formulated and diagnostically reported, so that the physician may access to this information.
This constitutes the new frontiers in the clinical pharmacology, in which the Pharmacology Unit of Cro Aviano represents a pioneer, because no guidelines were already available in the formulation of a consult.
The integrated pharmacology consult could be a winning approach in the age of precision medicine, now the goal will be to insert this approach in the daily clinical practice.

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