UNIVERSITÀ DEGLI STUDI DI SALERNO

Dipartimento di Medicina, Chirurgia ed Odontoiatria

Dottorato di Ricerca in Medicina Traslazionale dello Sviluppo e dell'Invecchiamento Attivo XXXI ciclo

Curriculum Medicina Traslazionale e Clinica

Tesi di dottorato

DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR THERAPEUTIC DRUG MONITORING

Coordinatore:

Ch.mo Prof. Palmiero Monteleone

Relatore: Prof.ssa Viviana Izzo

Candidato/a:

Marine Pingeon

Matricola:8800900012

Anno Accademico 2018/2019

Summary

1.1 Chapter I. Introduction. Going toward Personalized Medicine: the need of therapeutic drug monitoring 1
1.2 Variability in drug response 3
1.2.1 Biomarkers 3
1.2.2 Pharmacogenomics and pharmacogenetics 4
1.2.3 Pharmacokinetics and Pharmacodynamics 5
1.2.4 Pharmacokinetic and Pharmacodynamic variability 7
1.3 Therapeutic Drug Monitoring 12
1.3.1 Analytical Methods in Therapeutic Drug Monitoring 13
2.1 Chapter II. Hydroxychloroquine monitoring in the treatment of patients with Systemic Lupus Erythematosus. Introduction 16
2.1.1 Systemic Lupus Erythematosus 16
2.1.2 Genetics and epidemiology 16
2.1.3 Pathophysiology 17
2.1.4 Clinical manifestations 18
2.1.5 Clinical management 19
2.1.6 Hydroxychloroquine 19
2.1.7 Project aim 21
2.2 Materials and methods 22
2.2.1 Patients 22
2.2.2 Chemicals and reagents 23
2.2.3 Sample preparation 23
2.2.4 Preparation of standard calibration curves and quality control samples 24
2.2.5 Chromatography 24
2.2.6 Method validation 25
2.2.7 Mass spectrometry 26
2.2.8 Statistical analysis 26
2.3 Results and Discussion 27
2.3.1 Sample preparation and extraction 27
2.3.2 Optimization of HPLC-FL analysis 27
2.3.3 Method validation 29
2.3.4 Comparison between HPLC-FL and LC-MS/MS on a subset of clinical samples 32
2.3.5. Relationship between blood concentration of HCQ and QoL in SLE patients with inactive disease 34
2.4 Discussion 37
3.1 Chapter III. Ruxolitinib monitoring in the treatment of patients with Myeloproliferative Disorders. Introduction 41
3.1.1 Definitions 41
3.1.2 Idiopathic Myelofibrosis 41

3.1.3 Polycythemia Vera 43
3.1.4 JAK-STAT signaling pathway 43
3.1.5 Ruxolitinib 44
3.1.6 Project aim 45
3.2 Materials and methods 46
3.2.1. Chemicals and reagents 46
3.2.2 Chromatography 46
3.2.3 Sample preparation 46
3.2.4 Detection and quantification limits 47
3.2.5 Linearity and recovery 47
3.2.6 Intra- and inter-day variability 48
3.2.7 Stock solutions and sample stability 48
3.2.8 Method robustness 48
3.3 Results and discussion 49
3.3.1 Optimization of chromatographic conditions 49
3.3.2 Pre-analytical treatment 50
3.3.3 Method validation 50
3.4 Conclusions 54
4.1 Chapter IV. 5-Fluorouracil monitoring of oncologic patients in course of chemotherapy treatment. Introduction 56
4.1.1 TDM applied to chemotherapeutic agents 56
4.1.2 5-Fluorouracil 56
4.1.3 -Therapeutic drug monitoring of 5-Fluorouracil 58
4.1.4 Project aim 58
4.2 Materials and methods 60
4.2.1 Patients 60
4.2.2 Chemicals and reagents 60
4.2.3 Solution preparation 60
4.2.4 5-FU extraction 60
4.2.5 Preparation of calibration standards and quality controls 61
4.2.6 LC-MS/MS analysis 61
4.2.7 Method validation 62
4.3 Results and discussion 63
5.1. Chapter V. Neonatal monitoring of phenobarbital and caffeine. Introduction 71
5.1.1. Neonatal TDM 71
5.1.2. Phenobarbital 73
5.1.3. Caffeine 74
5.1.4. Project aim 75
5.2. Materials and methods 76

5.2.2. Chemicals and reagents 76
5.2.3. Patients 76
5.2.4. Preparation of stock and working solutions 76
5.2.5. LC-MS/MS conditions 76
5.2.6. Samples preparation 78
5.2.7. Method validation....78
5.2.8. Comparison between DBS and plasma 80
5.3. Results and discussion....81
6. Chapter VI. Conclusions…. 89
7. Bibliography…. 91

“Le bon sens de la vie humaine nous montre que la vie humaine est courte, et qu'il faut mieux faire de notre court passage sur terre quelque chose d'utile pour soi et pour les autres.”

“Il buon senso ci indica che la vita umana è breve, e che è meglio trasformare il nostro fugace passaggio sulla Terra in qualcosa di utile, per noi e per gli altri".

Dalaï Lama

Abstract

The U.S. Food and Drug Administration defines as Precision or Personalized Medicine (PM) an innovative therapeutic approach that tailors therapy and prevention on patients based on inter-individual variabilities in molecular or environmental features and in lifestyles. The major goals of PM are to maximize treatment efficacy and to reduce cost, toxicities and therapy failure rates by early identification of patients who might benefit or not of a specific treatment. In this scenario, therapeutic drug monitoring (TDM) is an important laboratory tool for PM because of the possibility to measure several drugs and bioactive molecules in human biological matrices. TDM is based on the hypothesis that in the majority of drugs, there is a relationship between administered dose and circulating concentration of unbound fraction - and between this concentration and observed pharmacological effects. TDM is recommended for drugs with significant inter-individual pharmacokinetic variability and an established relationship between blood concentrations and clinical efficacy and/or toxicity. Moreover, TDM is also advisable in special populations such as pregnant women and children. To date, liquid chromatography and immunometric assay are still considered the standard for molecule measurement in biological fluids; however, in recent years, LC tandem mass spectrometry (LC-MS) is gaining popularity because of the possibility of in-depth and multiplexed analysis with high selectivity and specificity.

During this Ph.D. program, we developed several high performance LC (HPLC)- and LC-MS/MS-based approaches for TDM of different drugs measured in various types of body fluids and validated according to EMA and FDA guidelines. In particular, we focused on:

1) TDM of hydroxychloroquine (HCQ) blood concentration, a drug with a wide therapeutic window. Our method was validated on a cohort of patients with Systemic Lupus Erythematosus treated with HCQ and blood concentrations were correlated to several clinical parameters, such quality of life. Moreover, TDM of HCQ was also used to monitor treatment adherence in those subjects.
2) TDM of a commonly used chemotherapeutic agent, the 5-fluorouracil (5-FU), which is known to have a narrow therapeutic window and a high toxicity
3) TDM of a new kinase inhibitor, Ruxolitinib, approved for the treatment of myeloproliferative hematologic disorders.
4) TDM of several drugs, such as caffeine and phenobarbital, in newborns who are at particular risk of uncorrected drug dosage. Due to the need to carry out analyses on very-small volume samples, we validated an analytical method using micro-sampling techniques such as the dried blood spot (DBS) sampling combined with LC-MS/MS analysis.

Riassunto

La U.S. Food and Drug Administration definisce con il termine Medicina di Precisione o Personalizzata (MP) un approccio terapeutico innovativo che identifica strategie terapeutiche e di prevenzione sul singolo paziente in base alla presenza di variazioni interindividuali in fattori biologici, ambientali e dello stile di vita. Gli obiettivi principali della MP sono quelli di aumentare l'efficacia terapeutica e di ridurre i costi, le tossicità e il tasso di fallimento terapeutico individuando precocemente i pazienti che possono beneficiare o meno di una specifica terapia. Il monitoraggio terapeutico dei farmaci (TDM) è quindi un importante strumento diagnostico per la MP perché permette di misurare farmaci e molecole bioattive in varie matrici biologiche. Il TDM si basa sull'ipotesi che per la maggior parte dei farmaci esiste un rapporto diretto tra dose somministrata e concentrazione circolante – e tra queste e l'effetto terapeutico. Per questo motivo, il TDM è raccomandato in tutti quei casi in cui sussiste una significativa variabilità farmacocinetica interindividuale ed una relazione nota tra concentrazioni plasmatiche ed effetto terapeutico o tossicità. Inoltre, il TDM è indicato anche in particolari popolazioni come le donne in gravidanza e i bambini. Ad oggi, la cromatografia liquida (LC) e le tecniche immunometriche sono ancora considerate lo standard per il dosaggio di molecole in fluidi biologici; tuttavia, negli ultimi anni, la LC accoppiata alla spettrometria di massa (LC-MS) sta rapidamente affiancando -e in alcuni casi soppiantando- le altre due tecniche grazie alla possibilità di effettuare analisi approfondite su numerose molecole in contemporanea, con alta selettività e specificità.

Nell'ambito del presente progetto di dottorato, sono stati sviluppati numerosi metodi analitici con analisi in high performance LC (HPLC)- o LC-MS/MS per il TDM di varie molecole in fluidi biologici e validati seguendo le linee guida della EMA e della FDA. In particolare, l'attenzione è stata focalizzata su:

1) TDM dell'idrossiclorochina (HCQ), un farmaco con un'ampia finestra terapeutica. Il metodo sviluppato è stato validato su una coorte di pazienti affetta da Lupus Eritematoso Sistemico in trattamento con HCQ e le concentrazioni plasmatiche sono state correlate con vari parametri clinici e con la qualità della vita. Inoltre, il TDM dell'HCQ è stato anche utilizzato per monitorare l'aderenza al trattamento di tali soggetti.
2) TDM di un chemioterapeutico largamente utilizzato quale il 5-fluorouracile, caratterizzato da una stretta finestra terapeutica e da grave tossicità.
3) TDM di un nuovo inibitore di chinasi, il Ruxolitinib, approvato per il trattamento di malattie ematologiche mieloproliferative.
4) TDM di varie molecole quali caffeina e fenobarbital in neonati che sono particolarmente a rischio di dosaggi terapeutici non corretti. In particolare, data la necessità di lavorare su

piccoli volumi, è stato messo a punto il TDM con metodica di micro-campionamento quale il dried blood spot accoppiato ad analisi in LC-MS/MS.

Chapter I

Introduction

1.1 Going toward Personalized Medicine: the need of therapeutic drug monitoring

Genetic and epi-genetic differences between individuals may induce significant variability in drug response [**1**]. Consequently, a therapeutic treatment based on the "one-size-fits-all" concept, which ignores the widely inter-individual variation that exists in response to medication, can be ineffective or even damaging. Nowadays medicine strives towards personalized therapy and "personalized medicine" (PM), which stands for therapy adaptation to the molecular characteristics of patient and its disease (Figure 1) [**2**].

PM challenges are to identify and classify subgroups of patients who differ in their susceptibility to a particular disease or their response to a specific treatment. Preventing or therapeutic intervention can then be foreseen for those who will actually benefit, sparing expense and side effects for those who will not. [**3**]. PM definition, commonly accepted and taken over by the European Medicine Agency (EMA), consists in administrating "the right dose of the right drug to the right person at the right time" [**4**]. From Ayurvedic medicine to Hippocrates, the concept of PM has long been known [**5-7**].

Even if the PM idea is not new, its current application is quite recent. Since 1990s technical advances are part of a historical evolution of biology and biomedicine, leading to a new turning point in personalized medicine. Today, -omics technologies offer an access to different molecular profiles and disturbances of cellular signaling pathways which seeks to understand all the molecular underpinnings of drug response or posology [**1,2**]. PM may thus be considered as an extension of traditional approaches to understand and treat diseases, but with a greater precision [**8**]. Several other important aspects, such as the decreasing rate of new medical product development and the increasing of healthcare costs generated by both aging population who lives longer and the increasing hospitalization due to adverse drug reactions (ADRs), encouraged manufacturers, authority and government to invest on PM.

ADRs are an important cause of morbidity and mortality worldwide, with a significant and increasing health and economic burden. Several meta-analysis studies conducted in the USA and in European countries found that the frequency of serious ADRs leading to hospitalization ranged, respectively, from 1.0 % to 16.8% and from 0.5% to 12.8%. Among hospitalized patients, the global incidence of serious ADRs was 6.7% and that of fatal ADRs was of 0.32% [**9,10**]. From 1999 to 2006, national USA Vital Statistics System data have shown that the rate of ADR-related deaths increased from 0.08 to 0.12 per 100,000 persons [**11**]. In the UK, an historical study on 20,000 hospitalized patients found that ADRs resulted in an average of eight days of hospital stay and were

associated with approximately € 706 million per year, including ADRs judged potentially avoidable. [**12**].

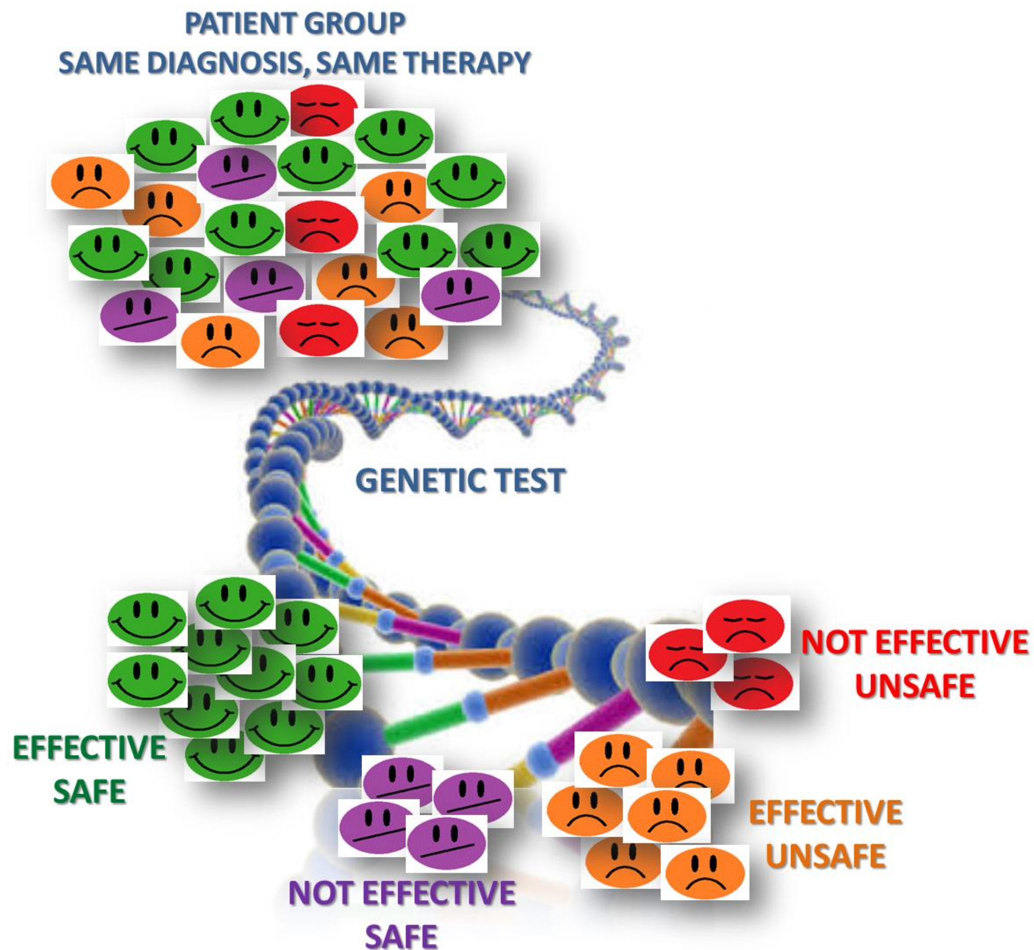


Figure 1. Personalized medicine. Personalized medicine promises to transform the delivery of healthcare to patients. Its aim is to evolve from a reactive "one-size-fits-all" system towards a system of predictive, preventive, and precision care. This picture depicts how personalized medicine could classify people into smaller subsets based on the therapy response from one large disease group. Genetic tests can help stratify patients in those who would respond effectively to a specific drug and those who would experience an ADR.

Another problem that healthcare system faces is the limited effectiveness of numerous treatments, especially those regarding chronic diseases. A substantial inter-variability to drug response in oncology field as well as for common conditions like hypertension, heart failure, depression, hypercholesterolemia and asthma, has indeed been observed [**13,14**]. As a result, the ability to rapidly discriminate between patients who will benefit from a given treatment and those who are at risk of significant adverse events could result in significant cost savings for the entire health system.

In this scenario, therapeutic drug monitoring would be a cheap and widely-applicable armored PM arm because, by using common simple instrumentations, such as liquid chromatography, or more complex apparatus, such as mass spectrometers, clinicians can rapidly adjust theoretical drug doses

in single patients in order to maintain an effective and safe serum/plasma trough concentration by maximizing efficacy while minimizing ADR incidence.

The need of PM tailored on each patient is of growing interest in scientific and non-scientific communities: the creation of several large-scale international projects such as the "Personalized Medicine Coalition", the "Genomics and Personalized Medicine Act" as well as ministerial commissions throughout Europe, demonstrate the growing interest of physicians, researchers, industry and government for this topic. However, we are still far from a real PM as many different fields need to deeply collaborate in order to combine epidemiology, genomics, molecular biology, proteomics, and metabolomics fingerprints which all might affect drug response in patients.

1.2 Variability in drug response

1.2.1 Biomarkers

The individual’s response to a drug depends on the complex interplay between environmental and genetic factors. There are multiple contributory factors which play a role in the drug response variability such as gender, age, diet, body mass, health status, epigenetic factors, concurrent therapy or environmental exposure to certain chemicals or toxins (*e.g.* cigarette smoke) [**1,15**]. To identify the ideal treatment and to adapt it to each patient it is therefore necessary to use specific tools to measure accurately and reproducibly the clinical manifestations of patient's health status. It was in 1998 that the word *biomarker* was defined for the first time by the *National Institute of Health* as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.” Even if biomarker use has been known for long time, recent advances in new high-speed technologies as genomic platforms, high-performance biochips or new generation of mass spectrometry, increase possibilities to identify new biomarkers for precocious diagnosis, prognosis and therapeutic monitoring [**16**].

Biomarkers are physiological, biochemical or molecular parameters that can be detected in tissue or biological fluids such as blood, urine, etc. Today, biomarkers represent a vast field that covers different areas of application ranging from pharmaceutical development to clinical medicine. [**17,18**]. They can be classified into three categories: prognostic, used to predict the natural course of a confirmed disease; predictive, used to predict the response of patient to a drug; pharmacodynamic, used to evaluate efficacy or toxicity to treatment and to modulate drug dosing. Predictive biomarkers are mainly used in hematology and oncology and consider both conventional environmental factors and dose selection. Indeed, immunohistochemistry tests such as Her2 protein in breast cancer or

pharmacogenomics tests as the dihydropyrimidine dehydrogenase (DPYD) test for 5-fluorouracil make it possible to identify patients with a pharmacokinetic/pharmacodynamic (PK/PD) risk such as ADRs or inefficient treatment [**19**].

1.2.2 Pharmacogenomics and pharmacogenetics

Genetic variations can affect how the body responds to certain medications [**20**]. Two main types of inter-individual variations have been found in the genome *i.e.* variable number tandem repeats (VNTRs) which occur in the non-coding DNA and is used for paternity testing, forensic science or DNA fingerprinting and single nucleotide polymorphisms (SNPs), which are the most important source of genome variation. These latter contribute for 0.1% of differences retrieved in gene sequences and are at the origin of interindividual variabilities; SNPs can lead to protein modifications that may be responsible of alteration of their stability, cell concentrations, metabolic activity or signaling properties [**21**].

Pharmacogenetics takes its first steps in the ‘50s and brings genetics, biochemistry, and pharmacology together. The first person to have mentioned that genetic variants can modulate drug action is the physiologist Garrod. Thereafter, a tide of reports suggested that ADRs could be genetically determined variations in enzyme activity. For example, hemolysis caused by antimalarials was recognized as being caused by inherited variants of glucose-6-phosphate dehydrogenase. Similarly, inherited changes in *DPYD* gene leading to low enzyme activity produce a greater bioavailability of the antimetabolite 5-fluorouracile, thus causing serious hematological, neurological and gastrointestinal toxicities. However, it was only after 1959 that F. Vogel proposed the term pharmacogenetics, which is defined as the study of the influence of genome variability in drug response [**22**]. Pharmacogenetics is aimed at determining the genetic differences in metabolic pathways that can affect individual responses to drugs [**23**]. In pharmacogenetics, the analysis of a specific gene, or group of genes, can be used to predict responses to a specific drug or class of drugs. However, rarely a single gene directly controls drug response or ADRs and advances in pharmacogenetics have remained limited. Emergence and development of Human Genome Project and genome science in 1990s [**24**] have then spawned a newer field, named pharmacogenomics [**25**]. The main aim of pharmacogenomics is to understand all of the molecular underpinnings of drug response. Pharmacogenomics carries the idea that variable drug response may reflect sets of variants within an individual or across a population. Pharmacogenomics is a broader application of genomic technologies for development of new drug and / or further categorization of existing drugs [**26**]. Even if current uses of pharmacogenomics are limited, it still offers new perspectives for the next years

including ***i)*** a better medication selection, predicting patients with risk of ADRs and those who will be likely to respond successfully, ***ii)*** a safer dosing option, since in some cases parameters approval of FDA is not sufficient, ***iii)*** improvements in drug development by reducing time and cost of R&D, thus preventing unsuccessful clinical trials and targeting a specific population for one specific medication.

Today, the boundary between pharmacogenetics and pharmacogenomics remains unclear and the debate in the scientific community is still very active. Indeed, both terms are often used interchangeably, and a unanimous and precise definition of either remains elusive [**27**].

1.2.3 Pharmacokinetics and Pharmacodynamics

Pharmacokinetic (PK) and Pharmacodynamic (PD) determine the clinical effect of drug therapy and can be resumed in what the organism does to the drug and what the drug does to the organism, respectively. Once a drug is administered, it is absorbed and distributed to its site of action, where it interacts with the target(s). Intensity and duration of drug response is determined not only by the PK processes, but also by the PD ones. Therefore, the variability in drug response is a result of the variability in either PK or PD processes, or a combination of both [**28**].

PK depends on the relationship between drug effective concentration in the body and its dose. Molecular mechanisms leading to a specific PK were summarized in the acronym L.A.D.M.E. that includes liberation phases, absorption, distribution, metabolism and excretion of the drug. Today, several evidences demonstrated the key role played by the patient's compliance, which should be considered as an additional parameter, thus leading to the more complete current acronym C.L.A.D.M.E.

Drug action begins with its administration and depends on its absorption pathway, which may be oral, intramuscular, subcutaneous or topical/transdermal. Obviously, the patient has an active role in the correct application of the therapeutic regimen; compliance concept describes the patient adherence degree with respect to medical prescriptions, and consequently underlines patient's responsibility in the success of any therapy [**29**]. Following drug administration, the bioactive compound must be liberated from its release system. Pharmaceutical formulation, physiochemical features of the drug - such as molecular weight and hydrophilicity/lipophilicity-, and the type of environment at the site of administration strongly influence the liberation process. Drug absorption mechanisms may be different before reaching the bloodstream: passive diffusion (para-cellular or facilitated), active transport and transcytosis. After absorption, the drug reaches the circulation and is distributed at the various compartments of the organism. Again, drug-related factors (*e.g.* molecular

size, acid dissociation constant…), the presence and location of drug transporters, protein binding (albumin and acid α1-glycoprotein), systemic pH, and tissue perfusion influence the distribution. Other parameters, as body composition in fat or water, nutritional and health status or age, have some effects on the drug distribution volume. Consequently, the way in which the drug will be distributed will affect drug concentration and consequently the PD. Within organism, a drug undergoes various metabolic processes, mainly occurring in the liver. These processes have the role to enzymatically transform the drug making it more hydrophilic and facilitating its excretion. Drug metabolism is mainly performed in two phases. Phase I enzymes introduce reactive or polar groups and are catalyzed by the superfamily of cytochrome (CYP)-450 monooxygenases. These enzymes catalyze reactions as oxidation, reduction, hydrolysis or hydroxylation. Phase II enzymes perform conjugation reactions such as glucuronidation, sulfonation, methylation. Phase II are synthetic reactions catalyzed by several enzymes, and are aimed at transforming metabolites in more polar compounds that can be more easily excreted. Phase I and phase II processes are also responsible for the so called "first pass effect" and the enterohepatic circulation. The ultimate step of C.L.A.D.M.E. consists of elimination phase, which is composed of two main routes: the renal route –characterized by glomerular filtration and tubular secretion - and the hepatic route. Elimination phase regulates the half-life of each drug, which corresponds to the time required to observe a decrease of fifty percent in the maximum plasmatic concentration obtained for that specific drug.

All these processes contribute in the definition of the bioavailability concept, which is the drug amount (expressed as percentage) in its active form that reaches the systemic circulation and can, then, reach the action site. After a single drug administration, monitoring of plasma concentration over the time allows to obtain PK parameters important to define the bioavailability concept such as the maximum concentration (Cmax) corresponding to a maximum time (Tmax), and the area under the curve (AUC) (Figure 2) [**30**].

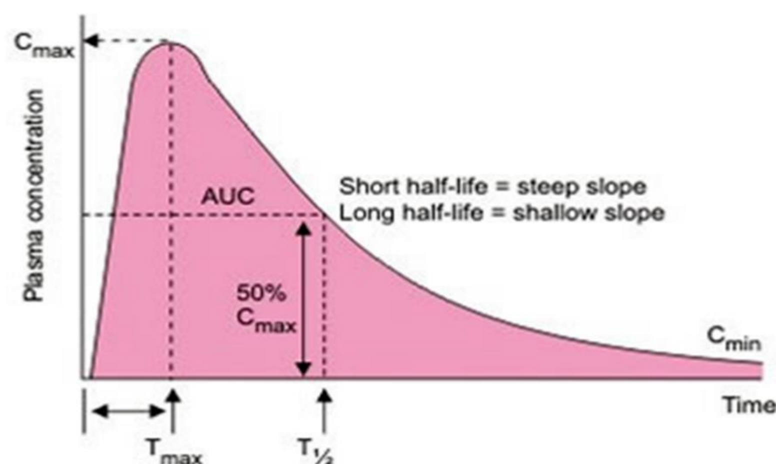


Figure 2. Typical bioavailability graph of an orally administrated drug.

Once undergone the different PK processes, the active principle reaches the target and can carry out its action on the biological systems thus determining the PD events. PD describes the relationship between drug concentration and the pharmacological action, including ADRs. Some drugs exert their function almost exclusively due to their chemical and physical characteristics; nevertheless, they generally interact with specific macromolecular systems like receptors, target proteins or ion channels, modulating or inhibiting normal biochemical processes. Drug-receptor interaction generate biochemical changes or physiological modifications that strongly influence drug response [**31**].

1.2.4 Pharmacokinetic and Pharmacodynamic variability

The large interindividual variability in drug response represents a major challenge in drug therapy, particularly for drugs with a narrow therapeutic index. Genetic factors represent the most important source of interindividual variability in drug response. Unlike non-genetic factors, genetic factors generally remain stable throughout a person's lifetime. Given the high SNP density, it is not surprising to see that virtually every protein involved in the drug PK and PD, such as drug-metabolizing enzymes, transporters and receptors will be eventually found to have genetic variation. Although genetic polymorphisms in drug metabolizing enzymes have generally been known for more than 50 years, genetic polymorphisms for transporters and receptors have only recently received attention [**32-35**].

PHARMACOKINETIC VARIABILITY. Many factors can contribute to the marked variability among patients in their plasma profiles following a fixed oral dose, including genetic and non-genetic factors. The former includes genetic variability in drug-metabolizing enzymes and drug transporter systems and the latter comprise gastrointestinal physiology and diet.

Genetic Variability in Drug-Metabolizing Enzymes. Genetic polymorphisms of drug-metabolizing enzymes may significantly contribute to the interindividual and interethnic differences in drug disposition and represents a major challenge in drug development. However, it is important to point out that although polymorphisms of drug metabolizing enzymes are generally considered undesirable and might be problematic, they are manageable in most cases. In this context many members of the superfamily of cytochrome P450 are involved such asCYP2D6, which has the largest phenotypic variation among the P450 enzymes, CYP2C19, whose impact has been highlighted by several studies and a meta-analysis in patients with coronary artery disease treated with the antiplatelet agent clopidogrel, CYP2C9, which is responsible for the metabolism of several drugs with narrow therapeutic indices (*i.e.* phenytoin and warfarin), and CYP3A4/CYP3A5 that, although accounting for approximately 30% of hepatic P450 content and being involved in over 50% of drug

metabolism, does not appear to have polymorphisms that result in absence of functional protein. Noteworthy, wide variability in CYP3A4 activity seems to be due in part to the large number of substrates capable of inhibiting or inducing the enzyme.

Genetic variability in drug transporters. With few exceptions such as anticoagulant drugs that act directly in the blood compartment, most drugs have to be transported from blood circulation to the action site in target tissue(s) in order to exert their pharmacologic activity. Once a drug enters the systemic circulation, it readily crosses several cell membranes and reaches the intracellular fluids of almost every organ and tissue. There are many processes by which drugs cross cell membranes, including passive (simple) diffusion and transporter-mediated influx and efflux transport. Kinetically, cellular distribution of drugs can be viewed as a two-step process arranged in series by influx (uptake) and efflux transport. Drug concentration in cells is thus determined by the difference between the rate of influx and efflux transport [**36**]. The uptake transporters include organic anion transporting polypeptide (OATP), organic anion transporter (OAT), and organic cation transporter (OCT) subfamilies, while the efflux transporters include multidrug resistance protein (P-glycoprotein; MDR1), multidrug resistance-related protein (MRP), and the breast cancer resistance protein (BCRP) families [**37-42**]. In the past 10 years, genetic polymorphisms have been identified for many uptake and efflux transporters, and there is increasing evidence that genetic polymorphisms in transporters may also contribute to drug absorption, disposition and response.

Although in general the effect of transporter polymorphisms on drug pharmacokinetics appears to be quantitatively less significant compared to that of drug metabolizing enzymes, the impact of transporter polymorphisms might be underestimated if the sole plasma concentration is monitored. An important lesson learned from the kinetic studies of transgenic animals is that genetic polymorphisms have a much greater impact on tissue distribution of drugs than on plasma concentration of drugs [**43,44**]. Therefore, together with the genetic polymorphisms of drug metabolizing enzymes, one should carefully assess the impact of transporter polymorphisms on drug pharmacokinetics to better predict drug efficacy and toxicity.

Non-genetic Pharmacokinetic Variability. Although genetic polymorphisms of both drug metabolizing enzymes and transporters are the major sources responsible for the observed interindividual variability in pharmacokinetics of drugs, many non-genetic factors, such as physiological, pathological and environmental factors, may also contribute significantly to the interindividual variability in ADME processes. Being absorbed primarily from the upper part of the small intestine, oral absorption of drugs is often affected by the gastric emptying time and small intestinal motility, which vary considerably between individuals, even within the same individual on different occasions [**45,46**]. Therefore, it is expected to see significant variations in oral absorption of drug at same dose level, and same formulation in the same subject during multiple drug dosing.

Both the rate and extent of drug absorption can vary considerably between individuals and within the same individual. Intraindividual variation in oral absorption is best exemplified by the study of azathioprine [**47**]. In a clinical study, the intraindividual (day-to-day) variability in the pharmacokinetics of azathioprine was determined in 10 renal transplant patients on two consecutive days after oral administration. In some patients, a 2- to 3-fold intraindividual difference in the AUC of azathioprine between the two days was retrieved. In this study, there was a 6-fold difference in the AUC between patients. Similarly, there was a 2- to 3-fold intraindividual variation in the AUC of cyclosporine in seven healthy volunteers after single oral doses on two occasions at 2-week interval [**48**]. Significant inter- and intra-individual variability in the oral AUC has also been reported for other drugs, such as nifedipine, doxorubicin, furosemide and chlorambucil [**49-52**]. Food intake has also a significant impact on drug absorption. Since the diet is so different among individuals, even within the same individual on daily basis, food intake is an important source responsible for the intra- and inter-individual variability in oral absorption of drugs. The effect of food on drug absorption is largely unpredictable. Depending on the type and size of a meal and the physicochemical properties of drugs, oral absorption of drugs may be reduced, delayed, increased, accelerated, or not affected by concomitant food intake [**53,54**]. Mechanisms related to food effects include direct effect on food-drug interactions and indirect effect on gastrointestinal physiology. The degree of food-drug interactions are determined mainly by the composition of food and the physicochemical properties of drugs. Dietary components may influence the dissolution and solubility of drugs and subsequent absorption. In some cases, irreversible interactions between food components and drugs, such as complexation and chelation interactions between drugs and metal ions in meals, may occur and decrease absorption. In addition, food may act as a physical barrier, preventing drug access to the absorptive surface of the intestinal tract. For drugs that are highly soluble and highly permeable, oral absorption is usually less sensitive to food effects, particularly when given with rapidly dissolving and immediate-release formulations [**55**]. Food can influence both the rate and extent of drug absorption by indirectly altering gastrointestinal physiology. Although food generally tends to delay gastric emptying, it has a stimulating effect on intestinal motility [**50,56**]. Delayed gastric emptying will delay absorption of drugs that are absorbed rapidly from small intestine. On the other hand, delayed gastric emptying might increase systemic availability of drugs that have relatively poor solubility by permitting more material to dissolve in the stomach before passing into small intestine, while drugs that are unstable in acidic pH are likely to be degraded because of prolonged residence in the stomach. In addition, food intake increases the gastric secretion of hydrochloric acid, intestinal secretion of digestive enzymes and bile secretion. If a drug’s solubility is pH- or bile-dependent, hydrochloric acid and bile secretion will have significant effect on drug absorption as exemplified by indinavir and rufinamide [**57,58**]. In addition, food intake may increase the splanchnic blood flow

leading to an increase in the bioavailability of drugs by decreasing first-pass metabolism. The indirect effect of splanchnic blood flow on drug absorption is best exemplified by food effect on the absorption of propranolol. The splanchnic blood flow-mediated food effects have also been reported for other drugs as well [**59-61**]. Food may have direct effect on drug metabolism also by inducing and inhibiting drug metabolizing enzymes. The metabolic clearance of antipyrine and theophylline was increased in healthy subjects by a high-protein low-carbohydrate diet for 2 weeks, suggesting an increase in enzyme activity [**62**]. Certain vegetables, including brussel sprouts, cabbage, broccoli and cauliflower contain chemicals that induce drug metabolizing enzymes and decrease drug bioavailability [**63**]. The lower plasma concentrations of antipyrine and theophylline after digestion of charcoal-broiled meat is believed to be due to the induction of CYP1A1/2 through the contamination of polycyclic hydrocarbons resulting from the incomplete combustion of meat drippings [**64**]. On the other hand, food may inhibit drug metabolism enzymes. Drinking grapefruit juice is known to increase the oral bioavailability of some drugs in humans by decreasing intestinal CYP3A4 protein expression [**65**]. Recently, it has been reported that fexofenadine absorption was reduced by ingestion of grapefruit juice [**66**]. The reduction of fexofenadine absorption might be due to the inhibition of uptake transporter by grapefruit juice.

In addition to food intake, environmental factors and pathophysiological changes, such as deterioration of renal function or progression of a chronic disease, could also contribute to the pharmacokinetic variability.

PHARMACODYNAMIC VARIABILITY. Unlike the dose-concentration relationship, a clear concentration-response relationship cannot always be obtained, due to the complexity of disease pathogenesis and the difficulty in evaluating the effective pharmacological response. Therefore, the knowledge of the genetic causes responsible for the interindividual variability in pharmacological response is not as advanced as that described above for drug-metabolizing enzymes.

Genetic Variability in Receptors. In spite of a large body of information on genetically polymorphic variations of receptor systems, our understanding about the pharmacologic consequences of these polymorphisms is still at early stage. There are still large gaps in our knowledge about how to utilize the information obtained from pharmacogenetic studies to explain the interindividual variability in drug response. First, many pharmacogenetic studies of the effect of receptor polymorphisms on drug response showed conflicting and inconsistent results. One of the reasons for the inconsistence is that most diseases have complex genetic traits, with multiple genetic and environmental components contributing to susceptibility. The complexity of gene-disease relationship can be best illustrated by a recent survey of genetic association studies. More than 600 positive associations between genes and diseases were reported; 166 had been studied more than 3 times, but only 3 had been consistently replicated [**67**]. These results strongly suggest that if the

disease-causing allele is not prevalent, it might not be easier to find a good relationship between a single genetic variant and drug response. Recent evidence suggests that determining haplotypes may be more informative than genotyping single variant in relating genetic factor to drug response [**68**]. The haplotypes of ß2-andrenergic receptor is a good example for predicting drug response [**69**]. While there was no association between the response to albuterol and any individual SNP of the ß2-andrenergic receptor in isolation, the presence of 5 haplotypes predicted well the albuterol response (increase in FEV1) in patients with asthma. Other possible reasons for the conflicting reports on the effect of polymorphisms of drug receptors may be related to the methods of measuring drug response and the power of sample size of study. With the advance of bioanalytical technologies, the effect of polymorphism of drug metabolizing enzymes on the plasma concentration of drugs can be very accurately measured. In contrast, the measurement of pharmacologic response is generally less accurate. Therefore, the inaccurate measurement of drug response could contribute significantly to the conflicting reports. In addition, the number of patients needed to detect drug-gene interactions is highly dependent on the type of trait (discrete or continuous) and the precision of drug response measurement [**70**]. Complex traits require a large number of patients to establish the relationship of drug-gene interactions.

Non-genetic Pharmacodynamic Variability. Although genetic polymorphisms of drug targets (receptors and enzymes) are the major sources responsible for the interindividual variability in pharmacodynamics, many nongenetic factors, such as psychological, pathological and environmental factors, may also contribute significantly to the interindividual variability in drug response. Among these non-genetic factors, psychological factor is probably the most intriguing aspect in drug treatment and represents a major source of non-genetic pharmacodynamic variability. In clinical trials, the pharmacologic response of a test drug is often evaluated against inactive substance known as placebo. Although the phenomenon underlying the placebo effect is not fully understood by science, it is believed that the placebo effect is psychological, due to a belief in the treatment. In contrast, a patient who disbelieves in a treatment may experience a worsening of symptoms, the so-called "nocebo effect" [**71**]. The nocebo effect may compromise the drug action when active drug is given. Clearly, depending on the patient's attitude towards the treatment, the effectiveness of the drug prescribed could be in more positive or negative direction. The notion that the placebo-induced pain reduction is mediated by an endogenous opiate-related mechanism is supported by a recent study on a total of 1183 participants by Sauro and Greenberg [**72**]. Placebo administration was associated with a decrease in self-report of pain, and injection of naloxone, an opiate receptor antagonist, reversed placebo-induced analgesia in those individuals experiencing placebo analgesia but had no effect in those who had no placebo pain reduction. The hypothesis that placebo-induced pain reduction is mediated via opiate receptor mechanism is further supported by the finding that proglumide, an

antagonist blocking cholecystokinin-induced excitation of midbrain dopaminergic neurons, enhanced the placebo analgesia [**73**]. Similar to the inhibitory effect of naloxone on placebo analgesia, placebo potentiation by proglumide occurred only in placebo responders, but not in non-responders.

1.3 Therapeutic Drug Monitoring

Therapeutic drug monitoring (TDM) is a clinical tool that allows to measure the concentration of a large variety of drugs, or more generally of different bioactive molecules in human biological matrices such as blood, plasma and urine, but also fluids such as serum, saliva and cerebral fluid and cell and tissue extracts [**74**].

TDM is based on the hypothesis that there is a definable relationship between the dose of drug administered and its concentration in blood and/or plasma - or more generally in biological fluids - and between this concentration and the observed pharmacological effects. However, the correlation between dose and concentration of a specific drug is not always as linear as it might seem and is generally characterized by several variables associated with its pharmacokinetics: mode of absorption, distribution, metabolism, excretion, concomitant pathologies, drug-drug interactions, pharmacogenetics factors etc [**75**]. Therefore, TDM is not a simple measurement and its output is not a mere numerical datum, but it consists of both the accurate evaluation of the drug concentration in the selected matrix, and the rational interpretation of the different factors for which this concentration has been reached [**76**].

In that light, a multidisciplinary approach is required to carry out a TDM study, and it has to be performed with the help of different professional orchestrating figures (doctors, pharmacists, nurses, biologists and chemists). This teamwork allows establishing the appropriate conclusions from clinical studies and optimizing therapeutic treatment, thus increasing its effectiveness and decreasing adverse effects [**77**].

TDM is now considered fully part of the new frontier of medicine, a personalized medicine in which the therapy is tailored taking in account all possible variables affecting a patient health status. Noteworthy, not every administrated drug is susceptible to benefit from concentration-based dosage adjustment in terms of improvement of therapeutic effect and/or of tolerance profile. The main criteria making a drug a suitable candidate to TDM are a significant inter-individual pharmacokinetic variability and an established relationship between blood concentrations and clinical efficacy and/or toxicity. TDM is generally considered for drugs that have to be administered on the long term (chronic conditions, *e.g.* new oral targeted anticancer drugs, antiviral agents against HIV infection, etc.), or for drugs constituting last resort treatments administered to critically ill patients, such as in intensive care units where appropriate exposure is key for maximizing efficacy while minimizing toxicity [**78**].

Some of the therapeutic classes presently recognized to benefit from TDM are listed in Table 1 [**74**].

Therapeutic classes	**Selected examples**
Immunosuppressants	ciclosporin, tacrolimus, everolimus, sirolimus, mycophenolate
Antiepileptics	phenobarbital, phenytoin, carbamazepine, valproate, lamotrigine, topiramate, levetiracetam, lacosamide, zonisamide
Anti-HIV drugs	darunavir, atazanavir, lopinavir, efavirenz, nevirapine, rilpivirine, maraviroc, raltegravir, elvitegravir, dolutegravir
Antivirals	ribavirin, ganciclovir, acyclovir
Antifungals	voriconazole, posaconazole, itraconazole, hydroxy-itraconazole, fluconazole, caspofungin, aniludafungin, micafungin, flucytocin
Antibiotics	gentamicin, amikacin, tobramycin, vancomycin, teicoplanin, meropenem, imipenem, meropenem, cefepime, piperacillin/tazobactam, amoxicillin, flucloxacillin, ceftazidime, ceftriaxone, cefuroxime, daptomycin, ciprofloxacin, levofloxacin, linezolid, tigecycline
Tuberculostatic drugs	isoniazid, rifampicin, pyrazinamide, ethambutol
Antimalarials	Quinine
Anticancer drugs	methotrexate, busulfan, mitotane, azathioprine, mercaptopurine, thioguanine, imatinib, nilotinib, dasatinib, bosutinib, sunitinib, sorafenib, erlotinib, gefitinib, lapatinib, vemurafenib, regorafenib, tamoxifen/endoxifen.
Antipsychotics, antidepressants	lithium, amisulpride, asenapine, mirtazapine, iloperidone, olanzapine, paliperidone, quetiapine, risperidone, citalopram, fluoxetine, fluvoxamine, paroxetine, sertraline

Table 1: Example of therapeutic drug classes currently subjected to TDM.

However, there are conditions suggesting the use of TDM, regardless of the used drug. In particular, when clinical evidences suggest a less than expected clinical response to therapy, or if over-dosage is suspected in the presence of clinical signs of toxicity. Moreover, when special patients (pregnant, children, newborns…) are subjected to drug therapy, TDM is always advisable. For these populations, the available data on drug effects, toxicity and metabolism are generally reduced because of the limited number of treatments and/or for the lack of clinical experimentation. Therefore, there is an increasing occurrence of TDM use in pediatrics, also in the case of therapies involving drugs with a quite wide therapeutic window [**79**]. Analogously, TDM has to be carried out for patients showing a borderline pharmacogenetic profile, suggesting a possible hyper-susceptibility to specific drugs, when it is not possible to use a different active compound [**80**].

1.3.1 Analytical Methods in Therapeutic Drug Monitoring

Different analytical approaches have been used so far to measure the concentration of a drug and/or its metabolites in biologic fluids or tissues. Although approaches as Surface Enhanced Raman Spectroscopy [**81**] or electrochemical biosensors have been proposed [**82**], generally TDM studies are carried out using immunoassay-based or chromatography-based techniques. However, the immunoassay approach suffers from the known limitations of antibodies-based methods (high costs, cross-reactivity, need of a specific antibody for each analyte), and on the other hand chromatography coupled with UV or fluorescence detection is poorly selective, requires complex sample treatments

and long analytical times, and is not always very sensitive.

Liquid chromatography (HPLC) and gas chromatography (GC) have been considered for years the "gold standard", in parallel with immunometric techniques, for the measurement of specific substances in biological fluids such as plasma/blood and urine. Since 1960s, HPLC has gained popularity because of its ability to separate with exceptional resolving power and quantitatively analyze complex samples in a rapid and cheap manner. In recent years, the ever-increasing use of methods based on mass spectrometry in liquid phase (LC-MS) has opened new horizons in clinical analysis. However, even though LC-MS allows deep analysis with high selectivity and specificity, this technique requires an initial money investment for very sophisticated instrumentations which need skilled staff and dedicated laboratory areas.

In the last 15 years the LC-MS/MS technology emerged for its impressive performances and has now became a key analytical tool for all modern clinical laboratories. High Performance and Ultra Performance Liquid Chromatography (HPLC and UPLC., respectively) coupled to MS/MS has transformed the way most drugs are now analyzed, accelerating the rate of analytical developments and hence playing a major role for the current deployment of TDM [**83**]. This technique (or better, this family of techniques) allows identification, structural characterization and quantitation of molecules that range in size from tens of daltons to hundreds of thousands of daltons. Qualitative analysis is based on the ability of a mass spectrometer to "weigh on the molecular scale" by determining the mass-to-charge ratio (*m/z*) of quasi-molecular-ions and fragment ions obtained by the ionization of the investigated compounds.

The LC-MS methods are characterized by a high versatility, allowing to analyze substances ranging from small hydrophobic molecules, to biomacromolecules, good sensitivity and a high speed of analysis. The high selectivity of LC-MS/MS makes it possible to simultaneously analyze many structurally unrelated analytes, within short times; therefore, it is possible to set-up numerous multiplex assays for the quantification of drugs from the same therapeutic class in a single run. Thanks to their sensitivity and selectivity, mass-based approaches can be easily applied to poorly purified samples, such as water-diluted urines.

The setting-up of assays by MS has become greatly facilitated by the availability of stable isotopically-labelled Internal Standards (IS) (deuterium, ^{13}C, ^{15}N) that compensates for deleterious matrix effects variably affecting biological samples, which may otherwise compromise the accuracy of the analytical method. For the assay of drugs in peculiar biological matrices, such as dried blood spots, the use of isotopically-labelled IS assures that unidentified, possibly variable, matrix effects are compensated for.

Chapter II

Hydroxychloroquine monitoring in the treatment of patients with systemic lupus erythematosus

2.1 Introduction

2.1.1 Systemic Lupus Erythematosus

The term "lupus erythematosus" was first introduced at the beginning of the 19th century to describe skin lesions. It took almost 100 years to understand that the disease was systemic and caused by an aberrant autoimmune response. Indeed, the immune system, which normally functions to protect against foreign invaders, is hyperactive during disease and antibodies against normal autologous tissues and organs are produced. Usually, immune responses are directed against skin, joints, kidneys, brain, heart, lungs, and blood [**84**]. To date, systemic lupus erythematosus (SLE) is defined as a chronic autoimmune syndrome characterized by a broad spectrum of clinical manifestations, ranging from periods of illness, or flares, to periods of wellness, or remission. Because of the lack of curative therapeutic strategies, early detection and treatment remain the key points for achieving a good clinical outcome and for reducing disease severity and the probability of progression.

SLE affects individuals of all ethnical groups especially Americans, Hispanics and Africans with a prevalence of 20-150 cases per 100,000 population. In addition, SLE has an increased prevalence in women of childbearing age with a female/male ratio of 9:1 [**85,86**].

Lupus is a complex disease of unknown etiology whose pathophysiology has been proposed to be due to a combination of genetic, environmental and hormonal factors, such as sun exposure, stress, some drugs and infectious agents.

2.1.2 Genetics and epidemiology

Female hormones may play an important role in lupus pathogenesis given that 90% of patients are women. Evidence shows that menopausal women with lupus who received hormone therapy have a greater risk to develop flares than women who received placebo [**87**]. A protective role of male hormones has been suggested; however, in trials with dehydroepiandrosterone (DHEA) the presence of flares was observed only in 16% of cases [**88**]. Therefore, the real impact of hormones on lupus pathophysiology is still unclear.

Genetic factors might confer a predisposition in SLE development [**89**]. Studies have shown an increased incidence in monozygotic twins compared to heterozygotic twins (25 % vs 2%, respectively) [**90,91**]. Genome-wide association study (G-WAS) analysis of lupus patients and their relatives have highlighted several potential genetic markers involved in the pathophysiology of the disease. In rare cases, SLE might be related to a single gene mutation. For example, the lack of C4 or C1q, proteins involved in the complement system, decrease the elimination of self-reactive B cells

[**92**], or necrotic materials [**93**]. However, the contribution of these single-gene hits minimally affects the probability to develop SLE, which is instead greatly improved by increasing the number of predisposing factors. The analysis of SNPs has revealed the presence of SLE-associated polymorphisms in intronic DNA regions encoding for immune–related genes [**94**], such as the immunosuppressive cytokine *IL-10*, transcription factor *STAT4*, or *TNIP1* gene encoding for the cellular inhibitor A20-binding protein. Several of these SNPs are also associated with other autoimmune diseases, such as rheumatoid arthritis or type I diabetes. [**95-97**].

DNA accessibility to transcription factors, and thus gene expression, is regulated by DNA methylation and histone modifications (acetylation and methylation), regulated by various epigenetic factors, such as deacetylation of *IL2* promoter by histone deacetylase 1 [**98**]. During SLE, regulatory regions of many lupus-related genes, such as CD40LG or CD70, are hypomethylated and subsequently their expression is upregulated, which may favor the hyperactivation of the immune system.

Few cases of SLE are reported to be drug-induced, the so-called drug-induced lupus (DIL); however, tissue and organ damage occurs similarly to SLE. Among drugs, procainamide, hydralazine, and quinidine are the most involved in the pathogenesis of this type of lupus. DIL symptoms are different from common SLE with only mild or few lupus-like symptoms, such as fever, malaise, weight loss, polyarticular arthralgia and symmetric myalgia. These symptoms worsen if the treatment is continued, otherwise a rapid resolution occurs after drug discontinuing. Therefore, this action provides a key (although retrospective) diagnostic tool [**99**].

Studies have described that viruses have epitopes similar to self-antigens; for example, EBNA-1 protein of Epstein-Barr virus can cross-react with the self Ro antigen, a common target of antibodies involved in the genesis of SLE. By contrast, some endemic infectious agents (malaria and some parasites) could play a protective role in the development of autoimmune diseases [**100,101**].

Among environmental factors, ultraviolet radiations are well-known lupus-related factors: indeed, photosensitive rash is a disease classification criterion of the American College of Rheumatology for diagnosis of SLE [**102,103**].

2.1.3 Pathophysiology

The hypothesis of a multifactorial pathophysiology of SLE is supported by the presence of immunological derangement in both humoral and cellular immune responses.CD4+ T lymphocytes have a crucial role in the pathogenesis of the disease contributing to B cell over-activation through an excessive production of IL-2, a key cytokine in regulation of immune cell proliferation and in

maintenance of self-tolerance [**104**]. In addition, pro-inflammatory Th17 cells by producing IL-6 and IL-17contribute to B cell activation and maturation in germinal centers [**105**].

In 2003, an American study performed on 130 subjects has highlighted the presence of specific antibodies in the blood of patients before the onset of clinical manifestations. The presence of different types of autoantibodies is a characteristic of SLE: antibodies against nuclear antigens (ANA or ANF) are the most common and react against native double or single stranded DNA (dsDNA or ssDNA), nucleoproteins, histones and others. Additional antibodies found in the blood of SLE patients are directed against cytoplasmic antigens, coagulation factors and several tissue antigens, such as liver or kidney. Moreover, the presence of autoantibodies is one of the main evidences of the autologous activation of B cells in the pathophysiology of SLE: it has been suggested that circulating autoantibodies are produced by B cells that lost their tolerance due to derangement of immune responses driven by dendritic and T cell activation [**106**].

The formation of immune complexes by aggregation of circulating autoantibodies is responsible of cellular damage because of their deposition on membranes, such as glomerular basement membranes or capillary endothelium, leading to complement activation and cellular necrosis [**107**]. In some cases, the presence of circulating anti-RH antibodies can cause hemolytic anemia by humoral response against red blood cells carrying the Rh factor on their surface. Likewise, antibodies against coagulation factors can interfere with hemostatic functions determining bleeding or thrombotic events [**108**].

2.1.4 Clinical manifestations

In early stages, clinical manifestations are non-specific and include fever, asthenia, anorexia, weight loss and generalized malaise. Clinical course of SLE may extremely vary based on severity or cycles of remission or activation, thus being a main challenge for SLE diagnosis for physicians [**109,110**].

The characteristic clinical feature is the “malar” or butterfly rash, an acute, erythematous and edematous eruption extending from nasolabial folds to the cheekbones. Other frequent skin/mucosal lesions are ulcers, alopecia, urticaria, Raynaud's phenomenon, purpura and acrocyanosis. However, these manifestations are also present in several autoimmune diseases, such as rheumatoid arthritis and connective tissue diseases. Musculoskeletal manifestations can occur in 90% of patients as symmetric inflammatory arthralgia with swelling and functional limitation of small and medium joints. In addition to joint involvement, myalgia and myositis often occur with non-specific muscle pain, sometimes associated with strength deficit and high serum concentrations of muscle enzymes.

Among all clinical manifestations, renal damage is a life-threating complication, and in early stage, damage is asymptomatic and can be accidentally discovered by routinely laboratory test. The presence of circulating anti-dsDNA autoantibodies and reduced levels of complement factors (C3, C4) can guide the differential diagnosis of Lupus nephritis. However, the goal standard for diagnosis remains the renal biopsy with histopathology for grading and evaluation of disease activity and / or the presence of irreversible damage [**109-112**]. Cardiovascular, enterogastric, and hepatic manifestations can also be observed in SLE patients.

2.1.5 Clinical management

To date, there are no curative therapies for SLE, even though progresses have been made in the management of symptoms and complications, improving both quality and life expectancy. Treatments are tailored based on disease severity and include non-steroidal anti-inflammatory drugs, HCQ, low or high doses of corticosteroids and several immunosuppressive agents (e.g. azathioprine, cyclophosphamide). Some selected subjects can benefit of biologic agents, such as rituximab or abatacept used as off-label drugs. Only in 2011, FDA approved the first biologic drug for SLE, Belimumab, an anti-Blys monoclonal antibodies [**113**]. However, HCQ represents a milestone in the treatment of SLE and is still recommended for prevention of flares and thromboembolic events, management of lipid and glucose metabolism, and to reduce tissue damage over time [**114**].

2.1.6 Hydroxychloroquine

Antimalarial drugs with a 4- aminoquinoline scaffold, such as quinine, or chloroquine (CQ), are still essential pharmacological tools in prevention and treatment of malaria [**115,116**]. Hydroxychloroquine (HCQ), first synthesized in 1946, was proposed as a safer alternative to CQ in 1955 [**117**]. Later, both CQ and HCQ were suggested as effective alternative for treatment of autoimmune rheumatic diseases [**118**]. To date, HCQ is one of the most prescribed drugs in patients affected by SLE. HCQ exerts its pharmacological action by preventing the occurrence of both flares and antiphospholipid antibody-dependent thrombotic manifestations, thus improving quality of life and survival of patients [**114,118-121**]. This drug is commonly administrated orally, as racemic sulfate salt, at a loading dose of 400mg/day followed by a maintenance dosage of 200–400mg/day [**122**]. Liver metabolizes HCQ into three active metabolites (Figure 3), *i.e.* desethylchloroquine (DCQ), desethylhydroxychloroquine (DHCQ) and bisdesethylchloroquine (BDCQ) [**123**].

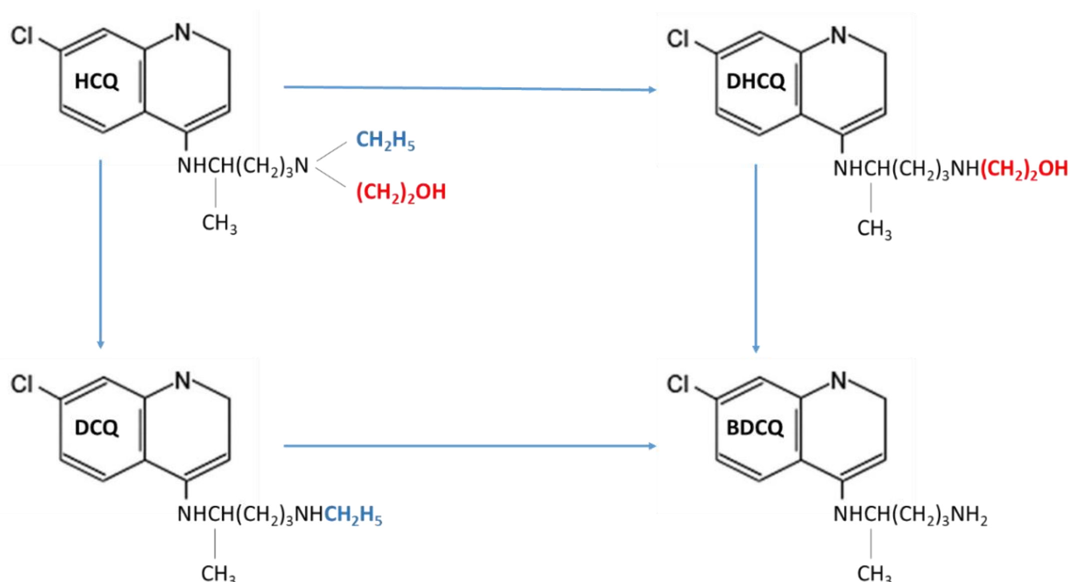


Figure 3. Molecular structure of hydroxychloroquine (HCQ) and its major metabolites: desethylhydroxychloroquine (DHCQ), desethylchloroquine (DCQ) and bidesethylchloroquine (BDCQ).

DHCQ, the major metabolite, is produced through the N- desethylation pathway, catalyzed by the enzymatic activity of the cytochromes (CYP) 2D6, 3A4, 3A5 and 2C8. Previous pharmacokinetic studies have shown that HCQ has a long elimination half- life of up to 40 days. Hence, the onset of action after initiating the therapy is slow and the steady state may be obtained only after 6months of treatment [**124,125**]. Moreover, the efficacy of this drug is strictly related to its trough concentration and maintaining plasma concentration above 1000ng/mL can reduce the frequency of flares, as described in the PLUS study [**126**]. High-responder subjects usually have increased blood concentration of HCQ ≥1250 ng/mL; while low responder patients show lower concentrations(100-750ng/mL) [**127**]; and non-responders very low blood HCQ levels (0–129ng/mL) also related to poor adherence to therapy [**128,129**]. For these reasons, monitoring blood concentration of HCQ is highly suggested for drug dose adjustment in order to increase responsiveness to therapy in SLE patients. Because the fine-tuned dosage of HCQ and its metabolites is emerging as one of the most critical points for an effective therapy, the availability of sensitive, fast and inexpensive analytical methods for qualitative and quantitative measurements of these compounds in body fluids is required for a good clinical management of SLE. Previously published methodologies although valid and well-designed show several drawbacks [**130-137**]. First, they rarely evaluate HCQ major metabolites, and thus lack the ability to perform comprehensive pharmacokinetic studies of all active molecules deriving from HCQ metabolism. In other cases, chromatograms did not display an optimal separation of all species of interest. Finally, methods providing the most

accurate results often require complex and very skilled analytical techniques (*i.e.* LC-MS/MS or sequential achiral–chiral HPLC), thus limiting their use in routinely laboratory practice.

2.1.7 Project aim

An ion- pairing high- performance liquid chromatography coupled with fluorescence detector (HPLC- FL) methodology was developed and optimized to efficiently separate and quantify HCQ, DHCQ, BDCQ and DCQ in peripheral blood. The method was validated and used for the TDM of SLE patients enrolled at the Rheumatology Unit of the University "Luigi Vanvitelli" of Naples (Italy). The same blood samples were also analyzed using a previously validated LC-MS/MS method and data were compared to those obtained from our HPLC technique [**133**].

After method validation, we correlated HCQ blood concentrations to quality-of-life (QoL) in SLE patients. Indeed, despite increasing interest, the extent of poor adherence to HCQ treatment, its main originating factors and the relationship with disease progression have not been yet sufficiently investigated in SLE patients with prolonged inactive disease. Therefore, this study was designed in collaboration with the Rheumatology Unit of the University "Luigi Vanvitelli" of Naples (Italy) to estimate the extent of and the main demographic, clinical and laboratory factors associated with HCQ non-adherence, and the relationship between outcomes and HCQ blood concentration in SLE patients with prolonged ($\geq$1 year) inactive disease.

2.2 Materials and methods

2.2.1 Patients

To evaluate circulating levels of HCQ and its metabolites, patients were enrolled at the Rheumatology Unit of the University of Campania ''Luigi Vanvitelli'', Naples (Italy) from November 2014 to April 2016, after written informed consent was obtained. The study was approved by the Ethics Committee of the University of Campania ''Luigi Vanvitelli''. Each patient had to satisfy the following inclusion criteria: diagnosis according to the 2012 classification criteria of the Systemic Lupus International Collaborating Clinics (SLICC); complete or clinical remission (with or without treatment) according to the preliminary Definitions of Remission in SLE (DORIS) criteria for at least one year; treatment with a stable dose of oral HCQ during previous six months. If taken, immunosuppressants and glucocorticoids had to be prescribed at a stable dose during the previous month. Exclusion criteria were: concomitant fibromyalgia; psychiatric disorders; or pregnancy.

On admission, medical history, physical examination and laboratory investigations were performed to assess disease activity according to the Safety of Estrogens in Lupus Erythematosus National Assessment SLE Disease Activity Index (SELENA-SLEDAI), and disease damage by the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index of SLE. Creatinine clearance was measured based on the Modification of Diet in Renal Disease (MDRD). Remission was defined according to the DORIS proposal as follows: complete remission without treatment, *i.e.* absence of clinical (SLE-related) manifestations, and/or serologic abnormalities (low C3 and/or C4, increasing dsDNA titer), only antimalarials allowed; complete remission on treatment, *i.e.* absence of clinical manifestations and/or serologic abnormalities, therapy with antimalarials, prednisone 5 mg/day and/or immunosuppressants allowed; clinical remission without treatment, *i.e.* absence of clinical manifestations, presence of serologic abnormalities, only antimalarials allowed; clinical remission on treatment, *i.e.* no clinical manifestations with serologic abnormalities, therapy with antimalarials, prednisone 5 mg/day and/or immunosuppressants allowed. The following parameters were also collected: height, weight, body mass index, time of the HCQ tablet intake, daily dose of HCQ, smoke status (current/past smoker). In addition, each patient was examined to assess the co-existence of fibromyalgia according to published diagnostic criteria, and the concomitant use of drugs for psychiatric problems was evaluated.

Each patient completed various tests: a visual analogue scale (VAS) for pain, fatigue and self-assessment of disease activity ranging from 0 (none) to 100 mm (very severe/active); a VAS for global health (GH) status ranging from 0 (poorest) to 100 mm (the best); the Italian version of Short-Form 36 (SF-36), summarized in two composite summary scores, the physical component summary

score (PCS) and the mental component summary score (MCS), ranging from 0 (poorest QoL) to 100 (highest); the Italian version of the Health Assessment Questionnaire Disability Index (HAQ-DI) from 0 (no disability) to 3 (highest disability); and The Hospital Anxiety and Depression Scale for level of anxiety and depression, where a score of 0 to 7 for either subscale was considered to be in the normal range.

Peripheral blood samples were collected to measure circulating concentrations of HCQ and DCQ, as described below. Treatment was prescribed according to latest published guidelines: the daily HCQ dose was up to 6.5 mg/kg. A follow-up visit was planned for each patient after six months (T6), when patients were re-assessed by clinical and laboratory testing together with a second peripheral blood drawing for measurement of HCQ and DCQ concentrations. The occurrence of a flare during the follow-up was assessed by the SELENA/SLEDAI flare composite score. Patients were considered non-adherent to therapy when HCQ levels were< 100 ng/ml.

2.2.2 Chemicals and reagents

HPLC grade water, methanol, diethyl ether and acetonitrile (ACN) were purchased from Romil (Waterbeach, Cambridge, UK). HCQ and its metabolites DCQ, DHCQ and BDCQ were obtained from LGC Standard GmbH (Milan, Italy); sodium dodecyl sulfate (SDS) and CQ were purchased from Sigma-Aldrich (St Louis, MO, USA). Twenty- five percent ammonia solution was obtained from Applichem (Darmstadt, Germany).

2.2.3 Sample preparation

Initial stock solutions of HCQ and CQ were prepared by dissolving powders in distilled water; DHCQ was prepared in DMSO, and DCQ and BDCQ in methanol (MeOH). Stock solution concentrations were 0.6 mg/mL for HCQ, 0.5 mg/mL for CQ, 10 mg/mL for DHCQ, 2.5 mg/mL for DCQ and 1 mg/mL for BDCQ. Working solutions were prepared by diluting stocks in HPLC-grade water. All solutions were stored at -20°C until use.

Heparinized whole blood samples were collected in BD Vacutainer tubes and stored at -20°C until use. For optimization and validation of the analytical procedure, whole blood samples were obtained from healthy volunteers from the Blood Establishment of the University Hospital “San Giovanni di Dio e Ruggi d'Aragona” of Salerno (Italy). After optimization and validation, the analytical procedure was used for measurement of HCQ levels in the blood of SLE patients.

All blood samples were processed and analyzed in the laboratories of the Department of Medicine, Surgery and Dentistry "Scuola Medica Salernitana", University of Salerno, Salerno (Italy). For compound extraction, 500 µL of blood were transferred to a 15 mL Falcon tube, 500 µL of water containing 0.13 µg/mL of CQ (used as internal standard [IS]) were added and briefly vortexed. Subsequently, 300 µL of 25% NH_3 and 4 mL of diethyl ether were added to each sample, vortexed for 2 min, centrifuged at 1200 g at 4°C for 5 min, frozen and maintained at -80°C for 10 min. The organic layer, which remained liquid after this procedure, was then easily collected and air-dried for at least 14 h at room temperature. Dried residue was dissolved in 500 µL of the mobile phase and centrifuged at 17,000 g at 4°C for 5 min; supernatant was then transferred to appropriate vials for acquisition.

2.2.4 Preparation of standard calibration curves and quality control samples

For calibration curve, a working solution containing HCQ, DHCQ, DCQ and BDCQ, each at a final concentration of 10 µg/mL, was freshly prepared in water from initial stock solutions. Serial dilutions were then prepared to obtain concentration of 10, 25, 100, 250, 500, 1000, 1600 and 2500 ng/mL of each analyte in a final volume of 500 µL of whole blood. Three quality controls (QC) were prepared by adding to whole blood from healthy volunteers the four analytes at a concentration of 200, 700 or 1500 ng/mL. Samples were then extracted according to our extraction procedure.

2.2.5 Chromatography

The chromatographic separation was achieved by reverse phase (RP)-HPLC using a Waters 1525 Model Binary Pump System equipped with a multi λ fluorescence detector (model 2475), a photodiode array detector (model 2998) and an autosampler (model 2707; Waters, Milford, MA, USA). Column used was an octadecyl silane Luna C18 100 (150 × 4.6 mm, 5 µm) column (Phenomenex). Samples were kept at room temperature in the autosampler and column oven was set at 40°C. Breeze software 2.0 (Waters) was used for peak analysis, integration and linear regression analysis of calibration curves. A water– methanol–acetonitrile (47:10:43 v/v/v) mixture containing 3.2 mM SDS was employed as mobile phase. The pH was adjusted to 9.4 with 25% NH_3 and 25% orthophosphoric acid. Chromatographic separation was achieved by isocratic elution with a flow rate of 1.0 mL/min. Analytes were revealed using fluorescence detector; excitation and emission wavelengths were set respectively at 320 and 370 nm. Compounds were identified comparing their

retention times with available commercial standards.

2.2.6 Method validation

The lower limit of detection (LLOD) was defined as the lowest concentration at which the analytical assay can reliably differentiate the signal of the analyte peak from background noise (signal- to- noise ratio ≥ 3). The lower limit of quantification (LLOQ) was considered as the lowest concentration at which imprecision was ≤20% and accuracy was ±20% of the nominal concentration, based on triplicate analyses characterized by a peak intensity at least 5 times higher than baseline noise.

Linearity was determined by plotting the peak area of analyte/internal standard ratio against the analyte concentrations; the resulting curves were fitted with a linear regression. Experimental concentrations were back-calculated using calibration curves to determine their deviation from the nominal ones. Mean standard deviation should not exceed 20% of nominal value.

The average recovery of HCQ and its metabolites was determined by comparing the experimental concentration measured in three samples containing specific amounts of each analyte (i.e. 200, 700 and 1500 ng/mL) pre- and post-extraction. Pre-extraction samples were prepared by adding each analyte to a final volume of 500 µL of whole blood followed by extraction. Post-extraction samples were obtained by extracting whole blood from healthy donors and then spiking the amounts of analytes required to obtain the desired concentrations in processed supernatants.

Matrix effects and possible carry-over were evaluated by analyzing samples obtained by extracting unspiked whole blood from healthy donors at the beginning of the analytical series, followed by analysis of samples containing high concentrations (700 or 1500 ng/mL) of the four analytes.

Intra- and inter-day precision and accuracy for each analyte were evaluated at three different concentrations (200, 700 and 1500 ng/mL) in samples extracted from whole blood. Three replicates for each concentration were analyzed in the same day. An aliquot of the same samples was analyzed after 1 and 4 days; during this time, samples were stored at +4°C. Precision was calculated using the following equation, and measured by percentage coefficient of variance (CV): CV% = (standard deviation/mean value) ×100. Accuracy was calculated using the following equation for percentage of relative standard error (RSE): RSE% = [(mean - theoretical value) / theoretical value] ×100.

Stability of stock solutions stored at −20°C was evaluated once a week for 6 months; 5 µL of each solution were diluted to 1 mL using HPLC- grade methanol and injected into the HPLC system. Peak areas at each timepoint were compared to those obtained at t =0. Similarly, stability of all

analytes in blood samples, stored at 4°C was evaluated for 5 days.

2.2.7 Mass spectrometry

LC-MS/MS analyses were performed using an Ultimate 3000 UHPLC coupled with a TSQ Endura mass spectrometer equipped by an electrospray ion source and a triple quadrupole analyzer (Thermo Fisher Scientific, Cambridge, MA, USA). LC-MS/MS analyses were performed using a previously validated method [139] with minor modifications. Briefly, compound separation was achieved using a Kinetex F5 column (100 × 2.0 mm, 2.6 µm; Phenomenex, Torrance, CA, USA) and a mixture of 0.1% formic acid in water (solution A) and 0.1% formic acid in acetonitrile (solution B) as mobile phase. The following gradient was used: 10% B from 0 to 1 min, linear gradient (10–37% B) from 1 to 3 min, 85% B from 3.5 to 5 min. Data were acquired in positive ion selected reaction monitoring (SRM) mode, using specific transitions for each compound: m/z 336 → 247 for HCQ, m/z 308 → 179 for DHCQ, m/z 292 → 179 for DCQ, m/z 264 → 179 for BDCQ and m/z 320 → 247 for the internal standard CQ. Peak areas were measured using the Quan Browser software (Thermo Fisher Scientific).

2.2.8 Statistical analysis

Statistical analysis was performed by MedCalc software, version 12.7.0. Comparison between results obtained by using either HPLC or mass spectrometry was performed using the Bland–Altman plot [**138**] and the Passing–Bablok linear regression [**139**]. Continuous variables were analyzed by unpaired Student's t-test, Mann-Whitney test, or paired t-test and Wilcoxon test, where appropriate. The chi-square or Fisher's exact test was applied for categorical variables. Spearman correlation was used to assess relationship between [HCQ] and continuous patient-related outcome variables. P values $\leq$0.05 were considered statistically significant. Univariate logistic regression analysis was constructed to assess factors associated with HCQ non-adherence, identifying as dependent variables an [HCQ] < 100 ng/ml. The factors found to be significant in univariate analysis ($p < 0.1$) were included in a multivariate model. For continuous predictive variables, odds ratios (ORs) expressed the risk associated with a one standard deviation (SD) increase for each continuous predictive variable.

2.3 Results and Discussion

2.3.1 Sample preparation and extraction

Great variability in HCQ blood concentrations was observed in patients treated with comparable doses of HCQ [**140**]. These variations are caused, among others, by drug binding to plasma proteins [**131**] and by HCQ internalization into erythrocytes [**141**]. For these reasons, whole blood is preferred to serum for quantification of HCQ and its metabolites for pharmacokinetic studies and to evaluate patient adherence to therapy [**135**].

For method development and optimization, whole blood from healthy volunteers was diluted with an equal volume of HPLC-grade water for red blood cell lysis. Because HCQ and its metabolites have a basic pKa, blood was alkalized with ammonia at a final concentration (v/v) of 5.8% to increase the extraction yield. Several solvents and mixtures of them at various ratios have been tested for organic extraction, such as dichloromethane, ethyl acetate, chloroform and diethyl ether. No significant differences in extraction yields were described (data not shown), and diethyl ether was chosen as the extraction solvent because of the good extraction yield and the fastest evaporation rate. The freezing step was included in the extraction procedure to reduce the solubility of HCQ and its metabolites in the aqueous phase, and for an easy recovery of the organic phase.

2.3.2 Optimization of HPLC-FL analysis

A mixture of ACN–MeOH–H_2O at a pH of 5.8 was initially used as mobile phase for the separation of standard solutions containing HCQ, its metabolites and the internal standard CQ. Different ratios of the three components were tested and a 47:43:10 (v/v/v) ratio was selected because three out of four total analytes were discriminated by HPLC-FL analysis (Figure 4A). However, HCQ and BDCQ seemed to co-elute in the second peak. To confirm the co-elution of the two species, an identical mixture lacking BDCQ was analyzed using the same method, showing a decreased intensity of the second peak. Moreover, peak tailing was evident for all analytes, thus negatively affecting resolution, sensitivity, and reproducibility of the method.

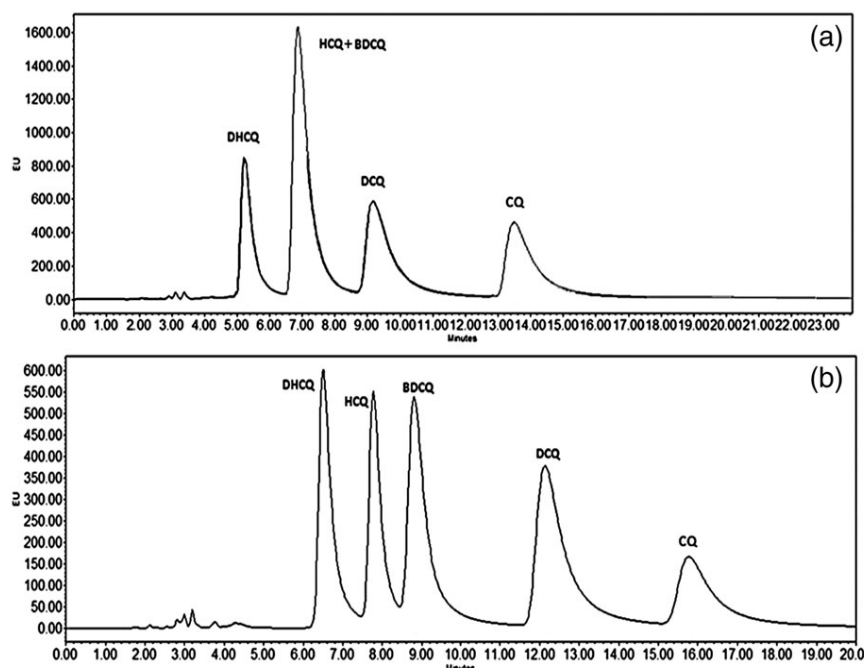


Figure 4. Presence of sodium dodecyl sulfate (SDS) in the mobile phase and corresponding effect on compounds separation. Chromatograms showing separation of analytes in the absence (a) or presence (b) of SDS. As shown, without SDS in the mobile phase, no effective separation between HCQ and BDCQ peaks is evident.

Next, the pH of the mobile phase was increased to 9.4to improve resolution and sensitivity of the method. Indeed, HCQ and its metabolites are basic compounds and at low or neutral pH values or with traditional silica materials, secondary interactions might increase the interactions between the analyte and residual silanols, thus causing peak tailing. For this reason, by increasing the pH of the mobile phase, we tried to prevent charging of both silanol groups and basic compounds [**142**]. Although peak tailing appeared to be effectively reduced, HCQ and BDCQ still co-eluted (data not shown).

To achieve the effective separation of HCQ and BDCQ, an ion pairing chromatography approach was applied [**143,144**], using SDS at a final concentration of 3.2 mM (0.092% w/v) in a mobile phase of ACN–MeOH–H_2O (47:43:10), pH 9.4 [**145**]. Basic compounds form an electrically neutral ion pair with sodium alkanesulfonates such as SDS, thus positively affecting the interaction of poorly retained molecules with the stationary phase. The presence of SDS in the mobile phase allowed the discrimination of all molecules, although peak tailing was still observed (Figure 4B).

To further improve peak shape and increase resolution, we also evaluated the influence of different temperatures on chromatographic elution. Column oven temperatures of 35, 40, 45 and 50°C were tested (Figure 5). The increase in temperature initially produced an improvement of peak shape and caused a reduction in the retention time of DCQ and CQ; however, exceeding 40°C worsened

analytes separation and induced a partial overlapping of HCQ and BDCQ peaks. For this reason, the column oven temperature was set at 40°C for further analysis.

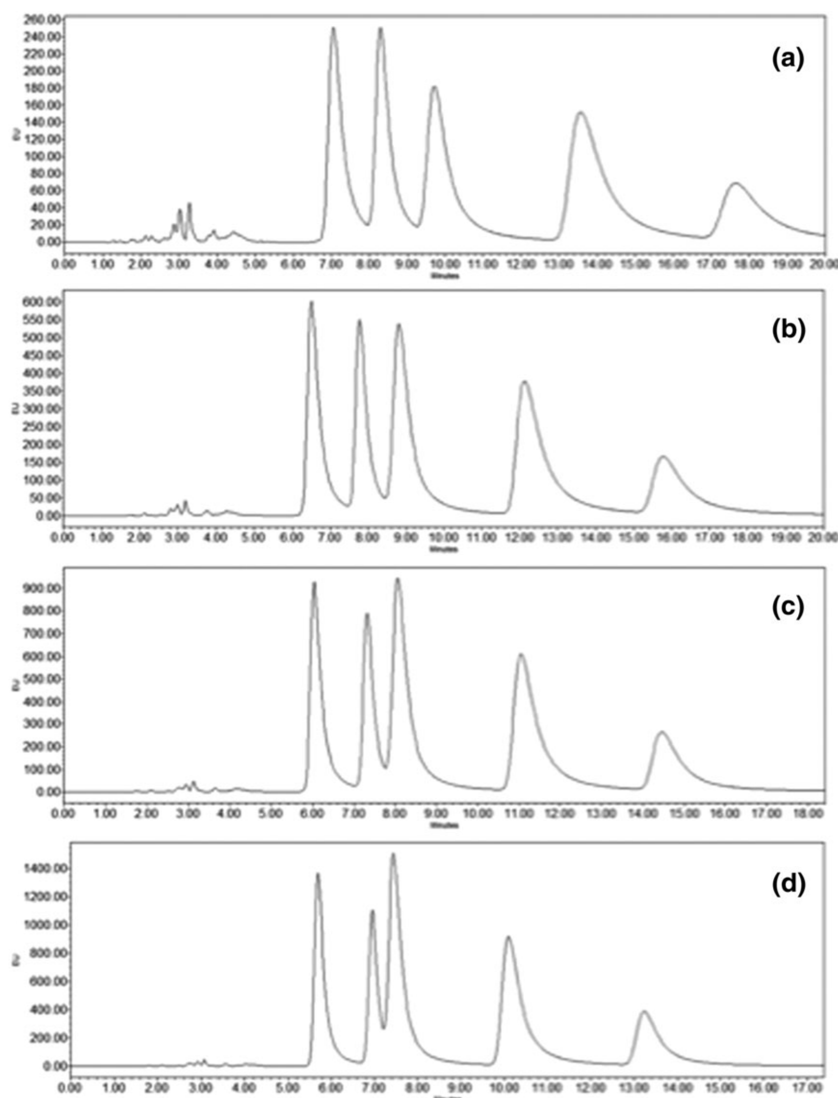


Figure 5. Temperature influence on analytes separation and retention times. Chromatograms show the separation of analytes at column oven temperatures of 35°C (a), 40°C (b), 45 °C (c) and 50 °C (d).

2.3.3 Method validation

The method was validated following the EMA and FDA guidelines for analytical procedure validation [**146,147**]. In particular, the following parameters were evaluated: selectivity; specificity; absence of carry-over; lower limit of detection (LLOD) and lower limit of quantization (LLOQ); linearity; matrix effect; extraction recovery; precision and accuracy; and short- and long-term stability.

LLOD and LLOQ were calculated as described in Section 2.2. A LLOD of 1 ng/mL was observed for all analytes; the LLOQ was 15 ng/mL for DHCQ and DCQ, and 20 ng/mL for HCQ and BDCQ. These LLOD and LLOQ values are similar or lower than those reported in literature [**120,134,148**]. Linearity was evaluated over a concentration interval ranging from LLOD to 2500 ng/mL for each compound (Figure 6). For each analyte, the peak area/IS ratio was plotted against the analyte concentrations with a 1/x weighting factor, and by fitting a linear regression.

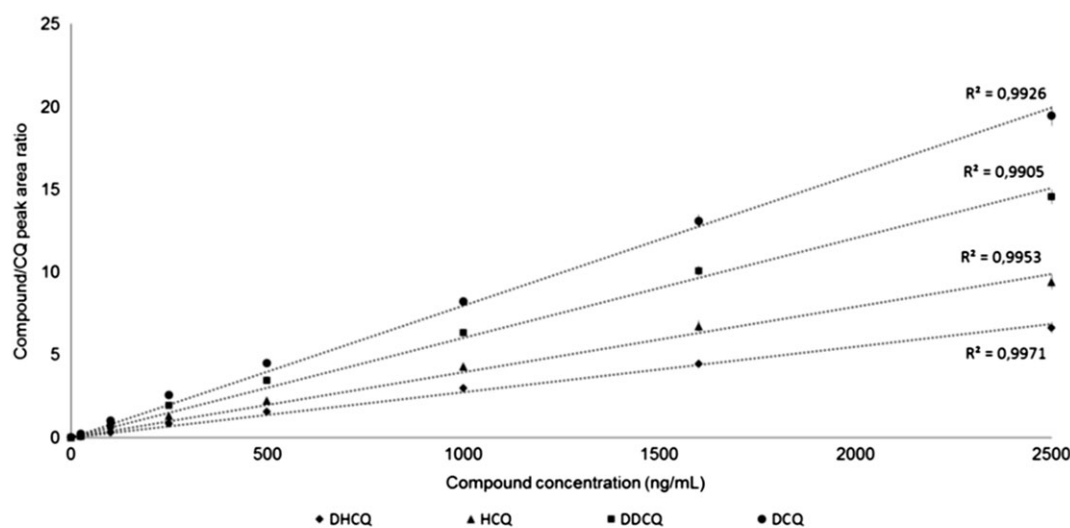


Figure 6. Calibration curves of HCQ and its metabolites. The ratios between peak areas measured for the four compounds at each concentration and the peak area measured in the same sample for the internal standard (CQ) were plotted as a function of the compound concentration. The coefficient of determination (R2) calculated for each calibration curve is reported.

Extraction recovery was calculated for each analyte, as described in Section 2.2, and results are reported in Table 2. Our data indicated that analyte recovery was slightly affected by initial concentration. However, it is important to note that analyte/IS ratios remained almost constant

	200 ng/mL		700 ng/mL		1500 ng/mL	
Compound	Recovery (%)	SD (%)	Recovery (%)	SD (%)	Recovery (%)	SD (%)
HCQ	33.4	3.4	54.2	5.8	55.2	7.2
DHCQ	26.7	4.0	48.4	7.6	49.3	3.9
DCQ	23.3	2.7	46.2	8.0	47.5	9.6
BDCQ	26.0	5.8	45.8	9.4	46.1	8.7
CQ	28.4	8.2	48.3	3.6	50.2	6.4

Table 2. Recovery of hydroxychloroquine (HCQ), desethylhydroxychloroquine (DHCQ), desethylchloroquine (DCQ), bisdesethylchloroquine (BDCQ), and chloroquine (CQ), after organic extraction, calculated at three different initial concentrations.

regardless of initial concentration (Table 3), thus resulting in a good linearity of the method over a wide concentration range.

The absence of possible contaminants and carry-over was confirmed by injecting blank blood samples at the beginning of each analytical series and immediately after high- concentration standards.

To evaluate the reproducibility of our method, we analyzed a mixture of all analytes at three different concentrations (200, 700 and 1500 ng/mL) over 4 days. Data reported in Table 4 show that our methodology allowed fora good intra- and inter-day precision and accuracy for all analyzed compounds.

Stock solution stability was also tested: a good stability for HCQ, DHCQ, DCQ, BDCQ and CQ was described at-20°C for more than 6 months. However, analytes in blood samples stored at 4°C showed a time-dependent decrease in signal intensity for all analytes, including IS. Therefore, an accurate and precise quantification of HCQ and its metabolites in blood could be reasonably performed up to the fifth day of storage at 4°C.

Compound	200 ng/mL	700 ng/mL	1500 ng/mL
HCQ	1.17	1.12	1.10
DHCQ	0.94	1.00	0.98
DCQ	0.82	0.96	0.95
BDCQ	0.91	0.95	0.92

Table 3. Metabolite/internal standard (CQ) peak areas ratio measured for HCQ, DHCQ, DCQ and BDCQ; each compound was analyzed at three different initial concentrations.

Compound	Initial concentrations (ng/mL)			Initial concentrations (ng/mL)		
	200	700	1500	200	700	1500
	CV intraday (%)			CV interday (%)		
HCQ	2.1	2.0	1.8	8.9	9.4	5.0
DHCQ	0.8	1.6	1.5	9.1	9.9	6.3
DCQ	0.9	0.7	2.0	9.7	9.1	5.7
BDCQ	1.0	1.1	2.0	9.7	8.9	5.8
	RSE intraday (%)			RSE interday (%)		
HCQ	8.0	3.2	4.7	2.2	0.4	0.5
DHCQ	1.5	5.6	4.8	10.0	1.2	1.0
DCQ	6.1	4.1	3.4	9.5	2.2	0.6
BDCQ	3.2	2.4	5.5	3.7	2.7	0.1

Table 4. Intra- and inter-day precision (CV) and accuracy (RSE) of HCQ, DHCQ, DCQ and BDCQ, obtained at three different initial concentrations (200, 700 and 1500 ng/mL). SD: standard deviation.

2.3.4 Comparison between HPLC-FL and LC-MS/MS on a subset of clinical samples

After optimization, our HPLC-FL methodology was used on a small subset of whole blood samples from SLE patients treated with HCQ. Each sample was processed as described above and analyzed by HPLC- FL. As already described in literature, a marked variability in the amount of HCQ and its metabolites was present among our cohort of treated patients (Table 5). According to HCQ metabolism [**123**], DCQ concentration was generally similar or slightly lower than that of HCQ in our patients, and the amount of the two less abundant metabolites (DHCQ and BDCQ) accounted for the 10–20% of that of HCQ. The only exceptions were patients 14 and 15, where very high HCQ levels were measured, and the amounts of DCQ, DHCQ and BDCQ were lower than expected.

Patient	Measured blood concentration (ng/mL)			
	HCQ	DHCQ	DCQ	BDCQ
1	145	143	23	15
2	728	520	119	95
3	705	504	100	85
4	263	240	41	27
5	486	332	50	42
6	114	84	NQ[a]	NQ[a]
7	758	570	130	131
8	1104	1020	231	152
9	99	128	NQ[a]	NQ[a]
10	901	885	171	137
11	1058	801	109	107
12	711	521	55	47
13	1252	1348	246	126
14	1896	953	138	81
15	1847	1169	160	172
16	594	523	62	50

Table 5. Blood concentration of HCQ, DHCQ, DCQ and BDCQ measured in systemic lupus erythematosus (SLE) patient samples. [a]: peak area below the LLOQ.

To evaluate the suitability of our approach for quantitative analysis of HCQ and its metabolites in clinical practice, the same samples were also analyzed by LC-MS/MS and results were compared to those obtained by HPLC-FL. Three concentration intervals were identified for the different species, corresponding to high, medium or low levels: for HCQ and DHCQ, 0–150, 151–700 and > 700 ng/mL; for less abundant compounds, 0–50, 51–150 and > 150 ng/mL. A good accordance between the two analytical approaches was described for more than 90% of measured values (58 out of 64) (Figure 7). In addition, the Bland–Altman model was used to evaluate the accordance between results obtained with LC–MS/MS and HPLC- FL for all analytes. This analysis also showed good correlation for three out of the four compounds, as the resulting confidence intervals were narrow

enough (about ±10%) for HCQ, DCQ and DHCQ (Figure 8). Similar results were also obtained applying the Passing–Bablok linear regression model (data not shown): good correlations (R^2,0.99-0.98) were observed for HCQ, DCQ and DHCQ, whereas a less favorable correlation was described for BDCQ ($R^2 = 0.88$). Noteworthy, the two methods were poorly comparable for the quantization of BDCQ, the less abundant metabolite. This difference might be related to the higher selectivity of LC-MS/MS method compared with HPLC- FL. As a consequence, although the HPLC-FL method we developed and validated remain an important tool for assessing HCQ concentration in clinical routine settings, at this stage we decided to perform further analysis on the whole cohort of patients using the LC-MS/MS method, and only HCQ and DCQ concentrations were evaluated.

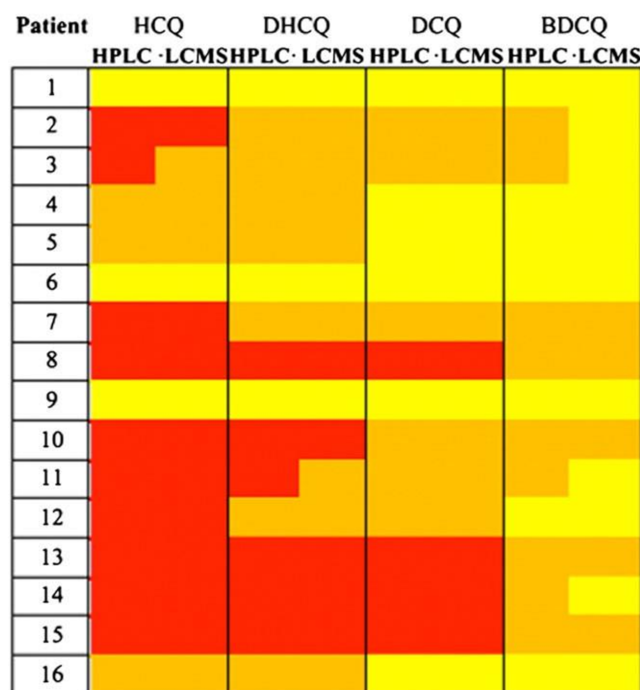


Figure 7. Heat map comparing the concentrations of HCQ and corresponding metabolites measured by HPLC and LC/MSMS techniques. High, medium and low levels for each compound are indicated in red, orange and yellow, respectively.

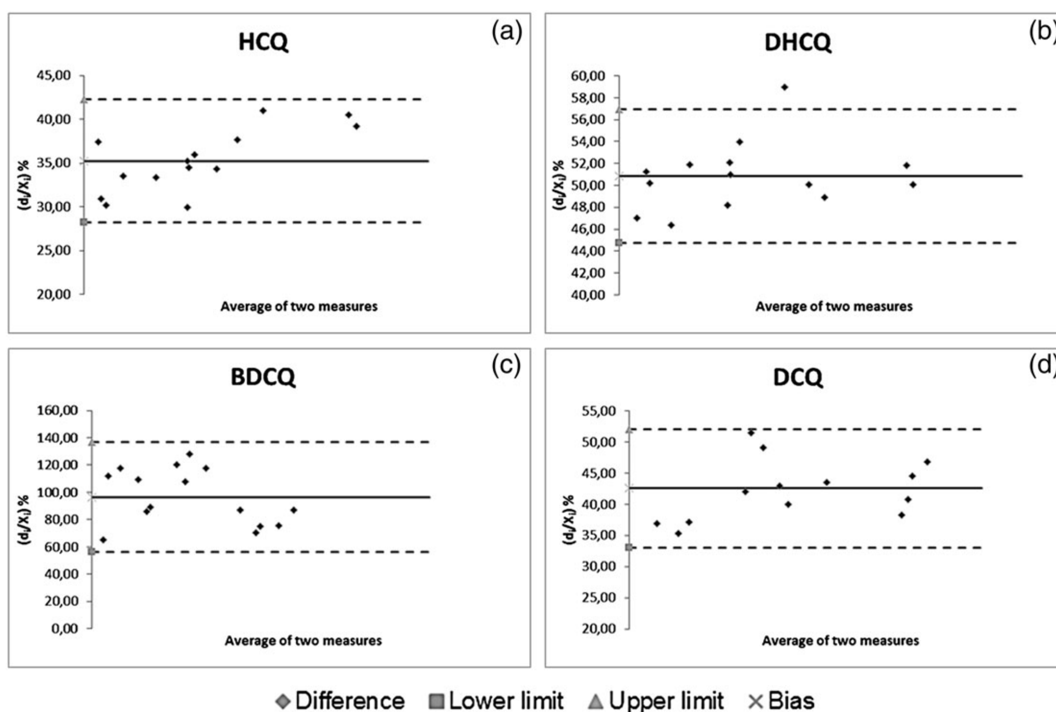


Figure 8. Bland–Altman plot(s) for HCQ, DHCQ, DCQ and BDCQ, obtained by plotting the average of the two measurements for each of the n samples (x axis) as a function of the percentage values of the differences in the averages (y axis).

2.3.5. Relationship between blood concentration of HCQ and QoL in SLE patients with inactive disease

Clinical and therapeutic features of enrolled patients are summarized in Table 6. Ninety-five % of patients were female, with a mean age of 41±11 years, a median disease duration of 15 years (range 2–37 years), and a median SLICC damage index of 0 (range 0–3). All patients were treated with HCQ and 61% of these patients received additional drugs for SLE treatment. The mean dose of HCQ per weight was of 5.3 ±1.2 mg/kg and a mean time from last pill intake was 9.8 ±8.7 hours. Median [HCQ] at baseline was 327 ng/ml (range 0–4003 ng/ml), and for [DCQ] was 47 ng/ml (range 0–650 ng/ml). There were no significant differences in HCQ levels between patients treated only with HCQ (median 285 ng/ml; range, 0–1723 ng/ml) or with additional medications (median 435 ng/ml; range, 0–4003 ng/ml). Similarly, no variations were described for DCQ levels between patients taking only HCQ (median 47 ng/ml; range, 0–239 ng/ml) or additional drugs (51 ng/ml; range 0–650 ng/ml). Twenty-four patients (29%) had undetectable blood [HCQ] (n = 17) or an [HCQ] < 100 ng/ml (n = 7; mean, 67±34; range 9–99 ng/ml), reflecting a very poor adherence to therapy. Additional four patients showed a [HCQ] < 200 ng/ml. No patient had undetectable [DCQ], a condition suggesting a very recent resumption of treatment in patients with dosable HCQ.

The therapeutic target for reducing occurrence of SLE within six months ([HCQ] > 1000 ng/ml) was found only in 11 (14%) patients.

	All patients n ¼ *83*	*[HCQ] < 100 ng/ml* n ¼ *24*	*[HCQ]* c *100ng/ml* n ¼ *59*	p *Adherent vs non-adherent*
Sexfemale	79 (95)	22 (92)	57 (97)	0.575
Age,years meanTSD	41T11	39T11	42T11	0.772
Disease duration, years median (range)	15 (2–37)	17 (2–29)	13 (2–37)	0.692
Remissionwithtreatment	51 (61)	16 (67)	35 (59)	0.708
SLICCdamageindexmedian(range)	0 (0–3)	0 (0–1)	0 (0–3)	0.385
Currentsmokers	31 (37)	9 (37)	22 (38)	0.816
Bodymassindex(kg/m²)meanTSD	25T5	24T4	26T5	0.828
Estimatedcreatinineclearance,ml/minmeanTSD	89T24	94T22	86T24	0.129
HCQprescribeddose/weight(mg/kg)meanTSD	5.3T1.2	5.4T1.2	5.4T1.2	0.710
[HCQ] ng/ml median (range)	327 (0–4003)	0 (0–99)	546.1 (101–4003)	<0.0001
[DCQ] ng/ml median (range) Additional treatment	47 (0–650)	0 (0–11)	79 (11–650)	<0.0001
Immunosuppressors	23 (28)	13 (54)	10 (17)	0.001
Azathioprine	10 (12)	5 (22)	5 (8)	
Mycophenolate mofetil	6 (7)	3 (12)	3 (5)	
Methotrexate	5 (6)	4 (17)	1 (2)	
Cyclosporine	2 (2)	1 (4)	1 (2)	
Glucocorticoids	40 (48)	11 (46)	29 (52)	0.974

Table 6. Clinical, demographic and laboratory features of 83 patients enrolled. a: If not otherwise specified, the values are the number (%) of patients. [HCQ]: whole blood concentration of hydroxychloroquine; [DCQ]: whole blood concentration of desethylchloroquine; SLICC: Systemic Lupus International Collaborating Clinics.

At baseline, median physical and mental component summary scores (PCS and MCS) were 47 (range, 12–62) and 45 (range, 18–66), respectively. The self-reported median VAS scores were as follows: VAS pain 17 mm (range, 0– 100 mm); VAS fatigue 33 mm (range, 0–100 mm); VAS GH

74 mm (range, 20–100 mm); and VAS patient self-assessment of disease activity 20 mm (range, 0–94 mm). The median HAQ-DI score was 0 (range, 0–2.125). All results of self-assessment scores are summarized in Table 7.

	All patients n ¼ *83*	*[HCQ] < 100 ng/ml* n ¼ *24*	*[HCQ]* c *100 ng/ml* n ¼ *59*	p *Non-adherent vs other patients*
PCS median (range)	47 (12–62)	50 (29–60)	44 (12–62)	0.021
MCS median (range)	45 (18–66)	48 (18–62)	45 (22–66)	0.755
HAQ-DI total score median (range)	0 (0–2.125)	0 (0–0.875)	0.625 (0–2.125)	*0.062*
Anxiety median (range)	14 (7–20)	15 (9–18)	14.5 (7–20)	0.772
Depression median (range)	12 (8–18)	12 (8–18)	11 (8–16)	0.08
VAS Pain, mm median (range)	17 (0–100)	3 (0–55)	25 (0–100)	0.011
VAS Fatigue, mm median (range)	33 (0–100)	23 (0–100)	44 (0–100)	0.212
VAS GH, mm median (range)	74 (20–100)	80 (32–100)	67 (20–100)	0.043
Patient's self-assessment of disease activity, mm median (range)	20 (0–94)	5 (0–70)	23 (0–94)	0.028

Table 7. Patients' related outcome measures in overall sample, in non-adherent and adherent patients. [HCQ]: whole blood concentration of hydroxychloroquine; PCS: Physical Component Summary score; MCS: Mental Component Summary score; HAQ-DI: Health Assessment Questionnaire Disability Index; VAS: visual analogue scale; mm: millimeter; GH: global health status. Statistically significant comparisons are in bold (p < 0.05).

A slight positive correlation was found between [HCQ] and both VAS pain (rho 0.275; p 0.012) and patient self-assessment of disease activity (rho 0.267; p 0.016). A negative correlation was described between [HCQ] and some parameters of the SF-36, such as limitations due to physical problems (expressed by the domain Role limits – Physical) (rho = –0.233; p=0.04) and body pain (rho– 0.228; p=0.04). Since these results could be influenced by the lowest [HCQ] found in non-adherent patients, we performed a second analysis excluding those patients; however, no significant correlations were observed at study entry and follow-up (data not shown).

Poor-adherent patients reported a better QoL compared to other patients in the following clinical features: physical domain (median PCS, 50 vs 44, poor-adherent vs others; p = 0.021); level of pain (median VAS pain, 3 vs 25 mm, poor-adherent vs others; p = 0.011); reported GH (median VAS GH, 80 vs 67 mm, poor-adherent vs others; p = 0.043); self-assessed disease activity (median VAS patient, 5 vs 23 mm, poor-adherent vs others; p = 0.028); and disability (median HAQ, 0 vs 0.625, poor-adherent vs others; p = 0.062). No significant differences were described between the two groups in fatigue, anxiety, depression or any available demographic or clinical features potentially influencing HCQ metabolism (HCQ dose/weight, body mass index, creatinine clearance, smoke). The number of non-adherent patients on concomitant immunosuppressive therapy was significantly higher than that of adherent patients (13/24 versus 10/59; p = 0.001); while, there was no difference in the number of glucocorticoid users (p = 0.974). At multivariate logistic analysis, the factors independently associated with non-adherence were the PCS (OR = 1.05; 95% confidence interval (CI) = 1.00–1.11; p = 0.038) and the concomitant use of immunosuppressants (OR = 4.35; 95% CI = 1.41–13.44; p = 0.010).

For what concerns the follow-up, of the 83 enrolled patients, 77 subjects (93%) attended the scheduled follow-up visit after a mean time of 7±2 months. Five of them (6%) had a minor flare (four skin rash; one skin rash and low platelet count); while, no major flare was observed. Occurrence of flare was not associated with any clinical or demographic features, reported outcome scores, low dsDNA positivity and/or hypocomplementemia (data not shown). Interestingly, patients who experienced flares had a median baseline [HCQ] of 284 ng/mL (range, 0–1723 ng/mL); while patients who did not flare had higher HCQ levels (median, 435 ng/mL; range, 0–4003 ng/mL) (p = 0.225). Fifty-four of patients on follow-up (70%) (14/20 non-adherent, 40/57 good adherent; p = 0.787) gave their consent to have another unscheduled blood sample to measure blood HCQ.

Patients labelled as non-adherent at study entry achieved a significant higher [HCQ] at T6 (median, 515 ng/mL; range, 0–1682 ng/mL) compared to baseline (median, 0 ng/mL; range, 0–99.4 ng/mL) (p < 0.0001). Adherent patients did not show significant variations between baseline levels (median, 515 ng/mL; range, 0–1682 ng/mL) and follow-up (median, 631 ng/mL; range, 0–2208 ng/mL) (p = 0.385).

2.4 Discussion

First aim of this part of this PhD project was to develop and validate a novel HPLC- based approach for qualitative and quantitative analysis of HCQ and its metabolites DHCQ, DCQ and BDCQ. The main strengths were the requirement of a commonly available instrumentation in clinical laboratories, and the complete chromatographic resolution of the four analytes. The last point was not trivial since HCQ and its metabolites share similar fluorometric and chromatographic properties; moreover, DCQ and HCQ blood levels are generally comparable in treated patients. Therefore, an incomplete separation between two or more peaks would seriously affect quantitative analysis. Our method showed linearity up to a concentration of 2500 ng/mL, which is the therapeutic range of HCQ and its metabolites, conferring to our method a high accuracy and sensitivity for monitoring these compounds in treated patients. Indeed, our method allowed an accurate estimation of a wide range of concentrations of all four analytes (from LLOQ up to 2500 ng/mL), both for the most represented (*i.e.* HCQ and DCQ) and for the less abundant species (i.e. BDCQ and DHCQ).

The efficacy of our method was evaluated and confirmed by analyzing blood samples from treated patients; the results obtained were in clear accordance with clinical observations and were correlated to those obtained with more accurate techniques such as LC-MS/MS. This latter was the analytical approach that was used for the analysis of the whole cohort of patients.

In the second part of this project, in fact, we investigated the prevalence of non-adherence to therapy in SLE patients with inactive disease by HCQ blood level measurement and by assessing QoL self-reported outcomes. One-third of patients with inactive disease did not take the drug properly; however, these patients were those with a better QoL, lower level of pain, lower self-rated disease activity, and in concomitant use of immunosuppressants. Flares within a six months follow-up occurred only in patients with low blood concentration of HCQ.

Poor adherence represents an important cause of treatment failure in patients affected by chronic diseases. Unfortunately, establishing the burden of non-adherence is not easy in clinical practice, and self-reported questionnaires are not reliable. For SLE patients on chronic HCQ, therapeutic HCQ monitoring can increase adherence to therapy. The rate of non-adherence assessed by HCQ blood measurement is highly variable (7-30%) in patients with active disease. In our study, a lower threshold of non-adherence was defined (i.e. [HCQ] < 100 ng/mL) in order to exclude any possible interference with factors known to influence HCQ blood concentration. A higher rate of HCQ non-adherence was described in our cohort of patients with inactive SLE; however, rates were similar to those reported for patients with active disease. Only 10% of patients reached the therapeutic target of [HCQ]≥ 1000 ng/ mL for reducing flare events. The number of relapses in our cohort was

comparable to that described by Costedoat-Chalumeau et al. (6% vs 12%), where low HCQ was the only predictor of SLE flares in a multivariate analysis. The discrepancy between a high rate of poor adherence and low numbers of flares could be explained by differences in concomitant treatments (*i.e.* corticosteroids, immunosuppressants) between the two cohorts, and/or by inclusion in our study of patients with prolonged inactive disease and, thus, less likely to relapse. However, we could only speculate that target blood concentration required to prevent flares in our subgroup of patients could be lower than 1000 ng/mL. Further larger studies with longer follow-up are required to confirm that lowering HCQ daily doses could effectively reduce flare rates and thus the risk of ADR such as retinopathy.

In the PLUS study, no associations between HCQ blood levels and QoL were described in SLE patients with sub-optimal blood concentrations of the drug (100-750 ng/ml). In our study, we assessed HCQ levels and QoL in patients with persistent inactive disease by measuring QoL not only by the standard SF-36 scale, but also by taking into account other parameters, such as fatigue, pain, patient self-rated disease activity, anxiety and depression. Patients with fibromyalgia and psychiatric disorders were excluded from the study in order to avoid biases on physical and mental status of patients with connective tissue diseases. We found that non-adherent patients were those rating their disease as less active, had a lower degree of pain and a better GH perception and QoL related to physical problems. The perception of a better health status might lead patients to skip regularly HCQ intake and subsequently to non-adherence to therapy, also according to previously published studies. In addition, patients taking immunosuppressants were more likely to skip HCQ intake probably because of their belief that immunosuppressants alone were sufficient to control disease or to avoid potentially harmful drug overuse.

According to previous findings, the rate of non-adherent patients decreased after enrollment in the study: during follow-up, only about 10% of our patients had very low HCQ levels compared to 33% of subjects at baseline. In addition, HCQ concentrations in non-adherent patients at study entry reached a value comparable to those with a better adherence at their follow-up visit. Interestingly, we observed an improved adherence despite the unawareness of patients to a second HCQ measurement at follow-up. This higher adherence might be due to the mere suspicion from patients to be more strictly monitored, and not related to patient's attitude to take the drug, knowledge of previous HCQ results or to occurrence of clinical interview. The present observation might represent an additional advantage of routinely measurement of HCQ blood concentration.

Although the assessment of blood HCQ concentrations is highly specific to identify non-adherent patients, sensitivity of the method is lower than specificity because therapeutic concentrations can be reached only after several days from starting drug intake. However, the simultaneously measurement of HCQ and DCQ could specifically identify patients who are re-

introducing the drug just right before the follow-up visit. Indeed, DCQ is generally undetectable in those subjects. In our cohort, only two patients with undetectable DCQ and suboptimal HCQ blood levels were found.

Limitations of our study were the short-term follow-up (six months), the small number of patients in the cohort, and the low number of events (flares) observed. These limitations did not allow us to identify clinical, serologic or reported outcomes associated with relapse, and to define a cut-off of HCQ blood concentration related to a lower risk of flares in patients with persistent inactive disease.

In conclusion, we found that an HCQ blood concentration above the standard non-adherence thresholds was not associated with a lower extent of pain, fatigue, mood disorders and perceived disease activity in SLE patients with inactive disease. Patients who felt better and those taking immunosuppressants were more prone not to take HCQ properly. Our preliminary data also suggested that the mere suspicion of having a routinely HCQ blood measurement effectively improved treatment adherence. However, our results confirmed the utility of performing routinely blood monitoring of HCQ and its metabolites in clinical practice. Studies with longer follow-up will help to better define the impact of a long-term correct intake of HCQ on clinical outcomes.

Chapter III

Ruxolitinib monitoring in the treatment of patients with myeloproliferative disorders

3.1 Introduction

3.1.1 Definitions

Myeloproliferative neoplasms (MPNs) or myeloproliferative disorders (MPDs) are a group of hematologic disorders sharing somatic mutations in signal-transduction pathways involved in hematopoiesis [**149**]. MPNs include polycythemia vera (PV), essential thrombocytosis (ET), chronic myelogenous leukemia (CML), and idiopathic myelofibrosis (IMF). [**150**]. In 2001, the World Health Organization, WHO has also included within the MPNs clinical entities known as Philadelphia chromosome negative or MPDc: chronic neuthrophilic leukemia (CNL); hypereosinophil syndrome (HES); chronic eosinophil leukemia (CEL); and systemic mastocytosis (SMCD) [**151**]. The incidence of MPNs is 3.5-12.6 cases per 100,000 habitants/year, with increased rates in adults and elderly.

MPNs are usually caused by chromosomal abnormalities or somatic mutations that constitutively activate signaling pathways involved in cell proliferation and survival. In other cases, growth factors and cytokines can enhance the downstream signaling, induce the upregulation of the anti-apoptotic factor Bcl-XL or the activation of STAT3/5 [**152-154**]. The genetic hit is at the hematopoietic stem cell (HSC) level leading to a growth factor-independent proliferation of the neoplastic clone that substitute the normal hemopoiesis in the bone marrow (BM) [**155**]. In most cases of Ph-negative MPNs, a somatic G>T mutation is present at the position 1849 of exon 14 of the Janus Kinase 2 (*JAK2*) gene leading to a valine to phenylalanine substitution in codon 617 (V617F). In 5-10% of cases, mutations can be present in genes encoding for the thrombopoietin receptor (*MPL*) in codon 515, substitution W>L/K/A, or deletions or insertions in exon 12 of *JAK2*. However, allele burden can differ among MPNs [**156**].

3.1.2 Idiopathic Myelofibrosis

IMF is a rare MPN with an incidence of 0.5-1.3 cases per 100,000 population/year, a mean age of onset of 60 years-old and similar incidence between males and females [**157**]. IMF can be primary or secondary to PV or TE [**158**]. The *JAK2* V617F mutations is present in 50-60% of cases, and the homozygous mutation is related to a worse prognosis and the presence of cytogenetic abnormalities. Somatic mutations in the calreticulin gene (*CALR*) account for another 20-30% of cases; while, mutations in MPL are less frequent (7-10%). As a result, more than 10% of patients do not have mutations in genes known to be associated to MPN pathogenesis and are defined as “triple negative”.

Clinical manifestations are: splenomegaly; presence of myeloid and erythroid progenitor cells in the peripheral blood; anisopoikilocytosis, as a variance in size and shape of a red blood cell; BM fibrosis; and extramedullary hemopoiesis in the spleen and liver [**159-161**]. In early stage of disease, 20% of patients are asymptomatic; however, complete blood counts (CBC) can show various grades of anemia, thrombocytosis or thrombocytopenia, and increased or decreased number of white blood cells. In late stage, patients show pancytopenia because of progressive BM fibrosis and hepato- and splenomegaly are present. In this stage, CD34+ blasts can appear in the peripheral blood and patients can rapidly progress to AML.

The BM of IMF patients is characterized by increased number of stromal cells, higher levels of extracellular matrix (ECM) proteins and fibrin, angiogenetic and osteosclerosis factors, and also increased levels of cytokines, such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor β (TGF-β), calmodulin, interleukin 1 (IL-1) and vascular endothelial growth factor (VEGF) [**162,163**]. These cytokines are mainly produced by monocytes and megakaryocytes which are supposed to play a major role in IMF pathogenesis as patients show megakaryocytic hyperplasia with dysplasia or necrosis, and presence of circulating megakaryocytic precursors. These cells show an increased number of α granules in the cytoplasm and their release causes an increased secretion of cytokines in the BM microenvironment altering the normal hemopoiesis and stimulating the deposition of fibrin [**164-166**]. In early stage, type III collagen is the main component of the ECM in the BM; while in later stages, fibrotic tissue is composed by fibronectin, tenascin and others. Frequency of monocytes is higher in patients with IMF compared to healthy volunteers and circulating levels of macrophage-colony stimulating factor (M-CSF) are increased in the plasma of patients [**167**].

There are no specific cytogenetic abnormalities for diagnosis of IMF, even though three alterations can be frequently found: deletion of the 13q related to a poor prognosis and increased frequency of AML progression [**168**]; deletion of the 20q; and trisomy of 1q [**169**]. Trisomy 8 and deletion of the 12p can also be detected and are related to a worse prognosis [**170**]. Some chromosomal abnormalities are also related to loss of heterozygosity (LOH) in oncogenes located on affected chromosomes, such as LOH of retinoblastoma-1 protein on 13q14 locus when del13q occurs and found in about 23% of IMF patients. However, the presence of cytogenetic abnormalities is always linked to a worse prognosis and a poor response to standard treatments [**171**].

The number of circulating $CD34^+$ cells is markedly increased in IMF compared to other MPNs and is a landmark for differential diagnosis. In addition, the number of circulating $CD34^+$ cells correlates with prognosis and is a marker of myeloid metaplasia [**172**]. Among $CD34^+$ cells, the most abundant in IMF are the megakaryocyte colony-forming units (MK-CFU); while less frequent are the GM-CFU, E-BFU, and GEMM-CFU.

3.1.3 Polycythemia Vera

Polycythemia Vera (PV) is a MPN disorder characterized by: increased red blood cells and also often by augmented number of white blood cells and platelets; BM hyperplasia of the erythroid, myeloid, and megakaryocytic precursors; erythropoietin (EPO)-independent cell proliferation; and presence of somatic mutations in *JAK2* gene, most frequently the V617F mutation (95% of cases). In Western countries, the incidence is 2.3-2.8 per 100,000/year and a male/female ratio of 1.2:1. In Italy, the prevalence is about 30 cases per 100,000 population; however, the real incidence may be higher because the disease may be misdiagnosed especially in elderly. The EPO-independent cell proliferation capacity was discovered *in vitro* in 1974 by Prchal et al. [**173**]; however, only in 2005, the somatic *JAK2* V617F mutation was identified and linked as driven mutation in IMF pathogenesis. The significance of allele JAK2 burden in the pathophysiology of the disease and in the development of thrombotic events is still largely unknown. Patients with PV have an increased risk to develop AML and long-lasting PV is more likely to progress to AML [**174**]. In several cases, a fibrotic phase can precede the leukemic phase. Other negative prognostic factors are age, presence of leukocytosis, administration of alkylating agents in combination with hydroxyurea, pipobroman or radioactive phosphor [**175**].

3.1.4 JAK-STAT signaling pathway

The Janus kinases (JAK) are a family of non-receptor tyrosine kinases that transduce the signal after cytokine stimulation through the JAK-STAT pathway. Molecular weight of JAK ranges from 120 to 140 kDa and proteins have seven homology domains (JH, JAK Homology): two C-terminal kinase and pseudokinase JH domains (JH1 and JH2); four N-terminus FERM domain (JH4 to JH7) for receptor association; and the SH2 domain formed by JH3 and part of JH4 with unknown function. The JAK family includes four members: JAK1, JAK2, JAK3 and TYK2. This latter is usually associated to cytokine receptors for interferons, IL-6, or IL-10 and involved in the Th2 immune response. JAK1 is also linked to interferon receptors and IL-2 and IL-6 receptors. JAK2 is involved not only in interferon and IL-3 signaling pathways, but also in the transduction of signals from single chain receptors such as EPO receptor or growth hormone receptor. JAK3 is specifically expressed in granulocytes and is exclusively associated with IL-2 receptor.

JAK activation is triggered by tyrosine phosphorylation in specific protein loops, which cause JAK dimerization on box1/box2 domains enriched in proline residues and close to cell membrane. Once activated, the signal is transduced by the Signal Transducers and Activator of Transcription (STAT) proteins. These proteins have a SRC2 (SH2) domain for dimerization, a DNA-binding

domain, and a trans-activation domain at the C-terminus. The STAT family includes seven members: STAT1, 2, 3, 4, STAT5A/B e STAT6. Various stimuli can activate JAKs inducing dimerization and phosphorylation through receptor binding, and then activated JAKs phosphorylate tyrosine residues on the cytoplasmic domains recruiting STAT proteins. After phosphorylation and dimerization, activated STATs translocate to the nucleus where they induce the expression of genes related to cell proliferation and survival or proinflammatory cytokines (Figure 9). Modifications in the JAK/STAT pathways are present in several autoimmune disorders and hematologic neoplasms. For example, JAK2/STAT5 signaling is hyperactivated in HSCs and T cells of patients with MPNs [**176,177**].

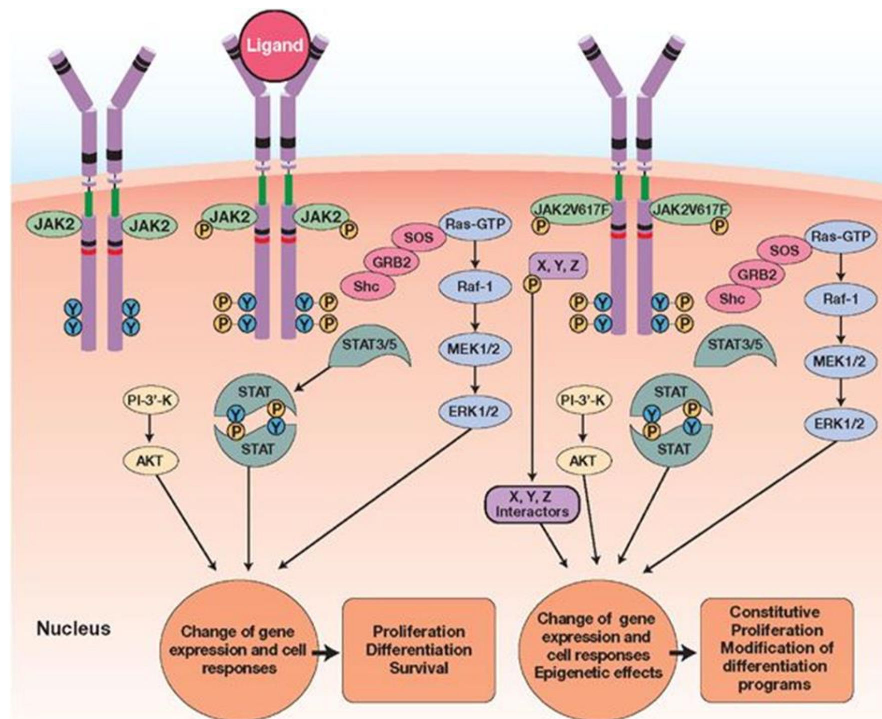


Figure 9. JAK-STAT signaling pathway in physiologic and pathologic conditions.

3.1.5 Ruxolitinib

Ruxolitinib (Figure 10), a selective JAK1/2 inhibitor [**178**], has been approved in 2011 by the Food and Drug Administration (FDA) for the treatment of patients with intermediate or high-risk IMF, including primary myelofibrosis, post-polycythemia vera myelofibrosis, and post-essential thrombocythemia myelofibrosis [**179**]. Initial approval of Ruxolitinib was based on the results of two phase III clinical trials: the controlled myelofibrosis study, with Oral JAK inhibitor Treatment (COMFORT)-I, and (COMFORT)-II [**180**]. All enrolled patients in COMFORT-I and II trials reached the primary endpoint of 24 or 48 weeks [**180,181**], respectively Results of these trials and of studies analyzing the 2- and 3-year follow-up data showed that Ruxolitinib provides significant and

durable clinical benefits and an overall improvement in quality of life for patients with advanced IMF or PV [185-188]. In particular, 51.4% of treated patients experienced a reduction in spleen size $\geq$35% compared to baseline, In addition, patients on the Ruxolitinib arm had a greater reduction of the risk of death (HR=0.48; 95% IC, 0.28-0.85; p=0.009), and an increased in overall survival compared to those treated with standard therapies (81% vs 61%, respectively) at 144 weeks of follow-up.

Ruxolitinib recommended starting and maintenance dose for IMF treatment depends on the baseline platelet count, and side effects are routinely managed through therapeutic dose adjustment and blood count monitoring [**184,186**]. However, the therapeutic efficiency and tolerability might be affected by the presence of genetic polymorphisms or drug–drug interactions. Furthermore, these drugs are administered orally and can be given over an extended period, thus occasionally leading to poor patient adherence [**187**]. As a result, patients might experience inadequate dose exposure and thus inefficacy of the drug; therefore, the need for a rapid and reliable method to evaluate Ruxolitinib concentration in biological fluids is emerging in recent years.

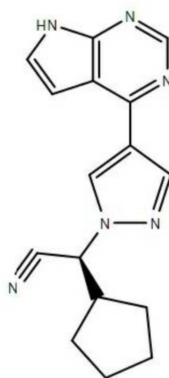


Figure 10. Chemical structure and mode of action of Ruxolitinib.

3.1.6 Project aim

Several methods have already been developed to quantify Ruxolitinib in its pharmaceutical forms [**188**] and in plasma samples [**189,190**] using liquid chromatography coupled to mass spectrometry (LC–MS/MS). This approach is associated with a considerably high sensitivity and specificity and remains the gold standard for simultaneous quantification of several tyrosine-kinase inhibitors [**189,190**]. However, LC–MS/MS has an high cost, requires skilled staff, and is not available in all clinical laboratories; therefore, high-performance liquid chromatography (HPLC) coupled with ultraviolet detection (HPLC–UV) or other spectroscopy detectors could be considered a valid and cost-saving analytical alternative [**191**]. For this part of my PhD thesis, we developed and validated a method for Ruxolitinib quantification in plasma using a reverse-phase high-performance liquid chromatography system equipped with a fluorometric detector (RP-HPLC-FL).

3.2 Materials and methods

3.2.1. Chemicals and reagents

HPLC grade water, methanol, and acetonitrile were purchased from Romil (Waterbeach Cambridge, GB); ortho-phosphoric acid from Biochem Chemopharma; and dimethyl sulfoxide and 2,3-diaminonaphthalene from Sigma-Aldrich. Ruxolitinib, obtained as a pure powder, was a kind gift provided by Novartis.

3.2.2 Chromatography

Chromatography was performed using a Waters 1525 Model Binary HPLC system equipped with a multiple wavelength fluorescence detector (Model 2475) and an autosampler (Model 2707). Samples in the autosampler were at room temperature and column oven at +30°C. Breeze 2.0 software was used for peak analysis, integration and for linear regression analysis of calibration curves. Separation was achieved using a Waters Symmetry C18 (4.6 x 75mm, 3.5µm) column, supplied with a Javelin column guard. A 67:33 (v/v) water:acetonitrile mixture at pH 4.8 with 25% ortho-phosphoric acid was used as mobile phase and at a 1.0 mL/min flow rate. For analyte detection, the excitation and emission wavelengths of the fluorescence detector were set to 320 and 386 nm, respectively.

3.2.3 Sample preparation

Ruxolitinib and 2,3-diaminonaphthalene (used as internal standard; ISTD) stock solutions were prepared by dissolving commercial powders in dimethyl sulfoxide at concentrations of 5 mg/mL and 1.2 mg/ mL, respectively. Stock solutions were diluted in 100% methanol for Ruxolitinib and in HPLC grade water for ISTD in order to obtain working solutions. All solutions were stored at -20°C until use.

Ethylenediaminetetraacetic acid (EDTA) blood samples were collected from healthy volunteers recruited at the Blood Bank of the University Hospital “San Giovanni di Dio e Ruggi d’Aragona” in Salerno (Italy) in accordance with the Declaration of Helsinki. For plasma collection, whole blood samples were centrifuged at 3,500*g* for 6 min, then plasma collected into a clean safe-lock tube and immediately stored at –20°C until use. Before extraction, 5 µL of a 2 µg/mL solution

of ISTD was added to 100 µL of each sample; this mixture was then added to 400 µL of 100% methanol directly on a vortex in a safe-lock 1.5 mL tube. After vortexing for 20 seconds, samples were centrifuged at 17,000*g* at 4°C for 10min. The organic layer was then collected, dried with a Savant Speed-Vac Concentrator (ThermoFisher), and dried residue was dissolved in 100 µL of 50% acetonitrile. After centrifugation at 17,000g at 4°C for 1min, supernatants were transferred to appropriate tubes for acquisition.

For calibration curve preparation, a working solution containing Ruxolitinib at a final concentration of 25 µg/mL was freshly prepared in water from the initial stock. Serial dilutions (500, 250, 100, 20, 1, 0.5, and 0.2ng/mL) were carried out in a final plasma volume of 100 µL in which 5 µL of ISTD at 2 µg/mL) were added. Molecules were then extracted according to the procedure described above. Quality controls (QCs) at concentrations of 100, 20, and 0.5 ng/mL were prepared using the same procedure.

3.2.4 Detection and quantification limits

LLOD and LLOQ were defined as detailed in Section 2.2.6. Briefly, the lower limit of detection (LLOD) was defined as the lowest concentration at which the analytical assay allowed to reliably differentiate the signal of the analyte peak (S) from background noise (N) ($S/N \geq 3$). The LLOQ was considered as the lowest concentration at which precision and accuracy were within 20% and a $S/N > 5$. To perform these measurements, samples were prepared at concentrations of 0.05, 0.1, and 0.2 ng/mL and analyzed in triplicates.

3.2.5 Linearity and recovery

Linearity was determined by plotting peak area of analyte/IS ratios as a function of analyte concentration and fitting the curve to a linear regression. Experimental concentrations were back-calculated using the calibration curve to evaluate their deviation from nominal values. The average recovery of Ruxolitinib from plasma was determined by comparing experimental concentrations from three samples subjected to pre- and post-extraction processing. Pre-extraction samples were obtained by directly adding Ruxolitinib at 0.5, 20, and 250 ng/mL concentrations to a final volume of 100 µL of blank plasma. These samples were then processed as described above. The post-extraction samples were prepared by spiking in an extracted blank plasma the quantity of compound required to obtain the same concentrations of pre-extraction samples.

3.2.6 Intra- and inter-day variability

Intra- and inter-day precision and accuracy were evaluated at three different concentrations (0.5, 5 and 40 ng/mL) in samples extracted from plasma. Three replicates for each concentration were extracted and analyzed in the same day; aliquots of the same samples were extracted and analyzed three times per run after 1 and 3 days. Samples were stored at -20°C until analysis. To evaluate precision, the coefficient of variations (%CV) was calculated, and accuracy by relative error (%RE) between nominal and measured concentrations.

3.2.7 Stock solutions and sample stability

Stock solutions of Ruxolitinib and ISTD were stable for more than six months at -20°C. Analyte stability in plasma samples was evaluated on three independent aliquots at three nominal concentrations. The short- (24h) and long-term (15 days) stability in plasma was assessed at +4°C, -20°C and/or room temperature. The stability of the extract after 48h at +4°C (temperature at which autosamplers usually operate) and following three freeze/thaw cycles was also evaluated. A sample was considered stable if the average of concentrations measured in each set of experiments was within ±15% of the concentration initially determined.

3.2.8 Method robustness

Robustness of our method was evaluated by introducing minimal voluntary according to the model proposed by Savic et al. [**192,193**]. The standard flow rate of 1.0 mL/min was changed by ±0.2mL/min, maintaining the other components of the mobile phase unaltered. The influence of column temperature was evaluated at 30°C, 35°C, and 40°C. Each experiment was performed in triplicate. Data analysis was carried out using a linear regression by a second-order polynomial model, in which flow and temperature were the variables. The number of theoretical plates, to evaluate column efficiency under tested conditions as by Dolan's formula [**194**].

3.3 Results and discussion

3.3.1 Optimization of chromatographic conditions

An HPLC-FL method suitable for the detection and quantization of Ruxolitinib in human plasma should be highly selective and sensitive for the target molecule. For this reason, excitation and emission wavelengths were carefully evaluated, and the 320 nm excitation/386 nm emission pair was selected for both the analyte and the internal standard because the highest signal intensity for Ruxolitinib was obtained using these parameters (Figure 11).

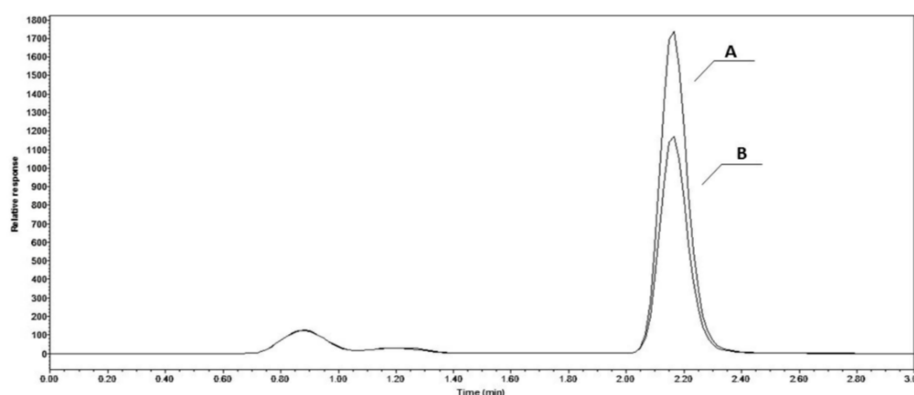


Figure 11. Selection of optimal fluorimeter wavelengths. The chromatogram shows the intensity obtained for Ruxolitinib with (A) excitation at 320nm and emission at 386nm compared to (B) excitation at 330nm and emission at 420nm. Analytical conditions: mobile phase consisting of 67:33 water:acetonitrile at pH 4.8 using a flow rate of 1.0ml/min.

Method efficacy strongly depends on the quality of chromatographic separation, which is determined by composition of the mobile phase. Initially, a ternary mixture of acetonitrile:methanol:H_2O (50:40:10), pH 2.9 with 0.001% trifluoroacetic acid (TFA) was used; however, Ruxolitinib retention time was too low (less than 1.00 min). An increase of pH value to 4.8 slightly improved retention time to approximately 1.2 min. Therefore, a mobile phase of 60:40 H_2O:acetonitrile at pH 4.8 was then used, giving a good separation between Ruxolitinib and other plasma peaks, but the compound partially co-eluted with the IS, as shown in Figure 12A. Different component ratios were tested, and the best results were obtained with a mobile phase composed of 67:33 H_2O:acetonitrile at pH 4.8, which provided a good separation of all peaks and an overall short run time (5.00 min) (Figure 12B,C).

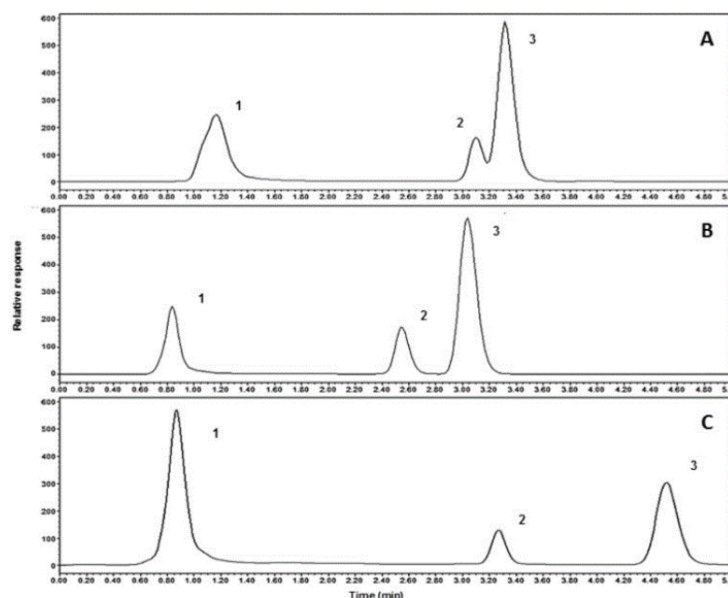


Figure 12. Mobile phase optimization. Chromatograms show the improvement in the separation of analytes peaks: (1) unspecific plasma residue, (2) internal standard, and (3) Ruxolitinib. Different water:acetonitrile ratios were evaluated in the mobile phase at pH 4.8: (A) 40:60, (B) 50:50, and (C) 67:33. A flow rate of 1.0ml/min was used in all experiments.

3.3.2 Pre-analytical treatment

The good selectivity achieved using the HPLC-FL method described above allowed the set-up of a simple pre-analytical treatment of plasma samples involving a methanol induced protein precipitation followed by air-drying and suspension in a suitable solvent. Various solvents were evaluated in order to identify the most effective suspension system. A 50:50 H_2O:acetonitrile mixture provided a satisfactory yield and extraction recovery for both the analyte and the ISTD (Table 8).

	0.5 ng/mL		20 ng/mL		250 ng/mL	
Compound	Recovery (%)	Relative standard deviation (%)	Recovery (%)	Relative standard deviation (%)	Recovery (%)	Relative standard deviation (%)
Ruxolitinib	90.9	6.7	88.2	7.8	94.0	1.1
2,3-Diaminonaphthalene	52.3	4.5	48.3	3.6	43.7	3.4

Table 8. Recovery after organic extraction for Ruxolitinib and 2,3-diaminonaphthalene calculated at three different initial concentration values.

3.3.3 Method validation

Our analytical procedure was validated according to the EMA guidelines (EMEA/CHMP/EWP/192217/2009 Rev. Corr. 2). No interferences from endogenous compounds were observed in either of the investigated biological fluids (data not shown). The injection of highly

concentrated samples (up to 1 μg/mL) followed by the analysis of blank samples did not show the presence of carry-over (Figure 13). Injection of blank blood samples was also performed at the beginning of each analytical series and immediately after high- concentration standards.

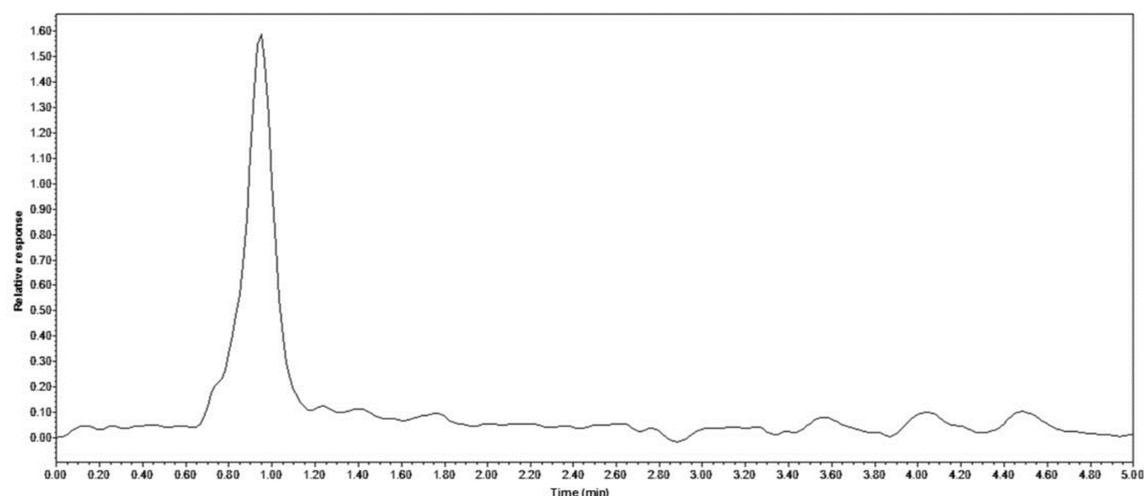


Figure 13. Potential carry-over among samples evaluated by loading a blank sample following the introduction of 1000ng/mL of Ruxolitinib.

The LLOD for Ruxolitinib in plasma was 0.05 ng/mL, and the LLOQ was 0.1ng/mL. The linearity was verified over a concentration range from 0.2 ng/mL to 500 ng/mL (n = 7) (Figure 14); correlation coefficient obtained ($R^2 = 0.9994$) confirmed the suitability of this analytical approach to measure a wide range of drug plasma concentrations. Accuracy and precision of the analytical were estimated intra- and inter-daily using plasma samples from healthy volunteers at three different Ruxolitinib concentrations (0.5, 5, and 40 ng/mL) across a linear range (Figure 14). As shown in Table 9, CVs were $<15\%$ and relative errors (RE) $< \pm 10\%$, confirming the reproducibility of our method. Analyte stability in plasma was evaluated d as described in the Materials and Methods section on three independent aliquots at three Ruxolitinib nominal concentrations, i.e. 0.3, 40, and 400 ng/mL, which corresponded, respectively, to three times the LLOQ, the middle part of the linearity range, and a value closer to the upper limit of the calibration curve.

	0.5 ng/mL		5 ng/mL		40 ng/mL	
	Coefficient of variation (%)	Relative error (%)	Coefficient of variation (%)	Relative error (%)	Coefficient of variation (%)	Relative error (%)
Intraday	7.5	−6.6	9.2	−8.0	0.8	−9.9
Interday	13.5	0.75	8.4	−5.1	3.0	−7.0

Table 9. Intra-/Inter-day precision and accuracy expressed, respectively, as the coefficient of variation and the relative error obtained for Ruxolitinib.

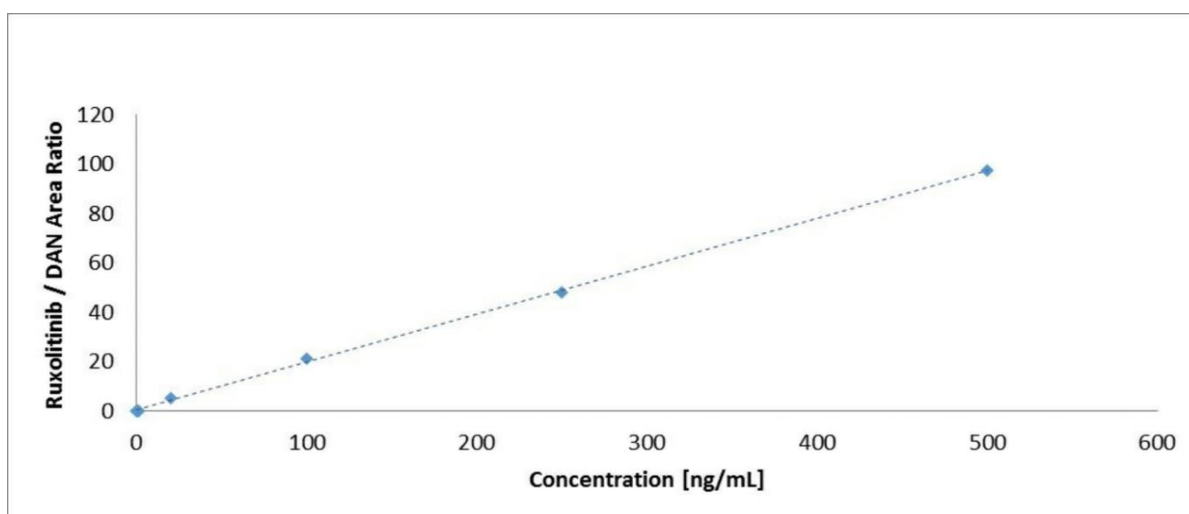


Figure 14. Linearity of the analytical response determined by plotting the ratio of Ruxolitinib peak area/2,3-diaminonaphthalene peak area as a function of Ruxolitinib concentration, and fitting the curve by linear regression. The resultant linear equation was y = 0.1938x0.3925 with a correlation coefficient (R^2) of 0.9994.

Plasma samples were stable at different temperatures and for different storage periods (Table 10), as CVs were within ±15%. Conversely, extracted samples did not satisfy this requirement, probably because the IS might be less stable than Ruxolitinib both in the stored and in the frozen/thawed extracted samples (data not shown). Therefore, samples should be preferably extracted right before the analytical session. The robustness tests highlighted that flow rate might influence chromatographic efficiency more than temperature values, as shown by variations in the number of theoretical plates (Figure 15). However, chromatographic efficiency remains satisfactory even after flow rate or temperature modifications, and those minimal variations did not significantly affect analyte quantization, thus allowing qualitative response to remain substantially unaltered.

	Percentage variation compared to the zero point (%)							
	Sample stability					Extracted analytes stability		
Nominal concentration (ng/mL)	24 h 25(±5) °C	24 h 4 °C	24 h −20 °C	15 days 4 °C	15 days −20 °C	24 h 4 °C	48 h 4 °C	Three freeze/ thaw cycles
0.3	−5.9	6.7	2.8	−0.4	12.7	−0.2	27.5	−2.2
40	8.2	6.3	7.2	4.6	8.6	−16.4	29.0	26.7
400	5.7	−0.5	0.1	9.8	10.3	−14.6	21.7	23.6

Table 10. Ruxolitinib stability at 0.3, 40, and 400ng/mL evaluated in plasma samples at different temperatures and time intervals.

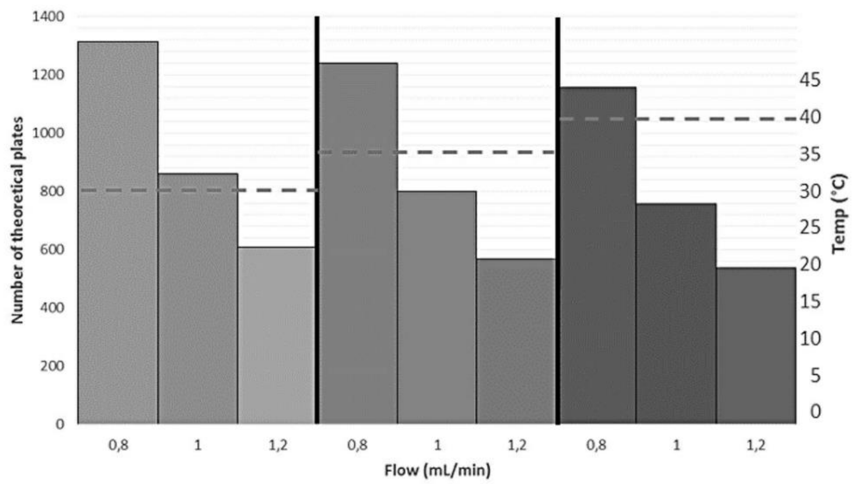


Figure 15. Robustness analysis. Number of theoretical plates plotted as a function of the flowrate values 0.8, 1, 1.2mL/min at different temperatures: light gray, 30°C; gray, 35°C; dark gray, 40°C.

3.4 Conclusions

The measurement of the effective plasma concentration of a specific drug plays a pivotal role in assisting the clinician in the decision-making process of keeping the current treatment by adjusting drug dosage or changing the type of drug molecule administered to the patient. This is particularly critical in case of treatment failure, severe side-effects, or suspected drug interactions.

JAK inhibitors therapeutic efficacy is known to be affected by a marked variability; therefore, regular monitoring of these compounds is required to ensure both an optimal response and the reduction of potential adverse effects. The correlation between therapeutic efficacy and plasma concentration has been widely studied for this class of molecules [**195-198**]. Nonetheless, an ongoing debate concerning the thresholds for other tyrosine-kinase inhibitor is still under way, as the existing studies are contradictory [**199**]. Therefore, further work concerning therapeutic monitoring of these drugs is strongly encouraged. The purpose of this paper was to optimize and validate a simple HPLC-FL method to measure Ruxolitinib concentration in plasma samples, even at low concentrations. The method was developed taking into account the chemical and physical properties of the drug; in fact, the low polarity (LogP of 2.1) of Ruxolitinib allowed the molecule to be analyzed by reverse phase liquid chromatography. The mobile phase composition was optimized to obtain two symmetric peaks (Ruxolitinib and internal standard) with a short time run. The coefficient of variation and relative error values for precision and accuracy studies and the high recovery rate of Ruxolitinib indicate that the proposed method is valid. There is no evidence of additional peaks that can interfere with Ruxolitinib or the internal standard, nor are there any interference peaks in extracted plasma samples. In conclusion, the feasibility and linearity of the results in plasma samples strongly support a widespread diffusion of this analytical method for the quantification of Ruxolitinib in myeloproliferative neoplasm patients.

Chapter IV

5-Fluorouracil monitoring of oncologic patients in course of chemotherapy treatment

4.1 Introduction

4.1.1 TDM applied to chemotherapeutic agents

Therapeutic Drug Monitoring (TDM) is used for dose adjustment of drugs with narrow therapeutic windows, such as chemotherapeutic agents with step dose–response relationship, in order to reduce toxicities, improve effects on targeted tissue and increased drug availability. In addition, TDM can decrease adverse drug reaction (ADR)-related morbidity, while increasing therapy efficacy and reducing costs for treatment of ADRs [**200,201**]. Anticancer drugs have also a significant inter-individual pharmacokinetic variability; for example, drug clearance is expressed as function of body surface area (L / h / m^2) or subject weight (L / h). In addition, the presence of polymorphisms, somatic mutations or other modifications that could influence pharmacokinetics is also responsible of inter-individual variability. Activity of anticancer drugs is mirrored by the area under the plasma concentration curve (AUC) versus time, which is reached in one or two days after a short intravenous infusion and before neutrophil and platelet counts at the nadir (lowest values) observed 1-2 weeks after chemotherapy. All of these characteristics make TDM suitable for measurement of anticancer drug levels in plasma samples [**202**].

4.1.2 5-Fluorouracil

5-FU, an uracil analogue where the hydrogen atom at the C5 position is substituted by a fluorine atom (Figure 16), is widely used in combination with other anticancer drugs for the treatment of several solid malignancies, such as colorectal, head and neck and breast cancers [**203**].

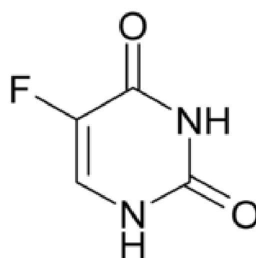


Figure 16. Chemical structure of 5-Fluorouracil.

The effects of 5-FU on response rates and patient survival is well established. Nevertheless, only 10-15% of patients with advanced colorectal cancer respond effectively to treatment [**204**]. The combined use of 5-FU with new CA such as irinotecan and oxaliplatin improved the response rates of advanced colorectal cancer of 40 to 50% [**205,206**]. 5-FU can rapidly enter into cells by facilitated

or passive transport mechanism [**207,208**] where it is converted into several active metabolites such as fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). These metabolites and not the 5-FU are responsible of the cytotoxic effects of the drug through DNA and RNA damage (Figure 17). Indeed, 5-FU metabolites are incorporated into macromolecules, such as DNA and RNA blocking gene and protein expression and DNA replication mechanisms.

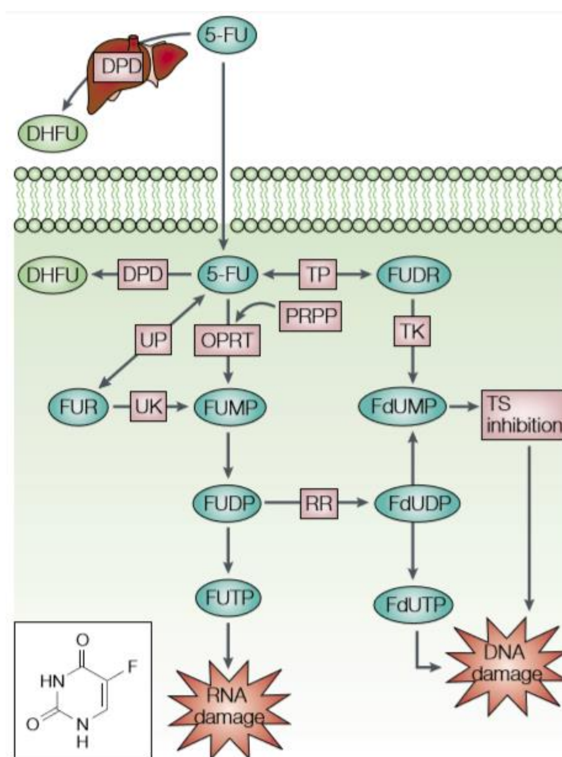


Figure 17. 5-FU metabolism [**209**]

The intracellular availability of 5-FU mainly depends on tissue catabolism: more than 80% of a single dose of 5-FU undergoes metabolic clearance [**210**] in the liver through the activity of the dihydropyrimidine dehydrogenase (DPD) enzyme [**211**]. DPD, an enzyme that catalyzes the rate-limiting step in fluorouracil metabolism, is encoded by the *DPYD* gene. In eukaryotic cells, in the first step of pyrimidine catabolism, the DPD enzyme reduces 5-FU to 5-fluoro-5,6-dihydrouracil (5-DHFU) that is rapidly converted by the dihydropyrimidinase to 5-fluoro-ureidopropionic acid (FUPA), and then to α-fluor-β-alanine (FBAL; the major urinary metabolite) by the β-ureidopropionase (Figure 18) [**212**].

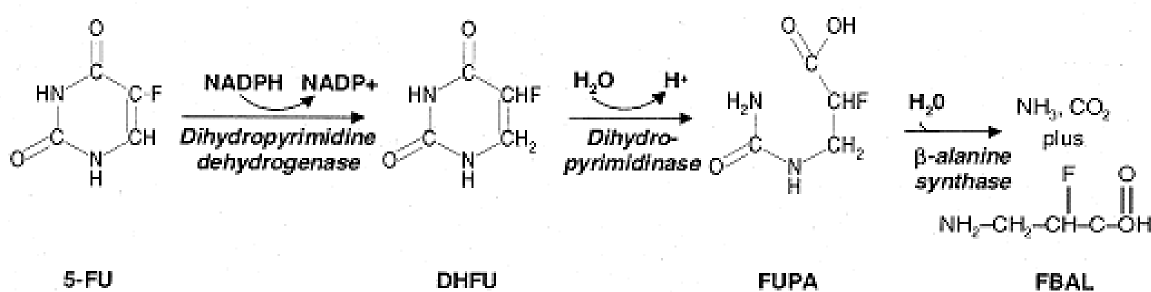


Figure 18. Metabolic breakdown of 5-FU [**213**]

4.1.3 -Therapeutic drug monitoring of 5-Fluorouracil

Pharmacokinetic variability of 5-FU depends on various factors such as age, gender, disease status, organ functions, drug-drug interactions, and also the activity status of the DPD enzyme [**214**]. Almost 5% of patients have DPD deficiency that causes 39-61% of all 5-FU-related severe toxicities [**215**]. Screening for *DPYD* gene variations can discover polymorphisms related to DPD deficiency and dramatically reduce 5-FU ADRs. More than 30 variants in the *DPYD* gene are annotated so far; however, only two polymorphisms are related to loss of function in DPD enzyme: *DPYD**2A and *DPYD**13 [**216**]. The *DPYD**2A variant at the spiking site between intron and exon 14 causes the production of a truncated protein, and in homozygosity there is a complete DPD deficiency which might expose patients to potentially life-threatening toxicities, such as bone marrow suppression and neurotoxicity [**217**]. In heterozygosity, the Clinical Pharmacogenetics Implementation Consortium set up a 5-FU dosage based on *DPYD* genotyping [**218**]. However, around 50% of patients treated with 5-FU experience various grade and type of toxicity without genotypic alterations in the *DPYD* gene, suggesting that other factors are implicated in the development of 5-FU-related ADRs [**216**]. Indeed, DPD expression is also regulated at the transcriptional and post-transcriptional levels [**219**]. For this reason, a correlation between genotyping and phenotyping is required to better evaluate enzymatic DPD activity before starting 5-FU treatment. One approach consists of plasma measurement of 5-FU concentrations; however, other strategies are under investigation, such as the evaluation of DHU/U ratio or uracil concentration measurement. The use of these markers is still limited in clinical practice because of the lack of validation studies on large cohorts [**219-224**].

4.1.4 Project aim

In order to increase the tolerable dose of 5-FU while minimizing drug-related toxicities, several strategies have been introduced in chemotherapeutic protocols, such as the addition of

leucovorin, a vitamin B9 derivative, or continuous intravenous infusion [**216,225,226**]. However, severe ADRs are still observed in 10-30% of patients without DPD deficiency treated with fluoropyrimidine monotherapy with a death rate of 0.5-1% [**215,219,227**]. This mortality rate is even higher when 5-FU is associated to other chemotherapeutic agents, such as irinotecan and oxaliplatin [**228**]. Therefore, the use of TDM have a relevant impact in clinical management of oncologic patients treated with 5-FU as monotherapy or in combination with other anticancer drugs. For this reason, we developed and optimized a LC-MS/MS method for quantization of 5-FU in plasma samples later validated on a small cohort of patients with colorectal, head and neck or breast cancers.

4.2 Materials and methods

4.2.1 Patients

Patients included in this study were enrolled at the Oncology Unit of the University Hospital “San Giovanni di Dio Ruggi d’Aragona”. Salerno (Italy). and ethylenediaminetetraacetic acid (EDTA) or heparinized blood samples were obtained after written informed consent was obtained. After centrifugation at 3.500*g* for 6 min. plasma was collected into a clean safe-lock tube and immediately stored at -20°C and processed within 48h.

4.2.2 Chemicals and reagents

UHPLC grade water. methanol. and acetonitrile were purchased from Romil (Waterbeach Cambridge. GB); dimethyl sulfoxide (DMSO). 5-FU. thiobarbituric acid (TBA) HRX and Phree column from Sigma-Aldrich.

4.2.3 Solution preparation

To obtain stock solution, 5-FU and TBA (used as internal standard; IS) powders were dissolved in DMSO to a final concentration of 1 mg/ml; work solutions were then obtained by diluting stocks in H_2O. All solutions were stored at -20° C.

4.2.4 5-FU extraction

For 5-FU extraction from plasma samples. 2 µL of IS solution at a concentration of 10 µg/mL were added to 198 µL of plasma (final concentration. 100 ng/mL). sample was vortexed and centrifuged at 13000 rpm for 2 min. Subsequently. 50 µL of supernatant were collected into a clean safe-lock tube and 200 µL of methanol were added while vortexing the sample. After 30 min incubation at RT with vortex step every 10 min. samples were subjected to centrifugation at 4°C at 17000g for 5 min; then 200 µL of supernatant were collected into a clean safe-lock and dried using a vacuum centrifuge for 50 min at 45°C. Pellets were dissolved in 40 µL of 5% methanol solution. vortexed and centrifuged at +4°C at 17000*g* for 1 min. Clear supernatants were then transferred in appropriate vials for LC-MS/MS analysis.

4.2.5 Preparation of calibration standards and quality controls

For calibration standards. a 5-FU solution at 500 µg/mL was freshly prepared in H_2O from stock solutions and serial dilutions (10. 1. 0.1 and 0.01 µg/mL) prepared in a final plasma volume of 500 µL. The range of concentrations was selected based on our LLOQ value and data previously reported in literature. Samples were then extracted according to the procedure described above.

4.2.6 LC-MS/MS analysis

LC–MS/MS analysis was carried out using an Ultimate 3000 UHPLC system (Thermo-Fisher. Waltham. MA. USA) coupled with a TSQ endure mass spectrometer (Thermo-Fisher) equipped with an electrospray ion source and a triple quadrupole ion analyzer. Chromatography was performed by injecting 5 µL of samples on a Phenomenex Luna Omega C18 (2.1 x 50 mm. 1.6 µm) and a mobile phase composed of 0.1% formic acid in water (A) and 0.1 % formic acid in acetonitrile (B). An elution gradient from 2% to 30 % of B over 3 min at 20°C at flow rate of 0.4 mL/min was used for compound separation.

Mass spectra were acquired in negative multiple reaction monitoring (MRM) mode. Specific transitions (Figure 19) were selected by direct injection for each analyte in order to maximize selectivity and sensitivity: 5-FU transitions 129→86 and 129→59; and IS 143→75. Peak area of 5-FU values were calculated only when both transitions were observed.

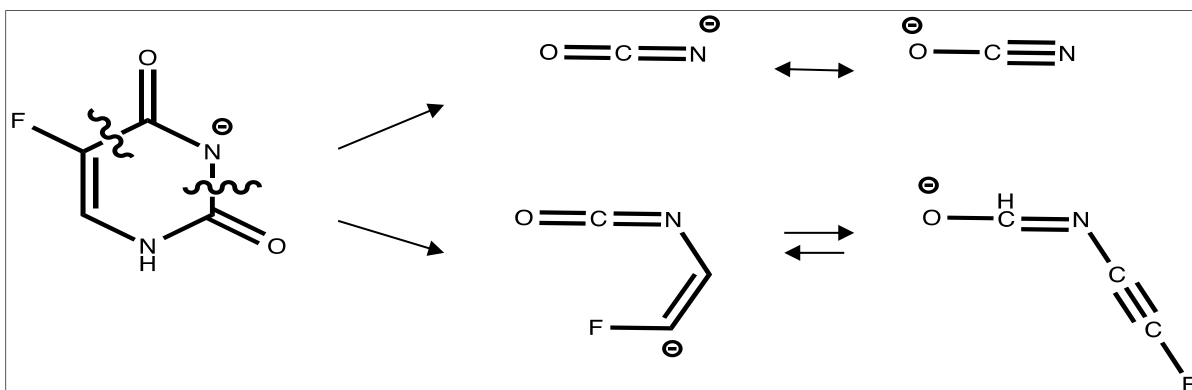


Figure 19. 5-FU fragmentation.

4.2.7 Method validation

LLOD and LLOQ were defined as described in paragraph 2.2.6, and, to this purpose. samples were prepared at various concentrations and analyzed in triplicates.

Linearity was determined by plotting peak area of analyte/IS ratios as function of the analyte concentration and by fitting the curve to a linear regression. Experimental concentrations were back-calculated using calibration curves to determine their deviation from nominal values.

Intra-day and inter-day precision and accuracy were evaluated at two different concentrations (0.1 and 1 µg/mL) in samples extracted from plasma. Five replicates for each concentration were extracted and analyzed three times per run. Then aliquots of the same sample were stored at +4°C and analyzed again (three times per day) after 24 (T24). 48 (T48) and 96 (T96) hours. Percent coefficient of variance (% CV) for each measurement was calculated to assess precision. and the percent of relative error (% RE) between nominal and measured concentrations was employed to evaluate accuracy of our method.

4.3 Results and discussion

Therapeutic Drug Monitoring of drugs with narrow therapeutic windows and higher inter-individual variability is mandatory to better tailor therapy on patients maximizing drug effectiveness while minimizing toxicities. In this part of my PhD thesis, we developed and optimized an analytical method for 5-FU measurement in plasma samples. This molecule is extremely unstable in whole blood and plasma at room temperature because of rapid degradation to dihydro-5-FU by the ubiquitarian dihydropyrimidine dehydrogenase enzyme (DPD). For this reason, blood samples should be kept on ice and plasma collected within few hours from drawing [**229**]. In addition, DPD inhibitors might be added to blood collection tubes in order to increase 5-FU stability. Noteworthy, an incorrect storage and manipulation could lead to a rapid 5-FU degradation thus leading to under-estimation of circulating drug levels and inappropriate drug dose adjustment.

Several analytical methods have been developed for measurement of 5-FU and its related compounds [**230**] by high-performance liquid chromatography (HPLC) using standard. reverse phase. or reverse-phase ion pairing techniques. Therapeutic ranges that can be defined using these methodologies are usually between 25-150 ng / ml (0.19-1.15 μM). However, by employing liquid chromatography coupled with mass spectrometry (LC-MS/MS) analysis limits of quantization have been lowered to 0.5 ng/mL (3.85 nM) or 10 ng/mL (0.08 μM) improving sensibility and specificity of 5-FU measurement and minimizing sample volume needed (100 μL of starting plasma sample volume) [**216**].

We developed and optimized a highly sensitive, specific and accurate analytical method for measurement of 5-FU and its related compounds in plasma samples by LC-MS/MS. The method was also validated in a cohort of patients with various types of cancers such as colon head & neck or breast neoplasms receiving 5-FU alone or combined with other anticancer drugs as their therapeutic protocol. Measurement of circulating levels of 5-FU and its metabolites could be used as additional tool in dose adjustment in patients treated with chemotherapeutic agents. However. polymorphisms in DPD, type of cancer, co-morbidities and concomitant use of other medications should be also considered for a better clinical management of oncologic patients.

First, selectivity was assessed by analyzing samples without 5-FU and ISTD but with other commonly used drugs. Based on the EMA guidelines the absence of interfering molecules is accepted when the signal at the same retention time of the target drug is less than 20% of the LLOQ for the analyte and less than 5% for the ISTD. For 5-FU and ISTD used in our method there were no interfering compounds. Specificity was evaluated based on the consistency of the retention time for a certain analyte across runs and the presence of both products (quantifier and qualifier) in a constant ratio for a precursor (Table 11). Our method showed high specificity for each analyte.

Molecule	Retention time	Transition I	Transition II	II/I ratio (%)
5-FU	0.43 min	129-42	129-86	13-17 %
ATB	0.38 min	143-75		

Table11. LC-MS/MS parameters for identification and quantization of 5-FU in plasma samples.

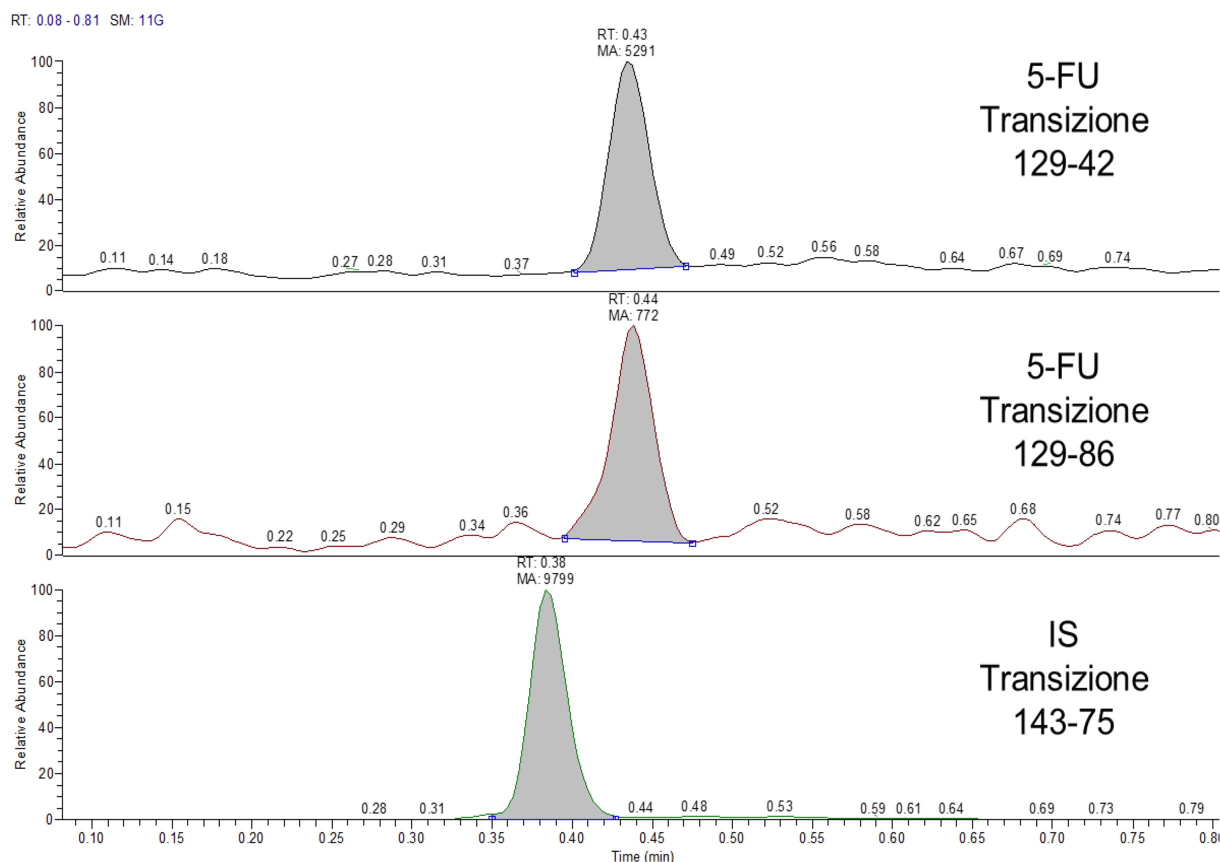


Figure 20. Chromatograms of 5-FU and IS by LC-MS/MS.

Next, various extraction procedures were tested in order to develop a methodology characterized by the highest extraction recovery. Injection of whole blood samples or plasma is not suitable in LC-MS/MS because lipids, proteins and salts can damage the instrumentation and also interfere in the measurement of targeted molecules. For this reason, a sample clean-up is required; however, compounds of interest do not need to be removed together with interferes. Plasma proteins were precipitated in acetonitrile (1:3. v/v); recovery was not satisfactory with this method (data not shown). A solid phase extraction was then performed using HR-X (Chromabond) or Phree (Phenomenex) columns (Table 12). In Phree columns, lipids and proteins strongly interact with the stationary phase and 5-FU, a highly hydrophilic molecule, can be eluted from columns. Using HR-X columns, the analyte first interacts with the stationary phase and is subsequently eluted using appropriate solvents. Using solid phase extraction, 5-FU (0.2 or 2 ng/mL) and IS were rapidly eluted and the extraction

yield was low: for Phree columns, extraction was <40% for 5-FU and 20% for IS; for HR-X columns, extraction yield was <20% for the analyte and 15% for the IS.

	MeOH	Phree	HRX
5FU (0.2)	64±5	32±4	16±7
5FU (2)	69±3	38±9	18±5
IS	56±8	20±5	12±3

Table 12. 5-FU and IS extraction rates using various procedures (methanol, MeOH; and solid phase extraction, Phree and HR-X).

Finally, an extraction procedure using methanol was employed in a sample/solvent ratio of 1:4 (v/v). Using this method, extraction rate for 5-FU at 0.2 ng/mL was 65% and at 2 ng/mL was of 70%; while the IS extraction rate was 56%. Methanol extraction showed the best recovery rate and the procedure was employed for further analysis (Figure 20 for chromatograms).

As first step for method validation, LLOD and LLOQ were defined according to the EMA guidelines. In our case, LLOQ was identified at 10 ng/mL, which was considered appropriate for our aim because our LLOQ was much lower than reported lower limits of 5-FU therapeutic window. Linearity was then assessed according to ranges reported in literature. In particular, 5-FU plasma levels at the steady-state should be between 2500-3000 ng/mL; lower or higher concentrations require dose adjustment as reported in Table 13. However, by LC-MS/MS analysis, a narrower window is proposed: 79-1791 ng/mL and a mean value of 400 ng/mL at the steady-state [**206**].

5-FU Plasma Concentration (µg/L)	AUC (mg×h×L^{-1})	5-FU Dose Adjustment (± % of previous dose)
< 500	<4	70
500-1000	4 to < 8	50
1000-1200	8 to < 10	40
1200-1500	10 to < 12	30
1500-1800	12 to < 15	20
1800-2200	15 to < 18	10
2200-2500	18 to < 20	5
2500-3000	20 to < 24	Unchanged
3000-3500	24 to < 28	-5
3500-3700	28 to < 31	-10
>3700	> 31	-15

Table13. Dose adjustment based on 5-FU plasma levels.

Based on literature, we assessed linearity of our method using concentrations between 10 ng/mL and 10 µg/mL obtaining good linearity ($R^2 = 0.9985$) (Figure 21).

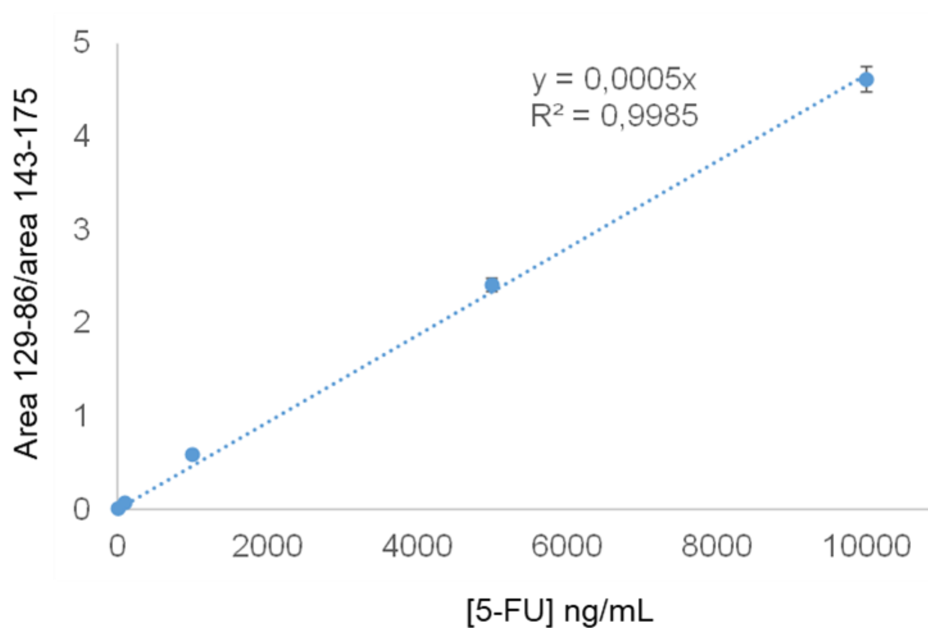


Figure 21. Range of linearity.

Precision and accuracy were evaluated as described above. In details, %CV values were <5% for all inter-day and intra-day quality controls, and %RSE were <3% for all samples, except for the intra-day quality control at 2µg/mL (Table 14). However, values were lower than the accepted cut-off of 15%, confirming that our method was precise and accurate. In addition, samples stored at +4°C showed a good stability.

Concentrations	INTRADAY		INTERDAY	
ng/mL	**% CV**	**% RSE**	**% CV**	**% RSE**
0.2	3.5	2.9	4.1	1.1
2	1.0	5.9	3.9	0.9
10	4.4	2.3	4.9	2.0

Table 14. Intra- and inter-day precision (CV %) and accuracy (RSE %).

For validation in a cohort of oncologic patients, blood drawings for TDM were performed at time 0 before 5-FU infusion; 3 to 18 min after the rapid injection; and 45 and 120 min after starting the infusion. Pharmacokinetic studies proposed that the steady-state is reached after 2 hours from the infusion; however, in our cases, we decided to consider 45 min as steady-state in order to collect blood for all patients who usually left the hospital setting after therapy. All patients had 5-FU levels lower than LLOQ before infusion (Table 15), confirming the drug is rapidly metabolized within cycles of chemotherapy. Values were highly variable at the rapid injection time ranging from 6,211 to 25,765 ng/mL. Drug concentrations were increasingly higher at 45 min and 2 h in patients without or with polymorphisms in the *DPYD* gene related to 5-FU metabolism. In particular, patients with *DPYD* polymorphism had higher 5-FU plasma concentrations at the steady-state suggesting alterations in drug metabolism between cycles (Table 16 and Figure 22). For patient B.N., 5-FU dose was reduced after the first cycle and drug levels were already undetectable at the beginning of the third cycle. In this case, TDM allowed tailoring therapy on patient B.N. reducing 5-FU toxicity due to higher plasma levels. Patient A.F. carrying a *DPYD* polymorphism showed higher 5-FU plasma levels even though drug dose was reduced already at the second cycle. This patient experienced severe drug-related adverse effects, which required drug discontinuation.

Patient	**Polymorphism**	**T0**	**Rapid (ng/mL)**	**T45 min (ng/mL)**	**2 h (ng/mL)**
F.G.	NO	0	9740	416	320
P.F.	NO	0	6758	332	152
M.C.	NO	0	14862	409	280
C.A.	NO	0	10150	385	220
S.L. 1	NO	0	9103	415	228
F.G.	NO	0	12313	535	323
S.L. 2	NO	0	8962	377	175
C.	NO	0	13975	469	386
B.N.1	DPYD*2A	0	12091	950	615
B.N.2	DPYD*2A	0	9340	392	183
V.P.1	DPYD*2A	0	10861	474	224
A.F.1	DPYD*2A	0	6211	811	X
B.N.3	DPYD*2A	0	25765	X	X
V.P.2	DPYD*2A	0	6624	342	X
A.F.2	DPYD*2A	0	9682	1093	X
A.F.3	DPYD*2A	0	6780	372	X

Table 15. 5-FU treated patients, *DPYD* polymorphisms and drug concentration at various timepoints.

Patient	**Cycles of chemotherapy**				
	1	**2**	**3**	**4**	**5**
B.N.	950*	392			
V.P.		474	342	372	
A.F.		811	1093	1048	934

Table16. Patients carrying DPYD polymorphisms and their corresponding 5-FU plasma levels at each cycle of chemotherapy at T45 min.

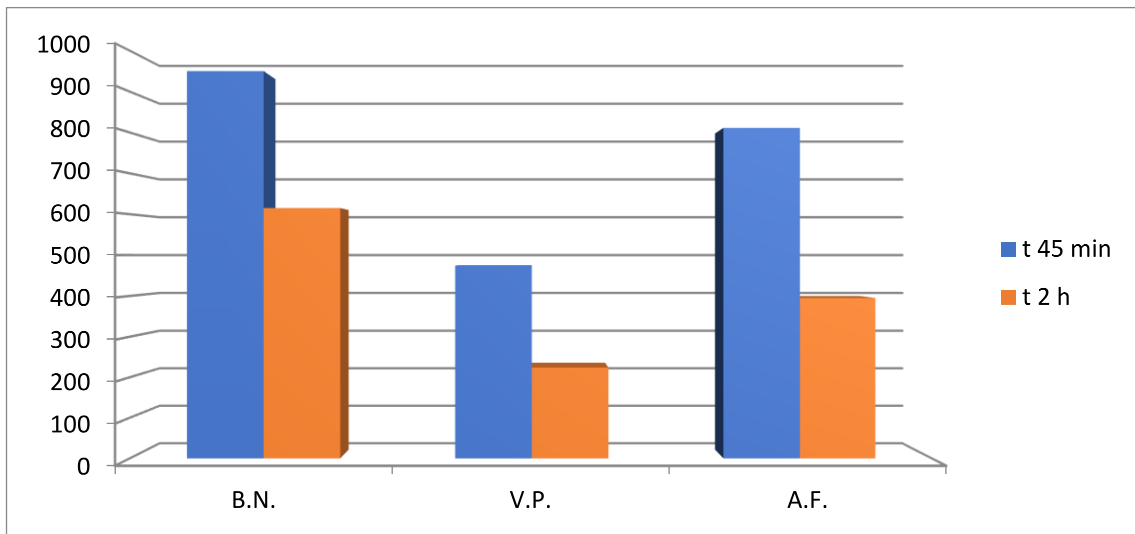


Figure22. 5-FU concentrations at 45 min and 2h in patients carrying the DPYD polymorphisms.

Chapter V

Neonatal monitoring of phenobarbital and caffeine

5.1. Introduction

5.1.1. Neonatal TDM

Human body is in a continuous change and neonates differ from children to teens to adults and elderly. These variations are responsible of modifications in pharmacokinetics (PK) and pharmacodynamics (PD) and thus different drug metabolism and elimination [**231**]. In addition, theoretical effective dosages are calculated on adults and empirically adjusted based on body weight, because of the lack of PK/PD study in term and preterm neonates that are characterized by an even greater inter-individual variability compared to adults [**232**] (Figure 23).

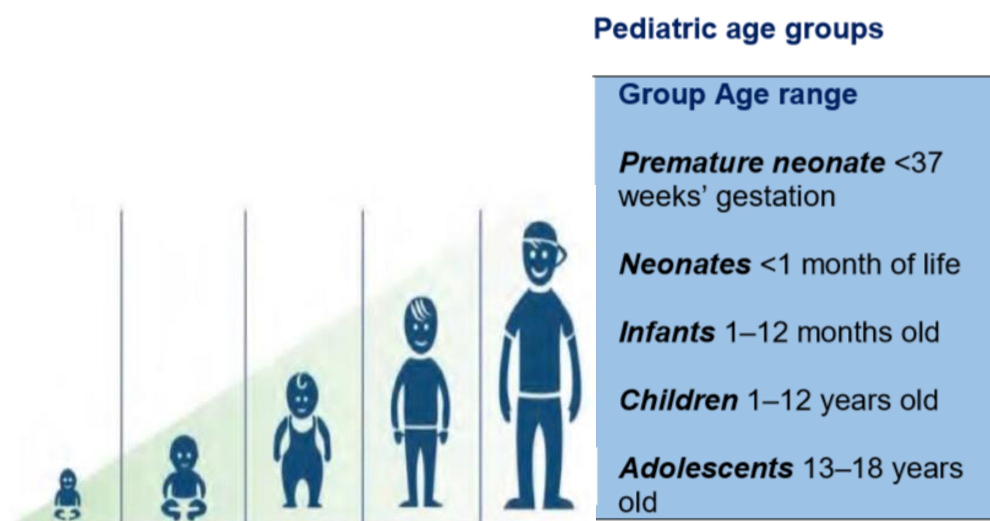


Figure 23. Pediatric age groups

For these reasons, quantitative analysis of drugs is needed to better tailor therapy in infants to increase efficacy and reduce drug-related toxicities [**233**]. However, therapeutic drug monitoring (TDM) requires large blood volumes that need to be drawn very frequently according to therapy protocols. By contrast, current guidelines from the Department of Health and Human Services recommend not to exceed a 2.5% of total blood volume/draw or 5% of total blood volume in a 30-day period. In order to reduce sample volume needed, microsampling techniques, such as dried blood spot sampling (DBS), have been developed for various analytical testing in neonates [**234,235**]. To date, DBS analysis coupled with liquid chromatography tandem mass spectrometry (LC-MS/MS) is the most common technique for detection of biomarkers in newborn screening studies, TDM, or illicit drug detection [**235,236**].

In pediatric population, age and body weight are highly related to PK parameters, such as body size and composition or liver and kidney functions. These physiological changes are responsible of variations in drug absorption and elimination in absence of other comorbidities or concomitant use

of various medications [231]. For example, in neonates, gastric pH is higher than adults (5 in newborns vs 2-4 in infants vs 1-2 in young adults) and acid gastric secretions are even lower in preterm compared to term neonates. In addition, milk intake increases alkalization of gastric pH. The higher pH negatively or positively influences drug absorption by increasing the ionization of molecules according to the acid/base balance. Indeed, most of the drugs, are weakly acidic or basic, such as β-lactams, and are highly absorbed in neonates; while acidic molecules, such as nalidixic acid, are less absorbed due to their presence in the ionized form. A slower gastric emptying or a larger extracellular and total body water space also lead to increased drug adsorption and distribution [232,237,238]. Other parameters that influence PK in neonates are: immaturity of the intestinal microbiota; reduced total area of intestinal mucosa; decreased activity of pancreatic enzymes; less concentrated bile acids; and a different bloodstream flow compared to adults [239]. In case of intramuscular administration, newborns have a reduced and delayed absorption of the drug because of a decreased perfusion, rate of passive transport through the endothelium or distribution volume. Moreover, newborns have a greater instability in vascular motility and a lower muscular mass and subcutaneous fat which contribute to the reduced absorption of the drug through intramuscular injections.

The distribution volume of a drug is also influenced by the total binding capacity of plasma proteins and by total body water space which continually change within the first years of life. In newborns, the total binding capacity of proteins is reduced because of lower levels of albumin or the presence of endogenous compounds, such as bilirubin, which compete for protein bindings. As a result, drugs have an increased circulating unbound fraction and, subsequently, a higher distribution volume [240].

Drug elimination is reduced in newborns because of the immaturity of liver and kidney functions. Phase I (hydroxylation, deacetylation and oxidation) and phase II reactions (conjugation) in the liver and glomerular filtration rates and tubular secretion in the kidneys are greatly reduced in newborns and even more in preterm neonates. Methyltransferase and sulfotransferase activities are increased in newborns; while, glucuronyl transferase and N-acetyltransferase-2 are greatly reduced, as well as cytochrome P450 functions are still not completely functioning. As a result, drugs have a longer half-life and higher plasmatic concentrations. Liver functions can also be affected by the presence of polymorphisms in genes encoding for enzymes related to drug metabolism [239]. Kidney functions reach the complete maturity only in late childhood; however, an adequate glomerular activity is observed only at 36 weeks of pregnancy, thus preterm neonates have an insufficient kidney function [240]. Finally, blood composition, such as a higher hematocrit (42-65%) compared to adults, can influence PK of drugs in preterm and term newborns [241].

5.1.2. Phenobarbital

Phenobarbital (PB) (Figure 24), introduced in clinical use in 1904, is one of the oldest effective anticonvulsants. PB, an anti-barbiturate with low toxicity, acts through the synaptic inhibition of GABA and by increasing the activation of the $GABA_A$ receptors leading to central nervous system (CNS) depression [**242,243**].

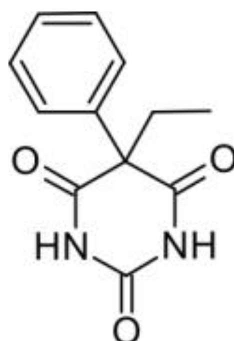


Figure 24. Phenobarbital

To date, PB remains an effective and safe anticonvulsant drug for prevention and treatment of neonatal seizures with a better efficacy and safety profile compared to other anticonvulsants, such as phenytoin and benzodiazepines [**244**]. The loading dose is 20 mg/kg intravenously and the maintenance dose is 3 to 4 mg/kg per day administered 12 to 24 hours after the loading dose. The therapeutic concentrations are 15-40 mg/mL. Non-responders generally receive additional doses of 5 to 10 mg/kg bolus and neonates with refractory seizures receive an additional dose up to a maximum plasma concentration of 100 mg/mL [**245**]. This therapeutic strategy allows the seizure control in 77% of newborns [**246**]. Even though PB has a safe profile, through concentrations $\geq$ 40 mg/mL can cause sedation, hypotension, arrhythmia, cerebral hypoperfusion and respiratory depression.

PB is metabolized by the cytochromes CYP2C9, CYP2C19, and CYP2E1 and conjugated metabolites are eliminated by the kidney; however, this drug is a strong cytochrome inducer causing decreased plasma concentration within 1-2 weeks of treatment [**242**]. PB has a long half-life (80-120 h) which is even longer in preterm infants which show also a higher distribution volume [**247,248**]. Body weight and distribution volume positively also correlate with total clearance of PB, which decreases by increasing in gestational age [**236,249**]

5.1.3. Caffeine

Methylxanthines, such as caffeine (Figure 25), are used for the treatment of apnea of prematurity (AOP) [**250-255**]. Caffeine has largely replaced other methylxanthines and is among the most used medications in preterm infants [**256,257**]. Caffeine, an adenosine receptor antagonist, stimulates the CNS by modulating several neuronal signaling pathways, such as noradrenaline or dopamine, and by stimulating the respiratory centers located in the brainstem [**258**].

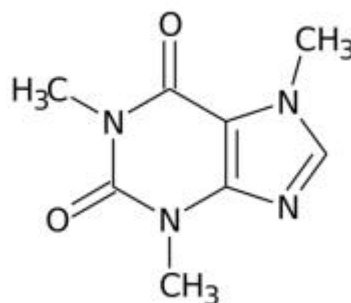


Figure 25. Caffeine

Very-low weight newborns (<28 weeks gestation or <1000 g) have an increased risk of apnea episodes and bronchopulmonary dysplasia (BPD), a chronic respiratory disease caused by respiratory distress syndrome and with high mortality and long-term pulmonary and neurodevelopmental morbidities. AOP is caused by the immaturity of respiratory control mechanisms, and the incidence is widely variable and is based on gestational age and birth weight [**259-265**] Since frequent apnea episodes can alter cardiorespiratory functions and neurocognitive development, caffeine is recommended for prevention and treatment of apnea events in preterm [**266**]. Indeed, early use of caffeine significantly reduces the incidence of BPD and the duration of respiratory assistance.

The loading dose is 20 mg/kg with slow intravenously infusion and the maintenance dose is of 5 mg/kg per day and can be increased at 10 mg/kg, if required. This protocol allows to reach the therapeutic range of 10-20 mg/kg [**267,268**]. AOP is not common after 34-week gestation, caffeine therapy should be continued until preterm infants are 34 to 36 week corrected gestational age and free of any apnea episodes for at least 8 days [**269**]. However, late discontinuation at 40 weeks can significantly reduce apnea episodes in preterm infants compared to standard discontinuation at 34-35 weeks gestation [**270**].

5.1.4. Project aim

In preterm infants, caffeine is employed for prevention and treatment of AOP; while PB is used for treatment and prevention of seizures. More than 50% of newborns are unresponsive to therapy and need additional loading doses; by contrast, higher PB levels can cause respiratory depression. TDM, a laboratory practice for measurement of serum/plasma drug levels, allows dose adjustment to minimize PB side-effects. However, TDM requires frequent blood drawing not feasible in preterm infants. Microsampling techniques, such as DBS, can minimize sample volume needed per analysis and could be a good sampling choice for TDM in newborns. In this part of my PhD thesis, we developed and optimized a DBS microsampling – LC-MS/MS method for the measurement of PB and caffeine in preterm infants [**271-274**].

5.2. Materials and methods

5.2.2. Chemicals and reagents

Formic acid. UHPLC grade water and methanol (MeOH) were purchased from Romil (Waterbeach Cambridge. UK). Caffeine. internal standards (IS) (Primidone. PRM; and caffeine C13. C13) and filter paper were from Sigma-Aldrich (St Louis. MO. USA). Phenobarbital (PB) solution was kindly provided by the Hospital "San Giovanni di Dio Ruggi d'Aragona" (Salerno, Italy).

5.2.3. Patients

Samples were obtained from neonates admitted to the Neonatal Intensive Care Unit. University Hospital "San Giovanni di Dio Ruggi d'Aragona". Salerno (Italy). Whole blood was collected by heel puncture and was directly deposited on filter paper. DBS were then placed in appropriate bags, transported to our laboratory, stored at +4°C and processed within 24 hours.

5.2.4. Preparation of stock and working solutions

Phenobarbital (PB) sodium solution for injection at initial concentration of 100 mg/mL was diluted to 10 mg/mL in physiologic solution (stock solution). Stock solutions for PRM and C13 were prepared in 100% MeOH at 1 mg/mL; while. caffeine was diluted at 1 mg/mL in physiologic solution. Work solutions were at 1 mg/mL in physiologic solution for PB and at 10 µg/mL in MeOH 100% for PRM. Caffeine and its isotope were diluted in physiologic solution at 250 µg/mL and in H_2O at a concentration of 10 µg/mL. All working solutions. as well as the initial stocks. were stored at -20°C.

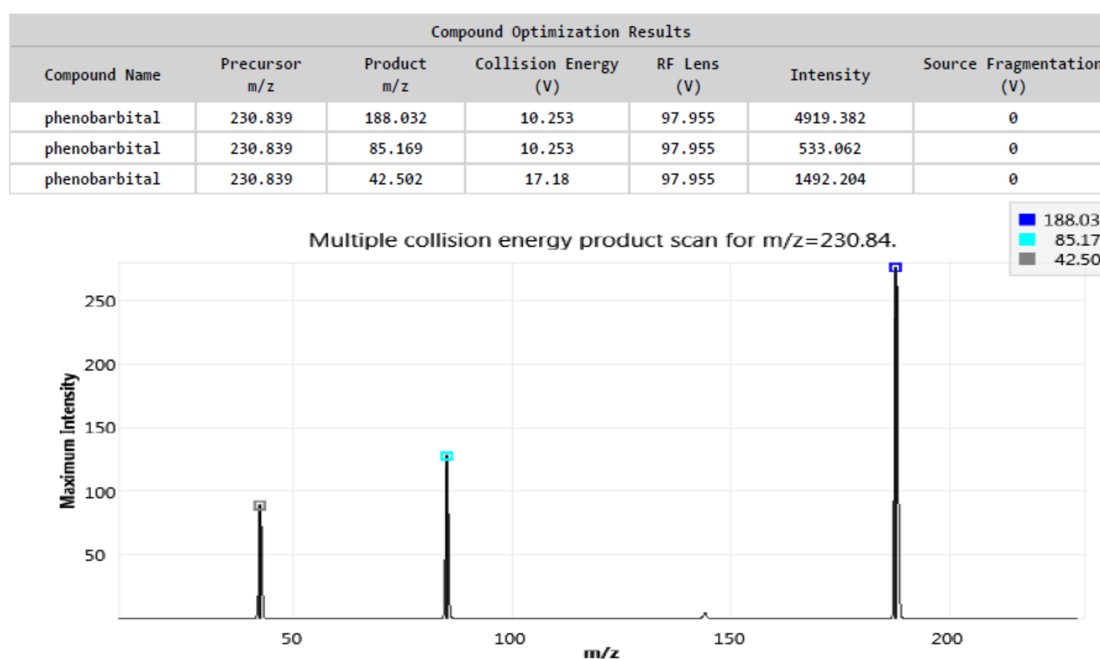
5.2.5. LC-MS/MS conditions

LC–MS/MS apparatus consisted of a Ultimate 3000 UHPLC system (Thermo-Fisher. Waltham. MA. USA) coupled with a TSQ endure mass spectrometer (Thermo-Fisher) equipped with an electrospray ion source and a triple quadrupole ion analyzer. Chromatographic separation of 20 µL to PB and 5 µL to caffeine was carried out using a Luna-Ω C18 Polar column (2.1 x 50 mm. 1.6 µm) and a mobile phase composed of a mixture of 0.1% formic acid in water (A) and 0.1 % formic acid in acetonitrile (B). An elution gradient ranged from 35% to 49.5 % of B in 0.7 min at 30°C for PB and from 5% to 20% of B in 1 min for caffeine. The flow was set at 0.4 mL/min.

Spectra were acquired in negative selected reaction monitoring (SRM) mode for PB and in positive SRM mode for PRM. Specific transitions were selected by direct injection in order to maximize selectivity and sensitivity: for PB. 230.8→188 and 230.8→85 with a collision energy (CE) of 10 V and an RFlens of 90; for PRM. 219.9→163 with a CE of 12 V and a RFlens of 85; for caffeine. 195→138 and 195→110 with CE of 23 and 22V. and RFlens of 134; for C13. 198→140 with CE of 18V and RF lens of 142 (Table 17 and Figure 26-27).

	MW (g/mol)	**Adduct [M+H]$^+$**	**Products (m/z)**	**Collision Energy (V)**	**RFlens**
Phenobarbital	229.8	230.8	188	10	90
			85	10	90
Primidone	218.9	219.9	163	12	85
			138	23	134
Caffeine	194	195	138	23	134
			110	22	134
Caffeine C13	197	198	140	18	142

Table 17. MS parameters.



Compound Optimization Results

Compound Name	Precursor m/z	Product m/z	Collision Energy (V)	RF Lens (V)	Intensity	Source Fragmentation (V)
phenobarbital	230.839	188.032	10.253	97.955	4919.382	0
phenobarbital	230.839	85.169	10.253	97.955	533.062	0
phenobarbital	230.839	42.502	17.18	97.955	1492.204	0

Figure 26. Tuning for PB.

Compound Optimization Results						
Compound Name	Precursor m/z	Product m/z	Collision Energy (V)	RF Lens (V)	Intensity	Source Fragmentation (V)
Caffeina	195.365	138.04	18.646	134.36	455272.197	0
Caffeina	195.365	110.111	22.287	134.36	99757.898	0
Caffeina	195.365	83.222	26.989	134.36	25977.936	0

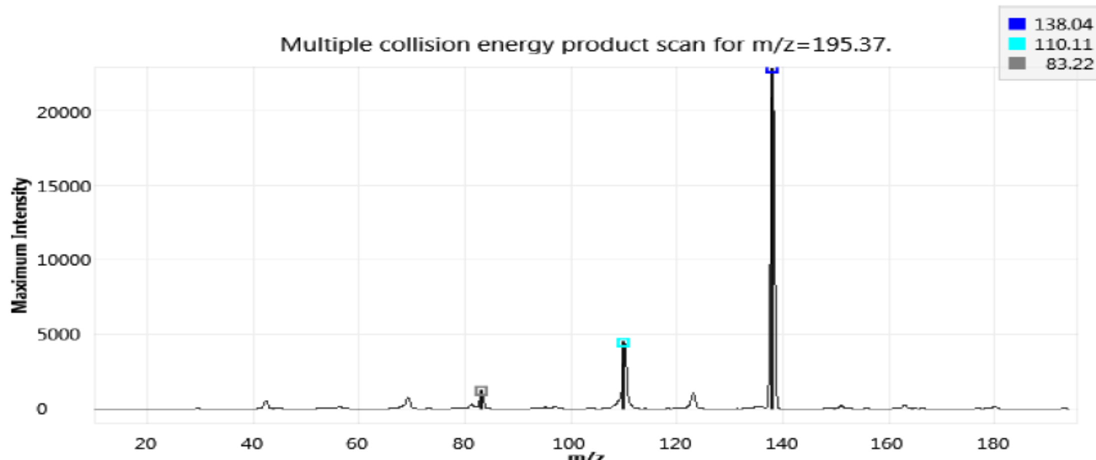


Figure 27. Tuning for Caffeine.

5.2.6. Samples preparation

Ethylene Diamine Tetraacetic Acid (EDTA) blood samples were obtained from healthy volunteers recruited at the Blood Bank of the University Hospital "*San Giovanni di Dio Ruggi d'Aragona*". Salerno (Italy). Briefly. 20 µL of whole blood were blotted on filter paper and air-dried at RT for 2 hours. Then DBS were cut out, placed in a 2 mL safe-lock tube and rehydrated with 50 µL of PRM at a final concentration of 100 ng/mL in UHPLC grade water. After vortexing, samples were incubated in a water bath at 37° C for 10 min, and then 200 µL of 80% MeOH were added, vortexed for 20 seconds and sonicated for 10 min. Supernatants were transferred to a clean tube and centrifuged at 17.000*g* at 4°C for 10 min. and 100 µL of clear supernatant placed in vials for LC-MS/MS analysis.

5.2.7. Method validation

The absence of carry-over was accepted when the signal for a certain analyte was less than 20% of the LLOQ and less than 5% for the IS. No carry-over was detected. Selectivity was assessed by analyzing whole blood samples without analytes but with other commonly used drugs, samples spiked with analytes at the LLOQ, and samples spiked with IS. Based on the EMA guidelines, the absence of interfering molecules is accepted when the signal at the same retention time of the target drug is less than 20% of the LLOQ for the analyte and less than 5% for the IS. For all analytes used

in our method, there were no interfering compounds. Specificity was evaluated based on the consistency of the retention time for a certain analyte across runs and the presence of both products (quantifier and qualifier) in a constant ratio for a precursor. Our method showed high specificity for each analyte. The matrix effect (ME), defined as modifications in ionization recovery of target analytes, is caused by the presence of interfering compounds present in the same matrix and co-eluting with studied molecules eventually leading to ion suppression. The FDA guidelines for validation of analytical procedures recommend evaluation of ME by post-column infusion of target analyte or post-extraction addition. In our case, we evaluated ME by comparing peak area of analyte/IS ratios in QC samples spiked after extraction to pure solutions at the same concentrations.

LLOD and LLOQ were defined as described in paragraph 2.2.6. For their estimation, samples were prepared at concentrations of 0.01. 0.025. 0.05. 0.1. 1 and 2 µg/mL and analyzed in triplicates.

Linearity was determined by plotting peak area of analyte/IS ratios as a function of the analyte concentration. and by fitting the curve to a linear regression. Experimental concentrations were back calculated using calibration curves to determine their deviation from nominal values.

For calibration standards, PB at final concentration of 100 µg/mL was freshly prepared in whole blood and serial dilutions (1. 5, 12.5, 25. 50 and 75 µg/mL) were obtained by using whole blood with a 10 % of dilution ratio with physiological solution. For caffeine. serial dilutions (0.05, 0.25, 1, 2, 4, 8, 16 e 32 µg/mL) were prepared in whole blood at 12.8% of physiological solution. Then 20 µL of each concentration were spotted on DBS and extracted as described above.

The average recovery of PB from DBS was determined by comparing experimental concentrations measured in three samples as pre- and post-extraction process. Pre-extraction samples were obtained by directly adding PB or caffeine in whole blood at specific amounts (1, 20 or 50 µg/mL for PB and 0.2, 3.2, 24 µg/mL for caffeine); post-extraction samples were prepared by spiking into an extracted matrix from DBS the amount of analytes required to obtain the desired concentration. Mean recoveries were calculated as the percentage difference between the quantity of PB recovered from the post-extraction and pre-extraction samples divided by the quantity of the PB spiked (post-extraction).

Intra-day and inter-day PB precision and accuracy of the method were evaluated at three different concentrations (3, 20 and 70 µg/mL) in samples extracted from DBS. For caffeine. precision and accuracy were evaluated at high (6, 12, 18, and 24 µg/mL) and low concentrations (0.2, 0.8, and 3.2 µg/mL). Three replicates for each concentration were extracted and analyzed twice in the same day (T0) and after 3 or 6 hours (T3 and T6, respectively). Aliquots of those samples were stored at +4°C and analyzed again (three times per day) after 24 (T24) and 48 (T48) hours. Percentage of coefficient of variance (%CV) was calculated for assessing precision and the percentage relative error (% RE) for accuracy.

To estimate samples stability on DBS, different conditions were evaluated for 3 concentrations: 3, 20 and 70 µg/mL for PB; and 0.25, 2 and 16 µg/mL for caffeine. DBS were directly placed in the fridge after blood spotting or left at RT for 2 hours and then placed at +4°C. Samples from both conditions were extracted and analyzed at T0, T24, T48 and after 15 days. In addition. samples were left at RT overnight and then extracted. Stability was evaluated by monitoring modifications in the relative peak intensities.

5.2.8. Comparison between DBS and plasma

To evaluate the correlation between plasma and DBS, PB or caffeine were added to whole blood at concentrations used for calibration curve. Afterwards, 20 µL of whole blood were directly spotted on filter paper while 250 of the same blood samples were centrifuged at 3000*g* at 4°C for 10 min and plasma collected. Then 50 µL for PB or 45 µl for caffeine of plasma were transferred to a clean tube and IS added at a final concentration of 5 µg/mL. Extraction was carried out adding 200 µL or 250 µL, for PB and caffeine respectively, of methanol to samples. After 30 min incubation at RT with a vortex step every 10 minutes, specimens were centrifuged at 4°C and 17000*g* for 5 min. An additional centrifuge step at 4°C at 17000g for 1 min was performed and supernatant was transferred in vial for LC-MS/MS analysis.

5.3. Results and discussion

As a first step for method optimization, we tested several chromatographic conditions for an optimal separation of the targeted compounds. A mobile phase composed of 0.1% formic acid in water (solution A) and 0.1% formic acid in acetonitrile (solution B) was employed, and an elution gradient from 35-49.5% of B in 0.7 min for PB and from 5-20% of B in 1 min for caffeine was used for separation (Figure 28-29). Total runtime was of 4.5 min.

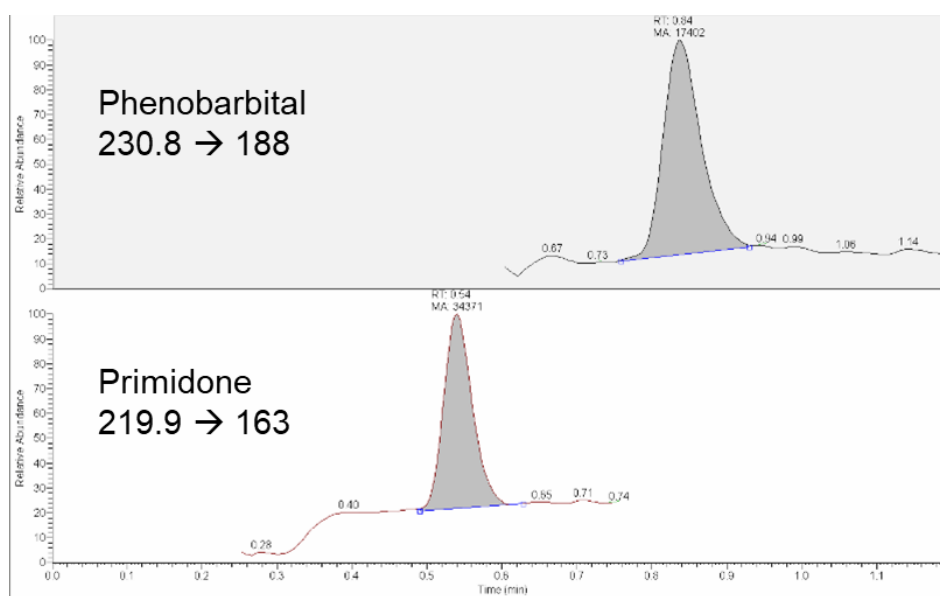


Figure 28. PB and PRM chromatograms

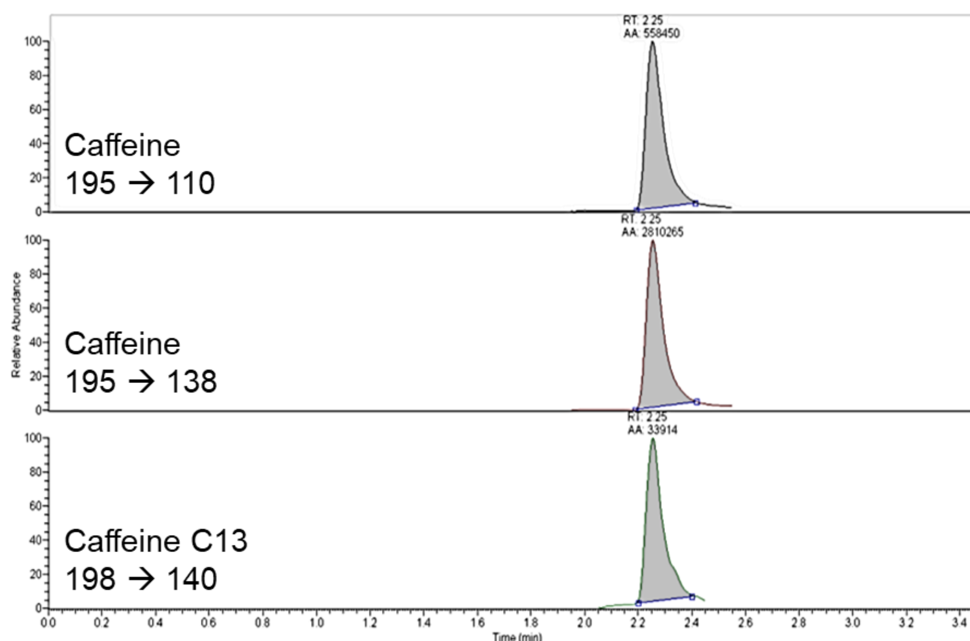


Figure 29. Caffeine and C13 chromatograms

Before LC-MS/MS analysis, body fluid samples should be cleaned up from lipids. proteins and salts to remove interferers and to avoid column damages. In addition, the use of DBS sampling

has several pre-analytical variables that need to be investigated during method optimization, such as blood spot volume for example. We first evaluated extraction procedure from DBS using a 100% methanol extraction as previously described [**275**]. After centrifugation. samples were dried at various temperatures in *Speed Vac* or under nitrogen flow. Pellets were then resuspended in MeOH from 10 to 100% concentration with or without 0.1% formic acid. Comparing the different conditions used, we found that the better results in terms of extraction yield were obtained when DBS were first rehydrated and then extraction was carried out using 80% methanol. Using this protocol. extraction yields for PB ranged from 65.5 to 85.3%. and for the IS was of 95.6% (RSD = 2%) (Table 18).

	1 µg/ml		20 µg/ml		50 µg/ml	
	CV (%)	RSD (%)	CV (%)	RSD (%)	CV (%)	RSD (%)
Phenobarbital	65.5	19.0	80.1	4.2	85.3	3.1

Table 18. Extraction yields for PB at various concentrations.

For caffeine. extraction yields ranged from 67.8 to 145% (Table 19); these high values rates are probably influenced by the presence of endogenous caffeine in the blood of volunteers.

Concentrations (µg/ml)	Recovery (%)
0.2	145 ± 7.3
3.2	82.9 ± 0.8
24	67.8 ± 2.5

Table 19. Extraction rates for caffeine.

We then validated the method based on the EMA guidelines. First, selectivity was assessed; the absence of interfering molecules is accepted when the signal at the same retention time of the target drug is less than 20% of the LLOQ for the analyte and less than 5% for the ISTD. Our analyses showed the lack of interfering compounds. Specificity was evaluated based on the consistency of the retention time for a certain analyte across runs and the presence of both products (quantifier and qualifier) in a constant ratio for a precursor. Our method showed high specificity for each analyte. LLOD and LLOQ were defined according to the EMA guidelines. In our case. LLOQ was identified at 1 µg/mL for PB and 500 ng/mL for caffeine. Next, linearity was assessed using six concentrations of the compound of interest in a therapeutic range, plotting peak area of analyte/IS ratios as a function

of analyte concentrations, and then fitting the data to a linear regression. For PB, the linear range was between 1 and 75 µg/mL; while. for caffeine was from 0.05 to 32 µg/mL (Figure 30).

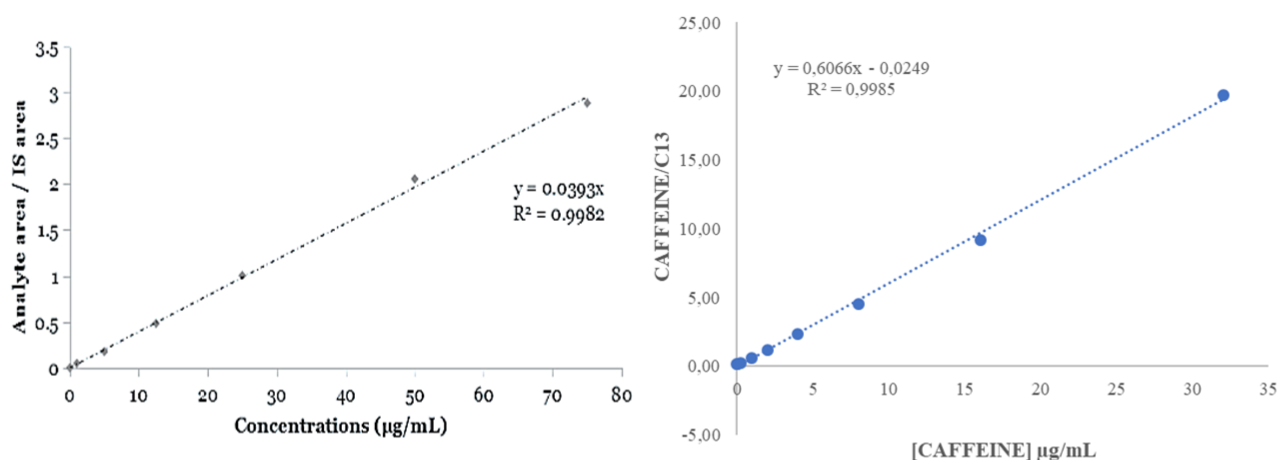


Figure 30. Linearity range for PB (left) and caffeine (right)

Precision and accuracy of the method were then investigated as previously described. For PB, %CV and %RSE were <15% for all concentrations of the quality controls (Table 20). For caffeine, the presence of the compound in the blood of volunteers hampered a reliable assessment of precision and accuracy of the method.

	INTRADAY		INTERDAY	
Concentrations µg/ml	CV%	RSE%	CV%	RSE%
3	7.23	5.16	9.36	2.12
20	3.63	3.57	7.36	2.98
70	5.46	26.02	8.61	23.92

Table20. Intra- and inter-day precision and accuracy of PB

Short- (24 and 48h) and long-term (15 days) stability was investigated for PB at various concentrations (3, 20 and 70 µg/ml) from DBS samples stored at +4°C right after spotting blood on filter paper or after 2h air-drying. Samples were then extracted and analyzed at 24/48h or 15 days. DBS were stable within 48h in both conditions but not after 15 days (Table 21).

Concentration µg/ml	Directly at 4°C			Stored at 4°C after drying		
	24h	48h	15 days	24h	48h	15 days
3	17.6	-14.8	-75.5	-1.4	10.6	-73.1
20	-11.1	-5.6	-77.1	-11.1	0.3	-79.9
70	-7.5	-5.8	-83.6	-11.6	0.2	N.d.

Table21. Stability of PB in different storage conditions.

We also investigated the stability of extracted samples stored at +4°C and analyzed at 3, 6, 12, 24 e 48 h (Table 22). All samples were stable except for specimens with PB at 3 µg/mL after 48h of storage.

Concentration (µg/ml)	Extracted samples stored at +4 °C				
	3h	6h	12h	24h	48h
3	2.59	6.38	10	10.43	17.19
20	3.9	6.24	8.04	9.52	14.2
70	8.48	9.33	4.42	11.98	10.87

Table22. Stability of PB

For caffeine, quality controls were prepared at concentrations of 0.25, 2 and 16 µg/mL, air-dried for 1h, and stored at room temperature for 1, 2, 3, 6, 9 and 12 days. DBS were then processed and analyzed. Samples were stable within three days from blood spotting on filter paper (Table 23).

Concentration µg/mL	**Day +1**	**Day +2**	**Day +3**	**Day +6**	**Day +9**	**Day +12**
0.25	-2	-2	-5	-28	-89	-47
2	0	-4	-10	-9	-86	-23
16	1	0	-10	-9	-87	-12

Table23. Stability of caffeine at RT

Storage of DBS at +4°C allowed to extend the stability of quality controls at 2 and 16 µg/mL for a six-day period (Table 24).

Concentration µg/mL	**Day +1**	**Day +2**	**Day +3**	**Day +6**	**Day +9**	**Day +12**
0.25	-5	-7	-6	-30	-23	-27
2	-1	-3	-6	-8	-9	-7
16	-2	-24	-10	-5	-15	-12

Table24. Stability of caffeine at a 4°C.

Reported methods for PB measurement have been optimized for LC-MS/MS analysis from plasma samples. For this reason, to confirm the reliability of our method using DBS samples, we correlated results from DBS to those obtained from plasma (Table 25). As expected, results from DBS samples were similar to those from plasma, thus confirming that this microsampling technique might be used as good alternative to peripheral blood drawing.

Concentrations (µg/ml)	DBS		Plasma		DBS *vs* plasma		
	Calculated concentration	RSD%	Calculated concentration	RSD%	Mean	SD	RSD%
1	1.1	16.8	0.8	5.1	0.9	0.2	20.6
20	15.3	6.6	14.2	3.2	14.8	0.8	5.2
50	45.2	1.1	40.7	3.8	42.9	3.2	7.5
70	64.2	1.7	78.6	4.9	71.4	10.1	14.2

Table25. Plasma vs DBS

Finally, our method was validated on DBS samples obtained from preterm neonates admitted to the Neonatal Intensive Care Unit, University Hospital "San Giovanni di Dio Ruggi d'Aragona", Salerno, Italy, and under PB or caffeine treatment. DBS samples in duplicate were prepared by directly spotting blood on filter paper after skin puncture of the heels. Samples were stored at +4°C for 24h before processing and analyzed in triplicate.

Blood for one neonate was collected for PB measurement (Table 26); however, spot volumes differ between duplicates thus resulting in different concentrations (0.365 e 0.632 µg/mL) below our LLOQ.

Duplicate	Area PB	Area PRM	PB/PRM	Mean	Calculated concentration (µg/ml)	SD	RSD%
	1398	96522	0.014				
A	1764	102200	0.017	0.016	0.365	0.001	9.5
	1565	104456	0.015				
	2814	101517	0.028				
B	2669	101592	0.026	0.027	0.632	0.001	2.7
	2774	103476	0.027				

Table26. LC-MS/MS analysis of neonate treated with PB

For caffeine measurement, three preterm neonates were enrolled (Table 27) and DBS sampling performed during maintenance therapy at the caffeine steady-state. Patient 1 received a loading dose of caffeine of 40 mg in a 30 min infusion, followed by a maintenance of 10 mg every 24h. Patient 1 experienced only few events of apnea and oxygen desaturation. Patient 2 had severe distress respiratory syndrome and received a loading dose of 23.4 mg in rapid infusion and a maintenance dose of 5.8 mg increased to 7.5 mg for prevention of apnea during mechanical ventilation. Patient 3 was a very-low weight birth preterm neonate who received mechanical ventilation and a loading dose of caffeine of 17 mg and a maintenance dose of 4.5 mg. For all neonates, trough caffeine concentrations were within the range of linearity.

UPN	Duplicate	Area Caffeine	Area IS	Ratio Caf/IS	Mean	Calculated concentration (ng/ml)	SD	RSD (%)
CAF1		3465153	86812	39.9				
	A	3827241	95011	40.3	42.6	**3228.8**	4.4	10.3
		4865548	102079	47.7				
		6226659	121573	51.2				
	B	4944421	114218	43.3	47.3	**3579.8**	5.6	11.9
		10110178	116335	86.9				
CAF2		1724391	103066	16.7				
	A	2307660	107271	21.5	20.5	**1549.7**	3.3	16.3
		2526876	109271	23.1				
		3144215	104560	30.1				
	B	3970257	104159	38.1	33.8	**2560.1**	4.1	12.0
		3387016	102046	33.2				
CAF3		3068617	109626	28.0				
	A	1297683	112264	11.6	18.9	**1433.4**	8.3	44.1
		1873314	108842	17.2				
		2968112	113814	26.1				
	B	2615516	113265	23.1	26.4	**1999.1**	3.5	13.1
		3406252	113558	30.0				

Table27. LC-MS/MS analysis in newborns treated with caffeine

In this study, we have developed and optimized a DBS-LC-MS/MS method for quantitative analysis of PB and caffeine in neonates. Our procedure was selective, precise and accurate for measurement of these compounds from DBS samples; however, validation on a larger cohort of patients is required at this stage.

Chapter VI

Conclusions

Personalized Medicine has the potential to tailor therapy with the best response and highest safety margin to ensure better patient care. By enabling each patient to receive earlier diagnoses, risk assessments, and optimal treatments, PM holds promise for improving health care while also lowering costs. The large inter-individual variability in drug response represents a major challenge in drug therapy, particularly for drugs with a narrow therapeutic index. This individual susceptibility may be due to peculiarity in the genetic sequence. However, many non-genetic factors, such as physiological, pathological and environmental factors, may also contribute significantly to the inter-individual variability in ADME processes.

TDM use to monitor the evolution of concentrations in biological fluids would be a fundamental tool to address this problem. It is based on the hypothesis that there is a definable relationship between the dose of drug administered and its concentration in blood and/or plasma - or more generally in biological fluids - and between this concentration and the observed pharmacological effects. However, the correlation between dose and concentration of a specific drug is not always as linear as it might seem, and is generally characterized by several variables associated with its pharmacokinetics. Several therapeutic classes have presently been recognized to benefit from TDM including antiepileptics, antibiotics, antimalarials or anticancer drugs, but there are conditions suggesting the use of TDM, regardless of the used drug. In particular, when clinical evidences suggest a less than expected clinical response to therapy, or if over-dosage is suspected in the presence of clinical signs of toxicity. When special patients like pediatric population, are subjected to drug therapy, TDM is always advisable. For these populations, the available data on drug effects, toxicity and metabolism are generally reduced, because of the limited number of treatments and/or for the lack of clinical experimentation. Different analytical approaches have been used so far to measure the concentration of a drug and/or its metabolites in biologic fluids or tissues. Liquid chromatography (HPLC) and gas chromatography (GC) have been considered for years the "gold standard", in parallel with immunometric techniques, for the measurement of specific substances in biological fluids such as plasma/blood and urine. However, since 15 years LC-MS emerged for its impressive performances and has now became a key analytical tool for all modern clinical laboratories.

During my Ph.D., we developed and validated some HPLC-based and LC-MS/MS-based approaches, aimed to carry out TDM of some drugs in different body fluids.

We have faced different problems, involving quite different active principles. First, the monitoring of drugs with a wide therapeutic window, but characterized by a frequent non-adherence to therapy (hydroxychloroquine-HCQ). The analytical method developed, based on the use of a widely diffused and medium cost equipment as HPLC, could find wide application in the control of patients suffering from chronic pathologies as SLE; for these patients, in fact, the main cause of

ineffectiveness of the treatment is the failure to correctly follow the therapeutic indications provided by the doctor.

The well-known problems associated with the use of drugs for anti-tumor therapy and the ever-increasing use of such drugs, led us to develop analytical methods aimed at monitoring this type of substances. We have therefore developed an analytical method for a new-generation anti-IMF drug (Ruxolitinib), believed to be safe, but for which cases of reduced efficacy have been reported. The HPLC-based approach developed proved to be efficient and easy to apply and could become a useful tool for choosing the correct individual dosage of the drug. It should also be emphasized that there is still a limited number of data related to Ruxolitinib safety profile; the availability of an analytic method like the one we developed could therefore allow to quickly and effectively increase the information on the dose-effectiveness-safety relationship for this drug.

Remaining in the field of the anticancer tumors, we focused on the TDM of one of the most used antiblastic chemotherapeutic drugs, 5-fluorouracil (5-FU). Its high toxicity, and the great inter-individual variability observed in the susceptibility to this drug, make therapeutic monitoring of patients treated with 5-FU mandatory. The *a priori* assessment of this drug dosage for each individual, carried out based on the pharmacogenetic information, does not always guarantee the efficacy and, above all, the safety of the therapy. The LC-MS / MS-based methodology we have developed and validated is very effective and robust, despite the intrinsic difficulty of analyzing such a small and polar molecule as 5-FU. Furthermore, the effective application of our methodology in a clinical setting allowed elucidating the molecular basis of some abnormal and unexpected responses to therapy.

Finally, we have started exploring the potential of TDM in the optimization of dosages of drugs administered to patients in neonatal age. This category of patients, in fact, is particularly susceptible to the risk of uncorrected drug dosage administration, due to multiple factors; first of all, the lack of specific information on the suitable amount of drug to deliver, since there are no clinical trials conducted on neonates; moreover, the great inter-individual variability that exists between patients who have an immature and rapidly evolving organism. From a technical point of view, the main difficulty in conducting TDM studies on neonates is the need to carry out analyses on very small volumes of biological fluids (especially blood). In our study, we validated an analytical method for monitoring drugs widely used in neonatal intensive care, such as caffeine and phenobarbital, based on the use of a micro-sampling system (DBS) and LC-MS/MS techniques. The achieved results suggested a good applicability of the methodology.

Bibliography

1. Mancinelli L, Cronin M, Sadée W. Pharmacogenomics: the promise of personalized medicine. AAPS PharmSci. 2000;2(1):E4.

2. Billaud M, Guchet X. L'invention de la médecine personnalisée : Entre mutations technologiques et utopie. Med Sci (Paris). 2015; 31(8-9): 797–803.

3. President's Council of Advisors on Science and Technology (PCAST). Priorities for Personalized Medicine.Septembre2008.https://bigdatawg.nist.gov/PCAST_Personalized_Medicine_Priorities.pdf

4. European Medicines Agency (EMA). Personalized medicine towards the market and patients: the approval process. European Medicines Agency's perspective. Mai 2011. https://ec.europa.eu/research/health/pdf/event06/13052011/marisa-papaluca-amati_en.pdf

5. Chatterjee B, Pancholi J. Prakriti-based medicine: A step towards personalized medicine. Ayu. 2011;32(2):141-6.

6. Joshi K, Ghodke Y, Shintre P. Traditional medicine and genomics. J Ayurveda Integr Med. 2010;1(1):26-32.

7. Sykiotis GP, Kalliolias GD, Papavassiliou AG. Pharmacogenetic principles in the Hippocratic writings. J Clin Pharmacol. 2005;45(11):1218-20.

8. Vogenberg FR, Isaacson Barash C, Pursel M. Personalized medicine: part 1: evolution and development into theranostics. P T. 2010;35(10):560-76.

9. Lazarou J, Pomeranz BH, Corey PN. Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. JAMA. 1998;15;279(15):1200-5.

10. Bouvy JC, De Bruin ML, Koopmanschap MA. Epidemiology of adverse drug reactions in Europe: a review of recent observational studies. Drug Saf. 2015;38(5):437-53.

11. Shepherd G, Mohorn P, Yacoub K, May DW. Adverse drug reaction deaths reported in United States vital statistics, 1999-2006. Ann Pharmacother. 2012;46(2):169-75

12. Pirmohamed M, James S, Meakin S, Green C, Scott AK, Walley TJ, Farrar K, Park BK, Breckenridge AM. Adverse drug reactions as cause of admission to hospital: prospective analysis of 18 820 patients. BMJ. 2004; 3; 329(7456):15-9

13.Spear BB, Heath-Chiozzi M, Huff J. Clinical application of pharmacogenetics. Trends Mol Med. 2001; 7(5):201-4

14. Abrahams E, Silver M. The case for personalized medicine. J Diabetes Sci. Technol. 2009; 1;3(4):680-4.

15. Vesell ES. Genetic and environmental factors causing variation in drug response. Mutat Res. 1991; 247(2):241-57.

16. Strimbu K, Tavel JA. What are biomarkers? Curr Opin HIV AIDS. 2010; 5(6):463-6.

17. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther. 2001; 69(3):89-95.

18. De Gruttola VG, Clax P, De Mets DL et al. Considerations in the evaluation of surrogate end points in clinical trials: summary of a National Institutes of Health Workshop. Control Clin. Trials 2001; 22(5):485-502.

19. Food drug Administration (FDA). Table of pharmacogenomic biomarkers in drug labeling. Aout 2014 http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083378.html

20. Ye S. Evolutionary path to the heart. Eur J Hum Genet. 2005; 13(2):132-3.

21. Nerenz RD. Pharmacogenomics and Personalized Medicine in the Treatment of Human Diseases, Molecular Pathology January 2018; Chapter 32: pp.731-743. DOI: 10.1016/B978-0-12-802761-5.00032-8.

22. Roden DM, Wilke RA, Kroemer HK, Stein CM. Pharmacogenomics: the genetics of variable drug responses. Circulation. 2011; 123(15):1661-70.

23. Motulsky AG, Qi M. Pharmacogenetics, pharmacogenomics and ecogenetics. J Zhejiang Univ Sci B. 2006; 7(2):169-70.

24. 1000 Genomes Project Consortium, Abecasis GR, Altshuler D, Auton A, Brooks LD et al. A map of human genome variation from population-scale sequencing. Nature. 2011; 26; 473(7348):544.

25. Marshall A. Genset-Abbott deal heralds pharmacogenomics era. Nat Biotechnol.1997; 15(9):829-30.

26. McCarthy AD, Kennedy JL, Middleton LT. Pharmacogenetics in drug development. Philos Trans R Soc Lond B Biol Sci. 2005 Aug 29; 360(1460):1579-88.

27. Grant SF. Pharmacogenetics and pharmacogenomics: tailored drug therapy for the 21st century. Trends Pharmacol Sci. 2001; 22(1):3-4.

28. Lin JH. Pharmacokinetic and pharmacodynamic variability: a daunting challenge in drug therapy. Curr Drug Metab. 2007; 8(2):109-36.

29. Colivicchi F, Abrignani MG, Santini M. [Therapeutic non-adherence: the hidden risk factor]. G Ital Cardiol (Rome). 2010 May;11(5 Suppl 3):124S-127S. Italian.

30. Gasco A, Gualtieri F, Melchiorre C. Chimica farmaceutica. 2015. Ambrosiana.

31. Annunziato L and Di Renzo GF, Trattato di Farmacologia. 2016. Idelson-Gnocchi.

32. La Du BN. Pharmacogenetics of drug Metabolism. Kalow, W. Ed., Pergamon Press. New York. 1991. pp.1-12.

33. Cascorbi I. Pharmacogenetics of cytochrome p4502D6: genetic background and clinical implication. Eur J Clin Invest. 2003;33 Suppl 2:17-22.

34. Marzolini C, Tirona RG; Kim RB. Pharmacogenetics: 2nd Edition. Kalow, W. Ed; Taylor & Francis. New York. 2005.pp. 109-155.

35. Weber WW. Chapter: Receptors. Pharmacogenetics: 2nd Edition, Kalow, W.; Ed., Taylor & Francis. New York. 2005. pp. 71108. 11-14

36. Lin JH. Tissue distribution and pharmacodynamics: a complicated relationship. Curr Drug Metab. 2006; 7(1):39-65.

37. Koepsell H, Endou H. The SLC22 drug transporter family. Pflugers Arch. 2004; 447(5):666-76.

38. Hagenbuch B, Meier PJ. Organic anion transporting polypeptides of the OATP/SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties. Pflugers Arch. 2004; 447(5):653-65

39. Leslie EM, Deeley RG, Cole SP. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol Appl Pharmacol. 2005; 1;204(3):216-37.

40. Kim RB. Transporters and xenobiotic disposition. Toxicology. 2002; 181-182:291-7.

41. Hagenbuch B, Meier PJ. The superfamily of organic anion transporting polypeptides. Biochim Biophys Acta. 2003; 1609(1):1-18.

42. Mizuno N, Niwa T, Yotsumoto Y, Sugiyama Y. Impact of drug transporter studies on drug discovery and development. Pharmacol Rev. 2003; 55(3):425-61.

43. Lin JH, Yamazaki M. Role of P-glycoprotein in pharmacokinetics: clinical implications. Clin Pharmacokinet. 2003; 42(1):59-98.

44. Lin JH, Yamazaki M. Clinical relevance of P-glycoprotein in drug therapy. Drug Metab Rev. 2003; 35(4):417-54.

45. Meyer JH. Physiology of the Gastrointestinal Tract. Johnson, I.R. Ed. Raven Press, New York. 1987. pp 613-629.

46. Weisbrodt NW. Physiology of the Gastrointestinal Tract. Johnson, I.R. Ed. Raven Press, New York. 1987. pp 631-663.

47.Ohlman S, Albertioni F, Peterson C. Day-to-day variability in azathioprine pharmacokinetics in renal transplant recipients. Clin Transplant. 1994; 8(3 Pt1):217-23.

48. Lindholm A, Henricsson S, Lind M, Dahlqvist R. Intraindividual variability in the relative systemic availability of cyclosporin after oral dosing. Eur J Clin Pharmacol. 1988; 34(5):461-4.

49. Soons PA, Schoemaker HC, Cohen AF, Breimer DD. Intraindividual variability in nifedipine pharmacokinetics and effects in healthy subjects. J Clin Pharmacol.1992; 32(4):324-31.

50. Jacquet JM, Bressolle F, Galtier M, Bourrier M, Donadio D, Jourdan J, Rossi JF. Doxorubicin and doxorubicinol: intra- and inter-individual variations of pharmacokinetic parameters. Cancer Chemother Pharmacol. 1990;27(3):219-25.

51. Grahnén A, Hammarlund M, Lundqvist T. Implications of intraindividual variability in bioavailability studies of furosemide. Eur J Clin Pharmacol. 1984; 27(5):595-602.

52. Hartvig P, Simonsson B, Oberg G, Wallin I, Ehrsson H. Inter- and intraindividual differences in oral chlorambucil pharmacokinetics. Eur J Clin Pharmacol. 1988; 35(5):551-4.

53. Welling PG. Effects of food on drug absorption. Annu Rev Nutr.1996; 16:383-415.

54. Fleisher D, Li C, Zhou Y, Pao LH, Karim A. Drug, meal and formulation interactions influencing drug absorption after oral administration. Clinical implications. Clin Pharmacokinet. 1999; 36(3):233-54.

55. Yu LX, Straughn AB, Faustino PJ, Yang Y, Parekh A, et al. The effect of food on the relative bioavailability of rapidly dissolving immediate-release solid oral products containing highly soluble drugs. Mol Pharm. 200; 1(5):357-62.

56. Chaudhuri TK, Fink S. Update: pharmaceuticals and gastric emptying. Am J Gastroenterol. 1990; 85(3):223-30.

57. Lin JH, Chen IW, Vastag KJ, Ostovic D. pH-dependent oral absorption of L-735,524, a potent HIV protease inhibitor, in rats and dogs. Drug Metab Dispos. 1995; 23(7):730-5.

58. Cardot JM, Lecaillon JB, Czendlik C, Godbillon J. The influence of food on the disposition of the antiepileptic rufinamide in healthy volunteers. Biopharm Drug Dispos. 1998; 19(4):259-62.

59. McLean AJ, McNamara PJ, duSouich P, Gibaldi M, Lalka D. Food, splanchnic blood flow, and bioavailability of drugs subject to first-pass metabolism. Clin Pharmacol Ther. 1978; 24(1):5-10.

60. Olanoff LS, Walle T, Cowart TD, Walle UK, Oexmann MJ et al. Food effects on propranolol systemic and oral clearance: support for a blood flow hypothesis. Clin Pharmacol Ther. 1986 Oct; 40(4):408-14.

61. Melander A, McLean A. Influence of food intake on presystemic clearance of drugs. Clin Pharmacokinet. 1983; 8(4):286-96.

62. Kappas A, Anderson KE, Conney AH, Alvares AP. Influence of dietary protein and carbohydrate on antipyrine and theophylline metabolism in man. Clin Pharmacol Ther. 1976; 20(6):643-53.

63. Pantuck EJ, Pantuck CB, Garland WA, Min BH, Wattenberg LW, Anderson KE et al. Stimulatory effect of brussels sprouts and cabbage on human drug metabolism. Clin Pharmacol Ther. 1979; 25(1):88-95.

64. Kappas A, Alvares AP, Anderson KE, Pantuck EJ, Pantuck CB, Chang R, Conney AH. Effect of charcoal-broiled beef on antipyrine and theophylline metabolism. Clin Pharmacol Ther. 1978; 23(4):445-50.

65. Lown KS, Bailey DG, Fontana RJ, Janardan SK, Adair CH et al. Grapefruit juice increases felodipine oral availability in humans by decreasing intestinal CYP3A protein expression. J Clin Invest. 1997; 99(10):2545-53.

66. Dresser GK, Kim RB, Bailey DG. Effect of grapefruit juice volume on the reduction of fexofenadine bioavailability: possible role of organic anion transporting polypeptides. Clin Pharmacol Ther. 2005; 77(3):170-7.

67. Hirschhorn JN, Lohmueller K, Byrne E, Hirschhorn K. A comprehensive review of genetic association studies. Genet Med. 2002 Mar-Apr; 4(2):45-61.

68. Evans WE, McLeod HL. Pharmacogenomics--drug disposition, drug targets, and side effects. N Engl J Med. 2003; 348(6):538-49.

69. Drysdale CM, McGraw DW, Stack CB, Stephens JC, Judson RS et al. Complex promoter and coding region beta 2-adrenergic receptor haplotypes alter receptor expression and predict in vivo responsiveness. Proc Natl Acad Sci U S A. 2000; 97(19):10483-8.

70. Luan JA, Wong MY, Day NE, Wareham NJ. Sample size determination for studies of gene-environment interaction. Int J Epidemiol. 2001; 30(5):1035-40.

71. Kienle GS, Kiene H. The powerful placebo effect: fact or fiction? J Clin Epidemiol. 1997; 50(12):1311-8.

72. Sauro MD, Greenberg RP. Endogenous opiates and the placebo effect: a meta-analytic review. J Psychosom Res. 2005; 58(2):115-20.

73. Benedetti F. The opposite effects of the opiate antagonist naloxone and the cholecystokinin antagonist proglumide on placebo analgesia. Pain. 1996; 64(3):535-43.

74. Decosterd LA, Widmer N, André P, Aouri M, Buclin T. The emerging role of multiplex tandem mass spectrometry analysis for therapeutic drug monitoring and personalized medicine. Trends in Analytical Chemistry. 2016; 84: 5-13.

75. Kang JS, Lee MH. Overview of therapeutic drug monitoring. Korean J Intern Med. 2009; 24(1):1-10.

76. Gross AS. Best practice in therapeutic drug monitoring. Br J Clin Pharmacol. 1998; 46(2):95-9.

77. Veringa A, Sturkenboom MGG, Dekkers BGJ, Koster RA, Roberts JA et al. In: TrAC-Trends in Analytical Chemistry. 2016. 84, Part B, p. 34-40 7 p.

78. Mercier T, Tissot F, Gardiol C, Corti N, Wehrli S, et al. High-throughput hydrophilic interaction chromatography coupled to tandem mass spectrometry for the optimized quantification of the anti-Gram-negatives antibiotic colistin A/B and its pro-drug colistimethate. J Chromatogr A. 2014; 1369:52-63.

79. Soldin OP, Soldin SJ. Review: therapeutic drug monitoring in pediatrics. Ther Drug Monit. 2002; 24(1):1-8.

80. Clarke NJ. Mass Spectrometry in Precision Medicine: Phenotypic Measurements Alongside Pharmacogenomics. Clin Chem. 2016 Jan; 62(1):70-6. (Jaworska et al 2016, Biosensor, 6:47)

81. Jaworska A, Fornasaro S, Sergo V, Bonifacio A. Potential of Surface Enhanced Raman Spectroscopy (SERS) in Therapeutic Drug Monitoring (TDM). A Critical Review. Biosensors (Basel). 2016; 6(3).

82. Alvau MD, Tartaggia S, Meneghello A, Casetta B, Calia G et al. Enzyme-Based Electrochemical Biosensor for Therapeutic Drug Monitoring of Anticancer Drug Irinotecan. Anal Chem. 2018; 90(10):6012-6019.

83. Adaway JE, Keevil BG, Owen LJ. Liquid chromatography tandem mass spectrometry in the clinical laboratory. Ann Clin Biochem. 2015; 52(Pt 1):18-38.

84. Duarte C, Couto M, Ines L, Liang MH. Epidemiology of systemic lupus erythematosus. In: Lahita RG, Tsokos G, Buyon J, Koike T, eds. Systemic lupus erythematosus. 5th ed. London: Elsevier, 2011:673-96

85. Danchenko N, Satia JA, Anthony MS. Epidemiology of systemic lupus erythematosus: a comparison of worldwide disease burden. Lupus. 2006; 15(5):308-18.

86. Petri M. Epidemiology of systemic lupus erythematosus. Best Pract Res Clin Rheumatol. 2002 Dec; 16(5):847-58.

87. Buyon JP, Petri MA, Kim MY, Kalunian KC, Grossman J, et al. The effect of combined estrogen and progesterone hormone replacement therapy on disease activity in systemic lupus erythematosus: a randomized trial. Ann Intern Med. 2005;142(12 Pt1):953-62.

88. Chang DM, Lan JL, Lin HY, Luo SF. Dehydroepiandrosterone treatment of women with mild-to-moderate systemic lupus erythematosus: a multicenter randomized, double-blind, placebo-controlled trial. Arthritis Rheum 2002; 46:2924-7.

89. Moser KL, Kelly JA, Lessard CJ, Harley JB. Recent insights into the genetic basis of systemic lupus erythematosus. Genes Immun 2009; 10:373-9.

90. Crow MK. Collaboration, genetic associations, and lupus erythematosus. N Engl J Med. 2008; 358(9):956-61

91. Sullivan KE. Genetics of systemic lupus erythematosus: clinical implications. Rheum Dis Clin North Am 2000; 26:22956.

92. Roozendaal R, Carroll MC. Complement receptors CD21 and CD35 in humoral immunity. Immunol Rev. 2007; 219:157-66.

93. Manderson AP, Botto M, Walport MJ. The role of complement in the development of systemic lupus erythematosus. Annu Rev Immunol 2004; 22:431-56.

94. Harley JB, Moser KL, Gaffney PM, Behrens TW. The genetics of human systemic lupus erythematosus. Curr Opin Immunol 1998; 10:690-6.

95. Gorman CL, Russell AI, Zhang Z, Cunninghame GD, Cope AP, et al. Polymorphisms in the CD3Z gene influence TCRzeta expression in systemic lupus erythematosus patients and healthy controls. J Immunol 2008; 180:1060-70;

96. Tan W, Sunahori K, Zhao J, Deng Y, Kaufman KM, et al. Association of PPP2CA polymorphisms with SLE susceptibility in multiple ethnic groups. Arthritis Rheum. 2011; 63(11):3521;

97. Gateva V, Sandling JK, Hom G, Taylor KE, Chung SA, et al. A large-scale replication study identifiesTNIP1, PRDM1, JAZF1, UHRF1BP1 and IL10 as risk loci for systemic lupus erythematosus. Nat Genet. 2009; 41(11):1228-33.

98. Tenbrock K, Juang YT, Leukert N, Roth J, Tsokos GC. The transcriptional repressor cAMP response element modulator alpha interacts with histone deacetylase 1 to repress promoter activity. J Immunol 2006; 177:6159-64;

99. Rubin RL. Drug-induced lupus. Expert Opin Drug Saf. 2015; 14(3):361-78.

100. Toussirot E, Roudier J. Epstein-Barr virus in autoimmune diseases. Best Pract Res Clin Rheumatol. 2008; 22(5):883-96.

101. Ramos-Casals M, Cuadrado MJ, Alba P, Sanna G, Brito-Zerón P, et al. Acute viral infections in patients with systemic lupus erythematosus: description of 23 cases and review of the literature. Medicine (Baltimore). 2008; 87(6):311-8.

102. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1982; 25(11):1271-7.

103. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum. 1997; 40(9):1725.

104. Gómez-Martín D, Díaz-Zamudio M, Crispín JC, Alcocer-Varela J. Interleukin 2 and systemic lupus erythematosus: beyond the transcriptional regulatory net abnormalities. Autoimmun Rev. 2009; 9(1):34-9.

105. Dardalhon V, Korn T, Kuchroo VK, Anderson AC. Role of Th1 and Th17 cells in organ-specific autoimmunity. J Autoimmun. 2008; 31(3):252-6.

106. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, et al. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. N Engl J Med. 2003; 349(16):1526-33.

107. Kimberly RP. Immune complexes in the rheumatic diseases. Rheum Dis Clin North Am. 1987; 13(3):583-96.

108. Giannouli S, Voulgarelis M, Ziakas PD, Tzioufas AG. Anaemia in systemic lupus erythematosus: from pathophysiology to clinical assessment. Ann Rheum Dis. 2006; 65(2):144-8.

109. Rugarli C. Medicina interna sistematica. 7° edizione. Edra Masson. 2015.

110. Longo DL et al. Harrison. Principi di medicina interna. 18° edizione. Volume 1. Casa Editrice Ambrosiana. 2016.

111. Robbins e Cotran. Le basi patologiche delle malattie. Patologia generale. VIII edizione. Elsevier. 2011.

112. Pontieri GM, Russo MA, Frati L. Patologia generale. IV edizione. Piccin. 2010.

113. Rahman A, Jayasinghe R, Rajendran S. Paradoxical embolism via a patent foramen ovale: an important mechanism of cryptogenic strokes. Circ Cardiovasc Imaging. 2008; 1(2):e9-e10.

114. Canadian Hydroxychloroquine Study Group. A randomized study of the effect of withdrawing hydroxychloroquine sulfate in systemic lupus erythematosus. N Engl J Med. 1991; 324(3):150-4.

115. Achan J, Talisuna AO, Erhart A, Yeka A, Tibenderana JK, et al. Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria. Malar J. 2011; 10:144.

116. Parhizgar AR, Tahghighi A. Introducing New Antimalarial Analogues of Chloroquine and Amodiaquine: A Narrative Review. Iran J Med Sci. 2017; 42(2):115-128.

117. Tzekov R. Ocular toxicity due to chloroquine and hydroxychloroquine: electrophysiological and visual function correlates. Doc Ophthalmol. 2005; 110(1):111-20.

118. Alarcón GS, McGwin G, Bertoli AM, Fessler BJ, Calvo-Alén J et al. LUMINA Study Group. Effect of hydroxychloroquine on the survival of patients with systemic lupus erythematosus: data from LUMINA, a multiethnic US cohort (LUMINA L). Ann Rheum Dis. 2007; 66(9):1168-72.

119. Akhavan PS, Su J, Lou W, Gladman DD, Urowitz MB et al. The early protective effect of hydroxychloroquine on the risk of cumulative damage in patients with systemic lupus erythematosus. J Rheumatol. 2013; 40(6):831-41.

120. Qu Y, Noe G, Breaud AR, Vidal M, Clarke WA et al. Development and validation of a clinical HPLC method for the quantification of hydroxychloroquine and its metabolites in whole blood. Future Sci OA. 2015; 1(3): FSO26.

121. Ruiz-Irastorza G, Egurbide MV, Pijoan JI, Garmendia M, Villar I et al. Effect of antimalarials on thrombosis and survival in patients with systemic lupus erythematosus. Lupus. 2006; 15(9):577-83.

122. Bell CL. Hydroxychloroquine sulfate in rheumatoid arthritis: long-term response rate and predictive parameters. Am J Med. 1983; 75(1A):46-51.

123. McChesney EW, Conway WD, Banks WF Jr, Rogers JE, Shekosky JM. Studies of the metabolism of some compounds of the 4-amino-7-chloroquinoline series. J Pharmacol Exp Ther. 1966; 151(3):482-93.

124. Munster T, Gibbs JP, Shen D, Baethge BA, Botstein GR et al. Hydroxychloroquine concentration-response relationships in patients with rheumatoid arthritis. Arthritis Rheum. 2002;46(6):1460-9.

125. Tett SE, Cutler DJ, Day RO, Brown KF. Bioavailability of hydroxychloroquine tablets in healthy volunteers. Br J Clin Pharmacol. 1989; 27(6):771-9.

126. Costedoat-Chalumeau N, Galicier L, Aumaître O, Francès C, Le Guern V et al. Hydroxychloroquine in systemic lupus erythematosus: results of a French multicentre controlled trial (PLUS Study). Ann Rheum Dis. 2013; 72(11):1786-92.

127. Costedoat-Chalumeau N, Amoura Z, Hulot JS, Hammoud HA, Aymard G et al. Low blood concentration of hydroxychloroquine is a marker for and predictor of disease exacerbations in patients with systemic lupus erythematosus. Arthritis Rheum. 2006; 54(10) :3284-90.

128. Costedoat-Chalumeau N, Amoura Z, Hulot JS, Aymard G, Leroux G, Marra D, Lechat P, Piette JC. Very low blood hydroxychloroquine concentration as an objective marker of poor adherence to treatment of systemic lupus erythematosus. Ann Rheum Dis. 2007; 66(6):821-4.

129. Costedoat-Chalumeau N, LE Guern V, Piette JC. Routine Hydroxychloroquine Blood Concentration Measurement in Systemic Lupus Erythematosus Reaches Adulthood. J Rheumatol. 2015; 42(11) :1997-9.

130. Iredale J, Wainer IW. Determination of hydroxychloroquine and its major metabolites in plasma using sequential achiral-chiral high-performance liquid chromatography. J Chromatogr. 1992; 573(2):253-8.

131. Lim HS, Im JS, Cho JY, Bae KS, Klein TA et al. Pharmacokinetics of hydroxychloroquine and its clinical implications in chemoprophylaxis against malaria caused by Plasmodium vivax. Antimicrob Agents Chemother. 2009; 53(4):1468-75.

132. Moraes de Oliveira AR, Sueli Bonato P. Stereoselective determination of hydroxychloroquine and its major metabolites in human urine by solid-phase microextraction and HPLC. J Sep Sci. 2007; 30(15):2351-9.

133. Soichot M, Mégarbane B, Houzé P, Chevillard L, Fonsart J et al. Development, validation and clinical application of a LC-MS/MS method for the simultaneous quantification of hydroxychloroquine and its active metabolites in human whole blood. J Pharm Biomed Anal. 2014; 100:131-137.

134. Tett SE, Cutler DJ, Brown KF. High-performance liquid chromatographic assay for hydroxychloroquine and metabolites in blood and plasma, using a stationary phase of poly(styrene divinylbenzene) and a mobile phase at pH 11, with fluorimetric detection. J Chromatogr. 1985; 344:241-8.

135. Tett SE, Cutler DJ, Day RO, Brown KF. A dose-ranging study of the pharmacokinetics of hydroxy-chloroquine following intravenous administration to healthy volunteers. Br J Clin Pharmacol. 1988; 26(3):303-13.

136. Wang LZ, Ong RY, Chin TM, Thuya WL, Wan SC et al. Method development and validation for rapid quantification of hydroxychloroquine in human blood using liquid chromatography-tandem mass spectrometry. J Pharm Biomed Anal. 2012; 61:86-92.

137. Williams SB, Patchen LC, Churchill FC. Analysis of blood and urine samples for hydroxychloroquine and three major metabolites by high-performance liquid chromatography with fluorescence detection. J Chromatogr. 1988; 433:197-206.

138. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet. 1986; 1(8476):307-10.

139. Passing H, Bablok. A new biometrical procedure for testing the equality of measurements from two different analytical methods. Application of linear regression procedures for method comparison studies in clinical chemistry, Part I. J Clin Chem Clin Biochem. 1983; 21(11):709-20.

140. Furst DE. Pharmacokinetics of hydroxychloroquine and chloroquine during treatment of rheumatic diseases. Lupus. 1996;5 Suppl 1:S11-5.

141. Nakagawa M, Sugawara K, Goto T, Wakui H, Nunomura W. Hydroxychloroquine binding to cytoplasmic domain of Band 3 in human erythrocytes: Novel mechanistic insights into drug structure, efficacy and toxicity. Biochem Biophys Res Commun. 2016; 473(4):999-1004.

142. Stella C, Rudaz S, Mottaz M, Carrupt PA, Veuthey JL. Analysis of basic compounds at high pH values by reversed-phase liquid chromatography. J Sep Sci. 2004; 27(4):284-92.

143. Chen X, Deng P, Dai X, Zhong D. Simultaneous determination of amodiaquine and its active metabolite in human blood by ion-pair liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2007; 860(1):18-25.

144. Schill G. High performance ion-pair chromatography. J Biochem Biophys Methods.1989; 18(4):249-70.

145. Bidló-Iglóy M. Application of sodium lauryl sulphate ion-pairing reversed-phase high-performance liquid chromatography in the separation of some isomeric and other closely related basic compounds in pharmaceutical analysis. J Chromatogr. 1990; 520:367-71.

146. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/analytical-procedures-and-methods-validation-drugs-and-biologics,

147. Guideline on bioanalytical method validation, European medicines agency 2011

148. Singh A, Roopkishora, Singh CL, Gupta R, Kumar Set al. Development and Validation of Reversed-Phase High Performance Liquid Chromatographic Method for Hydroxychloroquine Sulphate. Indian J Pharm Sci. 2015; 77(5):586-91.

149. Spivak JL. Myeloproliferative Neoplasms. N Engl J Med. 2017; 376(22):2168-2181.

150. Tefferi A, Guglielmelli P, Larson DR, Finke C, Wassie EA et al. Long-term survival and blast transformation in molecularly annotated essential thrombocythemia, polycythemia vera, and myelofibrosis. Blood. 2014; 124(16):2507-13.

151. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood. 2002; 100(7):2292-302.

152. Cross NC, Reiter A. Tyrosine kinase fusion genes in chronic myeloproliferative diseases. Leukemia. 2002; 16(7):1207-12.

153. Delhommeau F, Pisani DF, James C, Casadevall N, Constantinescu S et al. Oncogenic mechanisms in myeloproliferative disorders. Cell Mol Life Sci. 2006; 63(24):2939-53.16(7):1207-

154. Cross NC, Daley GQ, Green AR, Hughes TP, Jamieson C et al. BCR-ABL1-positive CML and BCR-ABL1-negative chronic myeloproliferative disorders: some common and contrasting features. Leukemia. 2008; 22(11):1.

155. Spivak JL, Barosi G, Tognoni G, Barbui T, Finazzi G et al. Chronic myeloproliferative disorders. Hematology Am Soc Hematol Educ Program. 2003:200-24.975-89.

156. Levine RL, Gilliland DG. JAK-2 mutations and their relevance to myeloproliferative disease. Curr Opin Hematol. 2007; 14(1):43-7.

157. Cervantes F, Passamonti F, Barosi G. Life expectancy and prognostic factors in the classic BCR/ABL-negative myeloproliferative disorders. Leukemia. 2008; 22(5):905-14.

158. Mesa RA, Verstovsek S, Cervantes F, Barosi G, Reilly JT et al. International Working Group for Myelofibrosis Research and Treatment (IWG-MRT). Primary myelofibrosis (PMF), post polycythemia vera myelofibrosis (post-PV MF), post essential thrombocythemia myelofibrosis (post-ET MF), blast phase PMF (PMF-BP): Consensus on terminology by the international working group for myelofibrosis research and treatment (IWG-MRT). Leuk Res. 2007;31(6):737-40.

159. Barosi G. Myelofibrosis with myeloid metaplasia. Hematol Oncol Clin North Am. 2003;17(5):1211-26.

160. Tefferi A. The forgotten myeloproliferative disorder: myeloid metaplasia. Oncologist. 2003;8(3):225-31.

161. Tefferi A. New insights into the pathogenesis and drug treatment of myelofibrosis. Curr Opin Hematol. 2006; 13(2):87-92.

162. Martyré MC, Romquin N, Le Bousse-Kerdiles MC, Chevillard S, Benyahia B et al. Transforming growth factor-beta and megakaryocytes in the pathogenesis of idiopathic myelofibrosis. Br J Haematol. 1994 ;88(1):9-16.

163. Martyré MC, Le Bousse-Kerdiles MC, Romquin N, Chevillard S, Praloran V et al. Elevated levels of basic fibroblast growth factor in megakaryocytes and platelets from patients with idiopathic myelofibrosis. Br J Haematol. 1997; 97(2):441-8.

164. Villeval JL, Cohen-Solal K, Tulliez M, Giraudier S, Guichard J et al. High thrombopoietin production by hematopoietic cells induces a fatal myeloproliferative syndrome in mice. Blood. 1997; 90(11):4369-83.

165. Taskin AL, Cohen-Solal K, Le Couedic JP, Abina MA, Villeval JL et al. Myelofibrosis: experimental models and human studies. Stem Cells. 1998; 16 Suppl 2:155-64.

166. Vannucchi AM, Bianchi L, Paoletti F, Pancrazzi A, Torre E et al. A pathobiologic pathway linking thrombopoietin, GATA-1, and TGF-beta1 in the development of myelofibrosis. Blood. 2005; 105(9):3493-501.

167. Gilbert HS, Praloran V, Stanley ER. Increased circulating CSF-1 (M-CSF) in myeloproliferative disease: association with myeloid metaplasia and peripheral bone marrow extension. Blood. 1989;74(4):1231-4.

168. Mesa RA, Hanson CA, Ketterling RP, Schwager S, Knudson RA, Tefferi A. Trisomy 13: prevalence and clinicopathologic correlates of another potentially lenalidomide-sensitive cytogenetic abnormality. Blood. 2009;113(5):1200-1.

169. Reilly JT. Cytogenetic and molecular genetic aspects of idiopathicmyelofibrosis. Acta Haematol. 2002;108(3):113-9.

170. Tefferi A. Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1. Leukemia. 2010;24(6):1128-38.

171. Dupriez B, Morel P, Demory JL, Lai JL, Simon M et al. Prognostic factors in agnogenic myeloid metaplasia: a report on 195 cases with a new scoring system. Blood. 1996;88(3):1013-8.

172. Barosi G, Viarengo G, Pecci A, Rosti V, Piaggio G, Marchetti M, Frassoni F. Diagnostic and clinical relevance of the number of circulating CD34(+) cells in myelofibrosis with myeloid metaplasia. Blood. 2001;98(12):3249-55.

173. Prchal JF, Axelrad AA. Letter: Bone-marrow responses in polycythemia vera. NEngl J Med. 1974;290(24):1382.

174. Marchioli R, Finazzi G, Landolfi R, Kutti J, Gisslinger H et al. Vascular and neoplastic risk in a largecohort of patients with polycythemia vera. J Clin Oncol. 2005;23(10):2224-32.

175. Tefferi A, Rumi E, Finazzi G, Gisslinger H, Vannucchi AM et al. Survival and prognosis among 1545 patients with contemporary polycythemia vera: an international study. Leukemia. 2013; 27(9):1874-81.

176. Anand S, Stedham F, Gudgin E, Campbell P, Beer P, Green AR, Huntly BJ.Increased basal intracellular signaling patterns do not correlate with JAK2genotype in human myeloproliferative neoplasms. Blood. 2011;118(6):1610-21.

177. Komura E, Tonetti C, Penard-Lacronique V, Chagraoui H, Lacout C et al. Role for the nuclear factor kappaB pathway in transforming growth factor-beta1 production in idiopathic myelofibrosis: possible relationship with FK506 binding protein 51overexpression. Cancer Res. 2005;65(8):3281-9.

178. Shilling AD, Nedza FM, Emm T, Diamond S, McKeever E et al. Metabolism, excretion, and pharmacokinetics of [14C] INCB018424, a selective Janus tyrosine kinase 1/2 inhibitor, in humans. Drug Metab Dispos. 2010; 38(11):2023-31.

179. Mesa RA, Yasothan U, Kirkpatrick P. Ruxolitinib. Nat Rev Drug Discov. 2012; 11(2):103-4.

180. Verstovsek S, Mesa RA, Gotlib J, Levy RS, Gupta V et al. A double-blind, placebo-controlled trial of ruxolitinib for myelofibrosis. N Engl J Med. 2012; 366(9):799-807.

181. Harrison C, Vannucchi AM. Ruxolitinib: a potent and selective Janus kinase 1 and 2 inhibitor in patients with myelofibrosis. An update for clinicians. Ther Adv Hematol. 2012; 3(6):341-54.

182. Cervantes F, Vannucchi AM, Kiladjian JJ, Al-Ali HK, Sirulnik A et al. Three-year efficacy, safety, and survival findings from COMFORT-II, a phase 3 study comparing ruxolitinib with best available therapy for myelofibrosis. Blood. 2013 Dec 12;122(25):4047-53.

183. Verstovsek S, Mesa RA, Gotlib J, Levy RS, Gupta V et al. Efficacy, safety and survival with ruxolitinib in patients with myelofibrosis: results of a median 2-year follow-up of COMFORT-I. Haematologica. 2013; 98(12):1865-71.

184. Bryan JC, Verstovsek S. Overcoming treatment challenges in myelofibrosis and polycythemia vera: the role of ruxolitinib. Cancer Chemother Pharmacol. 2016; 77(6):1125-42.

185. Alimam S, Harrison C. Experience with ruxolitinib in the treatment of polycythaemia vera. Ther Adv Hematol. 2017; 8(9):273.

186. Mesa RA, Cortes J. Optimizing management of ruxolitinib in patients with myelofibrosis: the need for individualized dosing. J Hematol Oncol. 2013; 6:79.

187. Josephs DH, Fisher DS, Spicer J, Flanagan RJ. Clinical pharmacokinetics of tyrosine kinase inhibitors: implications for therapeutic drug monitoring. Ther Drug Monit. 2013; 35(5):562-87.

188. Lakshmi B, Saraswathi K, and Reddy TV. Rp-hplc method development and validation for the analysis of ruxolitinib in pharmaceutical dosage forms. International Journal of Research and Reviews in Applied Sciences 2012; 2 (2):360–369.

189. Veeraraghavan S, Thappali S, Viswanadha S, Chennupati S, Nalla S et al. Simultaneous quantification of ruxolitinib and nilotinib in rat plasma by LC-MS/MS: application to a pharmacokinetic study. J Pharm Biomed Anal. 2014; 94:125-31.

190. Nandihalli, U, Tarnowski T, Cape S, Dorshorst D, and Ledvina A. Determination of Ruxolitinib in human plasma by LC-MS/MS. 2015. Paper presented at 9th Workshop on Recent Issues in bioanalysis, Miami, USA, April 13-17.

191. Charlier B, Pingeon M, Dal Piaz F, Conti V, Valentini G, Filippelli A, Izzo V. Development of a novel ion-pairing HPLC-FL method for the separation and quantification of hydroxychloroquine and its metabolites in whole blood. Biomed Chromatogr. 2018; 32(8):e4258.

192. Savic IM, Nikolic VD, Savic IM, NikolicLB, StankovicMZ. Development and validation of a new RP-HPLC method for determination of quercetin in green tea. Journal of Analytical Chemistry.2013; 68(10):906–911.

193. Savic IM, Nikolic VD, Savic IM, Nikolic LB, Stankovic MZ. Development and validation of HPLC method for the determination of amygdalin in the plant extract ofplum kernel. Research Journal of Chemistry and Environment.2012; 16(4):80-86.

194. Dolan JW. Column plate number and system suitability. LCGC.2016; 34(3):190–197.

195. Rousseau A, Marquet P. Application of pharmacokinetic modelling to the routine therapeutic drug monitoring of anticancer drugs. Fundamental and Clinical Pharmacology 2002; 16(4):253–262.

196. Lennard L. Therapeutic drug monitoring of cytotoxic drugs. British Journal of ClinicalPharmacology. 2008; 52(S1):75–87S.

197. Gao B, Yeap S, Clements A, Balakrishnar B, Wong M et al. Evidence for therapeutic drug monitoring of targeted anticancer therapies. J Clin Oncol. 2012;30(32):4017-25.

198. Shah RR, Morganroth J, Shah DR. Cardiovascular safety of tyrosine kinase inhibitors: with a special focus on cardiac repolarisation (QT interval). Drug Saf. 2013;36(5):295-316.

199. Herviou P, Thivat E, Richard D, Roche L, Dohou J et al. Therapeutic drug monitoring and tyrosine kinase inhibitors. Oncol Lett. 2016;12(2):1223-1232.

200. Masson E, Zamboni WC. Pharmacokinetic optimisation of cancer chemotherapy. Effect on outcomes. Clin Pharmacokinet. 1997;32(4):324-43.

201. Canal P, Chatelut E, Guichard S. Practical treatment guide for dose individualisation in cancer chemotherapy. Drugs. 1998;56(6):1019-38.

202. Paci A, Veal G, Bardin C, Levêque D, Widmer N et al. Review of therapeutic drug monitoring of anticancer drugs part 1--cytotoxics. Eur J Cancer. 2014;50(12):2010-9.

203. Capitain O, Boisdron-Celle M, Poirier AL, Abadie-Lacourtoisie S, Morel A et al. The influence of fluorouracil outcome parameters on tolerance and efficacy in patients with advanced colorectal cancer. Pharmacogenomics J. 2008;8(4):256-67.

204. Johnston PG, Kaye S. Capecitabine: a novel agent for the treatment of solid tumors. Anticancer Drugs. 2001;12(8):639-46.

205. Giacchetti S, Perpoint B, Zidani R, Le Bail N, Faggiuolo R et al. Phase III multicentre randomized trial of oxaliplatin added to chrono modulated fluorouracil-leucovorin as first-line treatment of metastatic colorectal cancer. J Clin Oncol. 2000; 18(1):136-47.

206. Douillard JY, Cunningham D, Roth AD, Navarro M, James RD et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. Lancet. 2000;355(9212):1372.

207. Wohlhueter RM, McIvor RS, Plagemann PG. Facilitated transport of uracil and5-fluorouracil, and permeation of orotic acid into cultured mammalian cells. J Cell Physiol. 1980;104(3):309-19.

208. Bajetta E, Carnaghi C, Somma L, Stampino CG. A pilot safety study of capecitabine, a new oral fluoropyrimidine, in patients with advanced neoplastic disease. Tumori. 1996;82(5):450-2.

209. Longley DB, Harkin DP, Johnston PG. 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer. 2003; 3(5):330-8.

210. Heggie GD, Sommadossi JP, Cross DS, Huster WJ, Diasio RB. Clinical pharmacokinetics of 5-fluorouracil and its metabolites in plasma, urine, and bile. Cancer Res. 1987;47(8):2203-6.

211. Diasio RB,Harris BE. Clinical pharmacology of 5-fluorouracil. Clin Pharmacokinet. 1989 Apr;16(4):215-37.

212. Sanno Y, Holzer M, Schimke RT. Studies of a mutation affecting pyrimidine degradation in inbred mice. J Biol Chem. 1970 Nov 10;245(21):5668-76.

213. Schilsky RL. Biochemical and clinical pharmacology of 5-fluorouracil. Oncology (Williston Park). 1998 Oct;12(10 Suppl 7):13-8.

214. Kaldate RR, Haregewoin A, Grier CE, Hamilton SA, McLeod HL. Modeling the 5-fluorouracil area under the curve versus dose relationship to develop a pharmacokinetic dosing algorithm for colorectal cancer patients receiving FOLFOX6. Oncologist. 2012; 17:296–302.

215. Meulendijks D, Henricks LM, Sonke GS, Deenen MJ, Froehlich TK et al. Clinical relevance of DPYD variants c.1679T>G, c.1236G>A/HapB3, and c.1601G>A as predictors of severe fluoropyrimidine-associated toxicity: a systematic review and meta-analysis of individual patient data. Lancet Oncol. 2015;16(16):1639-50.

216. Lee JJ, Beumer JH, Chu E. Therapeutic drug monitoring of 5-fluorouracil. Cancer Chemother Pharmacol. 2016;78(3):447-64.

217. Dean L. Fluorouracil Therapy and DPYD Genotype. 2016 Nov 3. In: Pratt V, McLeod H, Rubinstein W, Dean L, Kattman B, Malheiro A, editors. Medical Genetics Summaries [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 2012-. Available from http://www.ncbi.nlm.nih.gov/books/NBK395610/ PubMed PMID: 28520376

218. Caudle KE, Thorn CF, Klein TE, Swen JJ, McLeod HL et al. Clinical Pharmacogenetics Implementation Consortium guidelines for dihydropyrimidine dehydrogenase genotype and fluoropyrimidine dosing. Clin Pharmacol Ther. 2013;94(6):640-5.

219. Meulendijks D, Henricks LM, Jacobs BAW, Aliev A, Deenen MJ et al. Pretreatment serum uracil concentration as a predictor of severe and fatal fluoropyrimidine-associated toxicity. Br J Cancer. 2017;116(11):1415-1424.

220. Zhou ZW, Wang GQ, Wan de S, Lu ZH, Chen YB et al. The dihydrouracil/uracil ratios in plasma and toxicities of 5-fluorouracil-basedadjuvant chemotherapy in colorectal cancer patients. Chemotherapy.2007;53(2):127-31.

221. Wettergren Y, Carlsson G, Odin E, Gustavsson B. Pretherapeutic uracil and dihydrouracil levels of colorectal cancer patients are associated with sex and toxic side effects during adjuvant 5-fluorouracil-based chemotherapy. Cancer.2012;118(11):2935-43.

222. Galarza AF, Linden R, Antunes MV, Hahn RZ, Raymundo S et al. Endogenous plasma and salivary uracil to dihydrouracil ratios and DPYD genotyping as predictors of severe fluoropyrimidine toxicity in patients with gastrointestinal malignancies. ClinBiochem. 2016;49(16-17):1221-1226.

223. Ciccolini J, Mercier C, Evrard A, Dahan L, Boyer JC et al. Arapid and inexpensive method for anticipating severe toxicity to fluorouracil and fluorouracil-based chemotherapy. Ther Drug Monit. 2006;28(5):678-85.

224. van Staveren MC, van Kuilenburg AB, Guchelaar HJ, Meijer J, Punt CJ et al. Evaluation of an oral uracil loading test toidentify DPD-deficient patients using a limited sampling strategy. Br J ClinPharmacol. 2016;81(3):553-61.

225. Freeman K, Connock M, Cummins E, Gurung T, Taylor-Phillips S et al. Fluorouracil plasma monitoring: systematic review and economic evaluation of the My5-FU assay for guiding dose adjustment inpatients receiving fluorouracil chemotherapy by continuous infusion. Health Technol Assess. 2015;19(91):1-321, v-vi.

226. Gamelin E, Boisdron-Celle M. Dose monitoring of 5-fluorouracil in patients with colorectal or head and neck cancer--status of the art. Crit Rev Oncol Hematol. 1999;30(1):71-9.

227. Tsalic M, Bar-Sela G, Beny A, Visel B, Haim N. Severe toxicity related to the 5-fluorouracil/leucovorin combination (the Mayo Clinic regimen): a prospective study in colorectal cancer patients. Am J Clin Oncol. 2003;26(1):103-6.

228. Marques RP, Duarte GS, Sterrantino C, Pais HL, Quintela A et al. Triplet (FOLFOXIRI) versus doublet (FOLFOX or FOLFIRI) backbone chemotherapy as first-line treatment of metastatic colorectal cancer: A systematic review and meta-analysis. Crit Rev Oncol Hematol. 2017;118:54-62.

229. Murphy RF, Balis FM, Poplack DG. Stability of 5-fluorouracil in whole blood and plasma. Clin Chem. 1988; 34(7):1505

230. Breda M, Barattè S. A review of analytical methods for the determination of5-fluorouracil in biological matrices. Anal Bioanal Chem. 2010;397(3):1191-201.

231. Rotschafer JC, Andes RD, Rodvold AK. Antibiotic Pharmacodynamics. Humana press. 2016 eBook ISBN: 978-1-4939-3323-5

232. Rivera-Chaparro ND, Cohen-Wolkowiez M, Greenberg RG. Dosing antibiotics in neonates: review of the pharmacokinetic data. Future Microbiol. 2017; 12:1001-1016.

233. Roberts JA, Norris R, Paterson DL, Martin JH. Therapeutic drug monitoring of antimicrobials. Br J Clin Pharmacol. 2012;73(1):27-36.

234. Grüner N, Stambouli O, Ross RS. Dried blood spots--preparing and processing for use in immunoassays and in molecular techniques. J Vis Exp. 2015; 97:52619.

235. Zakaria R, Allen KJ, Koplin JJ, Roche P, Greaves RF. Advantages and Challenges of Dried Blood Spot Analysis by Mass Spectrometry Across the Total Testing Process. EJIFCC. 2016;27(4):288-317.

236. Pauwels S, Allegaert K. Therapeutic drug monitoring in neonates. Arch Dis Child. 2016;101(4):377-81.

237. O'Connor WJ, Warren GH, Edrada LS, Mandala PS, Rosenman SB. Serum concentrations of sodium nafcillin in infants during the perinatal period. Antimicrob Agents Chemother (Bethesda). 1965;5:220-2.

238. Silverio J, Poole JW. Serum concentrations of ampicillin in newborn infants after oral administration. Pediatrics. 1973;51(3):578-80.

239. Zanon D, Galenica pediatrica, I Corso superiore Sifo in farmacia clinica, edizione 2017-2018 ottobre 17-19, 2017, Catania (IT), pp. 1-122.

240. Kearns GL, Winter HS. Proton pump inhibitors in pediatrics: relevant pharmacokinetics and pharmacodynamics. J Pediatr Gastroenterol Nutr. 2003 Nov-Dec;37 Suppl 1:S52-9.

241. Hall EM, Flores SR, De Jesús VR. Influence of Haematocrit and Total-Spot Volume on Performance Characteristics of Dried Blood Spots for Newborn Screening. Int J Neonatal Screen. 2015; 1(2):69-78.

242. McNamara JO. In: Pharmacotherapy of the Epilepsies. Goodman & Gilman's The Pharmacological Basis of Therapeutics. 12th ed. L. Brinton, B. Chamber, B. Knoll man Eds. New York: 2011; pp 5934.

243. Ferrendelli JA. Pharmacology of antiepileptic drugs. Epilepsia. 1987;28 Suppl 3:S14-6.

244. Bartha AI, Shen J, Katz KH, Mischel RE, Yap KR et al. Neonatal seizures: multicenter variability in current treatment practices. Pediatr Neurol. 2007; 37(2):85-90.

245. Gilman JT, Gal P, Duchowny MS, Weaver RL, Ransom JL. Rapid sequential phenobarbital treatment of neonatal seizures. Pediatrics. 1989; 83(5):674-8.

246. Ouvrier RA, Goldsmith R. Phenobarbitone dosage in neonatal convulsions. Arch Dis Child. 1982; 57(9):653-7.

247. Neonatal Formulary. 6th ed. John Wiley & Sons, Limited European Distribution Centre New Era Estate, Oldlands Way Bognor Regis, West Sussex, PO22 9NQ, UK. 2011; pp 400-2.

248. Young TE, Mangum B. Neofax, A Manual of Drugs used in neonatal care. CNS Drugs. 23rd ed. Thomson Reuters, Montvale 07645, New Jersey, USA: 2010; pp 232-3.

249. Touw DJ, Graafland O, Cranendonk A, Vermeulen RJ, van Weissenbruch MM. Clinical pharmacokinetics of phenobarbital in neonates. Eur J Pharm Sci. 2000; 12(2):111-6.

250. Kuzemko JA, Paala J. Apnoeic attacks in the newborn treated with aminophylline. Arch Dis Child. 1973; 48(5):404-6.

251. Schmidt B, Davis PG, Roberts RS. Timing of caffeine therapy in very low birth weight infants. J Pediatr. 2014; 164(5):957-8.

252. Aranda JV, Gorman W, Bergsteinsson H, Gunn T. Efficacy of caffeine in treatment of apnea in the low-birth-weight infant. J Pediatr. 1977; 90(3):467-72.

253. Kreutzer K, Bassler D. Caffeine for apnea of prematurity: a neonatal success story. Neonatology. 2014;105(4):332-6.

254. Schoen K, Yu T, Stockmann C, Spigarelli MG, Sherwin CM. Use of methylxanthine therapies for the treatment and prevention of apnea of prematurity. Paediatr Drugs. 2014; 16(2):169-77.

255. Dobson NR, Hunt CE. Pharmacology review: Caffeine use in neonates: Indications, pharmacokinetics, clinical effects, outcomes. NeoReviews. 2013 ;14(11):e540-e550

256. Clark RH, Bloom BT, Spitzer AR, Gerstmann DR. Reported medication use in the neonatal intensive care unit: data from a large national data set. Pediatrics. 2006; 117(6):1979-87.

257. Hsieh EM, Hornik CP, Clark RH, Laughon MM, Benjamin DK Jr et al. Best Pharmaceuticals for Children Act—Pediatric Trials Network. Medication use in the neonatal intensive care unit. Am J Perinatol. 2014; 31(9):811-21.

258. Fredholm BB, Bättig K, Holmén J, Nehlig A, Zvartau EE. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol Rev. 1999; 51(1):83-133.

259. Mathew OP. Apnea of prematurity: pathogenesis and management strategies. J Perinatol. 2011; 31(5):302-10.

260. Di Fiore JM, Martin RJ, Gauda EB. Apnea of prematurity--perfect storm. Respir Physiol Neurobiol. 2013; 189(2):213-22.

261. Walsh MC, Morris BH, Wrage LA, Vohr BR, Poole WK et al. National Institutes of Child Health and Human Development Neonatal Research Network. Extremely low birthweight neonates with protracted ventilation: mortality and 18-month neurodevelopmental outcomes. J Pediatr. 2005; 146(6):798-804.

262. Groothuis JR, Gutierrez KM, Lauer BA. Respiratory syncytial virus infection in children with bronchopulmonary dysplasia. Pediatrics. 1988; 82(2):199-203.

263. Cristea AI, Carroll AE, Davis SD, Swigonski NL, Ackerman VL. Outcomes of children with severe bronchopulmonary dysplasia who were ventilator dependent at home. Pediatrics. 2013; 132(3):e727-34.

264. Ehrenkranz RA, Walsh MC, Vohr BR, Jobe AH, Wright LL et al. National Institutes of Child Health and Human Development Neonatal Research Network. Validation of the National Institutes of Health consensus definition of bronchopulmonary dysplasia. Pediatrics. 2005; 116(6):1353-60.

265. Hughes CA, O'Gorman LA, Shyr Y, Schork MA, Bozynski ME, McCormick MC. Cognitive performance at school age of very low birth weight infants with bronchopulmonary dysplasia. J Dev Behav Pediatr. 1999; 20(1):1-8.

266. McDonald FB, Dempsey EM, O'Halloran KD. Caffeine therapy for apnea of prematurity: Wake up to the fact that sex matters. Exp Physiol. 2018; 103(10):1294-1295.

267. Shenk EE, Bondi DS, Pellerite MM, Sriram S. Evaluation of Timing and Dosing of Caffeine Citrate in Preterm Neonates for the Prevention of Bronchopulmonary Dysplasia. J Pediatr Pharmacol Ther. 2018; 23(2):139-145.

268. Picone S, Bedetta M, Paolillo P. Caffeine citrate: when and for how long. A literature review. J Matern Fetal Neonatal Med. 2012; 25 Suppl 3:11-4.

269. Darnall RA, Kattwinkel J, Nattie C, Robinson M. Margin of safety for discharge after apnea in preterm infants. Pediatrics. 1997; 100(5):795-801.

270. Rhein LM, Dobson NR, Darnall RA, Corwin MJ, Heeren TC et al. Caffeine Pilot Study Group. Effects of caffeine on intermittent hypoxia in infants born prematurely: a randomized clinical trial. JAMA Pediatr. 2014; 168(3):250-7.

271. Allegaert K, van de Velde M, van den Anker J. Neonatal clinical pharmacology. Paediatr Anaesth. 2014; 24(1):30-8.

272. Kearns GL, Abdel-Rahman SM, Alander SW, Blowey DL, Leeder JS et al. Developmental pharmacology--drug disposition, action, and therapy in infants and children. N Engl J Med. 2003; 349(12):1157-67.

273. Smits A, Kulo A, de Hoon JN, Allegaert K. Pharmacokinetics of drugs in neonates: pattern recognition beyond compound specific observations. Curr Pharm Des. 2012; 18(21):3119-46.

274. Jacqz-Aigrain E, Leroux S, Zhao W, van den Anker JN, Sharland M. How to use vancomycin optimally in neonates: remaining questions. Expert Rev Clin Pharmacol. 2015; 8(5):635-48.

275. Barco S, Castagnola E, Moscatelli A, Rudge J, Tripodi Get al. Volumetric adsorptive microsampling-liquid chromatography tandem mass spectrometry assay for the simultaneous quantification of four antibiotics in human blood: Method development, validation and comparison with dried blood spot. J Pharm Biomed Anal. 2017; 145:704-710.

Pubblicazioni

1. Charlier B, Marino L, Dal Piaz F, **Pingeon M,** Coglianese A, Izzo B, Serio B, Selleri C, Filippelli A, Izzo V. Development and Validation of a Reverse-Phase High-Performance Liquid Chromatography with Fluorescence Detection (RP-HPLC-FL) Method to Quantify Ruxolitinib in Plasma Samples. Analytical Letters. 2019;52:8,1328-1339.

2. Charlier B, **Pingeon M**, Dal Piaz F, Conti V, Valentini G, Filippelli A, Izzo V. Development of a novel ion-pairing HPLC-FL method for the separation and quantification of hydroxychloroquine and its metabolites in whole blood. Biomed Chromatogr. 2018;32(8):e4258.

3. Izzo V, Charlier B, Bloise E, **Pingeon M**, Romano M, Finelli A, Vietri A, Conti V, Manzo V, Alfieri M, Filippelli A, Dal Piaz F. A UHPLC-MS/MS-based method for the simultaneous monitoring of eight antiblastic drugs in plasma and urine of exposed healthcare workers. J Pharm Biomed Anal. 2018;154:245-251.

4. Iudici M, Pantano I, Fasano S, Pierro L, Charlier B, **Pingeon M**, Dal Piaz F, Filippelli A, Izzo V. Health status and concomitant prescription of immunosuppressants are risk factors for hydroxychloroquine non-adherence in systemic lupus patients with prolonged inactive disease. Lupus 2018;27(2):265-272.

5. **Pingeon M**, Charlier B, De Lise F, Mensitieri F, Dal Piaz F, Izzo V. Novel Drug Targets for the Treatment of Cardiac Diseases. Curr Pharmacogenomics Person Med. 2017;15(1):32-44.

6. Bystry V., Reigl T., Krejci A., Demko M., Hanakova B., Grioni A., Knecht H., Schlitt M., Dreger P., Sellner L., Herrmann D., **Pingeon M.**, Boudjoghra M., Rijntjes J., Pott C., Langerak A.W., Groenen P.J.T.A., Davi F., Brüggemann M., Darzentas N.; EuroClonality-NGS. *ARResT/Interrogate: an interactive immunoprofiler for IG/TR NGS data.* Bioinformatics (2017) 33(3):435-437. doi: 10.1093/bioinformatics/btw634.

Abstracts presentati in Congressi Nazionali

1. Dal Piaz F, Charlier B, Marino L, **Pingeon M**, Izzo B, Vaccaro E, Manzo V, Conti V, Selleri C, Filippelli A, Izzo V. "Therapeutic Drug Monitoring of tyrosine kinase inhibitors: a novel RP-HPLC FL method to quantify ruxolitinib in plasma samples". 50° Congresso Nazionale della Società Italiana di Biochimica Clinica e Biologia Molecolare Clinica (SIBIOC), Napoli (Italia), 16-18 Ottobre 2018.

2. Izzo V, Conte C, Charlier B, **Pingeon M**, Coglianese A, Corbo G, Di Martino A, Iannaccone T, Filippelli A, Dal Piaz F. "Dried blood spot sampling coupled with tandem mass spectrometry for neonatal therapeutic drug monitoring". 50° Congresso Nazionale della

Società Italiana di Biochimica Clinica e Biologia Molecolare Clinica (SIBIOC), Napoli (Italia), 16-18 Ottobre 2018.

3. **Pingeon M**, Charlier B, Iudici M, Dal Piaz F, Finelli A, Izzo V, Filippelli A. Health status and concomitant prescription of immunosuppressants are risk factors for hydroxychloroquine non-adherence in SLE patients with prolonged inactive disease. 38° Congresso Nazionale della Società Italiana di Farmacologia, Rimini (Italia), 25-28 Ottobre 2017.

4. Izzo V, Charlier B, **Pingeon M**, Romano M, Finelli A, Conti V, Filippelli A, Dal Piaz F. Biological and environmental monitoring of antiblastic agents for workers safety evaluation. 49° Congresso Nazionale della Società Italiana di Biochimica Clinica e Biologia Molecolare (SIBIOC), Firenze (Italia), 16-18 Ottobre 2017.

Remerciements

Je me suis longuement demandée comment résumer en quelques lignes ces quatre dernières de ma vie qui se sont révélées riches de connaissances, d'émotions et de rebondissements. Quatre années durant lesquelles j'ai fait des rencontres formidables tant humainement que professionnellement. Quatre années qui n'ont pas toujours été « toutes roses » et au cours desquelles j'ai appris à repousser toujours plus loin mes limites dans l'unique objectif de servir ce métier passionnant qu'est la recherche. Il est évident que le fruit de tous ces efforts et sacrifices est le résultat d'une motivation acharnée qui ne s'est pas forgée sans une aide extérieure. C'est pourquoi je souhaite vous adresser quelques mots pour m'avoir aidée dans ce parcours.

Je souhaite tout d'abord remercier la **Prof. Amelia Filippelli** pour m'avoir accueillie au sein de son groupe de recherche et de m'avoir permis de réaliser ce doctorat.

Je souhaite bien évidemment remercier mes chefs, **Dr. Viviana Izzo** et le **Dr. Fabrizio Dal Piaz**. Je ne sais pas par où commencer tant l'aventure a été riche. Allons-y par étapes…

Viviana merci de tous ce que tu as fait pour moi lors de mon arrivée en Italie. Merci d'avoir permis que je ne me sente pas trop dépaysée et de m'avoir aidée dans ce premier parcours du combattant qui n'est autre que la bureaucratie. Merci d'avoir toujours été là dans les étapes de ma vie qu'elles soient heureuses ou non.

Viviana et Fabrizio, je vous remercie de m'avoir fait grandir tant sur le plan personnel que sur le plan professionnel même si cela nous a valu quelques discussions. Merci d'avoir été disponibles, compréhensifs et de m'avoir transmis votre savoir.

Je souhaite également remercier le **Dr. Bruno Charlier**. Nous sommes bien loin de nos débuts en tête à tête avec l'HPLC où tu m'appelais Wilson. Merci Bru, pour m'avoir enseigné les rudiments de l'HPLC et bien plus encore. Je sais que cela va faire un peu redondant comme l'est notre code génétique mais c'est bien parce que nous sommes un groupe de recherche que nous nous sommes tous serrés les coudes. Alors merci d'avoir été présent pour moi depuis le début de l'aventure. Comme quand tu t'es précipité à l'hôpital pour voir ta « Strapagnon ».

Je souhaite également remercier mes collègues de travail Le **Dr. Albino Coglianese**, la **Dr. Valentina Giudice** et la **Dr. Viviana Fortunato** qui font que chaque jour de travail est un jour de plaisir partagé.

Vale, merci de ton aide incommensurable lors des nombreuses traductions, relectures et de m'avoir sauvée plus d'une fois de mes traitements homéopathiques.

Albi, merci pour tout y compris les nombreuses heures de babysitting gratis. Tu es une personne pleine de qualités, reste comme tu es.

Vivi, merci de ton aide dans la relecture des remerciements et d'avoir été cette oreille attentive, reste celle que tu es.

Francesca bien que nous ayons passé peu de temps ensemble, je te remercie de ces petites attentions que tu as eues.

Je remercie aussi toutes les personnes qui ont fait un petit bout de chemin avec nous, que nous avons eu la chance de former et qui nous ont également permis de grandir. Marianna, Antonietta Carmela, Marisa, Elena…

Je remercie également **l'équipe de pharmacogénétique** de l'hôpital San Giovanni Dio e Ruggi D'Aragona qui sont toujours prêts pour un petit « casse-croute » tous ensemble.

Je souhaite également remercier mes amies Rossella et Zandra qui ont toujours été présentes pour moi et m'ont permis de rendre mon séjour en Italie agréable.

Rosse merci de m'avoir accueillie au sein de ta famille comme si j'en faisais partie. Ti voglio tanto bene.

Zandra, gracias por los dias que hemos trascorso juntos visitando y descubriendo este maravilloso país. Nos vemos pronto para descubrir el tuyo.

Enfin, je remercie ma famille et mon compagnon pour m'avoir toujours suivie dans mes choix même au prix de lourds sacrifices.

Une pensée toute particulière va à ma mère qui nous a toujours poussées à donner le meilleur de nous-mêmes, qui nous a appris que dans la vie rien ne tombe du ciel et que si l'on désir quelque chose il faut se battre. Merci **Maman** pour l'énergie que tu nous insuffles et de tes sacrifices pour avoir fait de nous qui nous sommes. Je t'aime.

Chouchou, je te remercie de m'avoir laissé aller jusqu'au bout de mes rêves, de m'avoir permis d'aller jusqu'au bout de mes ambitions sans jamais me ressentir le poids de mes choix. Je sais que ces quatre dernières années, aussi riches et belles qu'elles aient pu être au travers de la naissance de notre fils, de nos découvertes italiennes, ont également été le fruit de choix difficiles. Alors merci pour tout.

Papa, je te remercie d'affronter ce voyage et d'être présent pour moi ce jour-là.

Iris et **Coline** même si nous ne sommes pas toujours d'accords je suis fière de vous avoir comme sœurs. Je vous aime plus que tout. Alors plus de chute sur le tête Ok…

Papi, merci de m'avoir donné ce coup de pouce au début sans lequel je n'aurais jamais pu partir. Je ne te l'ai jamais vraiment dit mais tu comptes beaucoup pour moi.

Je n'ai plus qu'à vous remercier d'avoir partagé un bout de votre vie avec la mienne, et j'espère que l'histoire ne s'arrêtera pas ici et que nous aurons encore tant d'autres pages à écrire ensemble.

Rigraziamenti

Mi sono chiesta per molto tempo come riassumere in qualche riga questi quattro anni della mia vita che si sono rivelati ricchi di insegnamenti, di emozioni e di sconvolgimenti. Quattro anni durante i quali ho avuto degli incontri formidabili sia dal punto di vista umano che professionale. Quattro anni durante i quali non è stato tutto rose e fiori e durante i quali ho imparato a spingere oltre i miei limiti con l'unico obiettivo di fare questo mestiere appassionante che è la ricerca. È evidente che tutti i sacrifici e gli sforzi sono la risultante di una motivazione implacabile che è rimasta forte e viva anche grazie all'aiuto delle persone accanto a me. Per questo motivo voglio spendere delle parole per chi mi ha aiutata in questo percorso.

Innanzitutto voglio ringraziare la **Prof.ssa Amelia Filippelli** per avermi accolta all'interno del suo gruppo di ricerca e per avermi dato l'opportunità di lavorare con serenità durante tutto il periodo del mio dottorato.

Altrettanto sentiti sono i ringraziamenti per la **Dr.ssa Viviana Izzo** e il **Dr. Fabrizio Dal Piaz**, non saprei da dove iniziare talmente è stata ricca quest'avventura. Andiamo per steps…

Viviana grazie per tutto quello che hai fatto per me al mio arrivo in Italia. Grazie per aver "addolcito" il distacco dalla mia terra e avermi aiutato ogni volta che ne ho avuto bisogno come nelle pratiche burocratiche, come neomamma, come ricercatrice e come persona.

Viviana e Fabrizio, grazie anche di avermi fatto crescere personalmente e professionalmente attraverso i continui confronti e le discussioni sempre produttive. Grazie di essere stati disponibili, comprensivi e di avermi trasmesso il vostro sapere.

Voglio ringraziare anche il **Dr. Bruno Charlier**. È passato molto tempo da quando eravamo "tête-à-tête" di fronte all'HPLC e tu mi chiamavi Wilson. Grazie Bru, per avermi insegnato tutte le basi dell'HPLC e non solo. Lo so che sarò ridondante come il nostro codice genetico ma volevo anche ringraziarti per esserci stato anche al di fuori del laboratorio. Come quando sei corso in ospedale dalla tua "Strapagnon".

Voglio inoltre ringraziare i miei compagni di viaggio il **Dr. Albino Coglianese**, la **Dr.ssa Valentina Giudice** e la **Dr.ssa Viviana Fortunato** che hanno reso ogni giorno di lavoro un giorno di piacere condiviso.

Vale, grazie per il prezioso aiuto nelle numerose traduzioni e riletture ed avermi salvata dalle mie cure omeopatiche.

Albi, grazie di tutto ed anche per tutte le ore di babysitting gratis. Sei una persona piena di qualità, rimani come sei.

Vivi, grazie per il grande aiuto nella rilettura dei ringraziamenti e di essere stata una attenta ascoltatrice, resta quella che sei.

Francesca anche se abbiamo passato poco tempo insieme, ti ringrazio delle piccole attenzioni che hai avuto.

Ringrazio anche tutte le persone che sono state con noi anche solo per un breve periodo, che abbiamo avuto la fortuna di formare e che ci hanno permesso di crescere. Marianna, Antonietta, Carmela, Marisa, Elena.

Grazie anche all'**équipe di farmacogenetica** dell'ospedale San Giovanni di Dio e Ruggi D'Aragona con cui è sempre stato un piacere condividere del tempo e cibo insieme.

Voglio ringraziare le mie amiche Rossella e Zandra che sono state sempre presenti per me e mi hanno permesso di rendere il mio soggiorno in Italia piacevole.

Rosse, grazie di avermi accolta all'interno della tua famiglia come se ne facessi parte. Ti voglio tanto bene.

Zandra, gracias por los dias que hemos trascorso juntos visitando y descubriendo este maravilloso país. Nos vemos pronto para descubrir el tuyo.

Infine, grazie alla mia famiglia e al mio compagno, per avermi seguito nelle mie scelte anche al prezzo di pagare grandi sacrifici.

Une pensiero speciale va a mia madre che ci ha sempre spinti a dare il meglio di noi, che ci ha insegnato che nella vita niente è servito su un vassoio d'argento e che se desideriamo qualcosa dobbiamo conquistarla. Grazie, **Mamma** per l'energia che ci infondi e dei tuoi sacrifici per aver fatto di noi quelle che siamo ad oggi. Ti amo.

Chouchou, ti ringrazio per avermi dato la possibilità di realizzare i miei sogni, sostenendomi nella realizzazione delle mie ambizioni senza farmi sentire il peso delle mie scelte. So che questi ultimi quattro anni, per quanto ricchi e belli possano essere stati con la nascita di nostro figlio e le nostre scoperte italiane, sono stati anche il risultato di scelte difficili. Allora grazie di tutto.

Papà, ti ringrazio di affrontare questo viaggio e di essere presente questo giorno per me.

Iris e **Coline** anche se non siamo sempre d'accordo sono molto orgogliosa di avervi come sorelle. Vi amo più di tutto. Quindi niente più cadute dalla bici, Ok…

Nonno, grazie di avermi dato questa "spintarella" all'inizio senza la quale non sarei mai partita. Non te l'ho mai detto veramente ma sei una persona molto importante per me.

Mi rimane soltanto di ringraziarvi ancora d'aver condiviso un pezzo della mia vita con me, e spero che la storia non finisca qua e che avremmo ancora tante pagine da scrivere insieme.