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**Optimization of darunavir therapy through population
pharmacokinetic modeling and simulations**

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ABSTRACT

The infection by the human immunodeficiency virus (HIV) is typically treated using a combination of pharmacological agents targeting different stages of the viral cycle. Darunavir is a second-generation protease inhibitor that occupies a place of choice in this therapeutic arsenal and for which an important inter-individual pharmacokinetic variability has been described in the literature.

First, we characterized darunavir pharmacokinetics by developing a population model based on data collected in a large cohort of HIV patients. Alpha-1 acid glycoprotein level, sex, and genetic polymorphisms in the *CYP3A5* and *SLCO3A1* genes were found to be significant predictors of darunavir pharmacokinetics. The model was thoroughly evaluated using internal and external validation techniques.

The model also allowed us to simulate the effect of alternative dose regimens in populations representative of clinical practice. A reduction of the standard 800 mg once-daily dosage to 600 or 400 mg once-daily was found to be safe in a large proportion of patients, based on commonly defined pharmacokinetic targets for this molecule. On the other hand, intermittent therapy (five out of seven days) constituted an unsafe option in most subjects. Whether individual patients could benefit from these alternative regimens could be predicted by our model. Additionally, optimal sampling strategies for darunavir were derived, showing how to best design future studies or how to optimize therapeutic monitoring for this drug.

Finally, *in vitro* experiments helped determine the contribution of polymorphisms in the P-glycoprotein gene to the aforementioned variability. These experiments showed that neither the c.1199G>A polymorphism nor the haplotype defined by c.1236C>T, c.2677G>T and c.3435C>T influence intracellular accumulation of darunavir.

RÉSUMÉ

L'infection par le virus de l'immunodéficience humaine (VIH) est typiquement traitée par une combinaison d'agents pharmacologiques ciblant différentes étapes du cycle viral. Le darunavir est un inhibiteur de protéase de seconde génération qui occupe une place de choix dans cet arsenal thérapeutique et pour lequel une importante variabilité pharmacocinétique inter-individuelle a été décrite dans la littérature.

Premièrement, nous avons caractérisé la pharmacocinétique du darunavir en développant un modèle de population sur base de données collectées dans une large cohorte de patients VIH-positifs. Il a été démontré que le taux d'alpha-1 glycoprotéine acide, le sexe, et des polymorphismes dans les gènes *CYP3A5* et *SLCO3A1* étaient des prédicteurs significatifs de la pharmacocinétique du darunavir. Le modèle a été évalué en profondeur par le biais de techniques de validation interne et externe.

Le modèle a également permis de simuler l'effet de régimes posologiques alternatifs dans des populations représentatives de la pratique clinique. Une réduction de la dose journalière standard de 800 mg à 600 ou 400 mg a été démontrée sûre pour une large proportion de patients, sur base de cibles pharmacocinétiques couramment définies pour cette molécule. En revanche, la thérapie intermittente (cinq jours sur sept) ne constituait pas une option sûre pour la plupart des sujets. L'existence d'un bénéfice individuel de ces stratégies alternatives pourrait être prédite par notre modèle. Par ailleurs, des stratégies de prélèvement optimales pour le darunavir ont été définies, montrant comment concevoir de futures études de manière optimale ou comment optimiser le monitoring thérapeutique pour ce médicament.

Enfin, des expériences *in vitro* ont permis d'établir la contribution de polymorphismes dans le gène de la P-glycoprotéine à la variabilité précédemment mentionnée. Ces expériences ont montré que ni le polymorphisme c.1199G>A ni l'haplotype défini par c.1236C>T, c.2677G>T et c.3435C>T n'altèrent l'accumulation intracellulaire du darunavir.

LIST OF ABBREVIATIONS

3TC	Lamivudine
AAG	Alpha-1-acid glycoprotein
ABC	Abacavir
ABC (family)	ATP-Binding Cassette
AE	Adverse event
AIC	Akaike information criterion
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
ARV	Antiretroviral
ATV	Atazanavir
AUC	Area under the curve
AZT	Zidovudine
BIC	Bayesian information criterion
BLQ	Below the limit of quantification
BTG	Bictegravir
C ₀	Trough concentration
C3C	CYP3cide
CAR	Constitutive androstane receptor
CI	Confidence interval
CL	Clearance
COB	Cobicistat
C _{max}	Maximum concentration
CNS	Central nervous system
CSF	Cerebrospinal fluid
CYP	Cytochrome P450
DAD	Diode array detector
DBS	Dried blood spots
DDI	Drug-drug interaction
DNA	Deoxyribonucleic acid
DRV	Darunavir
DTG	Dolutegravir
EACS	European AIDS Clinical Society
EFV	Efavirenz
EMA	European Medicines Agency
ESI	Electrospray ionization
ETR	Etravirine
EVG	Elvitegravir
F	Bioavailability
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FTC	Emtricitabine
FIM	Fisher information matrix
f _u	Unbound fraction
GWAS	Genome-wide association study
HBV/HCV	Hepatitis B/C virus
HIV	Human immunodeficiency virus

HLM	Human liver microsome
IAS	International AIDS Society
IC	Inhibitory concentration
IDU	Injection drug user
IIV	Inter-individual variability
INSTI	Integrase strand transfer inhibitor
IOV	Inter-occasion variability
IQ	Inhibitory quotient
IQR	Interquartile range
k_a	Absorption rate constant
KCZ	Ketoconazole
MAT	Mean absorption time
MS	Mass spectrometry
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NPC	Numeric predictive check
NPDE	Normalised prediction distribution errors
PXR	Pregnane X receptor
NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
OATP	Organic-anion-transporting polypeptide
OFV	Objective function value
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PD	Pharmacodynamics
PEP	Post-exposure prophylaxis
PI	Protease inhibitor
PK	Pharmacokinetics
PrEP	Pre-exposure prophylaxis
Q	Intercompartmental clearance
q12h	Twice daily
q24h	Once daily
RAL	Raltegravir
RAM	Resistance-associated mutation
RCT	Randomized controlled trial
RNA	Ribonucleic acid
RPV	Rilpivirine
RTV	Ritonavir
RV	Residual variability
SCT	Short cycle therapy
SIV	Simian immunodeficiency virus
SLCO	Solute carrier organic anion
SNP	Single nucleotide polymorphism
$t_{1/2}$	Elimination half-life
TAC	Tacrolimus
TAF	Tenofovir alafenamide
TDF	Tenofovir disoproxil fumarate
TDM	Therapeutic drug monitoring
THF	Tetrahydrofuran
Tlag	Lag time
t_{max}	Time to maximum concentration

UGT	Uridine diphosphate glucuronosyltransferase
(UP)LC	(Ultra-performance) liquid chromatography
V	Volume of distribution
V_c / V_p	Central/peripheral volume of distribution
VL	Viral load
VPC	Visual predictive check
WHO	World Health Organization

LIST OF PUBLICATIONS

Publications pertaining to the present thesis:

- G. Stillemans, L. Belkhir, D.A. Hesselink, V. Haufroid and L. Elens, Pharmacogenetic associations with cytochrome P450 in antiretroviral therapy: what does the future hold?, *Expert Opinion on Drug Metabolism & Toxicology* 14 (2018), pp. 601–611
- G. Stillemans, L. Belkhir, B. Vandercam, A. Vincent, V. Haufroid and L. Elens, Exploration of reduced doses and short cycle therapy for darunavir/cobicistat in HIV patients using population pharmacokinetic modeling and simulations, *Clinical Pharmacokinetics* 60 (2021), pp. 177-189.
- G. Stillemans, L. Belkhir, B. Vandercam, A. Vincent, V. Haufroid and L. Elens, Optimal sampling strategies for darunavir and external validation of the underlying population pharmacokinetic model, *European Journal of Clinical Pharmacology* 77 (2021), pp. 607-616.
- G. Stillemans, H.P. Djokoto, K-A. Delongie, H. El-Hamdaoui, N. Panin, V. Haufroid, L. Elens, Effect of four *ABCB1* genetic polymorphisms on the accumulation of darunavir in HEK293 recombinant cell lines, *Scientific Reports* (Revision submitted, 2021).

1. INTRODUCTION

1.1. HIV and antiretroviral therapy

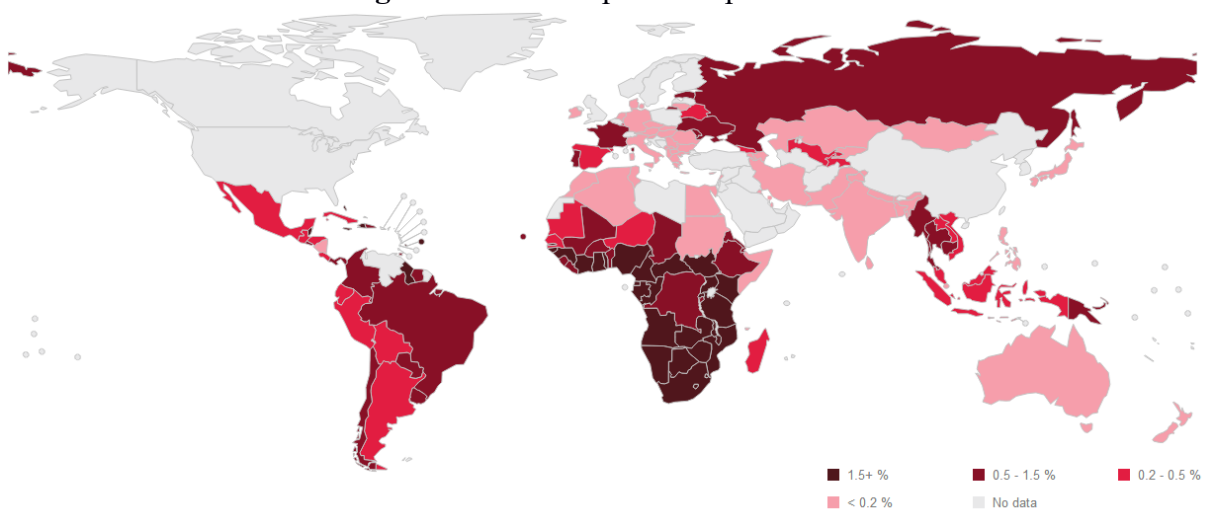
1.1.1. HIV

In the 1980s, the human immunodeficiency virus (HIV) was identified as the causal factor of the then poorly understood acquired immunodeficiency syndrome (AIDS) [1,2]. Since then, great insight has been gained into the physio-pathology of HIV infection, its underlying molecular and cellular mechanisms and how to best treat it. HIV is mainly transmitted by parenteral and sexual contact, or by vertical transmission from mother to child during pregnancy, delivery or breastfeeding. If left unchecked, HIV infection eventually leads to a complete depletion of the immune system and exposes patients to all manners of opportunistic infections and cancers.

1.1.1.1. Epidemiology

36.9 million people worldwide are infected with HIV, 1.8 million new infections occurred in 2017 and a million people die from it yearly [3]. These numbers are a reminder that despite considerable advances in medical science and access to healthcare, HIV remains a major healthcare issue. Furthermore, Africa is disproportionately affected by the pandemic (Figure 1), as the continent accounts for over 70% of all infections [3,4].

Figure 1. World map of HIV prevalence.



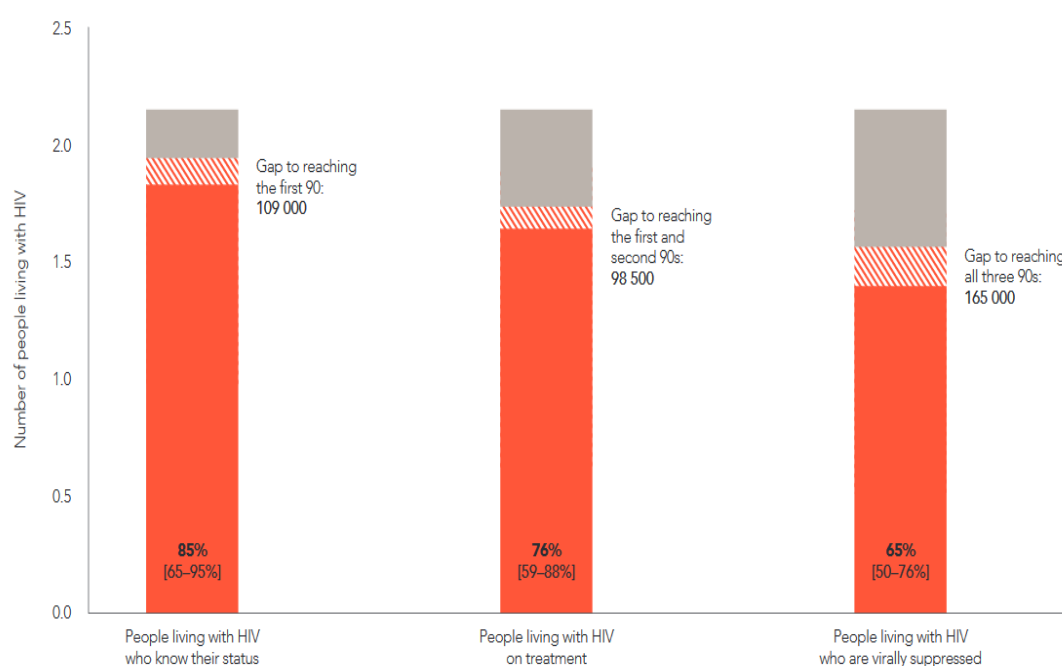
UNAIDS data (2017): Prevalence in adults (15-49). [5]

The epidemiology of HIV in Belgium is comparable to that in most Western countries (Figure 2). According to the latest statistics, the prevalence of HIV is between 0.1 and 0.2%, including individuals who ignore their status, believed to account for 15% of all HIV-positive subjects

[6]. Homosexual men, and heterosexual men and women from sub-Saharan Africa constitute the two major subpopulations of HIV patients in Belgium.

Thanks to antiretroviral therapy (ART), HIV patients may now live almost as long as uninfected individuals and consequently, the HIV population is an aging one – in Belgium, over a third of them is over the age of fifty [6]. The most recent guidelines support rapid initiation of ART in all patients, regardless of immune function at the time of diagnosis [7], which has led to a significant increase in the proportion of treated individuals. Fortunately, the continuum of care is encouraging: most diagnosed individuals are retained in follow-up, initiate ART and achieve an undetectable viral load (Figure 2).

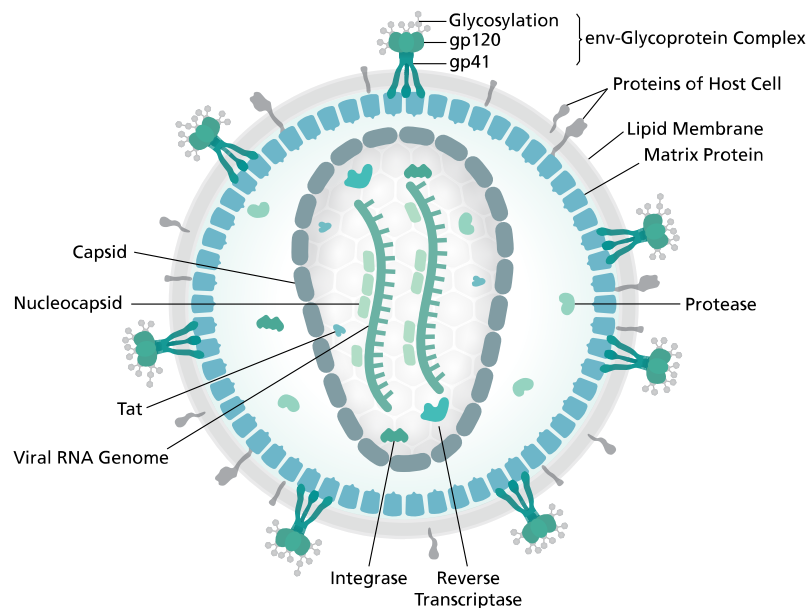
Figure 2. HIV testing and treatment cascade.



Source: UNAIDS special analysis, 2018; see annex on methods for more details.

Data for western and central Europe and North America, 2016. [3]

Figure 3. Structure of HIV [193]

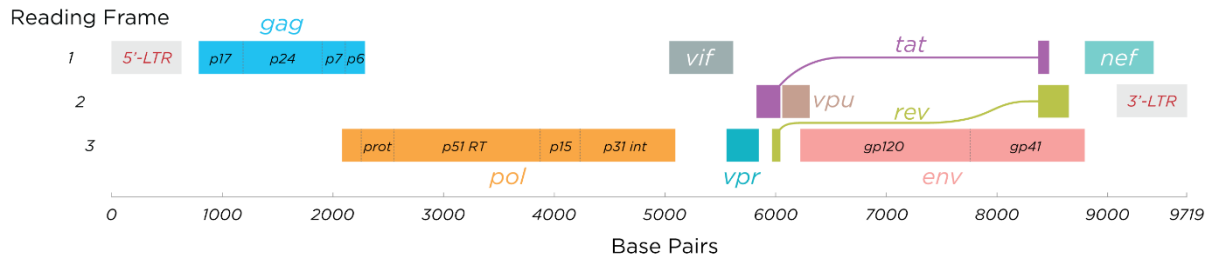


HIV is a retrovirus possessing two copies of single-stranded RNA (Figure 3). Its genome comprises 9 genes, some of which code for non-functional polypeptides that require cleavage into active proteins (Figure 4). The viral RNA and key proteins implicated in the viral cycle are enclosed in a capsid, itself surrounded by a protein matrix and a lipid envelope, with Env surface proteins embedded in it (Figure 3).

Genetically, HIV is closely related to the simian immunodeficiency virus (SIV) found in some monkeys (Figure 5). SIV strains are believed to have been independently transmitted to humans through contact with contaminated blood by hunters or bushmeat vendors in the early 20th century and to have mutated into two major subtypes, HIV-1 and HIV-2, through separate events. The strains that would give rise to HIV-1 originated from common chimpanzees [9] and possibly western lowland gorillas [10], while the HIV-2 progenitor came from sooty mangabeys [11].

Owing to its fast replication and high error rate during reverse transcription, HIV constitutes what is known as a quasispecies: a cluster of viruses that share common traits, yet differ enough that they may not be sensitive to the same treatments. HIV-1 strains are categorized in three major groups M, N and O, based on the sequence of the surface protein Env, and further subdivided into clades based on other genetic patterns (Figure 5).

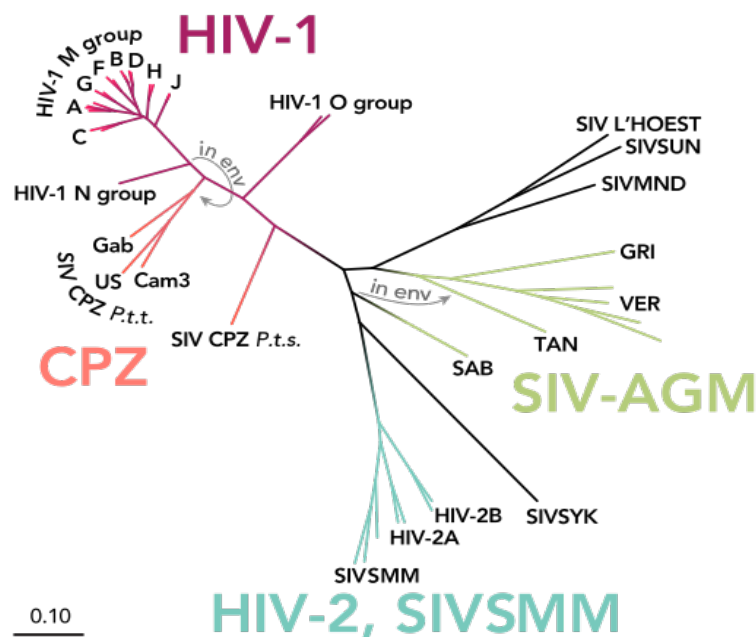
Figure 4. Genomic map of HIV-1. [8]



Gene	Proteins	Processed proteins	Function
<i>gag</i>	Gag	p17 p24 p7 p6	Matrix protein Capsid protein Nucleocapsid protein Virus particle budding
<i>pol</i>	Pol*	p10 p50 p15 p31	Protease Reverse transcriptase RNase H Integrase
<i>env</i>	gp160	gp120, gp41	Surface proteins
<i>tat</i>	Tat		Regulatory element
<i>rev</i>	Rev		Regulatory element
<i>nef</i>	Nef		Accessory regulatory protein
<i>vpr</i>	Vpr		Accessory regulatory protein
<i>vif</i>	Vif		Accessory regulatory protein
<i>vpu</i>	Vpu		Accessory regulatory protein

* as Gag-Pol polyprotein.

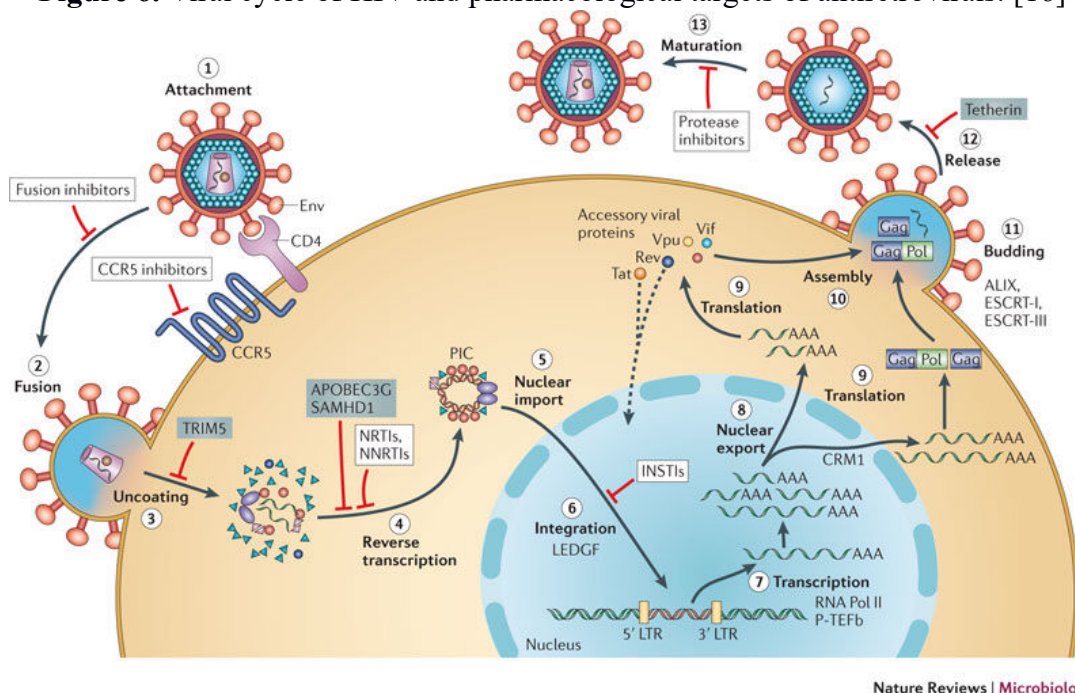
Figure 5. HIV phylogenetic tree [194]



Meanwhile, HIV-2 is endemic to Western Africa and much less virulent than HIV-1. For instance, rarely does the infection progress to AIDS. Therapeutic options are also more limited, as it is naturally resistant to certain drugs such as non-nucleoside reverse transcriptase inhibitors [12] and enfuvirtide [13]. Unfortunately, clinical trials of HIV-2 infection are few in numbers and treatment strategies remain undefined [14].

The viral cycle of HIV can be summarized as a five-step process: fusion and entry, reverse transcription, genome integration, translation, and finally maturation, assembly and release (Figure 6) [15].

Figure 6. Viral cycle of HIV and pharmacological targets of antiretrovirals. [16]



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INSTI: Integrase strand transfer inhibitor, N(N)RTI: (Non) nucleoside reverse transcriptase inhibitor.

The first step is fusion of HIV with the target cell through a complex sequence of interactions between viral and host cell proteins [17]. The Env surface protein of HIV is a glycosylated trimer of gp120 and gp41 that is anchored in the membrane, with gp41 being folded on the inner side. Env interacts with both a main receptor, CD4, and a co-receptor, either CXCR4 or CCR5 depending on the sequence of gp120. This dual co-receptor tropism ties into the ability of HIV to target both CD4⁺ T lymphocytes (through CXCR4), which are the main target of the virus, and macrophages (through CCR5), which are thought to play a role in the spread of the infection, although the exact mechanisms are still under investigation. The interaction between the receptor-binding section of gp120 and its target induces a conformation change in gp120 that exposes gp41, which then embeds itself into the host cell membrane [15,17]. Through subsequent folding of multiple gp41, a six-helix structure is formed, bridging the two membranes and forming a fusion pore. With the fusion process complete, the capsid is shed and the second step of the cycle may begin.

In the second step, the reverse transcriptase catalyzes the polymerization of viral RNA into double-stranded DNA [18]. The enzyme actually catalyzes three separate processes: RNA polymerization, DNA polymerization and digestion of nucleotide chains through its ribonuclease activity (RNase H). Using nucleotides and a specific transfer RNA primer (Lys3) from the host cell, the reverse transcriptase assembles an RNA-DNA hybrid, which is digested by the RNase H, releasing single-stranded DNA. Through its DNA polymerase activity, the reverse transcriptase then completes the synthesis of a double-stranded DNA molecule.

The third major step of the viral cycle is handled by the integrase, an enzyme that may exist in monomer form or in one of several polymer forms [19]. The integrase is responsible for binding the previously synthesized double-stranded DNA to form a nucleoprotein preintegration complex, processing this complex by cleaving two nucleotides at the 3' end, and transferring it to the nucleus through mechanisms which are not yet fully understood. Once inside the nucleus, the host's LEDGF (lens epithelium-derived growth factor) serves as a tether between the preintegration complex and the target DNA so that the integrase can catalyze the strand transfer and incorporate the viral DNA into the host cell genome, where HIV may lay dormant for many years. This integration may occur anywhere in the genome, although certain regions may be favored compared to others [19].

After translation of the viral genes by the cellular machinery of the host, several HIV proteins such as Gag and Gag-Pol polypeptides are in an inactive form (as detailed in Figure 4) and need to be cleaved by the viral protease in order to become fully functional [20]. This process is referred to as maturation and is thought to occur in parallel with the assembly and release of virions. Gag plays a key role in all of these processes: it binds both the plasma membrane and free Env proteins through its N-terminal domain, while the C-terminal domain binds accessory proteins. This allows the formation of microdomains in the plasma membrane that will lead to the budding of new viral particles, composed of a section of lipid membrane from the host with Env proteins embedded inside of it, and containing all necessary components of the virus (nucleocapsid, enzymes and accessory proteins). In this state, Gag acts as a scaffold of sorts, determining the shape of the particle. The maturation process involves the proteolysis of protein precursors, which generates functional proteins and alters the structure of the virion itself.

1.1.2. Antiretroviral therapy

From the viral cycle represented in Figure 6 (p.15), it is apparent that HIV replication can be blocked in a number of ways. Several therapeutic classes can be defined according to the key step targeted in the viral cycle (non-exhaustive list):

• Fusion inhibitors	Enfuvirtide
• CCR5 antagonists	Maraviroc
• Nucleoside reverse transcriptase Inhibitors (NRTIs)	Tenofovir, emtricitabine, abacavir, lamivudine, zidovudine
• Non-nucleoside reverse transcriptase Inhibitors (NNRTIs)	Efavirenz, nevirapine, rilpivirine, etravirine, doravirine
• Integrase strand transfer inhibitors (INSTIs)	Dolutegravir, raltegravir, elvitegravir, bictegravir, cabotegravir
• Protease inhibitors (PIs)	Darunavir, lopinavir, atazanavir

As previously mentioned, HIV displays an incredible genetic heterogeneity and a high potential for mutation. Consequently, ART regimens should aim for a high genetic barrier, that is, a low risk for the virus to develop resistance-associated mutations (RAMs). Using a combination of drugs from different pharmacological classes further reduces the probability of RAM emergence.

Guidelines differ in their recommendations, but a double NRTI backbone plus one INSTI, PI or NNRTI is often given as one of the preferred first-line regimen, as shown in Table 1. NRTI/INSTI dual therapy has recently been added in both EACS and IAS guidelines as well. Initial regimens are chosen based on multiple considerations, including results of viral resistance testing, presence of comorbidities (e.g., cardio-vascular diseases, HBV/HCV coinfection) and potential for drug interactions [21].

Table 1. Comparison of first-line ART in some guidelines.

ART	EACS 10.1 (2020) [21]	IAS 2020 [22]	WHO 2016 [7]
2 NRTIs + INSTI	ABC + 3TC + DTG TAF (or TDF) + FTC + DTG TDF + 3TC + DTG TAF + FTC + BIC TAF (or TDF) + FTC + RAL TDF + 3TC + RAL	TAF + FTC + BTG TDF (or TAF) + FTC + DTG TFV + 3TC + DTG ABC + 3TC + DTG TAF (or TDF) + FTC + RAL TDF + 3TC + RAL	TDF + 3TC (or FTC) + DTG
1 NRTI + INSTI	3TC + DTG	3TC + DTG (with some caveats)	
2 NRTIs + NNRTI	TAF (or TDF) + FTC + DOR TDF + 3TC + DOR TAF (or TDF) + FTC + RPV TDF + 3TC + RPV	TDF + 3TC + DOR TAF + FTC + DOR TAF + FTC + RPV TDF + 3TC + RPV TDF + 3TC + EFV (or EFV ₄₀₀) TDF + FTC + EFV (or EFV ₄₀₀)	TDF + 3TC (or FTC) + EFV AZT + 3TC + EFV (or NVP) TDF + 3TC (or FTC) + EFV ₄₀₀ TDF + 3TC (or FTC) + NVP
2 NRTIs + boosted PI	TAF (or TDF) + FTC + DRV/COB (or /RTV) TDF + 3TC + DRV/COB (or /RTV)	TAF + FTC + DRV/COB TDF + 3TC + DRV/COB	

First choice/recommended regimens in **bold font**, other recommended/alternative regimens in normal font. 3TC: Lamivudine, AZT: Zidovudine, BTG: Bictegravir, COB: Cobicistat, DOR: Doravirine, DRV: Darunavir, DTG: Dolutegravir, EACS: European AIDS Clinical Society, EFV: Efavirenz, EFV₄₀₀: Low-dose efavirenz (400 mg once daily), EVG: Elvitegravir, FTC: Emtricitabine, IAS: International AIDS Society, NVP: Nevirapine, RAL: Raltegravir, RPV: Rilpivirine, RTV: Ritonavir, TAF: Tenofovir alafenamide, TDF: Tenofovir Disoproxil Fumarate, WHO: World Health Organization.

Despite ART, HIV is never truly eradicated: the virus can persist in reservoirs and return soon after treatment is interrupted [23], meaning HIV is a chronic disease and ART a lifelong endeavor. The mechanisms of HIV persistence are not yet fully understood, but it has been hypothesized that poor penetration of ARVs in sanctuary compartments and continued low-level replication of the virus [24], as well as integration of viral genetic material into T lymphocyte DNA may be contributive [25,26].

Lifelong treatment increases the risk of long-term toxicity and represents a psychological burden for patients – and a financial one for healthcare systems. Therefore, there is growing interest in optimizing and simplifying current treatment guidelines, whether through a reduction in the number of ARVs, their doses or their frequency of administration. Specifically, treatment simplification aims to reduce costs, toxicity and potential for drug-drug interactions, in addition to improving patient compliance, through modification of either initial regimens or maintenance therapy [27,28]. Considering the number of infected individuals, such strategies could represent a major advance in ART. In particular, NRTI- and PI-sparing regimens, long-acting formulations, coformulation of ARVs in a single pill, lower doses, less frequent administrations and short cycle therapies (a type of planned treatment interruption) have been investigated. Some of these will be discussed further in the context of the present research work.

1.2. Darunavir

1.2.1. Overview

In 1995, the US Food and Drug Administration (FDA) approved the first HIV PI, saquinavir, which would soon be followed by ritonavir and indinavir. Further developments eventually led to the so-called second-generation PIs such as atazanavir, tipranavir and darunavir (DRV), the latter of which received FDA approval in June 2006. To increase plasma levels of DRV, it is always coadministered with a PK booster, a molecule that increases DRV exposure through inhibition of its metabolism and efflux. These boosters (ritonavir and cobicistat) have complex effects that will be discussed in greater detail in Boosters and drug interactions (p.27).

DRV is characterized by a high genetic barrier, which makes it a good choice for patients with incomplete treatment adherence or for those who have failed to achieve therapeutic targets using other regimens. Clinical trials support the use of DRV in combination with a background regimen in both PI-naïve and PI-experienced individuals: the ARTEMIS trial [29] established the efficacy and safety of DRV 800 mg once daily in comparison with lopinavir in the former, while the POWER [30–32] and TITAN [33] trials support the need for DRV 600 mg twice daily in experienced patients in order to reach adequate exposure. In pretreated patients with no PI RAMs, therapeutic success can actually be achieved even with the once daily dose, as shown in the ODIN trial [34]. For a summary of these studies, refer to Table 2 below. Current guidelines recommend the use of DRV as the third agent in addition to a double NRTI backbone (Table 1, p.17), although INSTI-based therapies are the optimal starting regimen for most patients according to the EACS and IAS guidelines, while the WHO relegate DRV to second- and third-line regimens due to its cost (compared to older PIs) and the lack of data in certain subpopulations [7].

A few groups of patients pose a particular challenge and are worth discussing here for the benefit of the reader. Even though they are not the focus of this thesis, it is important to provide an outline of why these groups require specific studies and guidelines.

The first of these groups is the pediatric population. DRV-based therapy is currently not recommended in children [7,35]. There is, however, some data from the DELPHI, DIONE and ARIEL trials that demonstrates the efficacy and safety of DRV in treatment-experienced adolescents and children older than 3 years, as well as in treatment-naïve adolescents [36–38], while a population pharmacokinetic (PK) model has been used to derive once daily dosing recommendations for younger treatment-naïve children [39] (Table 2). Under 3 years of age, DRV is contraindicated due its inherent toxicity and because of the unpredictable effect of boosters in subjects with immature CYP metabolism and an incompletely formed blood-brain barrier [40].

Table 2. Summary of main studies of DRV efficacy in adults and children.

Study	Details	n
ARTEMIS [29]	Phase III non-inferiority study in PI-naïve adults, randomized to TDF/FTC + DRV/RTV 800/100 mg q24h or LPV/RTV 800/200 mg (total daily dose, includes q24h and q12h dosing).	689 (DRV: 343)
POWER 1/2/3 [30–32]	Phase III studies of adult patients with prior ART, randomized to DRV/RTV 600/100 mg q12h (+ background regimen) or other regimen including another PI. Power 3 is a single arm study of DRV/RTV q12h.	591 (DRV: 467)
TITAN [33]	Phase III non-inferiority study of treatment-experienced, LPV-naïve adult patients randomized to DRV/RTV 600/100 mg q12h or LPV 400/100 mg q12h (+ background regimen).	595
ODIN [34]	Phase III non-inferiority study of PI-experienced adults experiencing viral failure, with no PI RAMs at baseline, randomized to a salvage regimen of DRV/RTV 800/100 mg q24h or 600/100 mg q12h (+ background regimen).	590
ARIEL [36]	Phase II study of DRV/RTV q24h and q12h (+ background regimen) efficacy and safety in treatment-experienced children (aged 3–6 years). Dose was adjusted based on weight and PK data.	21
DIONE [38]	Phase II study of DRV/RTV 800/100 mg q24h efficacy and safety in treatment-naïve adolescents (12–18 years) receiving a dual NRTI background regimen (AZT/3TC or ABC/3TC).	12
DELPHI [37]	Study of the efficacy of DRV/RTV 300–600/50–100 mg (+ background regimen) in treatment-experienced children (aged 6–18 years).	44

COB: Cobicistat, LPV: Lopinavir, RTV: Ritonavir.

Pregnant women constitute another difficult population to treat, both due to the risk of foetal toxicity and the potential for alterations of drug PK. Available data on the use of DRV during pregnancy is scarce [21]. Although placental transfer of the drug is limited [41], it was classified as category C (potential risk) by the FDA. Clinical data shows exposure is decreased during the second and third trimesters of pregnancy compared to the first trimester and postpartum, but total and unbound concentrations remain high enough to maintain antiviral activity in women using ritonavir-boosted DRV once [42] or twice daily [41,43]. In contrast with these observations, exposure was unacceptably low with the new once daily cobicistat-boosted DRV, which prompted the manufacturer to issue a warning [44,45].

HIV-2 infection can be treated using an NRTI backbone plus an INSTI or boosted PI [14]. Among PIs, DRV, lopinavir and saquinavir exhibit superior activity against this HIV subtype compared to other drugs of the same class [46,47].

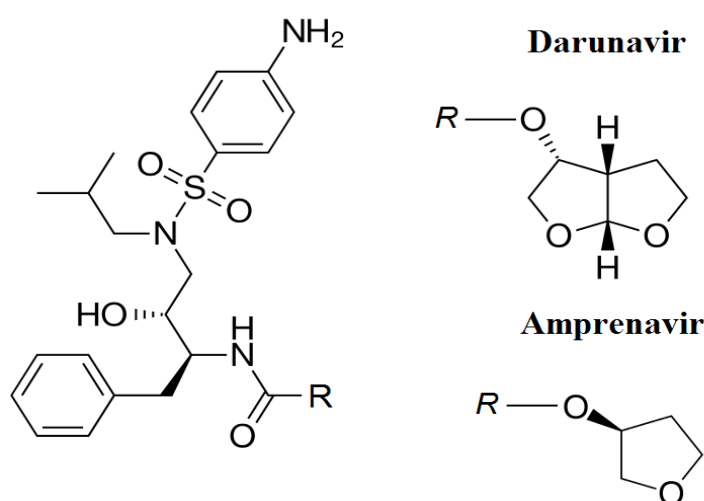
In addition to the treatment of active HIV infection, ARVs can also be used in prophylaxis. In pre-exposure prophylaxis (PrEP), ART is taken either continuously or on demand before engaging in risk behavior such as unprotected sex with people of unknown HIV status, while post-exposure prophylaxis (PEP) consists of administering ART after an accidental exposure. Tenofovir/emtricitabine is currently the prophylactic regimen of choice in both cases [22,48]. DRV could be a favorable candidate in this indication due to its virological (high efficacy and low risk of resistance) and PK (long half-life) properties. It is predicted to be effective in prophylaxis, while being less reliant on perfect adherence and more robust to PK variability compared to other PIs [49].

1.2.2. Mode of action and structure-activity relationship

The target of PIs is an aspartic protease encoded by the *pol* gene that functions in its homodimeric form, with each monomer being composed of 99 amino acids. This protease is implicated in the maturation of the virus, as explained in the section Virology (p.17) [15,20,50]: the protease catalyzes its own dissociation from the rest of Gag-Pol (this is referred to as autoprocessing), then cleaves both Gag and Gag-Pol polyproteins in several locations [15]. DRV is capable of blocking the catalytic activity of the protease, as well as its dimerization. This prevents maturation of the virion and renders it non-infective, but has no effect on cells that have already been infected.

Structure-wise, DRV is peptidomimetic drug and a chemical derivative of amprenavir (Figure 7), which itself was based on saquinavir. The only difference between DRV and amprenavir lies in the replacement of the terminal tetrahydrofuran (THF) cycle with a bi-THF moiety [51]. Its synthesis is the product of research into structural derivatives of PIs with less peptide-like traits and displaying a broad spectrum of activity against HIV strains.

Figure 7. DRV and amprenavir chemical formulaes



Since the catalytic site of the HIV protease is highly conserved (any deviation from the reference sequence would lead to a loss of viral fitness), drugs should be optimized for

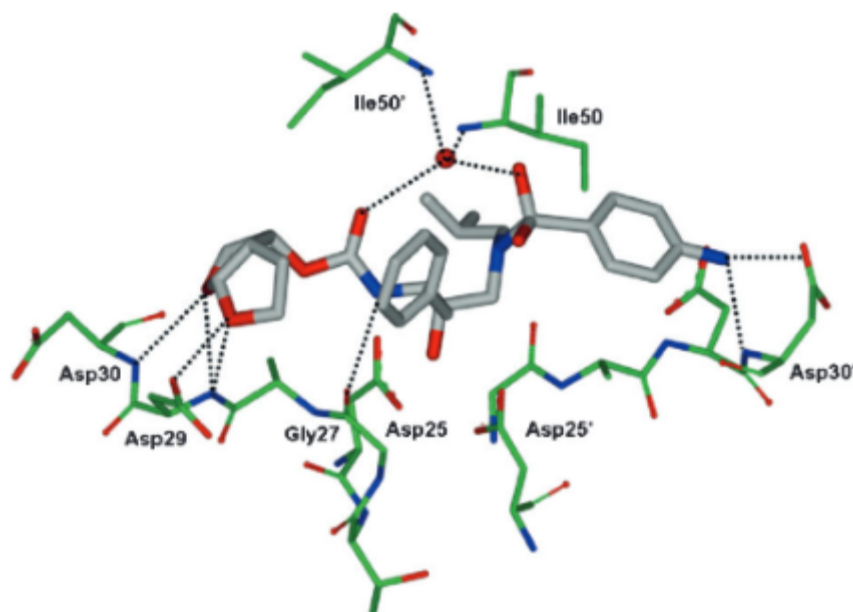
interactions in this region. The majority of PIs, DRV included, share the same scaffold and design efforts have typically focused on promoting interactions between key groups, especially the lateral chains of the drug, and residues close to the catalytic site of the enzyme [52]. Key interactions shared with most other PIs include the formation of several hydrogen bonds (Figure 8) [52]:

- between aspartate residues at the catalytic site (Asp25 and Asp25', one for each protease monomer) and the hydroxyl group of the PI;
- between two isoleucine residues (Ile50 and Ile50') and both the sulfonamide and carbamate groups of DRV, tetraordinated with a water molecule.

Interactions more specific of DRV include hydrogen bonds between:

- the oxygens of the bi-THF and Asp29 and Asp30;
- the amine group of the aminosulfonamide and Asp30';
- the amide and a glycine (Gly27).

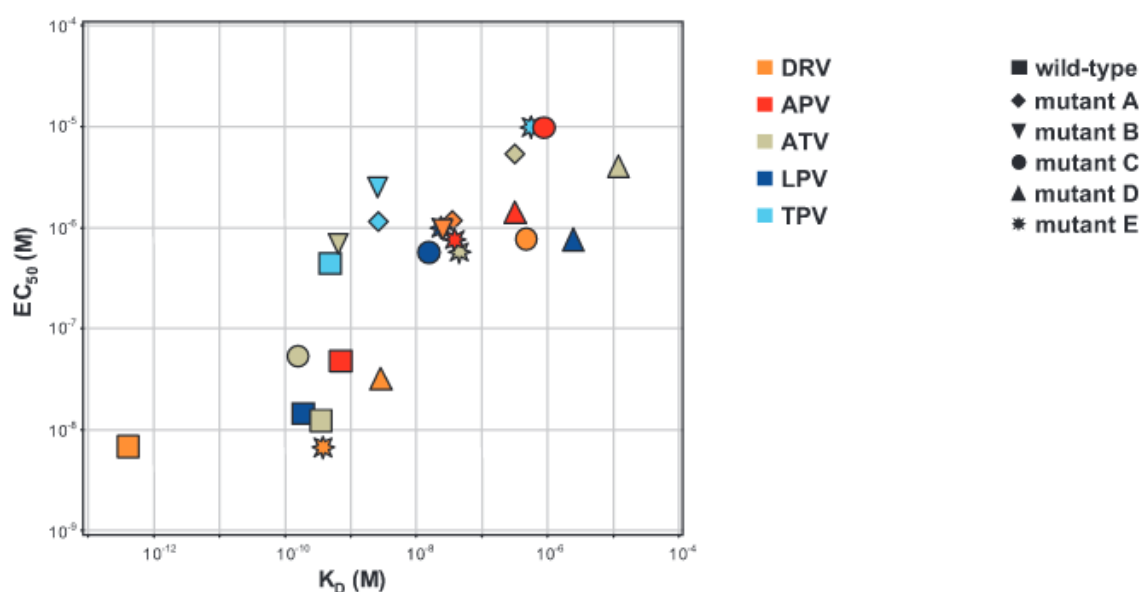
Figure 8. Binding of DRV to the HIV protease. [52]



DRV is characterized by a low IC_{50} , ranging from 1.2 to 8.5 nM (0.7 to 4.7 ng.ml⁻¹) for various HIV strains and cell lines, and a low dissociation constant of 4.5 pM [50,53,54]. DRV can be considered more potent and less susceptible to RAMs compared to older PIs (Figure 9).

On that topic, the emergence of RAMs is conditioned by the type of ART. For DRV, the primary PI mutations (occurring in the active site of the enzyme and having the greatest effects on viral susceptibility) are V11I, V32I, L33F, T74P and L89V, all of which are rarely encountered in clinical practice [54,56]. There is little cross-resistance with other PIs – which is not to say that other PIs cannot select for DRV RAMs, but rather that RAMs selected for by other PIs rarely affect DRV activity.

Figure 9. Comparison of binding affinity and antiviral activity for several PIs.



APV: Amprenavir, ATV: Atazanavir, LPV: Lopinavir, TPV: Tipranavir. Adapted from [55].

1.2.3. Clinical pharmacokinetics

When administered orally, DRV is readily absorbed: the peak concentration (C_{max}) is usually reached after 2.5 to 4 hours (t_{max}), although shorter absorption times, in the range of 1 hour, have also been reported [57,58]. Bioavailability (F) is low when unboosted (37%) due to incomplete absorption and extensive first-pass metabolism. Either way, the drug should be taken with a meal to increase the extent of absorption [57].

DRV is highly protein-bound in plasma (> 95%), predominantly to α -1-acid glycoprotein (AAG), and to a lesser extent albumin. This protein binding is not concentration-dependent over the range typically encountered in clinical practice (up to 10 mg.l^{-1}), but saturation of protein binding can occur at much higher concentrations: the unbound fraction (f_u) increases to 25% for a total DRV concentration of 18.5 mg.l^{-1} [59].

Distribution into the central nervous system (CNS) is limited: cerebrospinal fluid (CSF) concentrations represent under 2% of those found in plasma [60–64]. Likewise, intracellular penetration is low and can be quite variable: the PBMC (peripheral blood mononuclear cell) to plasma concentration ratio ranges from 0.1 to 6 [65–69] while the seminal plasma to blood plasma ratio is in the range of 0.1 to 0.2 [70–73]. It has been argued that due to the lower protein binding in tissues and the resulting higher f_u (> 80% in seminal plasma [74]), adequate exposure may be achieved despite this low tissular penetration.

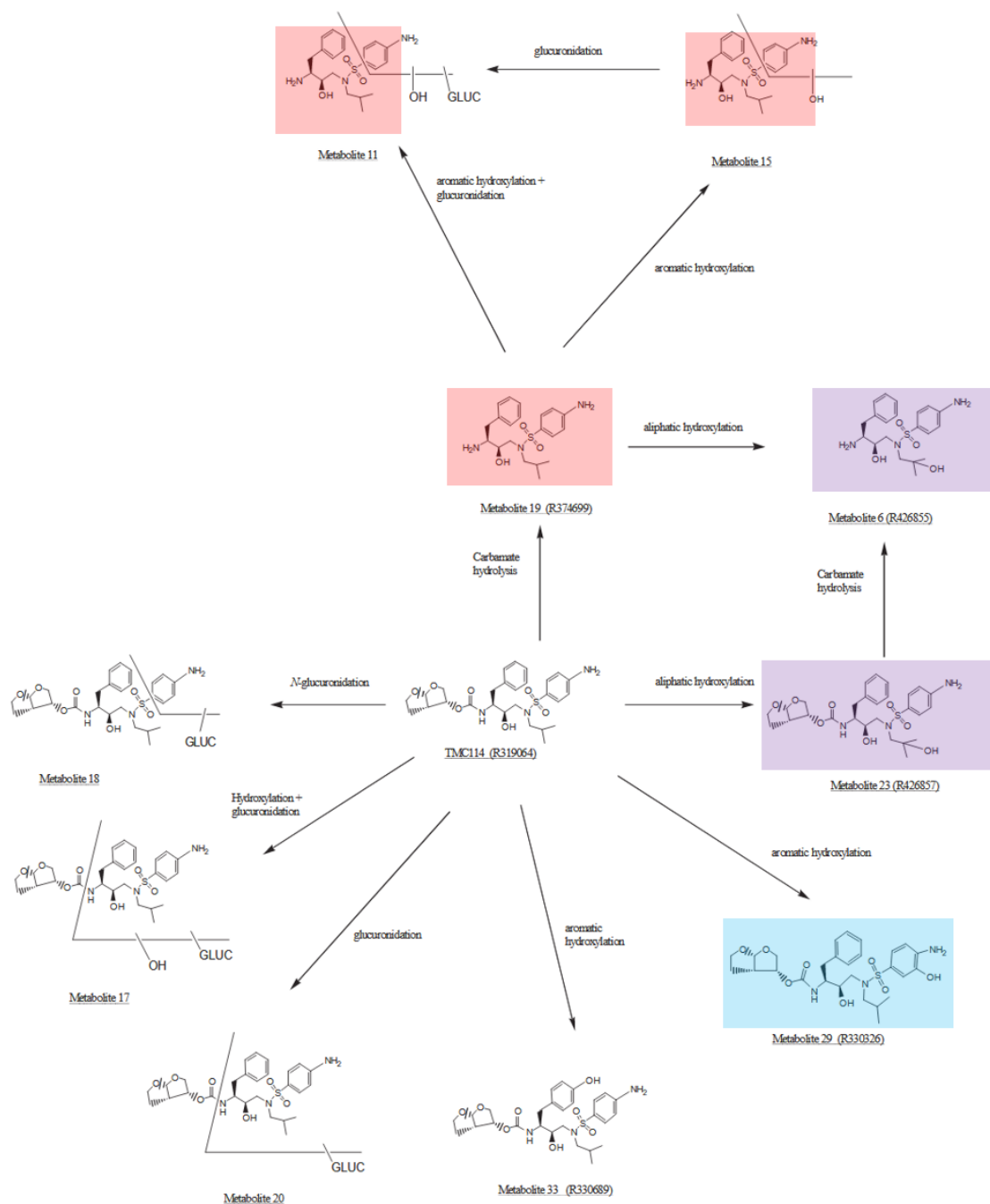
DRV is extensively metabolized through carbamate hydrolysis and aliphatic and aromatic hydroxylation (Figure 10), while renal excretion of the parent compound only accounts for approximately 10% of the dose when unboosted [54,59]. *In vitro* data shows that DRV metabolism is mainly mediated by CYP3A4 and CYP3A5, with contributions from CYP2D6

and CYP2C19 for the formation of certain metabolites, and little to no involvement of other CYP isoforms [59] (see also Annex 2, p.129). It should be noted that while some publications refer to “CYP3A” metabolism, the exact contribution of the various isoforms to the metabolism of DRV is difficult to establish, as it could depend on racial groups (due to differences in the frequency of genetic polymorphisms with reduced or neutered activity) and possibly drug interactions (on this topic, see also **Paper 1**). Further, different *in vitro* models will yield different results that may not always readily translate into *in vivo* estimates of clearance. Meanwhile, phase II biotransformation of DRV is less prevalent, although several glucuronides have been identified (M11, M17, M18, M20). None of the major metabolites display any antiretroviral activity. The plasma elimination half-life of boosted DRV is approximately 11 to 15 hours [40,75].

DRV exposure increases in a less than dose proportional manner over the range of doses initially investigated by the manufacturer. For instance, a ~60% increase in AUC was calculated for a 100% increase in total daily dose [76]. Several elements could explain this nonlinearity, namely the existence of saturable processes such as enzymatic biotransformation and influx/efflux transporters.

For a summary of its PK parameters in a variety of studies, see **Annex 4**.

Figure 10. DRV metabolic pathways in humans.



Major metabolites are highlighted (red / purple / blue: 13.2 / 9.8 / 4.5% of the dose). TMC114: darunavir. Adapted from [59].

1.2.4. Pharmacodynamics

The therapeutic target for DRV is defined based on an *in vitro* protein-binding adjusted IC_{50} for the wild-type protease of 0.055 mg.l^{-1} [53]. This value is arbitrarily multiplied by 10, resulting in a target of 0.55 mg.l^{-1} for treatment-experienced patients (perhaps because DRV remained effective *in vitro* against most mutant strains with a fold-change in its IC_{50} less than

10 [53]), whereas a cutoff of 2 mg.l⁻¹ has been proposed in some guidelines [77], perhaps based on the median trough concentration (C₀) measured in the ARTEMIS trial [78].

No clear relationship between DRV PK and PD has been established. This might in part be due to the use of complex multi-drug regimens in HIV patients. Still, in one study conducted in subjects harboring several PI RAMs, higher plasma concentrations were correlated with virological success [79]. Higher C₀ have also been measured in subjects using DRV monotherapy during periods of viral suppression compared to periods of detectable viremia, but this effect was weak and a sufficiently sensitive and specific target could not be defined [80]. PK was unrelated to PD in other studies [78]. The combination of PK and PD markers using an inhibitory quotient (IQ) may be a better predictor of treatment outcomes for PIs [62,81]. The IQ is defined as the ratio of the C₀ by a marker of viral susceptibility such as the IC₅₀ (phenotypic IQ) or the number of PI RAMs (genotypic IQ). No IQ threshold is presently defined for DRV.

1.2.5. Toxicity

DRV therapy is usually well tolerated. In pre-marketing clinical trials, the most frequently reported adverse events (> 10% subjects) were gastro-intestinal disorders (diarrhea, nausea), headaches and mild cutaneous reactions [75]. Other frequent (1-10%) side effects included lipodystrophy, increased lipid levels, increased transaminases, myalgia, fatigue and hypersensitivity reactions. Preclinical studies did not show the drug to have deleterious effect on fertility, nor was it teratogenic [59].

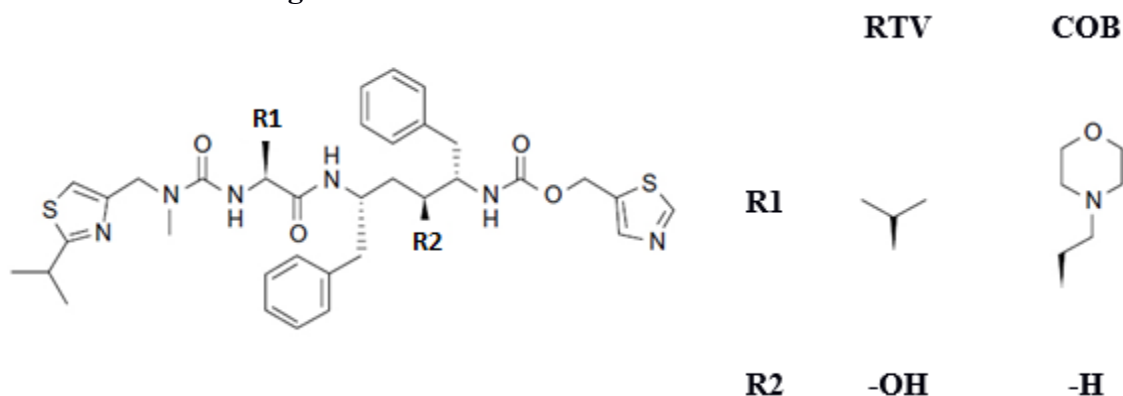
An elevation of lipid levels has been described as a class effect for PIs, though especially for older generation PIs [82]. Generally speaking, cardiovascular morbidity and mortality are higher in HIV-positive individuals compared to the general population due to an increased prevalence of risk factors such as hypercholesterolemia and hypertension, as well as HIV-related chronic inflammation. While treating the infection can reduce the cardiovascular risk in these individuals, alterations of lipid and glucose metabolism arising from long-term use of certain ARVs could be an issue. A review by the manufacturer of data from 19 clinical trials, as well as from pharmacovigilance and administrative claims databases did not produce any evidence of cardiovascular toxicity [83]. Patients using twice daily DRV were noted to have more advanced HIV disease and more comorbidities at baseline. Likewise, no cardiovascular toxicity of either DRV or ATV could be demonstrated in a long-term multicentric study in France [84]. Only the D:A:D study (a multi-cohort follow-up study of HIV patients) found a small increase in cardiovascular risk for long-term DRV-based ART compared to ATV-based ART, while lipid levels changed little and did not compound the cardiovascular risk [85,86]. The underlying mechanisms of this cardiovascular toxicity (if any) are not known.

1.2.6. Boosters and drug interactions

Due to its low bioavailability, DRV should be coadministered with a booster (also known as a pharmacoenhancer). Boosters increase exposure to the active molecule through inhibition of its metabolism and/or efflux. The first booster to be used was none other than ritonavir (RTV) (Figure 11), a first-generation PI that has fallen out of favor as a therapeutic agent but is used for its synergistic effect with PIs such as DRV, ATV and LPV. When combined with 100 mg of RTV q12h, the bioavailability of DRV increases from 37 to 82% and its elimination half-life is extended from 12 to 16 hours [75]. The “immediate” increase in pill burden is counterbalanced by the reduced frequency of administration of the active drug.

Later efforts led to the commercialization of cobicistat (COB) (Figure 11), a structural derivative of RTV with no anti-HIV activity of its own – thus completely removing the risk of drug resistance – as well as a more favorable toxicity profile [54]. It is co-formulated with DRV as Rezoista or Prezcobix (DRV 800 mg/COB 150 mg) in an attempt to improve patient compliance. The latest fixed-dose combination, Symtuza, further decreases the pill burden by associating three active drugs with a booster: DRV 800 mg/COB 150 mg/TAF 10 mg/FTC 200 mg. COB is also used to boost other ARVs such as ATV and EVG.

Figure 11. Structural formulae of RTV and COB.



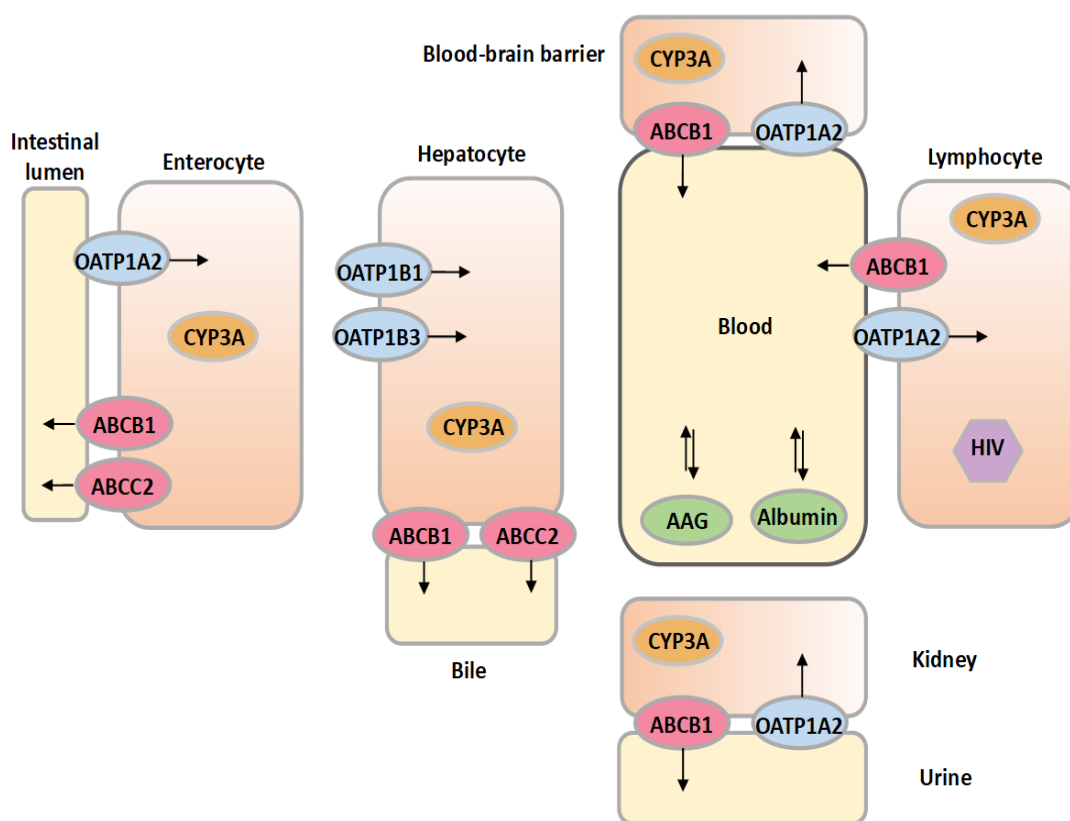
DRV therapy poses many challenges in terms of drug-drug interactions (DDIs) (see Annex 1 for a summary of clinically relevant DDIs). Indeed, DRV is a substrate of CYP3A as well as several influx (OATP1A2, OATP1B1, OATP1B3) and efflux (ABCB1, ABCC2) transporters (Figure 12) [87–90]. It is also an inhibitor of its own metabolism, an inhibitor of P-glycoprotein (encoded by the *ABCB1* gene), and an inducer of CYP3A mRNA and ABCB1 protein expression (Table 3). The addition of a booster, each with their own unique interaction profile, further complicates the management of HIV patients. The CYP-mediated biotransformation of DRV is more extensively discussed in Annex 3 (p.131).

Table 3. Biotransformation and transport of DRV and its boosters.

	Darunavir	Ritonavir	Cobicistat
CYP1A2	.	++	.
CYP2A6	.		
CYP2B6	.	+	-
CYP2C8	.	-	-
CYP2C9	.	-, ++	.
CYP2C19	<i>s</i>	++	.
CYP2D6	<i>s</i>	S, -	<i>s</i> , -
CYP2E1	.		
CYP3A4	S, --	S, --	S, --
CYP3A5	<i>s</i>	--	--
UGT1A1		-	-
ABCB1	S, -	--	-
ABCC1	-	--	.
ABCC2	S [?] , -	-	.
ABCC4		.	-
ABCG2	S [?] , -	.	.
OAT1		.	.
OAT3		-	.
OATP1A2	S		
OATP1B1	S, --	--	--
OATP1B3	S, --	--	--
OATP2B1	-	--	
OCT1		-	-
OCT2	.	--	-
OCTN1		-	-
MATE1	.	--	--
MATE2K		.	-
PXR	+	+	.

+: Weak inducer, ++: Strong inducer, -: Weak inhibitor, --: Strong inhibitor, S: Substrate, *s*: Substrate (minor contribution), .: Not a substrate / no effect, ?: Controversial, Blank: Unknown. Compiled from [54,59,87–99].

Figure 12. DRV cellular pathways.



AAG: Alpha-1 acid glycoprotein.

Boosting with RTV or COB greatly reduces CYP-based elimination (except for the formation of M33) and increases glucuronide formation. As a result, renal excretion of the parent compound also increases. The PK properties of DRV when administered alone and with RTV are compared in Table 4: RTV drastically increases exposure to DRV and reduces the inter-individual variability in half-life (note that the data shown in this table is collected from a small number of healthy volunteers and is not necessarily representative of actual patients). Originally, PK differences between RTV and COB were considered negligible [40,100–102]. However, the weaker boosting effect of COB turned out to be an important factor to consider, as previously shown in pregnant women [44]. Further, the DDI profile of COB is expected to be slightly different than that of RTV. For instance, the exposure of drugs that are metabolized by non-CYP3A isoforms or by UGT would be reduced by RTV but not by COB [99]. Therefore, switching boosters, while generally safe, could have unexpected consequences in some patients.

Table 4. Noncompartmental PK of DRV with and without RTV.

Parameter	Unboosted DRV	DRV/RTV
$t_{\max}$ (h)	0.5 (0.5–1.0)	0.75 (0.5–1.5)
$C_{\max}$ (ng.ml ⁻¹)	2730 ± 648	5125 ± 906
AUC _{0-∞} (ng.h.ml ⁻¹)	4746 ± 1647	50,760 ± 15,600
$t_{1/2}$ (h)	29.4 ± 45.3	13.5 ± 1.83

$t_{\max}$: Time to maximum concentration, $C_{\max}$: Maximum concentration, AUC: Area under the curve, $t_{1/2}$: Terminal elimination half-life. Data retrieved from [58]: “Data were obtained from healthy male subjects receiving a single 400-mg oral dose of [¹⁴C] darunavir either alone or with ritonavir (100 mg q12h). Values are mean ± S.D. except for $t_{\max}$, which are median (range) (n=4)”. Note the reduction of SD for $t_{1/2}$ with RTV.

1.2.7. Treatment simplification

As discussed in the last section of Antiretroviral therapy (p.17), various types of treatment simplifications have been evaluated on a theoretical level and in clinical practice, mostly in treatment-experienced individuals (Table 5, p.32).

For DRV specifically, several studies have demonstrated monotherapy to be a relatively safe alternative to standard tritherapy in stable patients with an undetectable viral load (VL), although an increased rate of viral blips or consecutive detectable VL were noted in some cases [100,103–109]. A recent meta-analysis of randomized clinical trials showed a small difference in favor of standard triple ART versus PI monotherapy if any kind of discontinuation was considered a failure, while this difference was no longer significant when a more restrictive definition of switch failure was used [105]. It could be argued that even in the event of virologic failure, no lasting consequences are expected since patients are unlikely to develop PI RAMs and intensification of ART with a double NRTI backbone (or another background regimen) allows re-suppression in the majority of subjects. DRV-containing dual therapy is also a viable option (Table 5, p.32), with the EACS 2020 guidelines mentioning DRV/3TC dual therapy as a potential switch strategy for virologically suppressed subjects, along with DTG/RPV, DTG/3TC and ATV/3TC (“supported by large randomized clinical trials or meta-analyses”), as well as DRV/RPV and DRV/DTG (“supported only by small trials”) [21]. DRV monotherapy is not yet recommended [21].

The daily dose of DRV could also be lowered from the current 800 mg to 600 mg once daily [107,110,111], or even to 400 mg once daily in virologically suppressed individuals [112,113] (Table 5, p.32). These results are not entirely surprising considering DRV already demonstrated good response rates with lower doses in pre-marketing clinical trials [32]. A small pilot study has even provided reassuring results on DRV 600 mg once daily monotherapy, as there were no viral rebounds and DRV plasma concentrations remained above the target value in all analyzed subjects [107]. It remains to be determined whether more patients could benefit from a reduced dose (including individuals with prior history of viral failure or RAMs) and if this is applicable to other ARV combinations than the ones already evaluated.

In the same vein as dose reduction, short cycle therapy (SCT) would require patients to alternate on- and off-treatment phases, each lasting a few days. Not only would this reduce costs and potential for adverse events, but it would also reduce the psychological burden ART places on patients' social and professional lives and could improve adherence [114]. Weekends-off regimens (5 days on/2 days off) have been successfully attempted for tenofovir- and EFV-based regimens [115–118], as have other types of SCTs for several NNRTI- and PI-based regimens, including DRV [119] (Table 5, p.32). Despite these planned treatment interruptions, NNRTI concentrations remained in the therapeutic interval, owing to their long plasma half-life. While this was not the case for DRV, it has been argued that its longer PD half-life could allow it to remain efficient during the off-treatment phases (see Mode of action and structure-activity relationship, p.21). In any event, this type of practice is not recommended at the moment [21].

Table 5. Simplification of DRV-based ART.

ART	Design / inclusion criteria	n	Follow-up duration	Virological endpoint	Results	Ref
DRV monotherapy	Prospective observational study of patients switching from 2 NRTIs and a third ARV, VL < 20.	28	48 weeks	Confirmed VL < 40	All remain virally suppressed. In subjects with available PCR, seminal plasma VL > 20 in 5%.	[103]
DRV monotherapy	Patients switching from DRV/RTV to DRV/COB monotherapy, VL < 20 for 24 weeks prior.	20	24 weeks	VL < 50	All remain virally suppressed. 20% detectable seminal plasma VL despite undetectable blood plasma VL (not significantly different from [103]).	[104]
DRV monotherapy	Prospective observational study of virally suppressed patients (for 24 weeks) switching to DRV/COB monotherapy for any reason.	234	96 weeks	Confirmed VL > 200	67.8% efficacy*. DRV C ₀ ~20% lower compared to historical controls (DRV/RTV monotherapy), but similar efficacy.	[100]
DRV monotherapy	Retrospective study of patients switching from DRV/RTV to DRV/COB monotherapy, VL < 50 at baseline.	173	58 weeks	Confirmed VL > 50 or switch after single VL > 50	5% failure and 6% discontinuation for non-virological reasons.	[120]
DRV mono/dual therapy	Switch from DRV/RTV/3TC dual therapy or DRV/RTV monotherapy to DRV/COB monotherapy.	162	48 weeks	Confirmed VL > 50	95.1% success rate*.	[106]
DRV or LPV monotherapy	Randomized to DRV or LPV monotherapy, or continuation of standard ART.	43	48 weeks	VL > 50	One failure in the LPV monotherapy arm. Non-inferiority could not be assessed due to the study being terminated. Worsening of lipid profile in monotherapy arms, but possible improvement of bone density with DRV monotherapy.	[108]
PI monotherapy (mostly DRV)	PI-experienced patients randomized to monotherapy or continuation of current ART, baseline VL < 50 for 24 weeks.	581	4 years	3 successive VLs > 50	38.5% failure rate (Kaplan-Meier analysis). Failure risk is highest early after monotherapy initiation. Predictors of failure: CD4 nadir, short time since suppression, ethnicity, VL assay sensitivity.	[109]
DRV monotherapy or DRV/3TC	Randomized trial comparing DRV mono- and bitherapy, baseline VL < 200 for 24 weeks.	265	96 weeks	Confirmed VL > 50	At 48 weeks, superiority of dual therapy versus monotherapy: 3% versus 24.8% failure*. Resuppression with adherence counseling or reintroduction of NRTIs.	[121]
DRV/3TC	Prospective, observational study of patients	106	96 weeks	Confirmed VL > 50	95% efficacy* at 48 weeks. 75% efficacy* at 96	[122]

	switching for renal/bone toxicity, intolerance or physician's decision.				weeks (mostly to avoid DDIs). Increased CD4/CD8 ratio.	
DRV/RPV	Retrospective study of patients switching from various types of mono-, bi- or tritherapy, baseline VL < 1000.	161	24 weeks	Confirmed VL > 50	86.2% success rate*. Re-suppression was possible with the same dual therapy, a different one or tritherapy.	[123]
DRV/RPV	RCT of virologically suppressed patients with stable PI-based ART.	42	96 weeks	VL < 50	Comparable success rate in both arms.	[124]
DRV/RPV	RCT of virologically suppressed patients (> 6 months) switching from triple ART to RPV/DRV/COB.	160	24 weeks	VL < 50	Dual therapy is non-inferior to triple ART.	[125]
DRV/RPV	Retrospective study of patients switching from mono-, bi- or tritherapy to RPV + boosted DRV, regardless of VL.	84	48 weeks	Confirmed VL > 50	87.7% efficacy, higher if baseline VL is < 50.	[126]
DRV/RAL	ARV-naïve patients randomized to DRV/RTV / TDF/FTC or DRV/RTV/RAL.	805	96 weeks	Composite endpoint of VL > 50, death and AIDS events	13.8% and 17.8% failure rate for standard and NRTI-sparing ART, respectively. Similar rates of AEs.	[127]
DRV/RAL	ARV-naïve patients.	112	48 weeks	VL > 1000 at 12 weeks or confirmed VL > 50 at 24 weeks or > 0.5 log increase in VL from week 4-12	16% and 26% failure rates at 24 and 48 weeks, respectively*. Failure correlated with baseline VL and subtherapeutic RAL levels.	[128]
DRV/ETR	Treatment-experienced and treatment-naïve patients.	54	48 weeks	VL < 50	89% success rate*.	[129]
DRV/ETR	Switch from various regimens, heavily pretreated patients, baseline VL < 1000.	99	24 weeks	VL < 50	89% success rate*.	[130]
DRV/ETR	Retrospective analysis of treatment-experienced patients switching to bitherapy.	68	2 years	Confirmed VL < 40	88.8% success rate. Correlation between failure and higher number of previous ART regimens.	[131]
DRV/DTG	Observational study of subjects switching to DRV/DTG, including those with treatment failure at baseline.	130	96 weeks	VL < 50	Baseline: 40% detectable VL. At week 96: 1.5% detectable VL, 17.7% VL 1-49 and 77.7% undetectable.	[132]
DRV/DTG	Retrospective analysis of treatment-experienced patients switching to bitherapy.	31	48 weeks	VL < 50	6.5% failure, all had detectable VL at baseline.	[133]

DRV/DTG	Switch study, patients suppressed at baseline, using COB or RTV boosting.	51	48 weeks	VL < 37	90% efficacy* and improvement of lipid parameters.	[134]
DRV/DTG	Retrospective study of heavily pretreated patients, suppressed for > 6 months, switching from any regimen.	50	2 years	VL < 50	98% success rate.	[135]
DRV/DTG	Retrospective study of treatment-experienced patients.	65	1 year	VL < 50	94% success, including 98 / 81% of those with undetectable / detectable VL at baseline.	[136]
PI-based dual therapies	Treatment-experienced patients switching from various triple ART.	376	5 years	Confirmed VL > 50	20.4% treatment discontinuation, including 10.1% failures.	[137]
4 days on/3 days off triple ART	Switch from triple ART, baseline VL < 50 for 48 weeks.	100	48 weeks	VL < 50	96% success rate.	[119]
DRV 600 mg once daily monotherapy	Switch from DRV 800 mg monotherapy, triple ART or other PI monotherapy.	31	48 weeks	VL < 50	No viral failure, 2 switches due to AEs.	[107]
DRV 600 mg once daily (including 1 monotherapy)	Case reports, treatment-experienced patients switching to ART containing low dose DRV.	7	Variable	VL < 50	Viral suppression achieved in those individuals.	[110]
DRV 600 mg once daily	Randomized to continue standard triple ART or reduce DRV dose.	100	48 weeks	Confirmed VL > 50	Comparable failure rates (including discontinuations for non-virological reasons). Comparable DRV C ₀ . Possible minor advantage in terms of AEs. Estimated cost savings: \$7000/patient/year.	[111]
DRV 400 mg once daily (RTV-boosted)	Switch from DRV 800 mg once daily, VL < 50 for 48 weeks and stable ART.	95	48 weeks	Confirmed VL > 50	91.6% success rate. Small improvement in frequency of gastrointestinal disorders.	[112]
DRV 400 mg once daily (RTV-boosted)	RCT of switch from LPV + 2 NRTIs to low dose DRV + 2 NRTIs, virally suppressed for 2 months, using LPV for 6 months prior.	300	48 weeks	VL < 50	96 and 94% of patients maintain viral suppression for the DRV and LPV arms, respectively. AEs more frequent with DRV (20 vs 5%).	[113]

AE: Adverse event, C₀: Trough concentration, ETR: Etravirine, LPV: Lopinavir, RCT: Randomized Controlled Trial, *: Intention-to-treat analysis. VL expressed in RNA copies/ml. Confirmed VL requires a second measurement. Unless otherwise specified, DRV can be either RTV- or COB-boosted.

1.3. Pharmacogenetics

1.3.1. Introduction

Genetic variation in genes coding for biotransformation enzymes, drug transporters, nuclear receptors, pharmacological targets and other entities is believed to play a role in the inter-individual variability that is described for many drugs. Pharmacogenetics (or, by extension, pharmacogenomics) is the discipline dedicated to studying the influence of these genetic variants on drug PK and PD in order to enable personalized medicine [138].

Variants with a minor allele frequency of at least 1% are referred to as genetic polymorphisms. Among these, single nucleotide polymorphisms (SNPs) are characterized by a change of one nucleotide, and, being a frequent type of variant in the human genome, they constitute a prime target of study for pharmacogenetics [139]. Other types of variants include insertions and deletions, as well as copy number variation (CNV), which are characterized by a variable number of repeats of a given sequence.

The change of a single nucleotide can alter the protein sequence that will be obtained through translation (missense variant) or even introduce a premature stop codon, resulting in a truncated protein (nonsense variant). In some cases, the change of nucleotide does not lead to a change of amino acid (due to codon degeneracy) – these are called synonymous SNPs. Further, SNPs may occur outside of the coding region, which can have effects through, e.g., mutation of promoters, silencers and enhancers, or introduction of alternate splice sites. Even so, SNPs (in the strictest sense of the word) represent but a small fraction of total genetic variation, as most polymorphisms occur at a low rate (in $< 0.5\%$ of the population) [139]. In other words, most variants are too rare to be considered SNPs.

In candidate gene studies, the selection of variants to investigate is operated by using prior knowledge derived from *in vitro* experiments for the drug of interest or clinical data from other drugs for which a variant has already been demonstrated to be predictive of PK, PD or toxicity. These studies can focus on anywhere from one to several dozen markers (sometimes more). However, to cover the full genetic diversity found in the human genome, high-throughput, more explorative, analyses called genome-wide association studies (GWAS) are required. The strength of a GWAS lies in the sheer number of variants that can be detected, although that is also a potential issue since the risk of type I error grows exponentially with the number of associations to be tested.

But even a GWAS is not guaranteed to fully describe PK/PD heterogeneity, as it is fundamentally multivariate: bar a few exceptions, such as thiopurine S-methyltransferase polymorphisms or prediction of abacavir hypersensitivity, single genes or single polymorphisms are not sufficient to explain the variability observed in individuals (on this topic, see our review for ARVs and CYP variants [140], p.131). Furthermore,

pharmacogenetic studies often face methodological limitations such as small effect sizes combined with small study populations [141].

1.3.2. Current evidence

To date, only a few small-scale candidate gene studies are available for DRV (Table 6). None of them showed genetic polymorphisms to be good predictors of inter-individual variability. One study found a marginal effect of two poorly characterized *SLCO3A1* intronic SNPs on plasma exposure, another one found a minor effect of *SLCO1A2* variants on CNS penetration, and our research group detected an effect of a well-known *CYP3A5* variant, but only in a subgroup of individuals with induced CYP metabolism.

Table 6. Pharmacogenetic studies of DRV.

Study design	SNPs	Effect	Ref
Population PK 75 patients, rich and sparse sampling	148 SNPs in 15 genes (<i>ABC</i> , <i>PXR</i> and <i>SLCO</i> families)	<i>SLCO3A1</i> rs8027174 and rs4294800 associated with slightly lower CL and with higher V_c , respectively (not clinically significant).	[142]
Population PK 60 patients, sparse sampling	36 SNPs in 15 genes (including <i>CYP3A</i> and <i>PXR</i>) (genotypes available for subsets of patients)	No effect of SNPs.	[143]
Single point PK (paired plasma and CSF) 41 patients	6 SNPs in <i>ABCB1</i> , <i>ABCC2</i> and <i>SLCO1A2</i>	Slightly higher (nonsignificant) CSF-to- plasma ratio for carriers of variant <i>SLCO1A2</i> alleles.	[144]
Single point PK 135 patients	<i>CYP3A5</i> 6986A>G	Reduced plasma concentrations in <i>CYP3A5*1</i> carriers receiving etravirine (no effect in other subjects).	[145]
Single point PK (paired plasma and PBMC) 19 patients (31 samples)	<i>ABCB1</i> 3435C>T	No effect of this polymorphism on plasma or PBMC concentrations.	[69]
Population PK 716 patients, sparse sampling	<i>CYP3A4</i> *22, <i>SLCO3A1</i> rs4294800, <i>PXR</i> rs2472677, <i>CAR</i> rs2307424, <i>SLCO1B1</i> 521T>C	No effect of these SNPs. <i>CYP3A5</i> *3 and <i>SLCO3A1</i> rs8027174 not analyzed due to not being in Hardy- Weinberg equilibrium.	[146]

CAR: Constitutive androstane receptor, CL: Clearance, PXR: Pregnane X receptor, V_c : Central volume of distribution.

A summary of all SNPs of interest is presented in Table 7. These have been chosen based on their functional effects and expected or demonstrated relevance to DRV PK, and some will be discussed further in **Paper 1** and **Paper 3**.

Table 7. List of single nucleotide polymorphisms of interest.

Gene	Nucleotide change	rs number	Effect	Minor allele frequency				
				African	American	East asian	European	South asian
<i>ABCB1</i>	c.1199G>A	rs2229109	Missense variant: Ser400Asn. Effect possibly substrate-dependent. [147]	0.003	0.024	0	0.033	0.009
	c.1236C>T ■	rs1128503	Synonymous variant. Effects are controversial (activity increased, decreased or unchanged) [147].	0.136	0.403	0.627	0.416	0.587
	c.2677T>G/A ■	rs2032582	Missense variant: Ser893Ala/Thr. Effects are controversial (activity increased, decreased or unchanged) [147].	0.020	0.369	0.398	0.410	0.592
	c.3435C>T ■	rs1045642	Synonymous variant, alters the timing of protein folding and decreases mRNA stability [148]. Effect possibly substrate-dependent.	0.150	0.428	0.398	0.518	0.575
<i>ABCC2</i>	g.99782821C>T	rs717620	5'UTR variant.	0.031	0.016	0.217	0.207	0.095
	c.1249G>A	rs2273697	Missense variant: Val417Ile.	0.189	0.159	0.096	0.204	0.272
	c.4544G>A	rs8187710	Missense variant: Cys1515Tyr.	0.162	0.055	0	0.071	0.017
<i>ABCG2</i>	c.421C>A	rs2231142	Missense variant: Gln141Lys, reduced activity.	0.013	0.141	0.291	0.094	0.097
<i>CYP3A4</i>	g.15389C>T	rs35599367	<i>CYP3A4</i> *22: Alternate splice variant, characterized by decreased functional mRNA and protein levels [149].	0.001	0.026	0	0.050	0.006
<i>CYP3A5</i>	g.6986A>G	rs776746	<i>CYP3A5</i> *3: Introduces a cryptic splice site, leading to a premature stop codon and truncated, inactive protein.	0.180	0.797	0.713	0.943	0.668
<i>SLCO1A2</i>	g.21335814C>T	rs4148977	Intron variant.	0.503	0.231	0.359	0.290	0.334
<i>SLCO1B1</i>	c.388A>G/T	rs2306283	Premature stop codon.	0.182	0.527	0.238	0.597	0.453
	c.521T>C	rs4149056	Missense variant: Val174Ala.	0.014	0.134	0.123	0.161	0.043
<i>SLCO3A1</i>	g.91941607G>T	rs8027174	Intron variant.	0.073	0.056	0.002	0.085	0.013
	g.91917464A>G	rs4294800	Intron variant.	0.334	0.316	0.078	0.338	0.231

Allelic frequencies are from the 1000 Genome Project, retrieved using Ensembl (<https://www.ensembl.org>) [134]. ■ In strong linkage disequilibrium.

1.4. Population pharmacokinetics

Pharmacometrics is a subdiscipline of pharmacology that applies mathematical modeling and simulations to drug PK/PD and pathophysiology in order to improve drug discovery, development and therapeutic use. One particular approach known as population pharmacokinetics has become a commonly accepted methodology for analyzing PK data [150,151]. Population models are compartmental models that represent the flow of drug between compartment units using systems of differential equations, supplemented with a statistical and probabilistic component to describe the variability in the population of interest.

One of their main advantages over the traditional, individual PK approach is that they can be used to obtain estimates of PK parameters even with sparse or incomplete data (even as few as one concentration per individual), which is often the case in the later stages of drug development and in post-marketing studies. They are not limited to descriptive analysis either. Simulations based on the model can be used to derive PK parameters in large virtual populations, to compute target attainment probabilities or to optimize experimental designs. Furthermore, models may be used to perform Bayesian estimation, wherein PK parameters are calculated in new individuals based on *a priori* information – the model – and *a posteriori* information – PK data and/or covariates collected during therapeutic drug monitoring (TDM) or clinical studies – to assess treatment response or to adapt the dose.

While software differ in their selection of estimation algorithms and various other features, the core modeling process remains fundamentally the same. The following sections describe a workflow for model development with particular attention to nonlinear mixed effects models.

1.4.1. Structural and covariate model

A structural model is the skeleton upon which all other components can be grafted. The term “structural model” is used to refer to the combination of the compartmental model and the statistical component, although it can also refer to the former only.

Absorption, distribution and elimination kinetics are most commonly described using zero- or first-order kinetics; more complex parameterizations may be needed to accurately describe saturable processes, pharmacologically active metabolites, etc. Although population models are often empirical in nature, model building can also be driven by knowledge of physiology, anatomy and physico-chemistry.

In mixed effects models, parameters of interest are described by a probability distribution using a typical value (the fixed effect) and a variance function (the random effect) [152]. To put it simply, fixed effects represent central tendencies and random effects represent variation. These parameters are used to describe PK processes such as the rates of absorption, distribution and elimination, but also the effects of covariates, and the PD or clinical response(s). For instance, assuming log-normal distribution of the PK parameter clearance:

$$CL = \theta_{CL} \times \exp(\eta_{CL})$$

where CL is the individual clearance, θ_{CL} is the fixed effect (typical value) and η_{CL} is the random effect. η are assumed to follow a normal distribution with mean 0 and variance ω^2 .

In the previous example, a single level of random effects is used. Variability can be further decomposed into multiple levels, typically including inter-individual variability (IIV), inter-occasion variability (IOV) and residual variability (encompassing analytical variability, errors in sampling time, model misspecification, etc.) [152].

Residual variability is usually modeled on the dependent variable (concentration or other biomarker), e.g.:

$$DV = PRED + \varepsilon$$

where DV is the dependent variable, PRED is the value predicted by the model and ε is the residual random effect. ε are assumed to follow a normal distribution with mean 0 and variance σ^2 .

Depending on the amount of data available, it may not be possible to resolve all variance components. For instance, if each subject is only sampled on one occasion, IOV cannot be estimated [152].

Once the individual PK parameters are known, univariate or multivariate regression according to any quantitative or qualitative variable can be conducted. The study of covariate relationships is perhaps one of the main draws of the population approach: it can improve the quality of model predictions and enables exploratory, semi-mechanistic analysis. Each covariate may be treated as a fixed or random effect (or both) and may be associated with model parameters through equations that can be purely empirical or attempt to describe some known biological reality. The order of covariate inclusion can be a trial and error process, be guided by existing knowledge, be automated through the use of certain algorithms, or one may perform full covariate modeling, wherein all covariates are included in a single step.

1.4.2. Model evaluation

When building a structural or covariate model, numerical, statistical and graphical methods are required to assess the goodness of fit – in other words, whether the model accurately describes the observed data. For the sake of clarity, we choose to refer here to the process of picking one model over another in the early stages of development as model *selection*, while the final process of gauging the performance of a model will be referred to as model *validation*, although both rely on (mostly) the same techniques and criteria.

The model that should be carried forward is not necessarily the most physiologically meaningful or the most elegant one – it is the model that is fit for purpose. One should favor

the model that describes the data the most accurately, while keeping their goal in mind. Should the model represent a physiological phenomenon? Is it going to be used to extrapolate the data to another, different type of population? How costly would a model misspecification be? While a wide array of diagnostic techniques are available, there is no consensus on how to best select and validate models, and one will find many disparities even in peer-reviewed literature [152,153]. Generally speaking, more stringent criteria should be applied when a model is built for predictive versus purely descriptive purposes, and demonstrating good model performance using multiple methods lends more credibility to the results, as there is no "universal" diagnostic for nonlinear models [154]. A non-exhaustive list of diagnostic methods, most of which are applicable to all stages of model development, will be presented in the next few paragraphs [152,154–156].

The likelihood function is the probability of observing the current data, given the model [154]. Oftentimes, the value of the objective function (OFV) is given, with the OFV being equal to $-2 \log(\text{likelihood})$. Thus, maximizing the likelihood is equivalent to minimizing the OFV. The OFV is a property of the data and is meaningless on its own, but a drop in OFV can be used to select one model over another one provided they are nested (i.e., one model is a reduced version of the other one, featuring fewer parameters but maintaining the same structure). A chi-squared test can be used to check whether two OFVs are significantly different. Objective functions provided by different algorithms or different software are not comparable, and neither are OFVs for models built on different datasets.

Criteria like the Bayesian information criterion (BIC) and Akaike information criterion (AIC) apply a penalty term to the OFV for every supplemental parameter introduced in the model, with the BIC being more conservative than the AIC [154]. They are not used for frequentist inference, and thus can be interpreted without a preset significance level. They are calculated as follows:

$$\text{AIC} = \text{OFV} + 2 p$$

$$\text{BIC} = \text{OFV} + p \times \ln(n)$$

where n is the number of observations and p is the number of parameters.

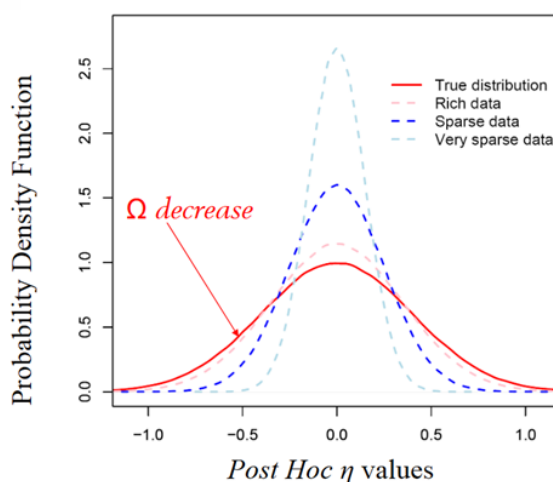
The OFV, BIC and AIC are convenient metrics and are frequently used in the process of model selection. However, they have no value as diagnostic tools since the numerical value itself does not tell us anything of the quality of the model, let alone why it is a good (or poor) model. On the opposite, most diagnostics described further in this section can provide at least some information regarding model shortcomings and how to fix them, and have their use both in model selection and validation.

The covariance matrices of fixed and random effects are useful for assessing correlation between parameters. A strong correlation between model parameters implies they cannot be estimated separately, which can be due to physiology (two phenomena that are not independent from one another), or a lack of data and overparameterization. Additionally, one can compute the precision of parameter estimates as standard errors (SEs) or confidence

intervals (CIs) based on the Fisher information matrix (FIM), which is minus the second derivative of the likelihood [157] (see also Optimal design, p.49). SEs are derived from the square root of the diagonal elements of the inverse of the FIM, then CIs are computed as $\text{mean} \pm 2 \text{ SEs}$. Alternatively, CIs can be obtained through simulation and resampling (see further in this section).

When the data is uninformative and there exists large inter- and/or intra-individual variability, model parameters and predictions may shrink toward a central value, as there is not enough information to fully separate subjects from one another (Figure 13) [152,158–160]. With very sparse designs, sampling times also contribute to some extent. Shrinkage translates into biased individual estimates, which limits the usefulness of many common model diagnostics, distorts parameter distributions and covariate relationships (masking true relationships, changing the shape of the relationship and leading to erroneous conclusions), and reduces the predictive power of the model.

Figure 13. Illustration of shrinkage. [161]



η : inter-individual random effect, Ω : variance/covariance of inter-individual random effects

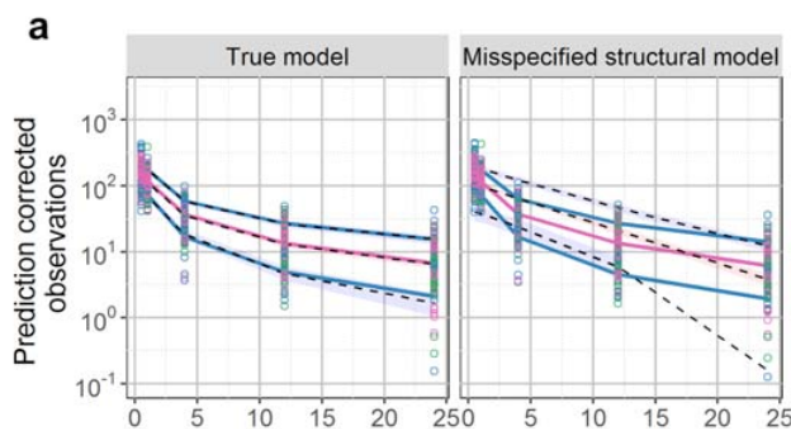
Model selection can also be operated based on reduction of unexplained variability (either in inter-, intra- or residual error), especially when adding covariates.

Graphical evaluation methods include plots of predicted versus observed concentrations (which are expected to be linearly correlated) or plots of residuals (calculated as the difference between observations and predictions) versus predictions or time. Such plots can be used to check for any tendency that would suggest model misspecification in different concentration or time ranges, or to look for outliers. Different methods for calculating residuals have been proposed, with some having better statistical properties than others [162].

Other diagnostics are based on Monte Carlo simulations that generate large amounts of datapoints using the model [152,156,163]. Intuitively, if the model fits the data well, there should be an agreement between data simulated from the model and observed data. Summary metrics of parameters (such as mean AUC) or percentages of outliers can be monitored, a

technique known as numeric predictive check (NPC). A visual predictive check (VPC) is the graphical equivalent of the NPC, wherein one typically plots observations along with a prediction interval defined by the median and some percentiles of simulated observations (Figure 14). Percentiles of observations and CIs may be layered on top of this to make the VPC more informative. Most observations should fall within predefined ranges, e.g., 90% of observations within the 90% prediction interval, and should be distributed evenly around the median [156]. Unbalanced sampling designs, very heterogeneous populations or clinical trials involving dose adaptation can make VPCs difficult to interpret or outright misleading. Data stratification according to subpopulations, dose regimens or covariates, or the use of prediction-corrected VPCs can help in this regard [155,164].

Figure 14. Example of VPC. [151]



Dots represent observations, dashed lines observation percentiles, the pink line the median of simulations, blue lines the 10th and 90th percentiles of simulations. In this example, the elimination phase is poorly described by the model on the right side, the median of simulations does not line up with the median of observations, and neither do the upper percentiles of observations and simulations.

Prediction discrepancies are another simulation-based metric that is conceptually similar to the VPC. For each observation, a large number of simulated datapoints are generated, and the percentile of the observation in this distribution is computed. Because observations (and simulations) within an individual are correlated, a decorrelation step must be performed, which yields normalized prediction distribution errors (NPDE) [165,166]. The normality of the NPDE distribution can be checked using graphical methods and appropriate statistical tests [166].

Methods such as the bootstrap are computationally intensive techniques that repeatedly and randomly resample the original dataset with replacement, not unlike a lottery in which tickets (individuals or observations) are drawn then placed back into the urn so they can potentially be drawn again, until a large enough sample is generated. These resampling methods are used to calculate bias and CIs of parameter estimates. A few variants exist: a nonparametric bootstrap takes a random subset of observed samples by omitting some of them, the semiparametric variant adds noise to this subset and the parametric bootstrap simulates from the actual posterior distribution of the data [152]. Alternatively, cross-validation repeatedly splits the original dataset in predefined proportions and fits the resulting subsets of data to the

final model. Unfortunately, these resampling techniques are unlikely to detect all types of model misspecifications [152].

All of the aforementioned simulation- and resampling-based techniques may be used to perform what is known as *internal* validation – because they do not involve new (external) data. In contrast, *external* validation applies these techniques to one or more new datasets, but since it is often not feasible to obtain new data solely for the purpose of validation, this second approach is more rarely used [150,152,153]. A compromise could be to split the original dataset into a learning and a validation set. A few ways to perform external validation are:

- Add the validation set to the learning set to check if parameter estimates are changed.
- Fix the model parameters and re-run it with the validation set. Assess the goodness of fit using residuals, NPDE, etc.
- Superimpose the validation set on simulated data from an internal validation to perform a VPC.

Finally, sensitivity analysis assesses model robustness: small changes to the data or to the initial estimates provided to the software should not significantly impact the results [152]. As all models make certain assumptions, whether in their structure or regarding the data they describe, the impact of these could be assessed through sensitivity analysis as well.

2. PERSONAL RESEARCH WORK

2.1. Objectives

As discussed in the introduction, DRV displays a large, poorly described inter- and intra-individual PK variability. Despite this variability, the same dosage is used in most patients, which could lead to over- or under-exposure, depending on the case. In this context, it is difficult to determine *a priori* whether alternative dose regimens, which could help reduce the drug burden, long-term toxicity and costs, among other things, would be adequate in a given patient.

The first main objective of this work was to develop a mathematical model to characterize DRV PK and how patient covariates can alter individual response to the treatment. To that end, clinical data was collected from a population of HIV-1 patients treated with DRV in combination with various other ARVs. In **Paper 1**, we detail the clinical trial design, the model building strategy and the results of the covariate analysis. The model was evaluated using standard goodness-of-fit metrics and diagnostics based on Monte Carlo simulations (**Paper 1**), followed by a rigorous external validation using a new dataset (**Paper 2**).

The second main objective was to use the model to simulate the effect of alternative dosage regimens for this drug and to determine whether these are safe from a PK point of view. The methodology and results of this approach are detailed in **Paper 1**.

In **Paper 2**, the same model was used to derive optimal sampling strategies, showing how much information is obtained using a variety of experimental designs. These were chosen to represent both typical designs for a sparse sampling PK study or for TDM.

Because clinical studies face certain methodological limitations (such as low sample size and confounding effects), we also aimed to use *in vitro* experiments to determine whether genetic polymorphisms in *ABCB1* could contribute to the PK heterogeneity of DRV, independently from other factors. This was the topic of **Paper 3**.

2.2. Population pharmacokinetics of darunavir

2.2.1. Introduction

We conducted a PK study of DRV in a cohort of HIV-positive patients in order to determine the extent of its PK variability and to identify covariates that are predictive of this variability. To achieve this goal, a population PK approach was used. This methodology was chosen for several reasons, which will be discussed here. Firstly, considering the number of patients that could realistically be recruited in a relatively short span of time and the fact that these would be ambulatory patients, thus only allowing for sparse sampling in most cases, population PK was deemed the best approach for data analysis. Secondly, population models would enable complex covariate modeling, allowing us to simultaneously study the influence of many individual and environmental factors. Thirdly, the model could then be used to conduct simulations for scenarios of interest to the clinician, such as extreme patient profiles or alternative dose regimens.

This model had to be validated internally and externally. Internal validation involves techniques based on Monte Carlo simulations, which is the norm for most models found in the literature. These are useful and require nothing more than computational time in terms of resources. However, they are suboptimal in their ability to detect model misspecifications as they rely on a form of cyclical reasoning: data is simulated based on the final distribution of PK parameters, itself obtained from observed data. For this reason, the use of new data (external validation) is seen as a more rigorous approach [150], although it requires more resources and is not without limitations – for instance, the type of subjects, the analytical method and the sampling scheme for the validation dataset should be comparable to those of the learning dataset [152]. Obviously, covariates used in the final model (if not the full range of covariates from the learning set) should also be available in the validation set. It is especially important to conduct this type of validation when attempting to use a model for predictive purposes.

Lastly, we used the model to propose accurate Bayesian estimators that would allow prediction of a patient's full PK profile based on a limited sampling strategy. Although population models can be used to obtain estimates of PK parameters from samples collected at random times, the use of an optimal sampling strategy can maximize the amount of information that is gathered over the course of a clinical study.

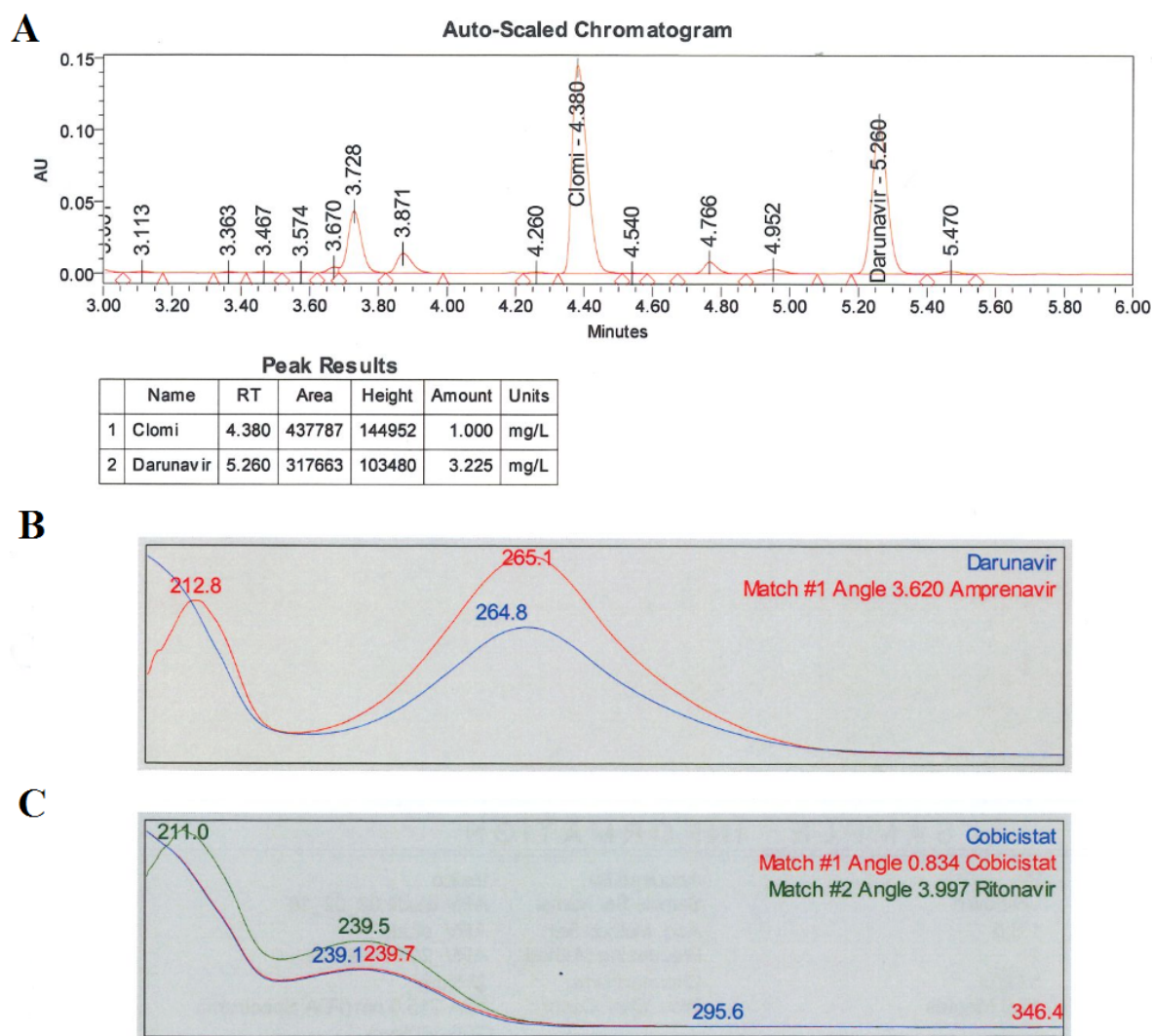
2.2.2. Methodological background

2.2.2.1. Drug quantification

DRV, RTV and COB were quantified using Ultra-Performance Liquid Chromatography (UPLC) coupled with a Diode Array Detector (DAD). This method has previously been validated and is routinely used for the TDM of several ARVs. Details of the analytical method

can be found in section 2.2 of **Paper 1** and examples of analyte chromatograms and UV spectra can be found below (Figure 15).

Figure 15. Chromatogram and UV spectra of DRV, RTV and COB.



(A) Chromatogram showing clomipramine (internal standard) and DRV peaks, their retention times (RT), peak areas/heights and concentrations (measured at 260 nm). (B) Spectra of DRV and amprenavir. (C) Spectra of RTV and COB. The COB sample (blue) is matched with its reference spectrum (red).

Liquid chromatography (LC) is a separation technique that relies on the difference of affinity of analytes for a mobile liquid phase, whose composition may be varied over time, and a stationary phase composed of micrometric particles, based on the respective physicochemical characteristics of all analytes. In normal phase, the stationary phase is more polar than the mobile phase; as can be expected, reverse phase is the exact opposite. The use of very high pressures (with high flow rates and small particle sizes) confers superior speed and resolution to the chromatographic technique, hence the name UPLC.

As they exit the LC column, analytes are identified by the DAD by comparing their UV spectra and retention time to those of known standards (Figure 15). Absorption of UV light mostly occurs in unsaturated chemical functions, conferring wide applicability to the technique. Unlike conventional UV detectors that are limited to a small number of analyzed wavelengths, diode arrays can simultaneously scan a wide range thereof. Generally speaking, DADs are cheap and robust, but they lack the high sensitivity of other detectors such as mass spectrometers (which will be discussed in the methodological background for **Paper 3**, p.88).

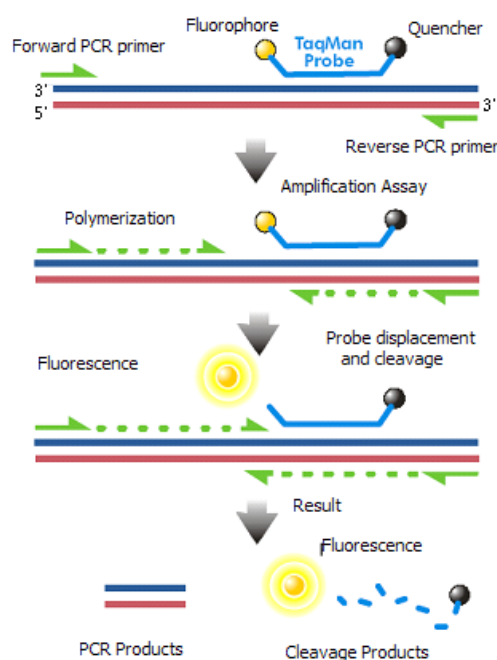
A calibration curve is used to determine the relationship between concentration and signal intensity (as surface area of each chromatographic peak) for a series of known concentration levels, then the concentration of each analyte in experimental samples is interpolated from the resulting equation. The addition in equal amounts to all processed samples of an internal standard, a substance that is chemically similar to the analyte of interest, increases the reliability of results by compensating for variations in, e.g., pipetting volumes.

2.2.2.2. Genotyping

Real-time polymerase chain reaction (PCR) is a molecular biology technique that amplifies the target DNA, with a wide range of applications, from identification of microorganisms to studies of gene expression in pathological conditions. PCR is more limited in scope than sequencing techniques but is very useful for interrogating small numbers of candidate SNPs.

DNA is first isolated from blood through non-specific binding to a silica membrane followed by several washing steps to eliminate all contaminants, before eluting the DNA. The recovered genetic material is amplified through a reaction catalyzed by a polymerase isolated from *Thermus Aquaticus* in the presence of necessary reagents, cofactors and fluorescent probes that specifically hybridize to the region of interest (Figure 16). Each probe carries a fluorescence emitter and a quencher. During polymerization, these two elements are separated, leading to a proportional increase in fluorescence intensity. Allelic discrimination is then performed by a clustering algorithm based on the relative fluorescence intensities of the various probes, each probe being specific of a given allele and associated to a distinctive fluorescent dye.

Figure 16. Real-time PCR with Taqman probe. [167]



2.2.2.3. Optimal design

PK study designs are often complex and costly in personnel and laboratory analyses, not to mention inconvenient for experimental subjects. Hence, it is desirable to reduce them to their simplest form while still ensuring objectives can be met. Indeed, with fewer subjects or fewer samples available per subject, model parameters may become biased or unidentifiable. Optimal design can help reach a compromise by comparing different strategies with regards to population sample size, the number of arms in a trial, the number and schedule of biological samples, dose levels, etc. Usually, all but one of these elements are fixed, and only, e.g., the sampling schedule, is optimized.

Theoretically, one could perform simulations for all scenarios of interest in order to find the optimal design, but the number of simulations required renders this type of approach infeasible in practice [168]. Instead, D-optimal design is a widely accepted approach for mixed effects models that involves optimizing the determinant of the FIM [169–173]. As mentioned in Model evaluation (p.40), standard errors of estimates are derived from the FIM, meaning the FIM directly reflects the accuracy of the model, and its determinant is a measure of the amount of information yielded by the experiment. As such, D-optimal design seeks the set of parameters that maximize the FIM for a given model. Time could be the parameter to optimize within constraints set by the user to ensure the design is practical, such as maximum number of subjects and samples, length of dosing interval and total observation period. Since biological samples cannot always be collected at precise timepoints, the effect of variations in sampling times can be assessed by constructing sampling windows using one of several methods (such as the one described in [174], which allows for windows of varying lengths around each timepoint).

All possible designs are evaluated by one or more search algorithms. Once optimal designs have been calculated, it is recommended to assess their performance using simulations and by calculating metrics such as the bias and precision on all parameters, including variance components.

Standard D-optimal designs were eventually extended to include IIV (and are thus referred to as population D-optimal designs) and IOV [175]. These may be advantageous in certain cases – for instance, the sampling scheme may comprise fewer points than a standard optimal design [170].

One major limitation of D-optimal designs is that they hinge on the underlying model being known with certainty; it is assumed there is no model misspecification and that the parameters are the “true” ones, which is invariably never the case. Designs that incorporate parameter uncertainty have been called Bayesian designs or robust designs. These include ED-optimal designs, that require knowledge of the full probability distribution of parameters [173].

2.2.3. Paper 1

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Exploration of Reduced Doses and Short-Cycle Therapy for Darunavir/Cobicistat in Patients with HIV Using Population Pharmacokinetic Modeling and Simulations

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Abstract

Background and objectives Protease inhibitors such as darunavir are an important therapeutic option in the anti-human immunodeficiency virus arsenal. Current dosage guidelines recommend using cobicistat- or ritonavir-boosted darunavir 800 mg every 24 h (q24h) in protease inhibitor-naïve patients, or ritonavir-boosted darunavir 600 mg q12h in experienced patients. However, darunavir displays a large, poorly characterized, inter-individual pharmacokinetic variability. The objectives of this study were to investigate the pharmacokinetics of darunavir and to elucidate the sources of its inter-individual variability using population pharmacokinetic modeling. Then, to determine the appropriateness of current treatment guidelines and the feasibility of alternative dosing regimens in a representative cohort of adult patients using simulations.

Methods Sparse pharmacokinetic samples were collected in 127 patients with human immunodeficiency virus type 1 infection, then supplemented with rich sampling data from a subset of 12 individuals. Data were analyzed using the nonlinear mixed-effects modeling software NONMEM. The effect of reduced doses (600 mg q24h and 400 mg q24h) or reduced frequency of administration (800 mg q24h for 5 days followed by 2 days of treatment interruption) was simulated.

Results Our model adequately described the pharmacokinetics of darunavir. Predictors of individual exposure were *CYP3A5**3 and *SLCO3A1* rs8027174 genotypes, sex, and alpha-1 acid glycoprotein level. No relationship was apparent between darunavir area under the curve and treatment efficacy or safety. For reduced dose regimens, darunavir concentrations remained above the protein binding-corrected EC₅₀ in the majority of subjects. More stringent pharmacokinetic targets were not reached in a significant proportion of patients.

Conclusions These results add to the growing body of evidence that darunavir-based therapy could be simplified to reduce costs and toxicity, as well as to improve patient compliance. However, the heterogeneity in pharmacokinetic response should be considered when assessing whether individual patients could benefit from a particular regimen, for instance through the use of population pharmacokinetic models.

Clinical Trial Registration ClinicalTrials.gov identifier: NCT03101644, date of registration: 5 April, 2017.

1 Introduction

According to international guidelines, protease inhibitors (PIs), in combination with a background regimen of two nucleoside reverse transcriptase inhibitors (NRTI), are a

first-line option for the management of human immunodeficiency virus type 1 (HIV-1) infection [1, 2]. Darunavir (DRV) is the preferred PI, but must be coadministered with a pharmacokinetic (PK) booster, either ritonavir (RTV) or cobicistat (COB), to maximize exposure. Standard dosages include DRV/RTV 800/100 mg every 24 h (q24h) or DRV/COB 800/150 mg q24h for PI-naïve patients [3] (or those pretreated with PIs but with no known resistance mutations) [4], and DRV/RTV 600/100 mg every 12 h (q12h) for patients presenting with PI resistance mutations [5–8]. One of the main advantages of DRV is its high genetic barrier to resistance as it binds to a highly conserved domain of the viral protease [9–11]. This makes it useful in patients with

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Key Points

A population pharmacokinetic model of darunavir was developed and validated. *CYP3A5* and *SLCO3A1* genotypes, alpha-acid glycoprotein level, and sex were predictors of darunavir pharmacokinetics.

Reduced dosages (600 mg every 24 h and 400 mg every 24 h) were predicted to be safe in the majority of patients. Our model could be used to assess changes in exposure for various alternative regimens in specific individuals.

incomplete adherence because subtherapeutic exposure is unlikely to result in the emergence of resistance mutations, making re-challenge with the same drug possible.

However, treatment with DRV is rendered delicate by its complex spectrum of drug–drug interactions (DDIs). Not only must certain drugs be avoided to ensure adequate exposure to antiretrovirals (ARVs), but DRV and its boosters can also inhibit (and sometimes induce) various cytochromes and transporters, thereby affecting other therapies [12–15]. Indeed, DRV is a substrate for cytochrome P450 (CYP) 3A; a substrate for efflux transports of the ABC family ABCB1 (P-glycoprotein) and ABCC2; a substrate for influx transporters of the OATP family OATP1A2, OATP1B1, and OATP1B3; and has the potential to inhibit CYP3A4, OATP1B1, and OATP1B3. Meanwhile, RTV and COB exert slightly different effects: both are capable of inhibiting CYP3A, OATP1B1, and OATP1B3. Ritonavir may also induce other CYP isoforms and pregnane X receptors, while COB is more selective in this regard [16].

Darunavir pharmacokinetics are highly variable between individuals, although the sources of this heterogeneity remain poorly understood. Single nucleotide polymorphisms (SNPs) in genes coding for biotransformation enzymes and transporters, DDIs, and other patient-specific covariates may all contribute to this phenomenon [17]. Previous studies have already highlighted the role played by certain individual factors such as alpha-glycoprotein level [18, 19], sex [20], weight [21], age [20], SNPs in *SLCO3A1* [19], and RTV exposure [21–23]. Our first aim was to use population pharmacokinetics to model this variability in a representative cohort of adult patients with HIV and to identify the best set of predictors. In addition, we evaluated whether changes in DRV pharmacokinetics were correlated with biomarkers of treatment efficacy (viral load and lymphocyte count) and toxicity (occurrence of adverse events).

Our second aim was to simulate the effect of alternative dose regimens. Simplified antiretroviral therapies (ARTs)

involving fewer drugs or lower doses have gained attention as they could help to reduce toxicity, reduce costs (therefore increasing access to the drug in developing countries), and improve convenience and patient compliance. A reduction in the daily dose of DRV/RTV from 800/100 to 600/100 mg q24h was demonstrated to be safe in virologically suppressed patients [24, 25], as was a further reduction to 400/100 mg q24h [26, 27]. Additionally, DRV 400 mg plus a NRTI backbone was found to be non-inferior to standard dose lopinavir plus a NRTI backbone [27]. Ritonavir- or COB-boosted DRV monotherapy has also seen some success [28–34], even with the lower 600-mg q24h DRV dosage [35]. Although this type of regimen may expose patients to a higher risk of viral blips, they may easily be re-suppressed by restoring their initial treatment due to the aforementioned pharmacological properties of DRV. Short-cycle therapy (SCT) is a different approach consisting of alternating on- and off-treatment phases for a few days each, which would significantly reduce the drug burden for the patient. Moreover, a lower pill burden has been correlated with better adherence in patients with HIV, which could help with long-term viral suppression [36]. Based on what few data are available, SCT for DRV-containing triple combination ART appears to be a viable option, despite low plasma concentrations of DRV during the off-treatment phase [37]. The effects of both reduced doses and SCT were investigated in the present study using simulations, based on the population PK model we developed.

2 Materials and Methods

2.1 Study Design

This was a prospective study conducted at the Cliniques Universitaires Saint-Luc (Brussels, Belgium). Blood samples were obtained during routine hospital visits, once every 3–6 months on average. One sample for the quantification of ARVs was drawn in a heparinized tube at each patient visit (dataset A). Self-reported drug intake time as well as blood sampling time were recorded. One additional sample for DNA extraction was drawn in an EDTA tube at the patient's first study visit.

Intensive sampling was performed in a subset of 12 individuals selected for their high treatment compliance based on available PK data and anamnesis by the physician. In those individuals, a dose journal over the previous 4 days was requested, dose intake on the day of sampling was witnessed by a nurse, and then eight samples were collected at pre-specified time points: pre-dose, 0.5, 1, 2, 3, 4, 5, and 6 h post-intake (dataset B).

2.2 Drug Quantification

Darunavir, RTV, and COB were quantified using a validated, ultra-performance liquid chromatography-diode array detection method [38]. Briefly, samples were spiked with the internal standard (clomipramine), buffered to alkaline pH, then a liquid-liquid extraction was performed using methyl *tert*-butyl ether. Samples were centrifuged and the supernatant was evaporated to dryness by heating at 40 °C under a stream of nitrogen. The residue was reconstituted in mobile phase and 5 µL was injected on an Acquity UPLC BEH C18 column. The mobile phase was a gradient of triethylammonium phosphate (pH 3) and acetonitrile. All reagents were of analytical grade.

The lower limit of quantification (LLOQ) was 0.1 mg/L for DRV and RTV. For COB, no LLOQ was formally defined but a threshold of 0.2 mg/L was used based on the signal-to-noise ratio. The method was linear over the range of 0.1–10 mg/L for all three analytes. Samples were analyzed in batches along with external quality controls (containing DRV and RTV) and internal controls (blank plasma spiked with known amounts of all analytes, as well as previously determined patient samples). For this analytical method, our laboratory successfully participates in an external quality assessment scheme organized by the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML, Nijmegen, the Netherlands); the trueness and precision of the method were within 10% for DRV and RTV. The stability of DRV in various conditions (autosampler, room temperature, freeze-thaw, and long-term storage) has been determined by other authors [39, 40].

2.3 Genotyping

DNA was isolated using the QIAamp® DNA mini-kit (Qiagen, Antwerp, Belgium) according to the manufacturer's instructions. Allelic discrimination was performed on a Step One Plus™ real-time polymerase chain reaction system using Taqman® genotyping assays (Fisher Scientific, Merelbeke, Belgium) for *ABCB1* rs2229109 and rs1045642; *CYP3A4* rs35599367; *CYP3A5* rs776746; and *SLCO3A1* rs8027174 and rs4294800. These SNPs were selected based on expected clinical relevance and results from previously published studies. Haldane's exact test (as implemented in the HardyWeinberg package for R) [41] was used to check for deviation from the Hardy–Weinberg equilibrium.

2.4 Modeling Software

Pharmacokinetic data were analyzed using the nonlinear mixed-effects modeling software NONMEM (version 7.4.3) [42]. Additional functionality was provided by PsN (version

4.7.0) [43]. NONMEM output was processed in R (version 3.5.2) [44] with the npde package (version 2.0) [45].

2.5 Structural Model

Dataset B was analyzed on its own then pooled with dataset A. For the pooled analysis, the PRIOR routine in NONMEM was used to specify a prior distribution of all fixed and random effects (except residual variability terms) based on final estimates of the model built on dataset B [46]. The reason we thus constrained parameter estimates was because it was assumed that dataset B would be more informative, especially regarding the absorption phase. Separate structural models were first developed for DRV and COB (development of a RTV model was not possible because of a lack of data). For each drug, a one- or two-compartment disposition model was fitted to the data. Models with first-order and delayed first-order absorption were evaluated. The interaction between COB and DRV was modeled according to a linear or $I_{\max}$ (Michaelis–Menten) function. To account for the nonlinear dose-exposure relationship of DRV [23, 47], dose-dependent and saturable clearance (CL) models were tested.

2.6 Covariate Model

The following covariates were investigated: age, body weight, race (subjects were classified as Caucasian or African, other races were treated as Caucasian), food intake with last DRV dose, plasma albumin and α 1-acid glycoprotein (AAG), genotypes, and DDIs. Genetic covariates have already been described in Sect. 2.3. For each SNP, subjects were stratified according to their number of functional alleles, or simply as expressors vs non-expressors in the case of *CYP3A5**3. For DDIs, concomitant drugs (ARVs and non-ARVs) were classified as CYP3A inhibitors, CYP3A inducers, or P-glycoprotein inhibitors based on published lists of interactions [48, 49]. Missing covariate values were substituted by the most recent measurement, the population median, or the most frequent category, as appropriate.

Covariates were first screened for univariate association with the empirical Bayesian estimates of PK parameters. The process of covariate stepwise selection was then partly automated using PsN, based on the decrease of the objective function value (OFV). The α thresholds for statistical significance were set to 0.05 for forward inclusion and 0.01 for backward elimination. The equations below were used to describe covariate relationships (for continuous covariates, linear and exponential models with normalization to the median were both considered), where *cov* covariate value, θ typical value, θ_{cov} covariate scale factor (0 for reference group), *P* model parameter.

Exponential : $P = \theta \times e^{\theta_{\text{cov}} \times (\text{cov} - \text{median})}$

Linear : $P = \theta \times (1 + \theta_{\text{cov}} \times (\text{cov} - \text{median}))$

Categorical : $P = \theta \times (1 + \theta_{\text{cov}})$

Association between model-derived area under the curve (AUC) and pharmacodynamic biomarkers was assessed using Wilcoxon tests or by calculating Spearman's rho, as appropriate. The viral load (>40 or <40 copies/mL, i.e., undetectable) and CD4+ and total lymphocyte counts were used as markers of efficacy, and the occurrence of adverse events at any point during the study, stratified by type of symptom (neurological or lipodystrophy), as markers of toxicity.

2.7 Goodness of Fit and Internal Validation

Goodness of fit was assessed using standard graphical methods (individual and population predictions vs observations, residuals vs observations and time). Reductions in unexplained variability, shrinkage, or standard errors of estimates were additional criteria for model selection. The model was validated by verifying the normality of the normalized prediction distribution errors. Parameter uncertainty was assessed by constructing 95% confidence intervals from 1000 nonparametric bootstraps.

2.8 Target Attainment

Target attainment probabilities were computed by simulating DRV steady-state PK profiles based on the original dataset (1000 simulations in the absence of residual error). Evaluated strategies were DRV 800 mg q24h, 600 mg q24h, and 400 mg q24h, and SCT 800 mg q24h for 5 days. Trough concentration (C_0) targets were set to 0.055 mg/L (protein binding-adjusted EC_{50} for wild-type HIV-1 protease, serving as the therapeutic target in PI-naïve patients with no resistance-associated mutations) [11], 0.55 mg/L (ten times the wild-type EC_{50} , serving as the target in PI-experienced patients), and 2 mg/L (recommended C_0 in PI-experienced patients) [50].

3 Results

3.1 Study Population

One hundred and twenty-seven patients were enrolled, 12 of whom underwent intensive sampling. A total of 309 sparse samples (dataset A) and 96 rich samples (dataset B) were collected. For sparse samples, the post-intake time range was 1–29 h (median 11.9 h). Concentrations were censored if the post-intake time was unknown (1.9%), if the patient

reported a lack of treatment compliance (0.3%), or because of analytical reasons (1%). Darunavir concentrations below the LLOQ (3.6%) were set to half of the LLOQ.

Summary characteristics of the cohort can be found in Table 1. At baseline, median DRV treatment duration was 4.2 years, 85.8% of patients were using COB-boosted DRV, and DRV 800 mg q24h was the most frequent dosage (91.3%), followed by 600 mg q12h (7.9%) and 1200 mg q24h (0.8%). The three most common ARTs in this cohort were: DRV/tenofovir/emtricitabine (22.0%), DRV/dolutegravir (18.1%), and DRV/dolutegravir/lamivudine (17.3%). Two patients (1.6%) were using DRV 800 mg q24h monotherapy. Frequent complaints possibly related to ART that occurred at least once during follow-up for a given subject included lipodystrophy (29.1%) and neurological symptoms (insomnia, fatigue, or headaches) (26.0%). Genotypes for pharmacogenes of interest are summarized in Table 2. All SNPs were in Hardy–Weinberg equilibrium with the exception of rs776746 (this deviation was no longer significant when analyzing African and Caucasian individuals separately, suggesting population stratification). Cytochrome P450 3A5 genotypes within each racial group matched their expected distribution (from, e.g., the 1000 Genomes Project) [51].

3.2 Structural Model

Darunavir kinetics were best described by a one-compartment model with first-order absorption and elimination. Inter-individual variability was described by an exponential error term, while a mixed (additive plus exponential) residual error was used. For dataset B only, the model fit was improved by the addition of a lag term to absorption ($\Delta\text{OFV} = -36$), and by the addition of a block diagonal matrix between random effects ($\Delta\text{OFV} = -12$). However, treating the daily dose of DRV as a covariate on CL or modeling a saturable CL did not, or prevented the model from converging. Neither the lag time nor the full random effect matrices was retained in the model developed on the combined dataset.

Similarly, COB kinetics were described by a simple one-compartment model. There appeared to be a positive correlation between DRV CL and COB CL (dataset B: $\rho = 0.33$, $p = 0.3$, see the Electronic Supplementary Material [ESM]). Because inclusion of this relationship in a joint model on dataset B did not improve the fit, the COB portion of the model was dropped at this stage.

3.3 Covariate Model

For dataset B alone, two covariates were retained in the final model: all other things being equal, volume of distribution (V) was decreased by 53% in subjects harboring the *CYP3A5**3 allele and by 70% in Caucasian individuals

Table 1 Cohort summary characteristics

	<i>n</i>	%
Patients	127	
Sampling		
Sparse	115	90.6
Sparse + rich	12	9.4
DRV dose		
800 mg q24h	116	91.3
600 mg q12h	10	7.9
1200 mg q24h	1	0.8
Booster		
COB	109	85.8
RTV	18	14.2
Age (years)		
Median (IQR)	55 (13)	
Sex		
Male	85	66.9
Female	42	33.1
Weight (kg)		
Median (IQR)	73 (17)	
Race		
Caucasian	67	52.8
African	55	43.3
Other	5	3.9
ART		
2 NRTIs/DRV	29	22.8
3TC/DTG/DRV	23	18.1
DTG/DRV	22	17.3
Other	53	41.7
DRV treatment duration (years)		
Median (IQR)	4.21 (4.82)	
Viral load, RNA copies/mL		
< 40	100	78.7
> 40	13	10.2
CD4 cell count (/μL)		
Median (IQR)	564.9 (348.3)	
Toxicity		
Lipodystrophy	37	29.1
Neurological symptoms	33	26.0
Drug–drug interactions		
CYP3A inhibitors	3	2.4
CYP3A inducers	7	5.5
P-glycoprotein inhibitors	5	3.9

All values given at baseline (except adverse events, for which at least one occurrence during follow-up was considered)

3TC lamivudine, ART antiretroviral therapy, COB cobicistat, CYP cytochrome P450, DRV darunavir, DTG dolutegravir, IQR interquartile range, NRTIs nucleoside reverse transcriptase inhibitors, RTV ritonavir

compared with African individuals. In the combined dataset, the *CYP3A5* phenotype was associated with CL instead, with CL being 19% higher in expressors compared with non-expressors. Additionally, for the combined dataset, CL and *V* were both decreased in subjects with higher AAG, CL was 21% lower in female individuals compared with male individuals, and *V* was increased by 81% in individuals harboring *SLCO3A1* rs8027174. Overall, AAG was the most relevant covariate based on the total reduction in OFV ($\Delta\text{OFV} = -45$), although its inclusion only decreased unexplained residual variability by 7% and 10% for CL and *V*, respectively. Final model parameters are displayed in Table 3. Details of the covariate selection process can be found in the ESM. There was no apparent correlation between DRV AUC and viral load ($W=582$, $p=0.45$), CD4 lymphocyte count ($\rho=0.09$, $p=0.3$), or occurrence of adverse events at any point during follow-up ($W=1687$, $p=0.97$ for lipodystrophy; $W=1613.5$, $p=0.42$ for CNS adverse events).

3.4 Goodness of Fit and Internal Validation

There was good adequation between observed and individual predicted concentrations ($\rho=0.87$), except for two outlier individuals with very high levels of AAG (2.66 and 2.2 g/L), thus low model-predicted CL, while their observed concentrations were actually low (see the ESM). A total of five further observations identified as outliers based on their weighted residuals were discarded, then the model was re-run. Figure 1 shows the goodness of fit after removal of these outliers. Normalized distribution prediction errors were normally distributed, with a mean of 0 and a variance of 1 (Fig. 2); p values for the Shapiro–Wilk, Wilcoxon signed-rank, and Fisher variance tests were 0.56, 0.84, and 0.79, respectively. Final parameter estimates were contained in the 95% confidence intervals generated through nonparametric bootstrapping (Table 3).

3.5 Target Attainment

Table 4 shows the probability of target attainment (PTA) for each scenario, based on a representative population. Darunavir C_0 remained above the recommended cut-off of 0.055 mg/L in the majority of subjects for the 600-mg q24h and 400-mg q24h strategies, while only 84.0% and 69.8% of individuals remained above 0.55 mg/L for these two respective strategies. The 2-mg/L target was reached in less than a third of patients, even with the standard once-daily dosage. Weekends-off SCT resulted in subtherapeutic concentrations

Table 2 Genotypes

Gene	rs	SNP	<i>n</i>	(%)	<i>D</i>	Notes
<i>ABCB1</i>	rs2229109	c.1199G>A			0.13	Missense variant
		G/G	116	91.3		
		G/A	8	6.3		
		A/A	0	0		
<i>ABCB1</i>	rs1045642	Missing	3	2.4	0.74	Synonymous variant, decreases mRNA stability
		c.3435C>T				
		C/C	61	48.0		
		C/T	53	41.7		
<i>CYP3A5</i>	rs776746	T/T	10	7.9	– 13.24**	<i>CYP3A5</i> *3: truncated protein
		Missing	3	2.4		
		g.6986A>G				
		*1/*1	33	26.0		
<i>CYP3A4</i>	rs35599367	*1/*3	33	26.0	0.16	<i>CYP3A4</i> *22: alternative splice variant
		*3/*3	58	45.7		
		Missing	3	2.4		
		g.15389C>T				
<i>SLCO3A1</i>	rs8027174	*1/*1	115	90.6	0.66	Intron variant
		*1/*22	9	7.1		
		*22/*22	0	0		
		Missing	3	2.4		
<i>SLCO3A1</i>	rs4294800	g.91941607G>T			– 1.11	Intron variant
		G/G	105	82.7		
		G/T	18	14.2		
		T/T	0	0		
<i>SLCO3A1</i>	rs4294800	Missing	4	3.1	– 1.11	Intron variant
		g.91917464G>A				
		G/G	56	44.1		
		G/A	53	41.7		
<i>SLCO3A1</i>	rs4294800	A/A	15	11.8	– 1.11	Intron variant
		Missing	3	2.4		

D Haldane's *D*, *mRNA* messenger RNA, *SNP* single nucleotide polymorphism

***p* < 0.05 (statistically significant)

of DRV in a significant portion of individuals: 39.3% did not reach even the lowest C_0 target.

4 Discussion

Using population pharmacokinetics, we leveraged rich and sparse data to estimate individual PK parameters of DRV. A one-compartment model described the data well, with CL and *V* being in the range of those previously estimated with one-compartment models in adult populations [22, 52]. The estimate of k_a was less precise, probably owing to a lack of data in the absorption phase, with its confidence interval encompassing lower values such as those found in Moltó et al. [19] and Dickinson et al. [53] all the way up to

values closer to those calculated in Dickinson et al. [21] and Arab-Alameddine et al. [22]. Earlier publications have also applied two-compartment models [18, 19, 21, 53, 54] and more complex absorption kinetics such as transition compartment models [55], but these were not supported by our relatively sparse PK data. Among the investigated covariates, AAG and sex, along with *CYP3A5* and *SLCO3A1* genotypes, were found to be predictive of DRV pharmacokinetics.

Darunavir CL and *V* both decreased with increasing AAG levels, which is consistent with previous reports of AAG affecting DRV pharmacokinetics [18, 19]. The clinical relevance of this association is unclear as total concentrations may be altered without any impact on unbound concentrations. In addition to the AAG level, the AAG phenotype is

Table 3 Final population pharmacokinetic model

	Estimate	RSE (%)	95% CI	Shrinkage (%)
Structural model				
CL/F ($L \cdot h^{-1}$)	12.9	5.3	11.3, 14.4	33
ω_{CL} (SD)	0.22	13.95	0.08, 0.28	
V/F (L)	152	21.1	107, 170	59
ω_V (SD)	0.33	16.36	0.05, 0.39	
k_a (h^{-1})	0.68	73.5	0.3, 1.2	63
ω_{ka} (SD)	0.60	22.27	0.52, 1.86	
Covariate model				
AAG on CL	− 0.61	37.9	− 0.72, − 0.43	
AAG on V	− 0.68	26.3	− 0.74, − 0.31	
Female sex on CL	− 0.21	27.3	− 0.31, − 0.08	
<i>SLCO3A1</i> $G > T$ on V	0.81	59.5	0.34, 2.23	
<i>CYP3A5</i> *3 on CL	− 0.16	35.5	− 0.28, − 0.05	
Residual variability				
$\sigma_{\text{exponential}}$ (SD)	0.281	27.7	0.15, 0.32	12
σ_{additive} (SD)	0.641	39.4	0.45, 0.97	12

AAG α 1-acid glycoprotein, CI confidence interval, CL clearance, CL/F apparent clearance, k_a absorption rate constant, RSE relative standard error (= standard error/parameter absolute value), SD standard deviation, V volume of distribution, V/F apparent volume of distribution, ω random effect (inter-individual variability), σ random effect (residual variability)

known to modulate the plasma pharmacokinetics of some PIs, which may also play a role [56].

Regarding pharmacogenetics, both the loss-of-function variant *CYP3A5**3 and Caucasian race were associated with smaller V based on the rich dataset. However, the latter association was no longer apparent in the whole cohort and was not retained in the final model. *CYP3A5**3 is commonly found in Caucasian individuals, which may result in *CYP3A4* being the major isoform responsible for DRV metabolism for that ethnicity, while it is far less frequent in African individuals, in whom both isoforms (*CYP3A4* and *CYP3A5*) may contribute to some extent. Indeed, in subjects receiving efavirine (a known CYP inducer), plasma exposure to DRV was previously found to be lower in carriers of the functional *CYP3A5**1 allele compared with *CYP3A5**3 carriers, suggesting that *CYP3A5* is also involved in DRV biotransformation [57]. It is also worth noting that race correlated with the presence of the *CYP3A4**22, *CYP3A5**3, and *ABCB1* 3435C>T alleles in this population. The fact that race remained a significant predictor in a model already including the *CYP3A5* genotype suggests there may be other race-specific factors that we did not identify. Additionally, the *SLCO3A1* rs8027174 variant was correlated with larger V in our dataset. This intronic polymorphism was previously associated with smaller CL; however, it is unknown if DRV is a substrate of *SLCO3A1* [19].

Lastly, DDIs were not identified as a significant covariate, most likely owing to the small number of clinically relevant interactions found in our patients (mostly limited to a few efavirine users) and because of RTV and COB boosting

blunting any inductive effects or exceeding the inhibitory effects of concomitantly administered drugs.

Next, we used our model to perform simulations of alternative dosing schemes. There is growing interest in reducing the dose of DRV in virally suppressed patients: it was recently demonstrated that the daily dose of DRV/RTV could be reduced from 800/100 to 400/100 mg q24h while maintaining adequate viral suppression in plasma and semen, although bioequivalence between the dosages could not be assessed [26, 54]. So far, it is unknown if a similar regimen could be used in patients using DRV/COB, especially as COB may not be quite as powerful as RTV. Moreover, dose reductions are typically performed in virally suppressed subjects. It would be important to assess their feasibility in more diverse groups of patients. With regard to ART simplification, one option would be “weekends-off” therapy (i.e., patients take ART for 5 days then interrupt for 2 days), although longer off-treatment phases may also be worth investigating.

In our simulations, for an unchanged COB dose, DRV 600 mg q24h and 400 mg q24h resulted in decreased exposure, but concentrations remained well above the target of 0.055 mg/L in the majority of subjects. Weekends-off therapy appeared unsafe from a purely PK point of view. Although concentrations fell below the cut-off of 0.55 mg/L in a significant portion of subjects, this value can be considered fairly conservative because it is already ten times higher than the typical wild-type EC_{50} , and consequently does not apply to PI-naïve individuals. Finally, when targeting a C_0 of 2 mg/L, the PTA was extremely low, even for the standard

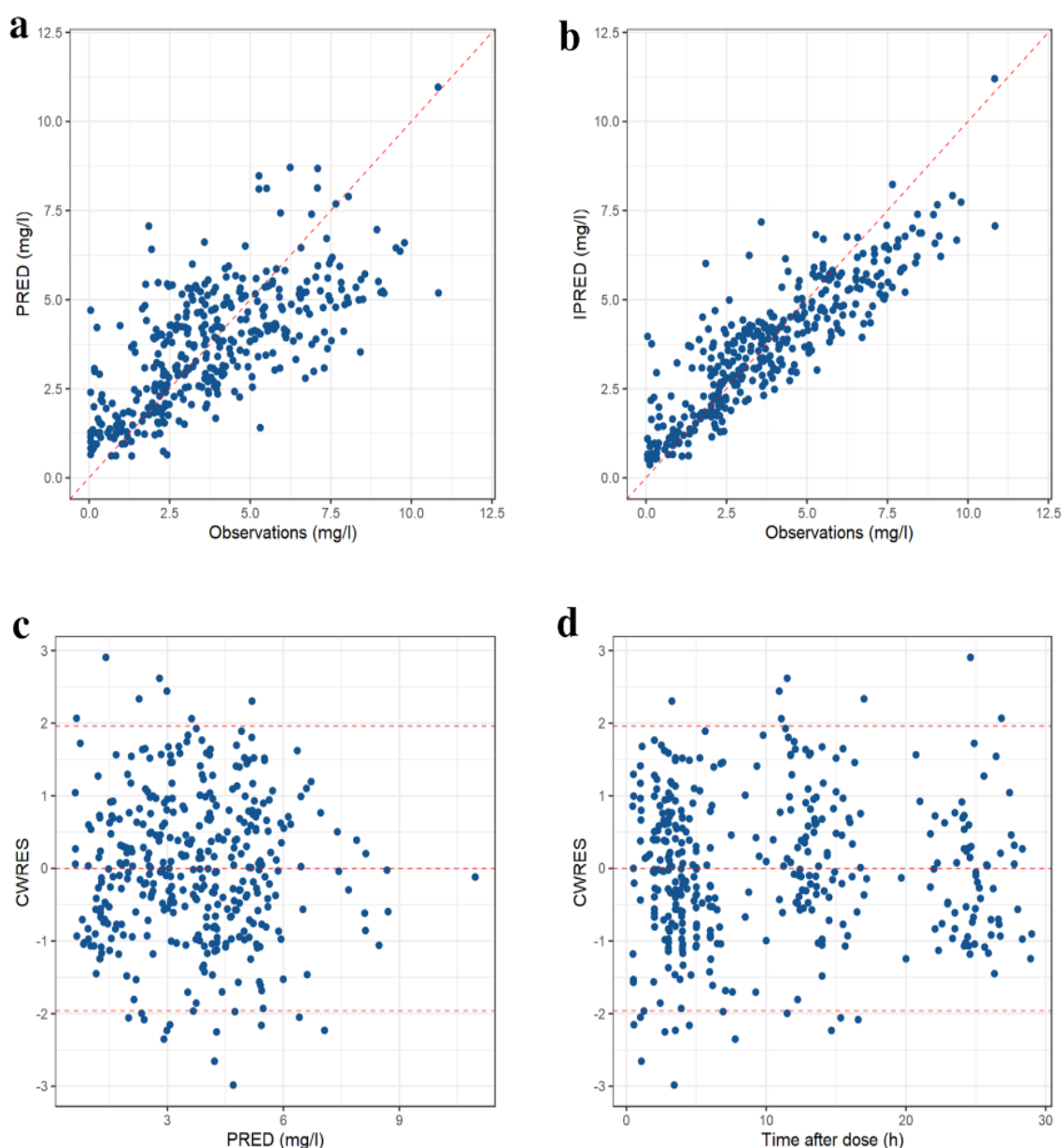


Fig. 1 Goodness of fit plots. **a** Population predicted concentrations (PRED) vs observations. **b** Individual predicted concentrations (IPRED) vs observations. **c** Conditional weighted residuals (CWRES) vs PRED. **d** CWRES vs time after dose

dosage. Interestingly, target achievement rates observed in our cohort were higher than the model-predicted rates: for q24h dosing, 7.1% and 25.7% of measured C_0 fell below 0.055 mg/L and below 0.55 mg/L, respectively (compared with the model-predicted probabilities of 0.7% and 10.4%). Among individuals with available C_0 , only four had detectable viremia (comprised between 40 and 63 copies/mL), and only one individual had what could be considered as a low DRV C_0 (0.246 mg/L). It is possible that in individuals with low DRV concentrations, concomitant ARVs ensured adequate anti-HIV activity, which would suggest that DRV is not a necessary component of the ARV regimen for those individuals, or it may be that the PK target itself is too stringent and that lower plasma concentrations are acceptable. Of

course, plasma exposure is only a surrogate for intracellular accumulation and target-bound DRV. These results should be contrasted with those from a 4 days a week intermittent ART study, in which virological failure was rare despite low DRV exposure [37].

Additionally, the simulations used to derive PTA in the present paper were performed on a dataset that is representative of a certain patient population; it may not necessarily reflect what would be observed in populations with completely different genotypes, for instance. Further, PTA for groups such as children and pregnant women would have to be calculated based on data obtained in those groups specifically because of how much they differ from a standard adult population. For example, dosing recommendations for

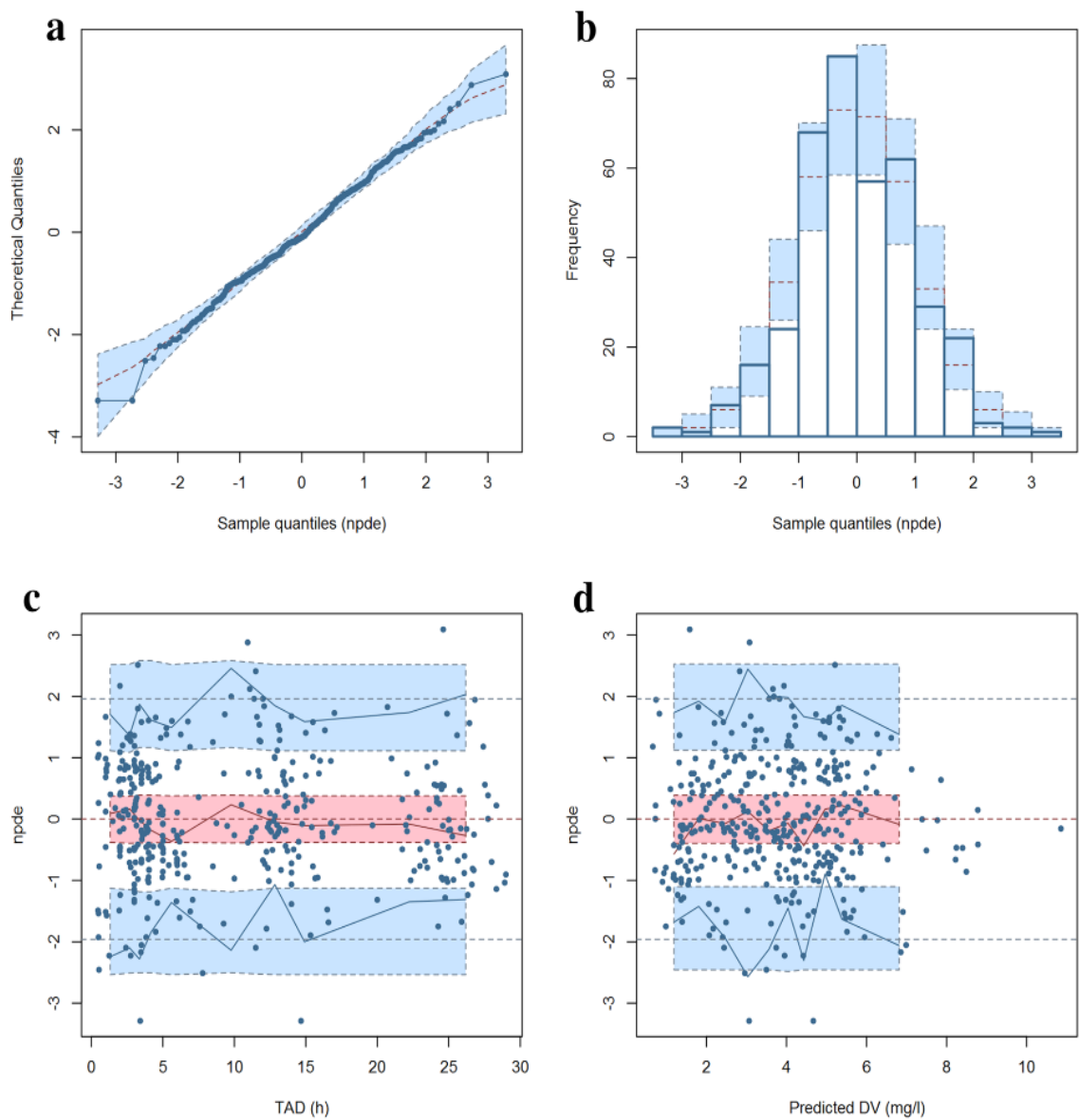


Fig. 2 Normalized distribution prediction error (NPDE). **a** *Q–Q* plot of NPDE. **b** Histogram of NPDE. Shaded area represents theoretical distribution. **c** NPDE vs time after dose. Shaded areas represent the prediction intervals associated with the 5th, 50th, and 95th percentiles. **d** NPDE vs predicted concentrations

Table 4 Probability of target attainment (PTA)

	800 mg q24h	600 mg q24h	400 mg q24h	800 mg q24h 5/7 days
C_0 (mg/L)	1.56	1.17	0.78	0.1
AUC_{0-24} (mg·h/L)	76.2	57.2	38.1	6.5
PTA (%)				
$C_0 > 0.055$ mg/L	99.3	99.1	98.6	60.7
$C_0 > 0.55$ mg/L	89.6	84.0	69.8	15.3
$C_0 > 2$ mg/L	33.2	16.4	3.6	1.3

C_0 and AUC_{0-24} are given as medians. For the 800 mg 5/7 days regimen, the C_0 is taken immediately before a new cycle (day 8) and the AUC is computed over day 7 to day 8
 AUC_{0-24} area under the curve from time 0 to 24 h, C_0 trough concentration, q24h every 24 h

DRV/RTV in children aged 3–12 years have been derived using a PK model that incorporated not only AAG, but also body weight, as is often the case for pediatric models [58]. For pregnant women, in whom changes in CYP activity and protein binding can alter PK processes, a recent population PK study showed that administering DRV/RTV q12h instead of the standard q24h dosing could compensate for the reduction in exposure caused by pregnancy, thereby ensuring therapeutic drug concentrations, although systematic dose adjustments might not be warranted [23].

There were a few limitations to our study, the first of which was the sparseness of PK data in most subjects, for whom one sample per dose interval was obtained at a random post-intake time. This resulted in high levels of shrinkage, especially on V and k_a , which could have prevented us from detecting all relevant covariate associations. Despite the addition of richer data and the use of a prior distribution, only a marginal improvement in model fit could be achieved and there was little change in individual parameter estimates. As a result, covariate associations should be regarded with caution. Further, there were few subjects using the 600 mg q12h dosage in our cohort. The limited amount of information on this dose level could be problematic for simulations, as DRV exposure (namely, the AUC) is known to increase less than dose proportionally [23, 47]. Consequently, extrapolation to lower doses using simulations may yield more pessimistic results than expected (i.e., predicted AUC will be lower than its true value), although this would not change our general conclusions.

While the relationship between dose and total exposure is nonlinear, that between dose and unbound exposure may be linear [23], which brings us to another limitation of our study: only total concentrations were measured. With DRV being highly protein bound, it has been argued that unbound concentrations could be a useful biomarker for therapeutic drug monitoring (TDM) [59]. If DRV is assumed to have a low extraction ratio, displacement of its binding to AAG could affect the total concentration without influencing the unbound concentration, rendering the interpretation of TDM more delicate. Ultimately, as TDM for this drug traditionally relies on total concentrations and as PTA targets are defined based on protein binding-adjusted EC_{50} , it was more rational to use the total concentration for the present work, but further studies are needed to establish the relationship between changes in total and unbound PK parameters, and how this could affect TDM of DRV. Lastly, most of the data used to build our model was collected in COB-boosted patients. While RTV and COB provide similar boosting effects, they are not identical and some minute differences have been demonstrated [16]; for instance, the boosting effect of COB was found to be insufficient in pregnant women [60]. Thus, model predictions could potentially be biased if applied to RTV-boosted patients.

5 Conclusions

Some individual factors were highlighted as potential predictors of DRV pharmacokinetics. Despite an important inter-individual variability, the probability of achieving commonly defined PK targets with reduced dose regimens was high, suggesting that this type of strategy could contribute to treatment simplification in a wide range of patients. Population PK models such as the one developed in this work could be used to predict changes in individual exposure based on a combination of TDM and covariate data.

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Compliance with Ethical Standards

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Conflict of interest Gabriel Stillemans, Leila Belkhir, Bernard Vandercam, Anne Vincent, Vincent Haufrond, and Laure Elens have no conflicts of interest that are directly relevant to the content of this article.

Ethics Approval The study protocol was approved by the local ethics committee (Comité d'Éthique Hospitalo-Facultaire des Cliniques Saint-Luc, approval number B403201731249). This study was conducted in accordance with the Declaration of Helsinki.

Consent to Participate Written informed consent was obtained from all participants before any study-related procedure.

Consent for Publication Not applicable.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code Availability Not applicable.

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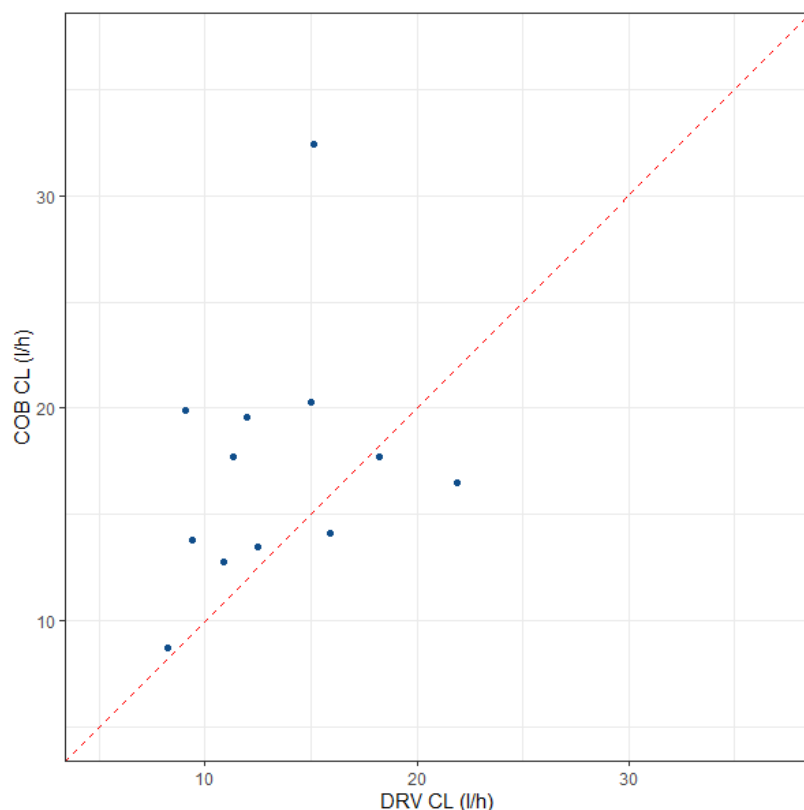
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SUPPLEMENTARY MATERIALS

Online Resource 1. DRV versus COB CL in dataset B



Online Resource 2. Covariate selection process

Covariate	Parameter	Equation	DF	Δ OFV	p
Forward search					
AAG	CL	Exponential	1	30.64969	3.09×10^{-8}
AAG	V	Exponential	1	14.45543	0.000144
<i>SLCO3A1</i> G>T	V	Categorical	1	7.23055	0.007167
Sex	CL	Exponential	1	6.50959	0.010729
<i>CYP3A5</i> *3	CL	Categorical	1	7.60685	0.005815
Backward search					
AAG	CL		1	-41.68951	1.07×10^{-10}
AAG	V		1	-12.77332	0.000352
<i>SLCO3A1</i> G>T	V		1	-8.01262	0.004645
Sex	CL		1	-11.86488	0.000572
<i>CYP3A5</i> *3	CL		1	-7.60685	0.005815

DF: degrees of freedom, Δ OFV: difference in objective function value.

Only covariates retained in the forward search are indicated, in their order of inclusion.

Associations tested:

- CL: Race, sex, *ABCB1* 1199G>A, *ABCB1* 3435C>T, *SLCO3A1* A>G, *SLCO3A1* G>T, *CYP3A4**22, *CYP3A5**3, age, body weight, albumin, AAG.
- V: Race, sex, *ABCB1* 1199G>A, *ABCB1* 3435C>T, *SLCO3A1* A>G, *SLCO3A1* G>T, body weight, albumin, AAG.
- k_a : Fasting status with last dose.

Online Resource 3. Outlier removal

#	Post-intake delay (h)	Concentration (mg.l ⁻¹)	Comment
1	18.13	9.178	
2	4.6	BLOQ	
3	12.33	BLOQ	
	13.42	BLOQ	
4	22.25	7.563	
5	3	2.361	High AAG (2.66 g.l ⁻¹)
6	2.75	2.214	High AAG (2.2 g.l ⁻¹)
7	3.42	BLOQ	

AAG: Alpha-1 acid glycoprotein, BLOQ: Below limit of quantification.

2.2.4. Paper 1 summary

- Using clinical data from 127 HIV-positive patients, we developed a population PK model of DRV.
- DRV CL and V decreased with increasing levels of AAG, CL was lower in females compared to males, CL was lower in *CYP3A5**3 individuals, and V was increased in carriers of *SLCO3A1* rs8027174.
- A universal dose reduction from DRV/COB 800/150 mg once daily to 600/150 mg once daily or 400/150 mg once daily appeared safe from a PK point of view. A 5 days on/2 days off dosing scheme could not be recommended.

Consult Annex 4 (p.142) for a comparison of previously published DRV population PK models.

2.2.5. Paper 1 – Additional data

In this section, we will provide some additional information, data and discussion regarding the PK study and the analyses described in **Paper 1**.

2.2.5.1. Intensive sampling cohort

An overview of the intensive sampling cohort (n = 12) is provided below, along with a comparison to the main cohort. Aside from differences related to inclusion criteria for the substudy, the 12 individuals were mostly comparable to the rest of the cohort.

Table 8. Sparse sampling cohort.

	Full cohort		Intensive sampling	
	n	%	n	%
Subjects	127		12	
Samples/patient (mean)	2.4*		8	
Dose				
800 mg q24h	116	91.3	12	100
600 mg q12h	10	7.9	0	0
1200 mg q24h	1	0.8	0	0
Booster				
COB	109	85.8	12	100
RTV	18	14.2	0	0
Sex				
Male	85	66.9	8	66.7
Female	42	33.1	4	33.3
Race				
Caucasian	67	52.8	8	66.7
African	55	43.3	4	33.3
Other	5	3.9	0	0

Age (years) median (range)	55.1 (21.2 – 76.3)		57.8 (31.4 – 77.8)	
BMI				
> 25	60	47.2	7	58.3
ARV combinations				
2 NRTIs/DRV	29	22.8	4	33.3
DTG/3TC/DRV	23	18.1	3	25.0
DTG/DRV	22	17.3	3	25.0
Other	53	41.7	2	16.7
Viral load				
< 40 ml ⁻¹	106	83.5	12	100
> 40 ml ⁻¹	13	10.2	0	0
CD4+ T lymphocytes (μl ⁻¹) median (range)	565 (74 – 1395)		589 (139 – 886)	
ABCB1 1199G>A				
GG	116	91.3	10	83.3
GA	8	6.3	1	8.3
AA	0	0	0	0
ABCB1 3435C>T				
CC	61	48.0	4	33.3
TC	53	41.7	6	50.0
TT	10	7.9	1	8.3
CYP3A4				
*1/*1	115	90.6	11	91.7
*1/*22	9	7.1	0	0
*22/*22	0	0	0	0
CYP3A5				
*1/*1	33	26.0	3	8.3
*1/*3	33	26.0	1	25.0
*3/*3	58	45.7	7	58.3
SLCO3A1 g.91941607G>T				
GG	105	82.7	8	66.7
GT	18	14.2	2	16.7
TT	0	0	0	0
SLCO3A1 g.91917464G>A				
GG	56	44.1	6	50.0
GA	53	41.7	4	33.3
AA	15	11.8	1	8.3

* 1 sample per dose interval, multiple dose intervals in the study.

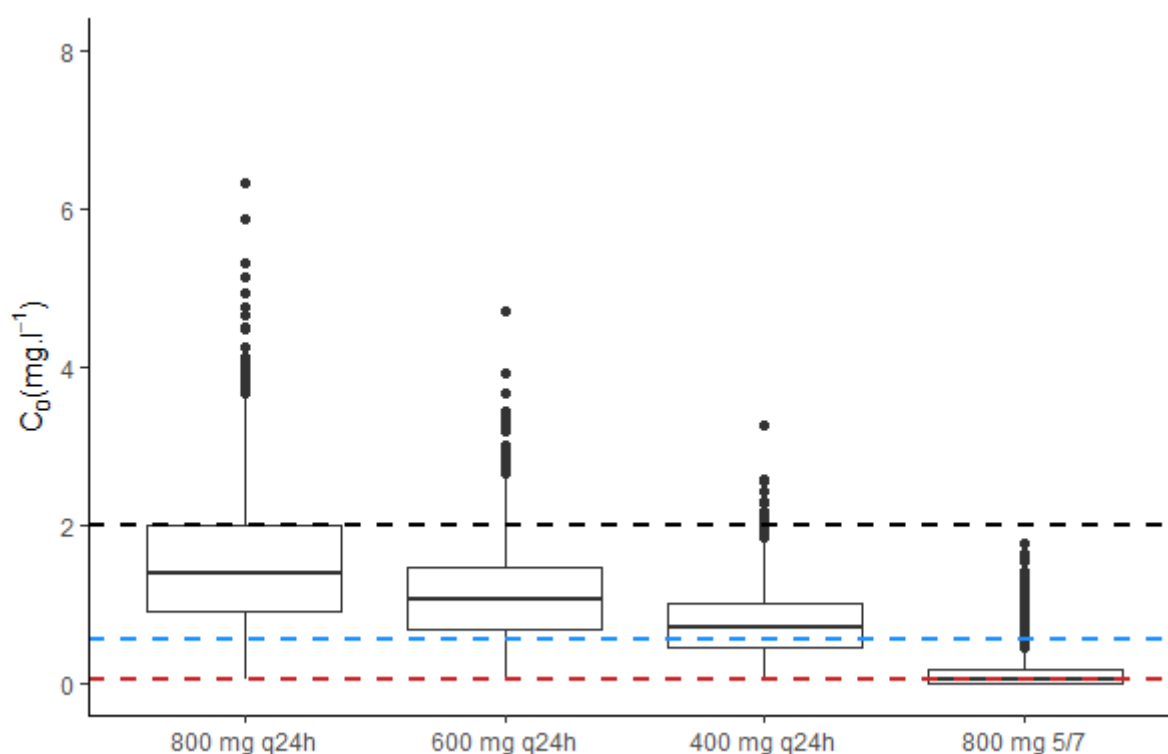
2.2.5.2. Target achievement rates

As discussed in **Paper 1**, target achievement rates provided in Table 4 are population-level PTAs. These should be understood as the total percentage of the population that achieves a given target. It says nothing of PTAs in specific subpopulations or individual PTAs for

patients with specific sets of covariates (that is what individual-level simulations and Bayesian estimation are for). It also says nothing of the magnitude of target achievement (how much higher than the target each individual is). This second point deserves to be briefly addressed here.

C_0 and AUCs provided in Table 4 can be used to gain a general overview of simulated target achievement. The boxplots below show the actual distribution of C_0 for each scenario, based on 100 simulations, with the 0.055, 0.55 and 2 mg/l targets indicated by colored lines.

Figure 17. C_0 distribution under each simulated scenario



As mentioned in the discussion of **Paper 1**, observed target achievement rates were slightly lower than model-predicted PTAs. Observed C_0 ranged from 0.1 (the limit of quantification, excluding samples below this value) to 5.3 mg/l (median = 1 mg/l, SD = 1.1 mg/l). 7.1% and 25.7% of available C_0 were below 0.055 and 0.55 mg/l, respectively. These values include all C_0 for q24h dosing in both the main study and intensive sampling substudy, measured at times ranging from 20 to 30 hours post-dose.

2.2.5.3. Effect of adherence on exposure

When performing simulations in **Paper 1**, one of the assumptions that was made was that of perfect adherence. Adherence can be broadly defined as whether patients take their treatment in the manner it was prescribed, including the correct drug, dose, frequency of administration and duration of treatment. However, adherence is not necessarily perfect at all times. Based on the data in our possession, it is obvious that some of our patients followed a strict drug

intake schedule while others were more flexible and irregular. It is also known that some subjects forget to take their medication and, in some cases, interrupt their entire ART for periods of time ranging from a few days to several months. The prevalence of these phenomena is unknown, but the latter is expected to be relatively rare, as it would easily be detected through viral load monitoring. Therefore, let us consider a simple case: the patient occasionally forgets to take their drug but roughly maintains the prescribed frequency of administration. This is assumed to be the most frequent case encountered among our patients.

One might be interested in knowing how non-adherence, especially these occasional lapses in adherence, can impact PTAs under various alternative dosing regimens. It is obvious that lower adherence can result in lower exposure and thus put patients at risk of under-exposure, but defining to what extent that is true is not so straightforward, for reasons that will be explained later in this section.

The 800 mg SCT regimen we studied in **Paper 1** is equivalent to missing two consecutive doses. In other words, this already gives us an idea of how much this type of non-adherence can impact PTAs (defined as the probability of achieving a C_0 target). C_0 and PTAs were computed in the manner described in **Paper 1** for the 800 mg q24h, 600 mg q24h and 400 mg q24h regimens (SCT was not considered due to the low base PTA, as well as the fact that this regimen *requires* missing doses), using 100 rounds of simulations, and relaxing the assumption of perfect adherence by having a single missed dose (which is the same as a 6/7 days regimen). The results of this analysis are presented in **Table 9**.

Table 9. Target achievement rates for incomplete adherence scenarios.

Simulated						
Dose regimen	800 mg q24h		600 mg q24h		400 mg q24h	
Missed last dose	No	Yes	No	Yes	No	Yes
C_0 (mg/l)	1.39	0.28	1.04	0.21	0.69	0.14
PTA (%)						
$C_0 > 0.055$ mg/l	99.3	84.9	99.8	80.8	99.5	74.1
$C_0 > 0.55$ mg/l	89.6	28.2	83.0	19.4	64.4	9.2
$C_0 > 2$ mg/l	33.2	1.2	9.3	0.3	1.2	0

As expected, at the population level, a missed dose dramatically decreases the PTA for the next C_0 , and this is especially true for more stringent PK targets. The reduction in exposure may not be clinically relevant, depending on which target is applicable to the patient and depending on their individual characteristics. The scenario discussed here is pessimistic: normally, not every patient would be non-adherent to such an extent. The numbers presented in **Table 9** are illustrative and are not meant for clinical forecasting. A more relevant approach would be to compute the PTA for a new patient under various adherence scenarios, or perhaps using a model explicitly including non-adherence.

A different type of scenario would involve multiple missed doses (but not necessarily consecutive ones) over a given period of time. While modeling non-adherence patterns is beyond the scope of this work, one might for instance suppose that the last dose before a patient's visit would be missed less frequently due to the white coat adherence effect. One could also suppose that missed doses within an individual follow a particular pattern (such as being more frequent during the weekend or more frequent in succession). It would also be logical to assume that adherence is not a randomly distributed variable: some individuals are more prone to have a low adherence than others, though the patterns of non-adherence in an individual might be random themselves (i.e., missing a dose does not modify the probability of missing another dose). Some of these elements can be accounted for using more elaborate methods – for instance, Markov mixed effects models [195]. Irregular drug intake patterns (e.g., near-complete adherence but without following a rigid q24h schedule) would be even more representative of true patient behavior, though it is not expected that they would have as much of an impact as missed doses.

2.2.6. Paper 2

G. Stillemans, L. Belkhir, B. Vandercam, A. Vincent, V. Haufroid and L. Elens, Optimal sampling strategies for darunavir and external validation of the underlying population pharmacokinetic model, *European Journal of Clinical Pharmacology* 77 (2021), pp. 607-616.



Optimal sampling strategies for darunavir and external validation of the underlying population pharmacokinetic model

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Abstract

Purpose A variety of diagnostic methods are available to validate the performance of population pharmacokinetic models. Internal validation, which applies these methods to the model building dataset and to additional data generated through Monte Carlo simulations, is often sufficient, but external validation, which requires a new dataset, is considered a more rigorous approach, especially if the model is to be used for predictive purposes. Our first objective was to validate a previously published population pharmacokinetic model of darunavir, an HIV protease inhibitor boosted with ritonavir or cobicistat. Our second objective was to use this model to derive optimal sampling strategies that maximize the amount of information collected with as few pharmacokinetic samples as possible.

Methods A validation dataset comprising 164 sparsely sampled individuals using ritonavir-boosted darunavir was used for validation. Standard plots of predictions and residuals, NPDE, visual predictive check, and bootstrapping were applied to both the validation set and the combined learning/validation set in NONMEM to assess model performance. D-optimal designs for darunavir were then calculated in PopED and further evaluated in NONMEM through simulations.

Results External validation confirmed model robustness and accuracy in most scenarios but also highlighted several limitations. The best one-, two-, and three-point sampling strategies were determined to be pre-dose (0 h); 0 and 4 h; and 1, 4, and 19 h, respectively. A combination of samples at 0, 1, and 4 h was comparable to the optimal three-point strategy. These could be used to reliably estimate individual pharmacokinetic parameters, although with fewer samples, precision decreased and the number of outliers increased significantly.

Conclusions Optimal sampling strategies derived from this model could be used in clinical practice to enhance therapeutic drug monitoring or to conduct additional pharmacokinetic studies.

Keywords Population pharmacokinetics · Darunavir · HIV · External validation · Optimal sampling strategy · Limited sampling strategy

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Introduction

We previously developed a population pharmacokinetic (PK) model for darunavir (DRV), an HIV-1 protease inhibitor used in antiretroviral therapy (ART), based on data collected in a large cohort of ambulatory patients using cobicistat- (COB) or ritonavir- (RTV) boosted DRV [1]. DRV PK are characterized by major inter-individual variability. Among investigated co-variables, those that contributed the most to this variability were alpha-1 acid glycoprotein (AAG), sex, *CYP3A5**3, and *SLCO3A1* rs8027174 variants. This PK model was used to conduct simulations of alternative dosage regimens, including a reduction of the daily dose from 800 mg q24h to 600 mg q24h, 400 mg q24h, or 800 mg 5 days in a row followed by

2 days of treatment interruption. The model was evaluated and validated using standard goodness of fit metrics (precision, shrinkage) and graphical methods (plots of predictions and residuals), normalized prediction distribution errors (NPDE), and bootstrapping. These techniques are considered sufficient in most cases, but so-called external validation methods, which require a new dataset, remain the superior approach when one wishes to ensure the quality of model predictions, which is paramount when this model is to be used for predictive purposes [2]. To provide additional information regarding the accuracy and robustness of our model, we decided to perform external validation using a new dataset. Based on this model, we will also derive optimal sampling strategies (OSSs) that would allow one to obtain accurate estimates of PK parameters with as few samples as possible. A single concentration, such as the trough concentration (C_0), may not be sufficient to reliably assess individual exposure, but as the number of collected samples increases, so do laboratory and personnel costs, not to mention the inconvenience it represents for the patient. Therefore, an OSS is necessary to strike a balance between accuracy and convenience/feasibility, both in terms of the optimal number of samples and the optimal timing of sample collection. One method for obtaining OSSs is based on multiple regression models [3]. However, these have several limitations: they are not PK models and usually only predict one variable (e.g., AUC), they do not explicitly take inter- and intra-individual variability into account, they cannot be used to extrapolate to different types of patients, and the OSS can only include timepoints that were included in the original design. Bayesian methods are a more elegant and efficient approach that has been introduced to population PK. Among them, D-optimal design, which is based on optimization of the Fisher information matrix, is the most frequently employed [4, 5]. DRV OSSs will be calculated according to a D-optimal design and then further evaluated through Monte Carlo simulations.

Materials and methods

Population PK model

The model used throughout the present paper has been extensively described in a previous publication [1]. Briefly, this model was developed using NONMEM (version 7.4.3) [6] based on a dataset comprising 127 HIV-positive patients (learning set). DRV PK was described by a one-compartment model with first-order absorption and first-order elimination. The final model included the following covariates: AAG, sex, *CYP3A5**3, and *SLCO3A1* rs8027174. A prior distribution had been set on all fixed effects and inter-individual random effects, based on a subset of rich pharmacokinetic data.

Validation dataset

The validation set was composed of data collected between November 2012 and April 2016 at the Cliniques universitaires Saint-Luc (Brussels, Belgium) as part of a study by Belkhir et al. [7]. Original approval for data collection was granted by the local ethics committee (Comité d'Éthique Hospitalo-Facultaire des Cliniques Saint-Luc, approval number B403201214460). One hundred eighty DRV plasma concentrations were measured in 164 individuals (one sample per dose interval) using the same liquid chromatography-diode array detector method that was used for the learning set [8]. One concentration was excluded because the post-intake time was unknown. Genotypes were unavailable in 18 subjects (11.0%), whereas AAG was not available at all. Genetic covariates were assumed to be missing at random for baseline covariate analysis and then substituted by the most frequent genotype for the patient's race for PK modeling and simulation. Drug-drug interactions that could alter DRV concentrations were summarized for each patient by the presence or absence of at least one drug belonging to each of the following groups: CYP3A inhibitors, CYP3A inducers, and P-glycoprotein inhibitors, using the methodology outlined in our previous publication [1]. There was some patient overlap between the datasets: 51 individuals contributed data to both studies. Baseline covariates were compared using Fisher's exact test for categorical covariates or Wilcoxon's rank-sum test for continuous ones. Statistical analyses were conducted in R (version 3.5.2) [9].

External validation

An external validation procedure similar to the one used in Krekels et al. [10] was applied. First, the model was refitted to the validation data with all parameters fixed to their final estimates, Bayesian estimation was performed, and the goodness of fit was assessed. PsN (version 4.7.0) was used for handling NONMEM runs [11]. NPDE, which are a more appropriate metric for validation compared to standard weighted residuals, were computed for the validation set based on 1000 simulations from the final model, by means of the npde package (version 2.0) [12–14]. Their distribution was compared to the reference distribution using graphical methods and appropriate statistical tests: the Wilcoxon signed-rank test (H_0 : mean = 0), Fisher variance test (H_0 : variance = 1), and Shapiro-Wilk test (H_0 : normal distribution). Additionally, a prediction-corrected visual predictive check (pcVPC) was constructed from these 1000 simulations, and the observed concentrations from the validation set were overlaid on the prediction intervals. Finally, the validation and learning sets were merged, and the model refitted to this combined dataset. For subjects who contributed data to both studies, their data

points were considered separate occasions. 95% CIs were computed from 1000 bootstrapped datasets.

Optimal sampling strategy

Sampling strategies were evaluated according to the D-optimality criterion in PopED for R (version 0.4.0) using a sequential combination of adaptive random search, line search, and BFGS algorithms [15, 16]. Because both the learning and the validation set were primarily composed of PK data collected at random timepoints, there was no initial design in the traditional sense of the word (as in, a specific set of timepoints for observations). Consequently, it was not possible to evaluate and optimize it using D-optimality without making strong assumptions. However, the subset of rich PK data, which was originally used to define a prior distribution on all parameters, could be used as a substitute for the full learning set. Therefore, the initial design in PopED mirrored the rich PK substudy in terms of measured parameters, sample size, sampling scheme, and dose regimens: 12 patients treated with DRV 800 mg q24h, sampled 8 times over 6 h (C_0 , $C_{0.5}$, C_1 , C_2 , C_3 , C_4 , C_5 , C_6) at PK steady-state. Designs could include up to four points, sampling times were constrained to be identical in all subjects, and other aspects of the design (e.g., sampling density, population size, dose regimens) were kept fixed. Sensitivity analysis was conducted by perturbing each fixed effect by $\pm 30\%$, one at a time.

To further evaluate these strategies, the final model in NONMEM was used to perform Monte Carlo simulations in order to generate a new virtual population. Two hundred fifty PK profiles were generated for each subject from the learning set, using their baseline covariate values to derive their PK parameters. The reference area under the curve over 24 h (AUC_{ref}) was obtained from each individual's daily dose and clearance. Individual Bayesian estimates of clearance were then computed using the timepoints selected in PopED (rounded to the nearest discrete value to obtain near-optimal timepoints), and the corresponding AUCs were derived (AUC_{OSS}). The performance of each strategy was assessed by calculating Spearman's ρ between AUC_{ref} and AUC_{OSS} for all n subjects, as well as prediction errors (PEs), the mean percentage error (MPE), and the root mean square percentage error (RMSPE) using the following equations:

$$PE = \frac{AUC_{ref} - AUC_{OSS}}{AUC_{ref}}$$

$$MPE = 100\% \times \frac{1}{n} \sum PE$$

$$RMSPE = 100\% \times \sqrt{\frac{1}{n} \sum PE^2}$$

Values of 15% RMSPE were considered acceptable [3, 17]. Additionally, the proportion of AUCs predicted within $\pm 15\%$ of the reference value was computed.

Results

Datasets

Summary statistics of the learning and validation sets are provided in Table 1. All subjects in the validation set were using RTV-boosted DRV since this data was collected prior to the commercialization of COB, while subjects in the learning set used COB-boosted DRV for the most part (85.8% of subjects). In both cases, DRV 800 mg q24h was the most frequent dosage (91.3% and 67.1% of individuals in the learning and validation sets, respectively), followed by 600 mg q12h (7.9% and 29.3%). Concomitant antiretrovirals differed significantly, in part due to the different time frames in which the two studies were conducted: the most frequent combinations in the learning set were lamivudine/dolutegravir/DRV (18.1%), tenofovir/emtricitabine/DRV (18.1%), and dolutegravir/DRV (17.3%), while in the validation set, tenofovir/emtricitabine/DRV (48.2%) and abacavir/lamivudine/DRV (16.5%) made up a majority of ARTs. Genotype frequencies were comparable. Finally, while the validation set only included single concentration data collected at random post-intake times, the learning set also included 12 PK profiles over 6 h (Fig. 1). On average, post-intake times were longer in the validation set.

External validation

Since AAG was not available in the validation set, this covariate had to be removed from the final model, which led to a 43 points increase in the objective function value (OFV), and this reduced model was validated instead. It was considered preferable to remove AAG entirely because imputation methods could not be easily applied to substitute all the missing values without distorting the AAG distribution that would have been observed, also keeping in mind the possibility of correlation between AAG and other variables. Other than the increase in OFV, this reduced model was very similar to the final model in terms of goodness of fit, with only one individual prediction being noticeably shifted (Online Resource 1 and Online Resource 2).

Standard goodness of fit plots obtained after fixing all parameters to their final estimates are displayed in Fig. 2. With the exception of two high concentrations (13.6 and 14.01 mg Γ^{-1}) that were severely underpredicted, there was decent agreement between observations and

Table 1 Summary characteristics of the learning and validation sets

	Learning set		Validation set		<i>p</i>
Patients	127		164		
Samples	405		180		
DRV dosage					< 0.05*
300 mg q12h	0	0%	1	0.6%	
600 mg q24h	0	0%	1	0.6%	
600 mg q12h	10	7.9%	48	29.3%	
800 mg q24h	116	91.3%	110	67.1%	
900 mg q24h	0	0%	1	0.6%	
1200 mg q24h	1	0.8%	3	1.8%	
Booster					< 0.05*
RTV	18	14.2%	164	100%	
COB	109	85.8%	0	0%	
Post-intake delay (h)					< 0.05*
Median (range)	6.3 (0.5–29)		14.8 (2–31.5)		
q24h dosing	6.9 (0.5–29)		16.2 (2.3–31.5)		
q24h dosing (sparse only)	12.3 (1–29)		16.2 (2.3–31.5)		
q12h dosing	3.8 (2.2–27.5)		13.7 (2–25.8)		
Age (years)					< 0.05*
Median (IQR)	55 (13)		48 (14)		
Sex					0.62
Male	85	66.9%	105	64.0%	
Female	42	33.1%	59	36.0%	
Race					0.26
Caucasian	67	52.8%	101	61.6%	
African	55	43.3%	60	36.6%	
Other	5	3.9%	3	1.2%	
Concomitant ARVs					< 0.05*
Tenofovir	35	27.6%	92	56.1%	
Emtricitabine	28	22.0%	84	51.2%	
Lamivudine	42	33.1%	46	28.0%	
Abacavir	8	6.3%	29	17.7%	
Nevirapine	0	0%	2	1.2%	
Etravirine	7	5.5%	18	11.0%	
Rilpivirine	7	5.5%	0	0%	
Dolutegravir	79	62.2%	4	2.4%	
Raltegravir	7	5.5%	43	26.2%	
Maraviroc	21	16.5%	15	9.1%	
Drug-drug interactions					0.66
CYP3A inhibitors	3	2.4%	8	6.3%	
CYP3A inducers	7	5.5%	21	16.5%	
P-glycoprotein inhibitors	5	3.9%	8	6.3%	
CYP3A5 g.6986A>G					0.58
*1/*1	33	26.0%	37	22.6%	
*1/*3	33	26.0%	32	19.5%	
*3/*3	58	45.7%	77	47.0%	
Missing	3	2.4%	18	11.0%	
SLCO3A1 g.91941607G>T					0.73
GG	105	82.7%	127	77.4%	
GT	18	14.2%	19	11.6%	
TT	0	0%	0	0%	
Missing	4	3.1%	18	11.0%	

ARVs antiretrovirals

*Statistically significant difference ($p < 0.05$). All covariates given at baseline, except post-intake delay (given for all timepoints)

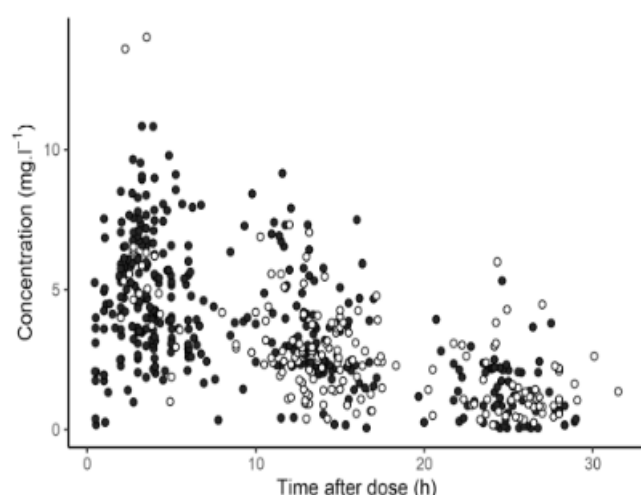


Fig. 1 Pharmacokinetic data in the learning and validation sets. Observations from the learning set are represented with black circles and validation set with white circles

predictions, although trends toward over- and underprediction were noted for the lower and upper ends of the concentration range, respectively. No obvious trend was apparent in the conditional weighted residuals. Meanwhile, NPDE deviated from their expected normal distribution (Fig. 3). The p values for the Wilcoxon signed-rank test, Fisher variance test, and the Shapiro-Wilk test were 0.1, 0.39, and 0.01, respectively. Thus, the global adjusted p value (accounting for multiple testing) was 0.04. A pcVPC of the final model predictions versus the validation data is presented in Fig. 4: most observations lied within the 95% prediction interval, but due to the low number of data points at higher concentrations, these were characterized by a large uncertainty, especially during the absorption phase.

Merged data analysis

Model parameters remained similar after re-estimation on the merged learning/validation set (Table 2). Median bootstrapped parameters were comparable, with noticeably large CIs on k_a and the effect of *SLCO3A1* genotype on V/F . The difficulty in obtaining accurate estimates of k_a can be attributed to the lack of samples in the absorption phase. Goodness of fit was similar to that of the learning set, with a trend toward underprediction of high concentrations and, in the case of NPDE, a departure from normality (Online Resource 3 and Online Resource 4).

Optimal sampling strategy

A design including three points was found to be equivalent to the base design of our clinical study. The D-optimal design included C_1 , C_4 , and C_{19} , with a correlation coefficient of 0.93, an MPE of 1.4%, an RMSPE of 12.1%, and 81.2% AUCs predicted with less than 15% prediction error (Table 3). Late post-intake times

may not be convenient for sampling, as a patient who usually takes their medication early in the morning would need to either be sampled during the night or change their drug-taking habits; hence, we also assessed the effect of substituting C_{19} by a pre-dose sample (C_0). The objective functions calculated when evaluating a C_1 - C_4 - C_{19} and C_0 - C_1 - C_4 design in PopED were comparable ($\Delta = 0.081$), and the latter strategy yielded similar results in NONMEM as well (Table 3), showing that the C_{19} may be substituted with a C_0 if it is more convenient. It was not possible to compute the objective function in PopED for a one- or two-point design, perhaps indicating that these were not sufficiently informative. Regardless, potential one- and two-point OSSs were evaluated in NONMEM by assuming they would include the same times selected for the three-point OSS (i.e., one-point designs were C_0 , C_1 , or C_4 , and two-point designs were C_0 - C_1 , C_0 - C_4 , and C_1 - C_4). The MPE remained low in all evaluated scenarios, likely due to positive and negative prediction errors canceling each other out (Fig. 5). Meanwhile, the RMSPE was less than 15% for both three-point OSSs, as well as the C_0 - C_1 and C_0 - C_4 two-point OSSs. The percentage of AUCs predicted within $\pm 15\%$ of the reference value increased substantially with the number of samples: from 55 with a C_1 only to 81.2% with the optimal design (Table 3, Fig. 5). 86.8% of AUC_{ref} within a clinically meaningful range of 50 to 130 $mg\ h\ l^{-1}$ (equal to the 95% prediction interval in the learning set) were accurately predicted with the optimal design, while bias grew for AUCs outside of this range for every strategy. Prediction errors did not appear to be correlated with individual covariate values based on visual inspection (data not shown). Lastly, sensitivity analysis showed that estimates of CL , V , and k_a in the initial design mostly affected the timing of the last sample, which ranged from 16 to 24 h, depending on the case.

Discussion

Basic goodness of fit metrics and internal validation techniques relying on Monte Carlo simulations are the norm for the validation of population PK models, while external validation is more rarely conducted, despite contributing a useful piece of information [2]. The validation set used for the present work was similar to the learning set in terms of sample size, which allowed us to capture the magnitude of the inter-individual variability that is observed in clinical practice, and thus to assess the robustness of our model over a wide range of concentrations. However, the two datasets differed in several ways. The first major difference was the lack of COB users in the validation set since it predates the introduction of this booster in the therapeutic arsenal. However, the results of the external validation actually show that a model developed mostly on DRV/COB data can reliably be used to predict individual parameters in DRV/RTV users. The second difference was the absence of AAG, which had been used as a covariate in our original model. This meant that external

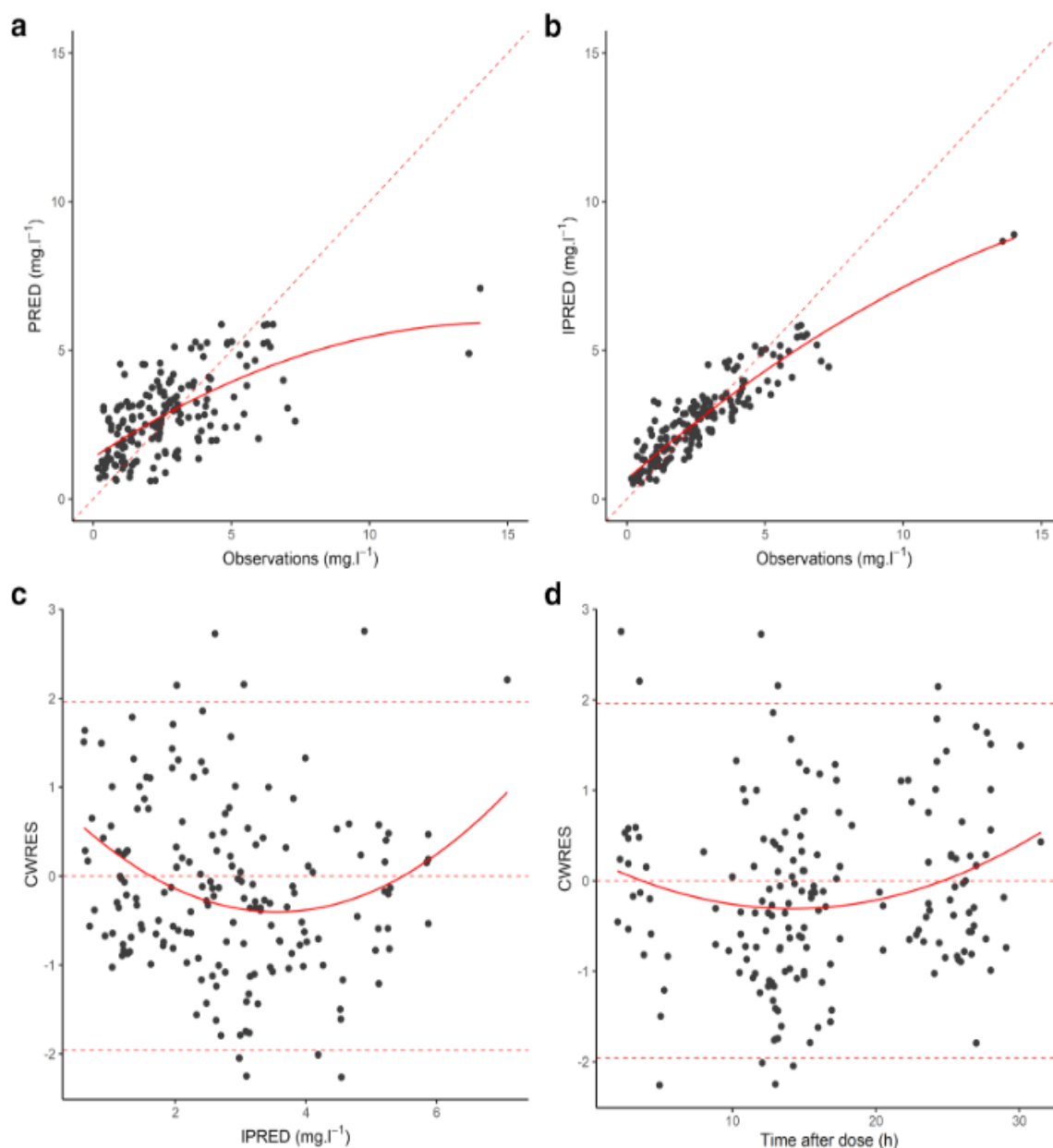


Fig. 2 Goodness of fit plots for the validation set. **a** Population predictions (PRED) versus observed concentrations. **b** Individual predictions (IPRED) versus observed concentrations. **c** Conditional weighted residuals (CWRES) versus PRED. **d** CWRES versus time after dose.

Dashed line is the line of identity (panels **a** and **b**) or the reference CWRES range assuming a normal distribution (panels **c** and **d**), and continuous line is the LOESS fit line (all panels)

validation had to be performed not on the final model, but on an earlier iteration that did not include AAG. Despite a significant increase in the objective function when this covariate was removed from the model, the goodness of fit remained comparable in terms of visual trends on standard plots, parameter estimates, and precision. PK data from the validation set was also characterized by slightly longer post-intake times, although this was partly due to the presence of twelve 6-h PK profiles in the learning data, which skewed the median. Nevertheless, there were fewer samples available during the early PK phase for that dataset. The last major difference was in the ARV backbone and concomitant medications. Since most coadministered ARVs are not known to cause major PK interactions with DRV, disparities in ART should not be an issue as far as model

predictions are concerned [18]. The only exception is etravirine, which was the perpetrator drug in all cases of CYP induction in the learning set and in 86% of cases in the validation set (followed by nevirapine, 9.5%, and amiodarone, 4.8%), and which was used twice as often in the validation set compared to the learning set. Although the model did not incorporate a drug interaction component, the number of subjects with potentially induced CYP metabolism was low, and their individual predictions did not appear biased.

After fixing model parameters, the goodness of fit was reassessed with the validation data. General trends were captured well, but concentrations at the upper end of the observed range ($\geq 10 \text{ mg.l}^{-1}$) were poorly predicted. This model misspecification may have been due to the lack of data at high concentrations or

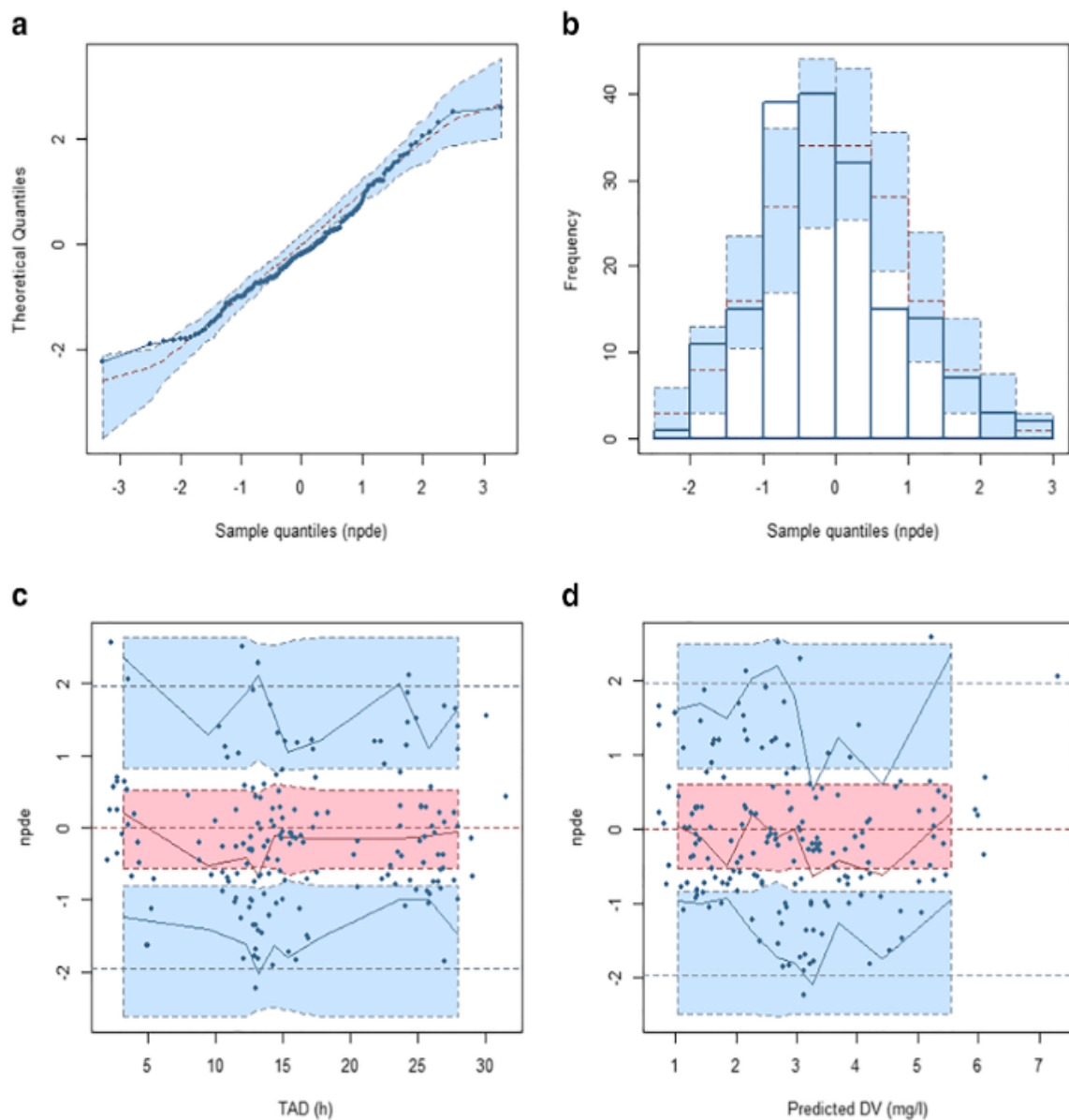


Fig. 3 NPDE for the validation set. **a** Q-Q plot. **b** Histogram of NPDE. Theoretical distribution represented as shaded bars. **c** NPDE versus time after dose (TAD). Prediction intervals represented as shaded areas.

Observations plotted as circles and observation percentiles as solid lines. **d** NPDE versus PRED

Fig. 4 Prediction-corrected visual predictive check for the validation set. Circles represent observed concentrations, solid line is the median of simulations, dashed lines are the 5th and 95th percentiles of simulations, and shaded areas represent CIs around simulation percentiles

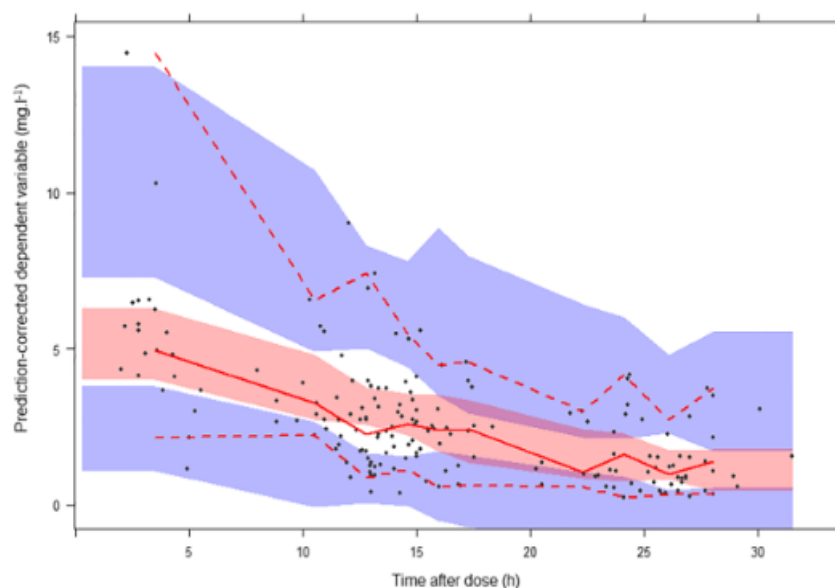


Table 2 Comparison of model parameters with learning, merged, and bootstrapped data

Parameter	Learning set	Merged set	Bootstrapped data
CL/F (l h^{-1})	12.6	12.4	12.3 [11.1–13.8]
ω_{CL} (sd)	0.238	0.248	0.240 [0.162–0.291]
V/F (l)	137	147	144 [110–170]
ω_{V} (sd)	0.353	0.308	0.292 [0.069–0.427]
k_{a} (h^{-1})	0.545	0.724	0.704 [0.371–1.419]
ω_{ka} (sd)	0.575	0.712	0.826 [0.335–1.762]
θ_{sex} on CL	−0.198	−0.151	−0.153 [−0.258 to −0.046]
θ_{CYP3A5} on CL	−0.192	−0.126	−0.131 [−0.230 to −0.029]
θ_{SLCO3A1} on V	0.991	0.697	0.756 [0.230–1.848]
$\sigma_{\text{exponential}}$ (sd)	0.306	0.334	0.325 [0.265–0.382]
σ_{additive} (sd)	0.611	0.539	0.548 [0.349–0.715]

Parameters given as fixed or random effect. Bootstrapped parameters given as median [95% CI]. CL/F, apparent clearance; V/F, apparent volume of distribution; k_{a} , absorption rate constant; ω , inter-individual variability; σ , residual variability

the lack of a key covariate that could explain these outliers. Simulation-based diagnostics such as the NPDE and VPC yielded more nuanced results, showing moderate deviation from the reference normal distribution in the case of NPDE, and large CIs in the case of the VPC. The large CIs could be due to the heterogeneous sampling design, as few samples were collected in certain time ranges, which also made it difficult to find an appropriate binning strategy. Meanwhile, parameter estimates remained almost unchanged by the addition of the validation set to the learning set, confirming model robustness. In any event, this analysis highlights the usefulness of simulation-based diagnostics in assessing model performance.

Using this model, we then evaluated limited sampling strategies for DRV, which could be used to enhance TDM or to design further PK studies. To our knowledge, no such strategies have been proposed in the literature. Should the clinician only need a rough idea of patient adherence, a single C_0 can readily be used and interpreted according to recommended levels for PI-naïve or PI-pretreated patients. However, due to the important inter-

individual variability that exists for DRV, this C_0 may be difficult to interpret, and low concentrations would not necessarily be indicative of low adherence. Furthermore, if a C_0 cannot be obtained at the time of TDM, a population model may be used to extrapolate it from available information or to derive a more complete measurement of drug exposure, which could for instance be used to assess whether the patient is eligible for alternative dosage regimens or to investigate possible causes of under- or over-exposure. The model we previously proposed is applicable to any HIV patient under DRV-based therapy, regardless of concomitant antiretrovirals, but requires AAG measurement and genotyping for two single nucleotide polymorphisms. In case additional laboratory costs and analysis time are an issue, a model without these covariates may also be used without a significant loss of predictive power, as we have shown in the present paper by validating a model which did not feature AAG. In any event, to obtain a precise estimate of individual PK parameters, samples should be as informative as possible—this is where an OSS can come in useful. Different strategies were evaluated using D-optimal design and keeping practical limitations in mind. While it is possible to first derive an OSS and to then evaluate it on a new validation set by comparing the parameters one obtains using the entire validation set with those obtained using only the previously determined optimal times, this requires the validation data to include all points selected in the OSS. This was unfortunately not the case here, as only sparse concentrations were collected in our patients, whereas the OSS included multiple points per individual. Instead, a simulation approach was used: AUCs were simulated based on the learning set (once again, the validation set could not be used due to the lack of AAG), and we assessed how accurately this parameter could be predicted using each strategy of interest. The AUC was chosen as the parameter to be estimated because it reflects total exposure and it (or CL) is the parameter one would mostly be interested in predicting, although, from a clinical point of view, it has yet to be convincingly demonstrated that AUC is a better marker of exposure than the C_0 or, for that matter, that there is a correlation between DRV exposure and efficacy [1, 19, 20]. If only a single sample can be obtained, C_0 was found to be the best choice. The two-point OSS was C_0 and C_1 , while the three-point

Table 3 Comparison of sampling strategies with regard to AUC calculation

Strategy	ρ_{AUC}	MPE_{AUC} (%)	$\text{RMSPE}_{\text{AUC}}$ (%)	AUCs < 15% PE (%)
C_1 – C_4 – C_{19}	0.93	1.4	12.0	81.2
C_0 – C_1 – C_4	0.92	0.7	12.8	79.2
C_0 – C_1	0.88	−0.9	14.7	73.2
C_0 – C_4	0.90	0.4	13.7	75.3
C_1 – C_4	0.75	0.1	18.8	58.9
C_0	0.82	−1.7	17.1	65.0
C_1	0.69	−1.5	20.7	55.3
C_4	0.72	−0.2	19.7	56.2

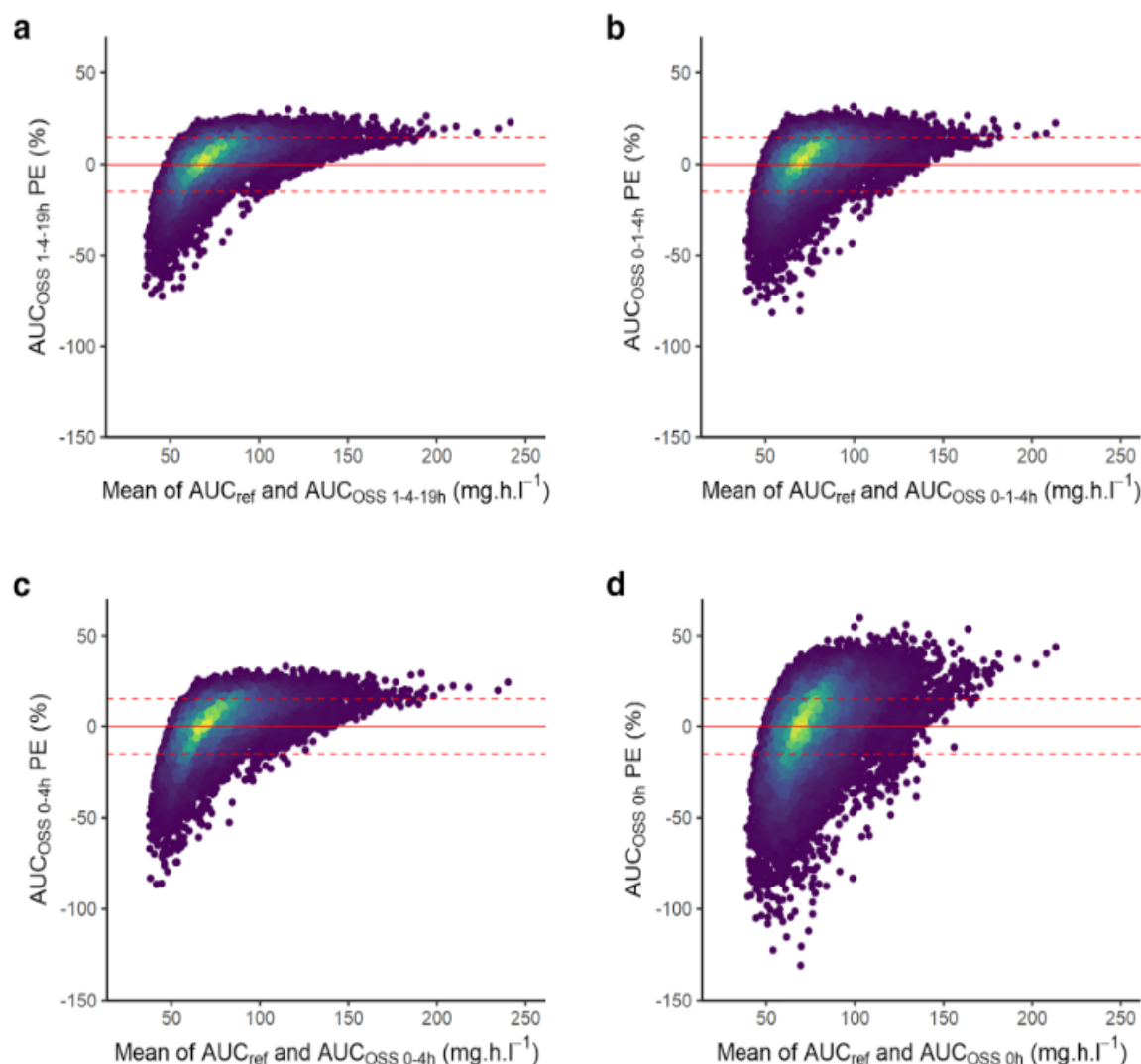


Fig. 5 Bland-Altman plots of predicted AUCs for select strategies. Point density indicated by the color scale, from dark (least dense) to light (most dense), with the scale being proper to each panel. **a** C_1 - C_4 - C_{19} (optimal

design). **b** C_0 - C_1 - C_4 (near-optimal design). **c** C_0 - C_4 (best two-point design). **d** C_0 (best one-point design)

OSS was C_0 , C_1 , and C_4 . Although C_1 - C_4 - C_{19} was initially found to be the optimal strategy, sensitivity analysis showed some variation in the timing of the last sample depending on input parameters, but using a C_0 instead did not appear to have much influence, and a C_0 is likely the most convenient option for clinicians. All three OSSs were found to be adequate in terms of bias and precision, but extreme values of AUC were more poorly predicted with sparser designs (although AUCs within a clinically relevant range were more often predicted accurately). The three-point design, while imperfect, provided the most information over a larger range of AUCs.

Conclusion

A previously published population PK model for DRV was validated using an external dataset. Results confirm its robustness and lend more credibility to model-derived predictions. Several sampling strategies were evaluated, among which a three-point

strategy (either C_0 , C_1 , and C_4 or C_1 , C_4 , and C_{19}) was the most adequate. These strategies could be used as a starting point for the design of further PK studies in order to maximize the amount of information that can be gathered, especially in resource constrained settings.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00228-020-03036-2>.

Authors' contributions All authors contributed to the study conception and design. Gabriel Stilleman performed data analysis and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethics approval Not applicable. The present work uses data from two previous studies, for which ethics approval had been granted by the Comité d'Éthique Hospitalo-Facultaire des Cliniques Saint-Luc, approval numbers B403201731249 (learning data) and B403201214460 (validation data).

Consent to participate Not applicable.

Consent for publication Not applicable.

Code availability Not applicable.

Abbreviations AAG, alpha-1-acid glycoprotein; ART, antiretroviral therapy; ARV, antiretroviral; AUC, area under the curve; BFGS, Broyden-Fletcher-Goldfarb-Shanno; C_{tr} , trough concentration; COB, cobicistat; CWRES, conditional weighted residuals; DRV, darunavir; IPRED, individual prediction; MPE, mean percentage error; NPDE, normalized prediction distribution error; OFV, objective function value; OSS, optimal sampling strategy; PE, prediction error; PI, protease inhibitor; PK, pharmacokinetic; PRED, population prediction; RMSPE, root mean square percentage error; TDM, therapeutic drug monitoring; (pc)VPC, (prediction-corrected) visual predictive check

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SUPPLEMENTARY MATERIALS

Supplementary Material 1. Effect of AAG removal on model fit

Full model				Reduced model		
OFV				660.683		
Structural model	Estimate	RSE (%)	Shrinkage (%)	Estimate	RSE (%)	Shrinkage (%)
CL/F (l.h ⁻¹)	12.9	5.3		12.6	5.5	
ω_{CL} (SD)	0.22	14.0	33.3	0.24	14.1	31.7
V/F (l)	152	21.1		137	16.3	
ω_V (SD)	0.33	16.4	59.0	0.35	17.1	56.6
k_a (h ⁻¹)	0.68	73.5		0.55	43.5	
ω_{k_a} (SD)	0.60	22.3	63.1	0.58	22.3	62.7
Covariate model						
AAG on CL	-0.61	37.9		/	/	
AAG on V	-0.68	26.3		/	/	
Female sex on CL	-0.21	27.3		-0.20	30.0	
<i>SLCO3A1</i> G>T on V	0.81	59.5		0.99	46.1	
<i>CYP3A5</i> *3 on CL	-0.16	35.5		-0.19	28.3	
Residual variability						
$\sigma_{\text{exponential}}$ (SD)	0.281	13.9	11.5	0.306	13.4	11.7
σ_{additive} (SD)	0.641	19.7	11.5	0.611	22.9	11.7

CL/F: Apparent clearance, k_a : Absorption rate constant, OFV: Objective function value, RSE: Relative standard error (= standard error/parameter absolute value), V/F: Apparent volume of distribution, ω : Random effect (inter-individual variability), σ : Random effect (residual variability).

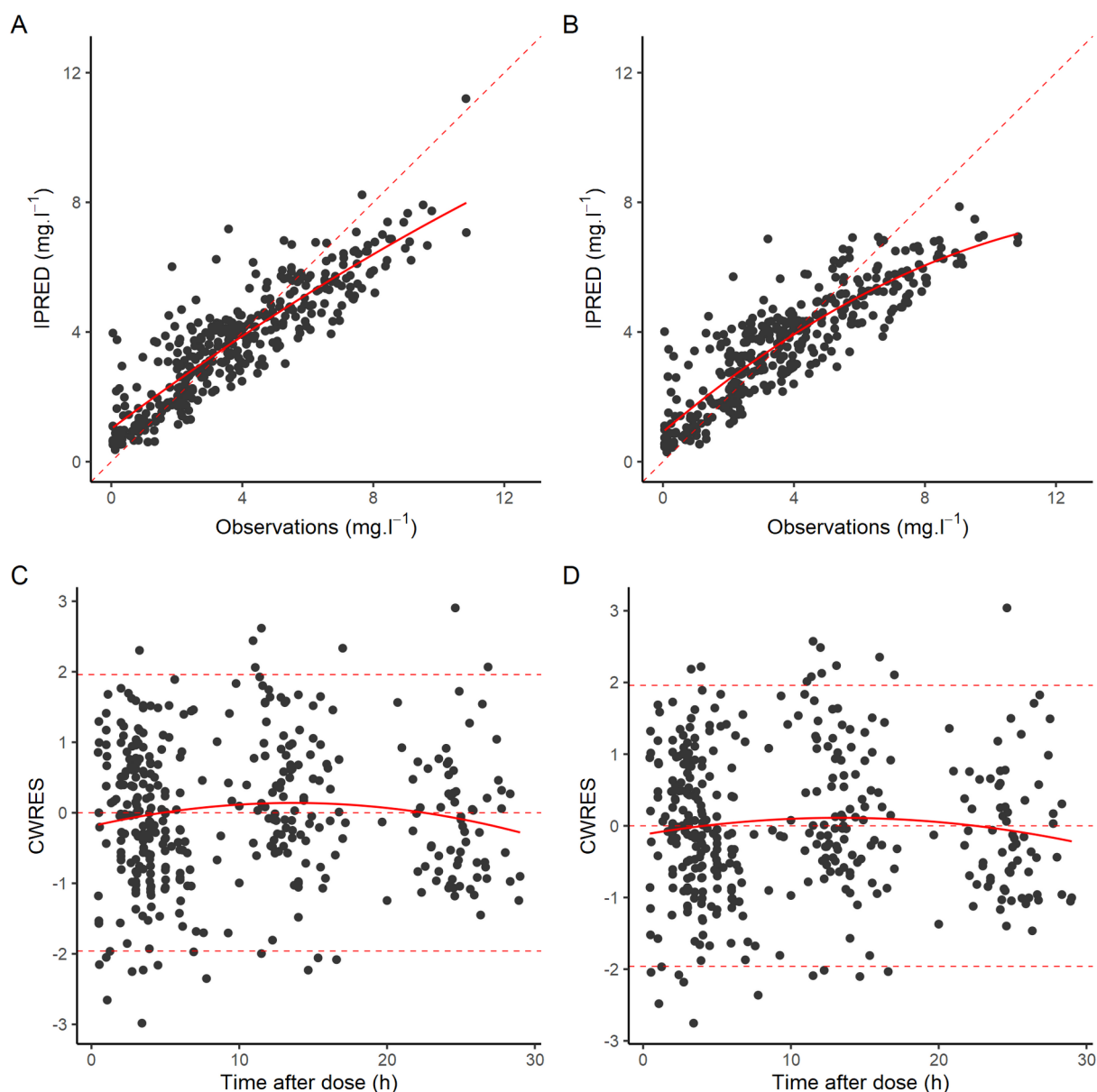


Figure S1. Goodness of fit after removal of AAG. (A) Individual predictions (IPRED) versus observed concentrations, full model. (B) IPRED versus observed concentrations, reduced model. (C) Conditional weighted residuals (CWRES) versus time after dose, full model. (D) CWRES versus time after dose, reduced model. Dashed red line is the line of identity (panels A and B) or the reference CWRES range assuming a normal distribution (panels C and D), continuous red line is the LOESS fit line (all panels).

Supplementary Material 2. Goodness of fit for merged dataset

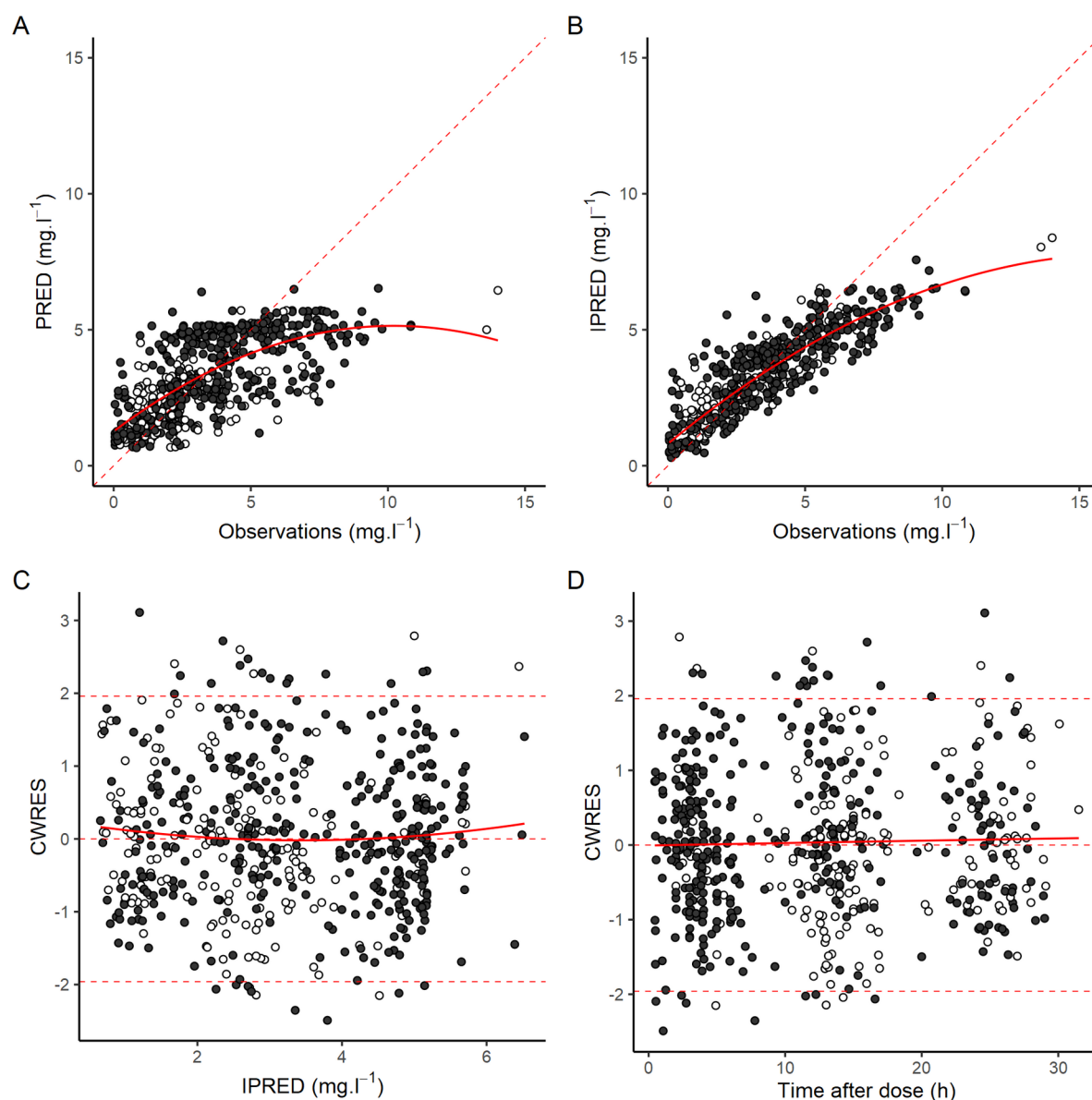


Figure S2. Goodness of fit plots for merged dataset. (A) Population predictions (PRED) versus observed concentrations. (B) Individual predictions (IPRED) versus observed concentrations. (C) Conditional weighted residuals (CWRES) versus PRED. (D) CWRES versus time after dose. Learning set represented with black circles, validation set with white circles. Dashed red line is the line of identity (panels A and B) or the reference CWRES range assuming a normal distribution (panels C and D), continuous red line is the LOESS fit line (all panels).

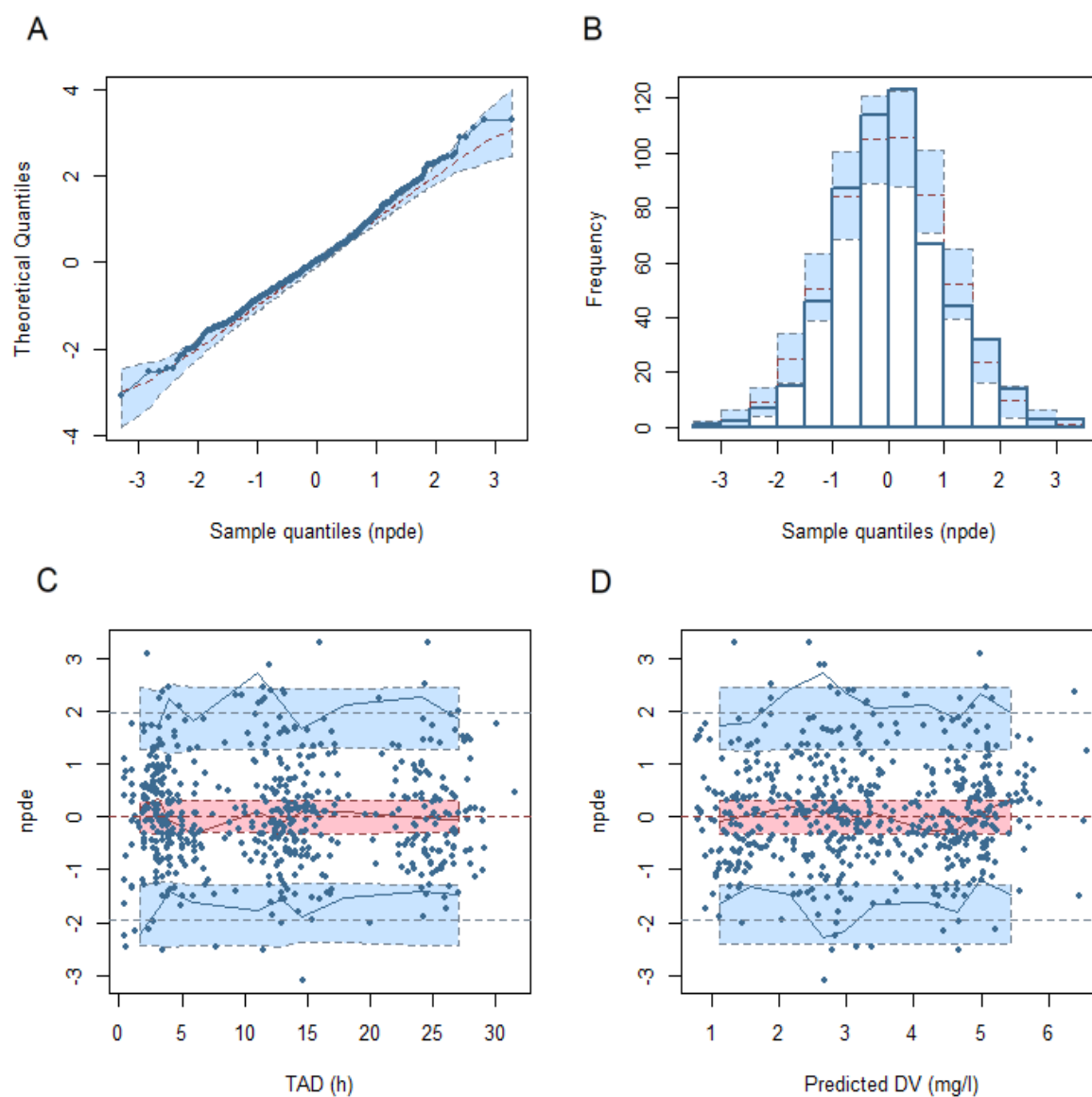


Figure S3. NPDE for merged dataset. **(A)** Q-Q plot. **(B)** Histogram of NPDE. Shaded area represents theoretical distribution. **(C)** NPDE versus time after dose (TAD). Shaded areas represent the prediction intervals associated with the 5th, 50th and 95th percentiles. **(D)** NPDE versus PRED.

Test	p-value
Wilcoxon signed-rank test	0.0853
Fisher variance test	0.674
Shapiro-Wilk test	0.0106*
Global adjusted	0.0317*

* p-value < 0.05

2.2.7. Paper 2 summary

- The model from **Paper 1** was externally validated using a new dataset of 164 patients. It was shown to have adequate performance, even when applied to a new, different population.
- Optimal sampling strategies were determined. A three-point strategy, including 1 h, 4 h and either 19 or 24 h post-dose showed the highest performance. Two- and one-point strategies were also valid options, though depending on how extreme the individual PK were, such strategies would more often lead to biased results.

2.3. ABCB1 efflux of darunavir

2.3.1. Introduction

DRV is a known substrate and inhibitor of the P-glycoprotein (ABCB1), begging the question of whether SNPs in *ABCB1* could affect the transport of this drug. At this stage, it should be reiterated that PK boosters are strong inhibitors of ABCB1 and that this interaction (among other factors) makes it difficult to isolate the effect of polymorphisms, which is probably why our clinical study, as well as previous ones, did not show *ABCB1* variants to be strong predictors of DRV PK (Table 6, p.30). On top of this booster effect, there is some controversy over the very nature of these SNPs: pharmacogenetic studies related to drugs in different classes have found certain variants to be responsible for increased, decreased or unchanged ABCB1 activity (for a review of this information, see [147]). A substrate-specific effect has been suggested as an explanation for some of these puzzling results. In order to clarify our *in vivo* findings, an *in vitro* accumulation study in recombinant cell lines was carried out.

Among the dozens of *ABCB1* variants that have been described, four in the coding region are of particular interest due to their functional effects and allelic frequencies (see Table 7, p.38). As *ABCB1* c.1236C>T and c.2677G>A are in strong linkage disequilibrium with *ABCB1* c.3435C>T, it makes more sense to investigate the haplotype they define, which is considered to capture most of the genetic variability for *ABCB1* [147]. A fourth, rarer SNP (*ABCB1* c.1199G>A) was investigated as well, separately from the effect of the aforementioned haplotype. Recombinant HEK293 (human embryonic kidney) cell lines expressing these variants or the wild-type ABCB1 have previously been developed by our group [176,177].

In the following section, cell lines will be labeled according to which *ABCB1* variant they express (variant alleles in red):

1199	1236	2677	3435	Denomination
G	C	G	C	HEK _{WT} (wild-type)
G	C	G	T	HEK _{CGT} (single variant)
G	T	T	T	HEK _{TTT} (triple variant)
A	C	G	C	HEK _{1199A} (single variant)

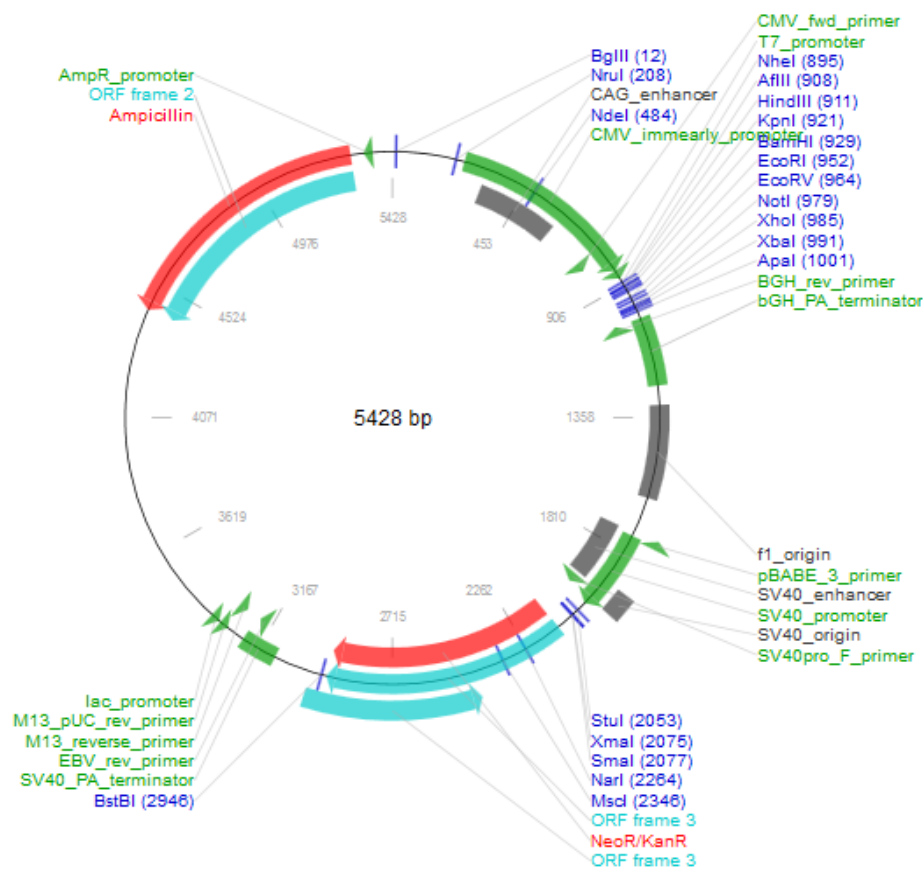
2.3.2. Methodological background

2.3.2.1. Recombinant cell lines

While it is possible to observe drug efflux in cells constitutively expressing the transporter of interest, overexpression systems make the task easier – and using site-directed mutagenesis, cells may be made to express any variant the researcher is interested in. As the present work was not focused on generation of recombinant cell lines, but use of existing ones, only a brief summary of the methods used to generate them will be given here.

HEK293 cells present several advantages: they are easily cultured, adhere well to plastic surfaces, express mostly low endogenous levels of transport proteins [178] and are compatible with multiple modes of transfection. In our case, the transfection vector was a plasmid carrying the wild-type *ABCB1* gene, a variant *ABCB1*, or neither (empty vector). More specifically, the pcDNATM 3.1 vector (Figure 18) enables high levels of expression in mammalian cell lines through the use of the CMV promoter. To ensure cell uptake of the plasmid, it also carries the neomycin resistance gene (Figure 18), allowing selection of cells by cultivating them in the presence of G418, an aminoglycoside antibiotic that blocks protein synthesis in eukaryotic cells [179].

Figure 18. Schematic representation of the pcDNATM vector. [167]



There are many methods of transfection, including physical and chemical techniques (heat shock, electroporation, lipofection, etc.) and techniques using a virus as a vector. Transfection models can be broadly divided into transient and stable ones, depending on how long the cell retains the ability to express the gene of interest. Transient expression models, in which the DNA sequence is expressed directly from the plasmid, can last up to a few days before the number of cellular divisions grows too large and the vector of interest becomes too diluted within the population. Meanwhile, stably transfected models incorporate the sequence of interest into genomic DNA, which allows the cells to continuously express it, even after many

cell divisions, provided an appropriate selection medium is used to eliminate cells that have lost the trait of interest. In our case, stably transfected cell lines had been generated by lipofection, which involves fusion of a liposome carrier with the target cell membrane, and selected using an appropriate antibiotic, as described earlier. Stable recombinant cells can be maintained in culture, frozen in batches and thawed whenever necessary, making them a very convenient tool.

Before further experimentation, the expression of the protein of interest should be assessed using techniques such as flow cytometry, immunofluorescence and quantitative Western Blot. As our cell lines had already been fully characterized before cryogenization [176,177], flow cytometry was deemed sufficient to ensure that *ABCB1* expression levels were comparable across cell lines. The basic concept will be briefly described here (assuming a membrane-bound antigen, as is the case for *ABCB1*, and therefore no need for a membrane permeabilization step). First, cells are tagged using an antibody-bound fluorochrome, such as fluorescein isothiocyanate (FITC). Then, a tagged cell suspension is passed through a capillary where it is struck by one or more laser beams. By reading the intensity of light scattered in different directions (forward scatter, side scatter), it is possible to get an estimate of cell size and granularity. The population of interest can be gated – that is, events are filtered according to the forward and side scatter values to separate living cells from dead cells, debris, cell clumps, etc. Within that population, the fluorescence intensity corresponding to the fluorochrome tag indicates the proportion of cells expressing the protein of interest. To assess the nonspecific binding of antibodies, an isotype control may be used: these are generic antibodies of the same isotype and bearing the same dye used in the experiment. Cell autofluorescence is easily assessed using a sample without any added antibody. Using a similar technique, cells may also be sorted based on whether they express the protein – this is referred to as fluorescence-activated cell sorting (FACS). FACS may be used to ensure a sufficient rate of positivity for the protein interest (a threshold of 80% was used in this case).

2.3.2.2. Mass spectrometry

Since intracellular drug concentrations tend to be far lower than plasma concentrations, the sensitivity of the analytical method used for **Paper 1** was not low enough for accumulation experiments. Instead, a mass spectrometer detector was used.

The core concept of MS is the identification of analytes based on their mass-to-charge ratio (m/z). For this explanation, we will focus on the setup used in this work, i.e., a tandem quadrupole mass spectrometer coupled with LC.

In the first step, the eluent is eliminated and the analyte is ionized using one of several techniques, e.g., electrospray ionization (ESI), which applies a high voltage to the solvent in aerosol form. Unlike other ionization techniques, ESI does not usually fragment analytes much. The mass spectrometer itself is composed of three successive chambers. In each one rests a quadrupole composed of four parallel rods through which runs an electric current, generating an oscillating magnetic field. Ion selectivity results from differing m/z ratios, as

only ions of specific ratios will be able to pass through the quadrupole for a given voltage (and by varying this voltage, the device can scan over a wide range of ratios). The first chamber of the triple quadrupole plays the role of a mass filter by selecting ions based on their m/z ratio, the collision cell fragments parent ions through collision within a gas phase, and the third chamber allows the user to isolate and identify resulting fragments. The experimenter will usually track one specific m/z transition for analyte quantification, but multiple transitions per analyte may be monitored to further increase specificity.

2.3.3. Paper 3

G. Stillemans, H.P. Djokoto, K.-A. Delongie, H. El-Hamdaoui, N. Panin, V. Haufroid*, L. Elens*, Effect of four ABCB1 genetic polymorphisms on the accumulation of darunavir in HEK293 recombinant cell lines, Scientific Reports (Revision submitted, 2021).

* Contributed equally to this work

ABSTRACT

The intracellular penetration of darunavir, a second-generation HIV protease inhibitor, is limited by the activity of the efflux P-glycoprotein (ABCB1). ABCB1 expression and/or activity levels can vary between individuals due to genetic polymorphisms including the c.1199G>A, c.1236C>T, c.2677G>T and c.3435C>T variants, which could in part explain why the pharmacokinetics of darunavir are so variable from one individual to another. While a few clinical studies have failed to demonstrate an influence of these polymorphisms on darunavir pharmacokinetics, drug-drug interactions and methodological limitations may have prevented them from revealing the true influence of *ABCB1* variants. In this work, we report on the intracellular accumulation of darunavir in recombinant HEK293 cell lines expressing wild-type ABCB1 or one of several variants: ABCB1 1199A, ABCB1 3435T, and ABCB1 1236T/2677T/3435T. We demonstrate that while ABCB1 expression limits intracellular accumulation of darunavir, there is no significant difference in efflux activity between cells expressing wild-type ABCB1 and those that express any of the studied variants.

INTRODUCTION

Darunavir (DRV) is a second-generation protease inhibitor used as part of a multi-drug cocktail in HIV therapy [1,2]. Its pharmacokinetics (PK) are known to be highly heterogeneous, with the plasma AUC varying more than 5-fold between individuals receiving the same dosing regimen [3]. Many individual factors could potentially contribute to this variability, including genetic variations in key biotransformation enzymes and transporters involved in the disposition of DRV. As far as transporters are concerned, DRV is a substrate of influx transporters of the SLCO family (SLCO1A2, SLCO1B1 and SLCO1B3) and efflux transporters of the ABC family (ABCB1, ABCC2) [4–6]. The *ABCB1* gene, which encodes the P-glycoprotein, is of particular interest [7]. ABCB1 efflux limits drug penetration in enterocytes, therefore decreasing the oral bioavailability of DRV, which is low when administered alone [8]. This is one of the reasons why DRV is always coadministered with a PK booster such as ritonavir (RTV) or cobicistat (COB), which reduces first-pass metabolism and systemic clearance of DRV, mainly through inhibition of CYP metabolism and ABCB1 activity. ABCB1 also controls drug accumulation in lymphocytes, the active site of antiretrovirals, thereby directly limiting the ability of DRV to reach its target. It is also expressed in other organs, including the blood-brain barrier, meaning it could limit penetration in the central nervous system, which has been described as a sanctuary site for HIV. And while single nucleotide polymorphisms (SNPs) that alter the expression and/or activity of *ABCB1* have been described and could potentially explain – at least in part – the important PK variability that characterizes DRV, their exact contribution remains unclear. Functional *ABCB1* SNPs of interest include *ABCB1* c.1199G>A, c.1236C>T, c.2677T>G/A and c.3435C>T (**Table 1**). Both *ABCB1* c.1236C>T and c.2677T>G/A are in strong linkage disequilibrium with c.3435C>T, and the haplotype they define is considered to capture most of the genetic variability for *ABCB1* [9]. There is some controversy over the impact of these variants on the PK of several drugs, perhaps due to differences in studied populations and experimental protocols, but also due to substrate-specific effects, which explains why observations made for one drug cannot be directly transposed to another, even if they are both known substrates of this efflux transporter. Moreover, even when *in vitro* associations are found, these do not necessarily translate into *in vivo* associations probably due to compensatory mechanisms, confounding factors or DDIs [10].

Nucleotide change	rs number	Description	MAF		
			Euro	Asian	African
c.1199G>A	rs2229109	Missense variant: Ser400Asn. Effect are possibly substrate-dependent.	3%	< 1%	< 1%
c.1236C>T	rs1128503	Synonymous variant. Controversial effects.	42%	59-63%	14%
c.2677T>G/A	rs2032582	Missense variant: Ser893Ala/Thr. Controversial effects.	41%	40-59%	3%
c.3435C>T	rs1045642	Synonymous variant. Alters the timing of protein folding and	52%	40-58%	15%

decreases mRNA stability. Effect are possibly substrate-dependent.

Table 1. *ABCB1* polymorphisms of interest. Euro: European, MAF: minor allele frequency. MAFs are from the 1000 Genomes Project [11]. Asian MAFs include South and East Asians.

The influence of some of these *ABCB1* variants on DRV PK has been investigated in clinical studies [3,12–14], but they did not appear to be good predictors of inter-individual variability. However, the inhibitory effect of RTV and COB on ABCB1 could limit the *in vivo* effect of genetic variants for this transporter towards DRV [6]. Combined with small sample sizes and other confounding factors, this makes it difficult to delineate the true contribution of *ABCB1* variants. Therefore, we decided to study the intracellular accumulation of DRV in human embryonic kidney cells (HEK293) overexpressing the wild-type ABCB1 or one of several variants (ABCB1 1199A, ABCB1 3435T, and ABCB1 1236T/2677T/3435T) to clarify this point.

MATERIALS AND METHODS

Chemicals and reagents

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin and enzyme-free cell dissociation buffer were purchased from Gibco (Thermo Fisher); G418 from Roche; flow cytometry antibodies from BD Biosciences (FITC mouse anti-human CD243, clone 17F9, reference 557002; and FITC mouse IgG2b κ isotype control, clone 27-35, reference 555742); DRV, deuterated DRV (DRV-d9) and COB from Toronto Research Chemicals. All chemicals used in drug quantification were of analytical grade.

Characterization of cell lines

The generation and characterization of recombinant cell lines have been described in previous work conducted by our lab [15,16]. Briefly, HEK293 cells were transfected with plasmids carrying wild-type *ABCB1* (1199G-1236C-2677G-3435C, HEK_{WT}); variants 1199G-1236C-2677G-3435T (HEK_{CGT}), 1199G-1236T-2677T-3435T (HEK_{TTT}) or 1199A-1236C-2677G-3435C (HEK_{1199A}) obtained using site-directed mutagenesis; or an empty vector (HEK_{control}). HEK293 cells feature low endogenous levels of expression of ABCB1, ensuring that all ABCB1 expression originates from the transfection process. This model has previously been extensively characterized using flow cytometry, Western blot, and fluorescence microscopy, and validated using reference substrates and inhibitors of ABCB1 [15,16]. Since the same cells were used, only flow cytometry was used to re-characterize them. After thawing, cells were grown in DMEM, FBS 10% and penicillin/streptomycin 1% at 37°C in the presence of 5% CO₂. After at least seven days of growth in the presence of the selection antibiotic G418 (1 g/l), ABCB1 expression was assessed by flow cytometry: 0.5×10^6 cells were collected by centrifugation and washed twice with ice-cold buffer (PBS, FBS 1%, EDTA 1 mM) then re-suspended in buffer supplemented with 10% anti-ABCB1 antibody, 10% isotype control or in buffer with no antibody, and left to incubate for 45 minutes on ice and in the dark. Finally, the cells were washed with buffer and resuspended before being analyzed using a BD FACSVerse

flow cytometer (for characterization) or BD FACS Aria III (for cell sorting) and the BD FACSuite software. Additional data analysis and plotting were carried out in FlowJo (version 10.6.1).

Intracellular accumulation experiments

0.35×10^6 cells were seeded on poly-L-lysine-coated 24-well plates and incubated overnight. The next day, DRV dilutions in DMEM were prepared from a stock solution and added in each well at a final concentration of 0.5, 1, 2.5, 5 or 10 mg/l (final volume: 500 μ l). These values were chosen to cover the range of total plasma concentrations found in patients treated with COB- or RTV-boosted DRV. Cells were incubated in triplicate at 37°C in the presence of 5% CO₂ for 2 hours. The plates were then centrifuged for 5 minutes at 450 g, 4°C, then kept on ice for the remainder of the experiment to block drug efflux. Cells were washed twice with 500 μ l of ice-cold PBS, then 400 μ l of a mixture of methanol/water 60/40% (v/v) containing 20 ng/ml DRV-d9 was added and cells were detached by scratching the surface of the well. Cell suspensions were kept at -20°C until quantification.

Cell viability

The viability of HEK cells in the presence of increasing concentrations of DRV (1, 5 and 10 mg/l) was assessed using the WST-1 assay according to the manufacturer's instructions. Cells were grown in 96-well plates and viability was assessed separately for the HEK_{control} and HEK_{WT} groups. Absorbance was measured at 450 nm after 0.5, 1, 1.25, 1.5 and 1.75 hours of incubation.

Drug quantification

DRV intracellular concentrations were determined using an adapted version of a previously published liquid chromatography-tandem mass spectrometry (LC-MS/MS) method [17]. Cell suspensions were vortex mixed, sonicated for 5 minutes, placed on an orbital shaker for 2 hours and centrifuged for 10 minutes at 10500 g. The supernatant was transferred to a vial for injection and the pellet was set aside for protein quantification. Calibrators ranging from 1.25 to 125 ng/ml were prepared in a similar fashion. Chromatographic separation was achieved on a Waters UPLC BEH C18 1.7 μ m column (2.1 $\times$ 50 mm) maintained at 40°C. The injection volume was 5 μ l and the flow rate was 0.5 ml/min. The mobile phase consisted of a gradient of water/formic acid 0.1% (mobile phase A) and acetonitrile (mobile phase B), starting with 95% A and 5% B, ramping up to 80% B over 6 minutes, then returning to 5% B at 6.1 minutes and remaining at this ratio until the end of the run (total run time: 8 minutes). The MS system was a Xevo TQS-micro tandem quadrupole mass spectrometer (Waters). The following ion transitions were monitored: 548.2 > 392.3 for DRV and 557.3 > 113 for DRV-d9.

Protein quantification

Total proteins were quantified in pellets using the BCA kit (ThermoFisher Scientific) according to the manufacturer's instructions. Absolute DRV concentrations were normalized by total protein content.

Statistical analysis

Each set of experiments were performed thrice. Normalized intracellular concentrations at each dose level were compared using one-way ANOVA ($\alpha = 0.05$) followed by Tukey's HSD test. All statistical analyses were carried out in R (version 3.6.3) [18].

RESULTS

Flow cytometry

ABCB1 expression in our model was confirmed by flow cytometry analysis. After sorting, all cell lines expressed comparable levels of ABCB1 ($> 80\%$) except for HEK_{control}, which, predictably, were characterized by low levels of expression (**Figure 1**).

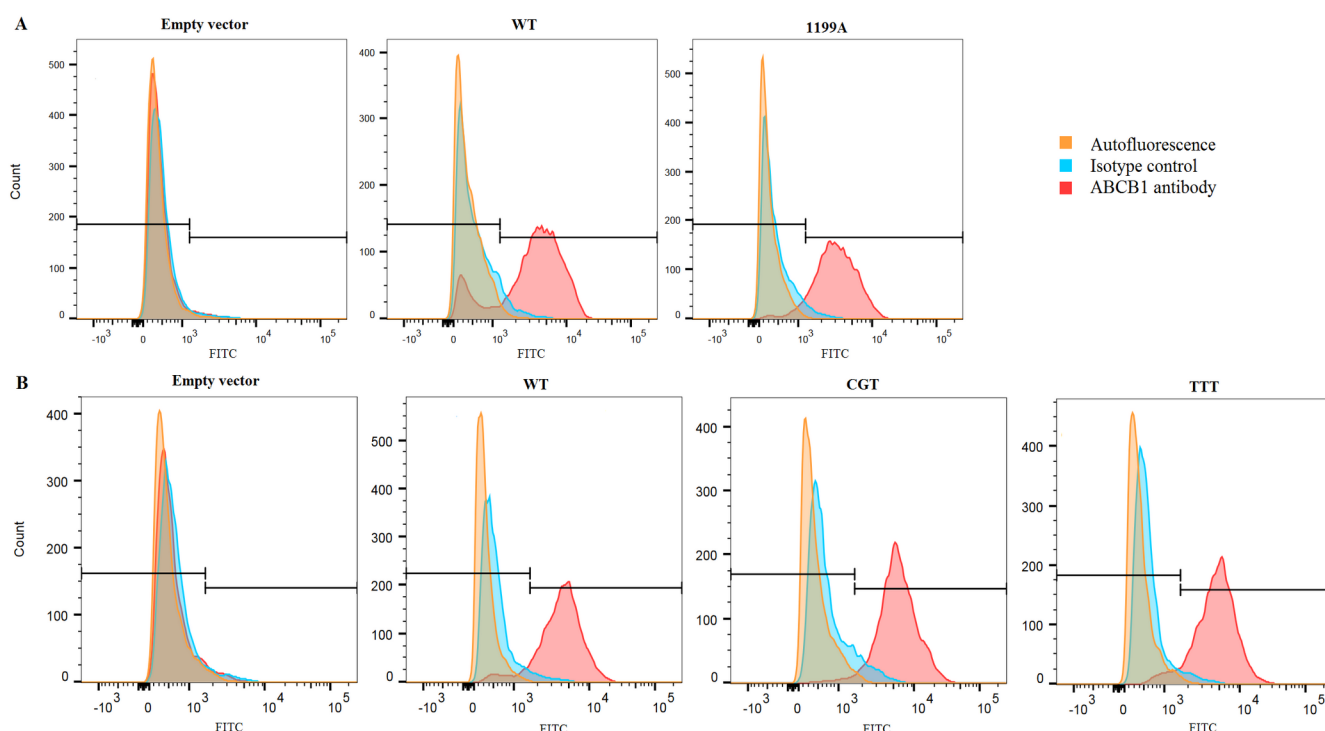


Figure 1. ABCB1 expression assessed by flow cytometry for cells stained with ABCB1 antibody, isotype control and no staining (autofluorescence). (A) Histograms of HEK_{control}, HEK_{WT} and HEK_{1199A}. (B) Histograms of HEK_{control}, HEK_{WT}, HEK_{CGT} and HEK_{TTT}. FITC: Fluorescein isothiocyanate.

Intracellular accumulation

Intracellular concentrations of DRV, adjusted by the total protein content of the sample, markedly differed between cell lines: they were significantly lower in all ABCB1-expressing cells compared to control cells transfected with an empty plasmid in all experiments (**Figure 2** and **Figure 3**), confirming that DRV is a substrate of ABCB1 and that drug efflux greatly limits cellular penetration in this model. There was no difference in intracellular concentrations between cells expressing the c.1199G or the c.1199A variant ($p = 0.89, 0.98, 0.81, 0.38, 0.56$ at the 10, 5, 2.5, 1 and 0.5 mg.l⁻¹ dose levels, respectively) (**Figure 2**). Likewise, concerning the *ABCB1* haplotype defined by the c.1236C>T, c.2677G>T and c.3435C>T variants, intracellular concentrations did not significantly differ between CGC

(wild-type) versus CGT cells ($p = 0.85, 0.66, 0.37, 0.95, 0.20$), CGC versus TTT ($p = 0.99, 0.16, 0.74, 0.93, 0.98$), or CGT versus TTT ($p = 0.7, 0.64, 0.9, 0.68, 0.12$) (**Figure 3**).

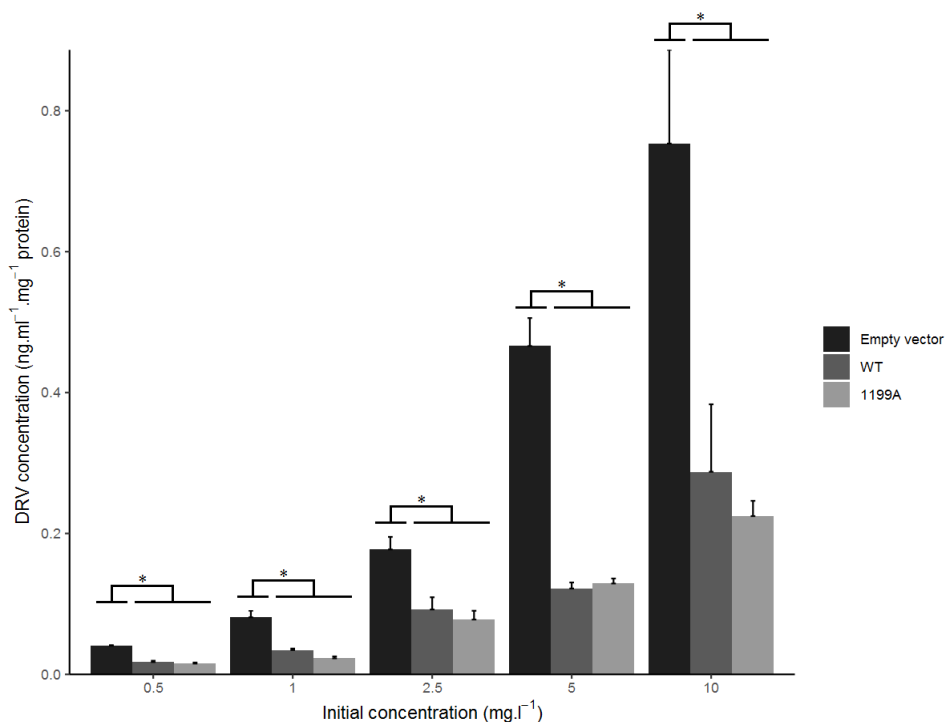


Figure 2. Intracellular protein-normalized DRV concentrations in HEK_{control} (empty vector), HEK_{WT} and HEK_{1199A} cells at several dose levels after 2 hours of incubation. Results reported as mean + standard error ($n = 3$). * denotes statistically significant difference ($p < 0.05$) between groups.

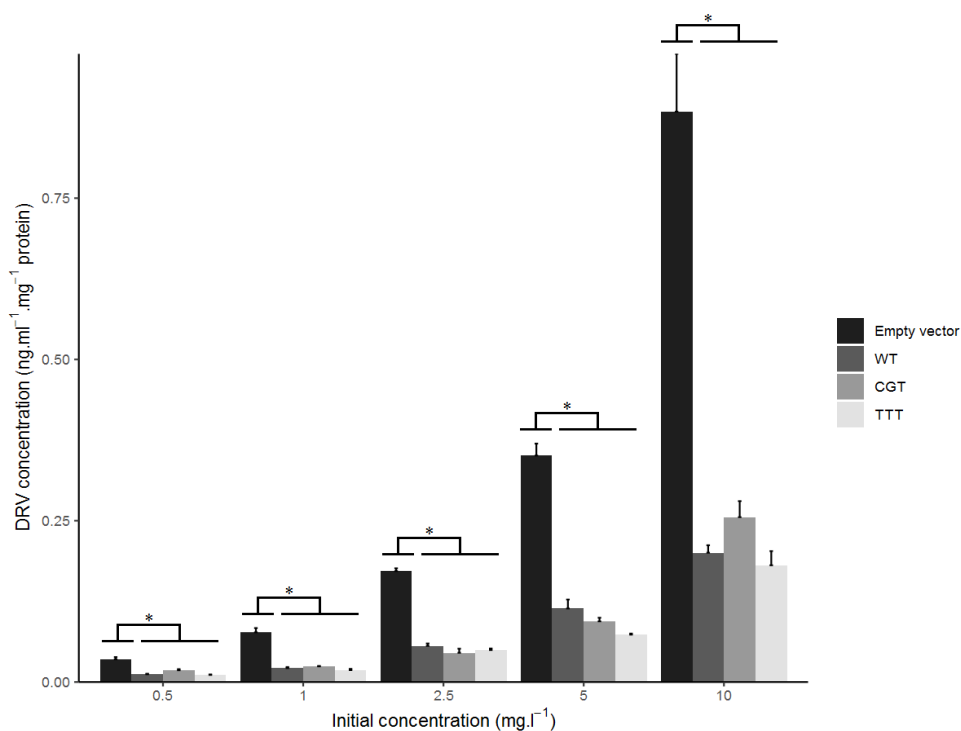


Figure 3. Intracellular protein-normalized DRV concentrations in HEK_{control} (empty vector), HEK_{WT}, HEK_{CGT} and HEK_{TTT} cells at several dose levels after 2 hours of incubation. Results reported as mean + standard error (n = 3). * denotes statistically significant difference (p < 0.05) between groups.

Cell viability

Cell viability was unaffected by DRV at concentration levels ranging from 1 to 10 mg/l. The results of these experiments are displayed in **Figure 4**.

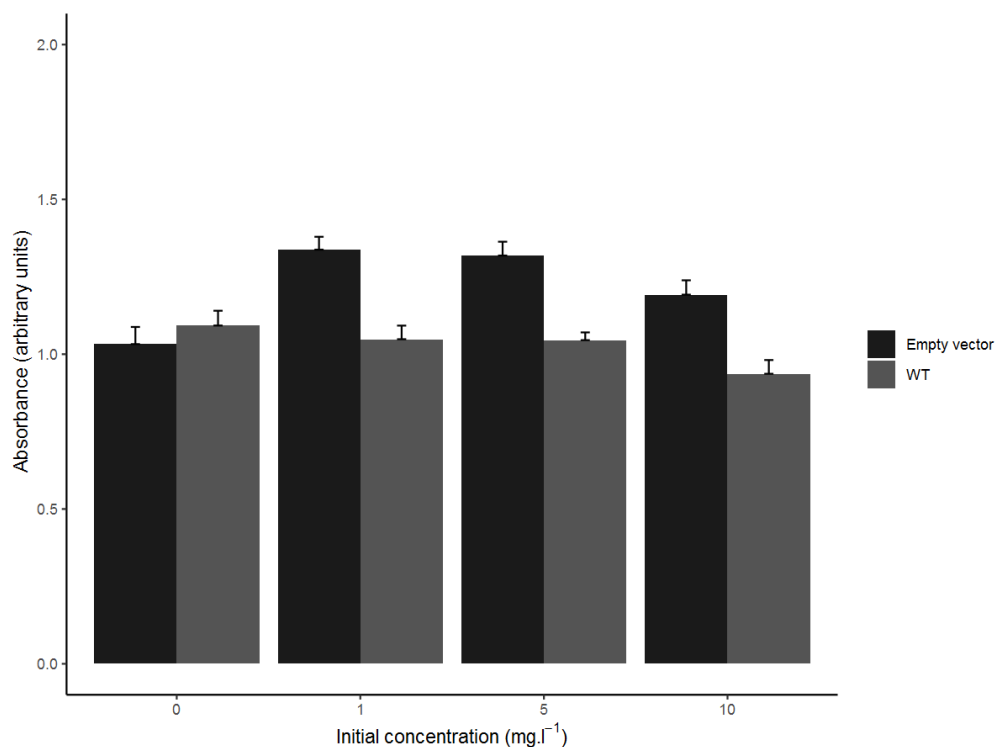


Figure 4. WST-1 cell viability assay results in HEK_{control} (empty vector) and HEK_{WT} cells at several dose levels (0 being the control condition) after 1.75 hours of incubation. Results reported as mean absorbance + standard error (n = 6).

DISCUSSION

Several pharmacogenetic studies have attempted to determine the influence of *ABCB1* variants on DRV pharmacokinetics. Calcagno *et al.* studied the paired PK of DRV in plasma and cerebrospinal fluid (CSF) in 41 patients and showed that there was no influence of *ABCB1* c.1236C>T, c.2677G>T or c.3435C>T on DRV penetration in the central nervous system, as assessed by the ratio of concentrations between the CSF and plasma [14]. Moltó *et al.* developed a population PK model of DRV on 75 subjects and assessed the influence of 148 SNPs in 15 genes, including *ABCB1* c.1236C>T and c.2677G>T, neither of which showed a significant effect on PK parameters [13]. PK parameters were similarly unaffected by the c.1236C>T and c.3435C>T variants in a small group of 25 patients in another population study [19]. Moreover, Nagano *et al.* found that the *ABCB1* c.3435C>T variant was not correlated with concentrations of DRV in either plasma or peripheral blood mononuclear

cells (PBMCs) in a group of 19 patients [12]. Finally, we recently published a population PK model developed on 127 patients for which neither *ABCB1* c.1199G>A nor *ABCB1* c.3435C>T were correlated with DRV PK parameters [3]. Although these studies were not necessarily powered with a specific set of covariates in mind, they suggest that genetic polymorphisms in *ABCB1* either do not alter protein activity toward this molecule, or that other, unidentified factors mask or neuter the effect of *ABCB1* variants *in vivo*. RTV- or COB-based boosting likely has a role to play since both of these boosters are capable of inhibiting *ABCB1* to some degree, but other factors may also contribute. Besides these considerations related to DRV based therapy, there have been conflicting results in the literature regarding the effect of these genetic variants not only *in vivo* but also *in vitro*. For instance, Woodahl *et al.* showed that *ABCB1*-mediated efflux for older protease inhibitors such as lopinavir, amprenavir, indinavir, saquinavir and RTV was increased in cells expressing the c.1199A variant compared to the wild-type protein [20]. Meanwhile, tumor resistance to anticancer agents was increased in c.1199A cells for certain agents such as vinblastine, vincristine, paclitaxel and etoposide, with fold-changes ranging from 1.9 to 11 depending on the drug, but was unchanged for doxorubicin [21]. Dessilly *et al.* also showed that the c.1199G>A variant modulated the intracellular accumulation of tacrolimus, but not that of ciclosporine [15]. Further, they showed that the 1236-2677-3435 haplotype had little to no effect on the accumulation and activity of several tyrosine kinase inhibitors (dasatinib, nilotinib and ponatinib), whereas the variant *ABCB1* displayed lower activity toward imatinib [16]. Sennesael *et al.* also showed that there was no effect of any of the four aforementioned SNPs on the efflux of the anticoagulant drug rivaroxaban [22]. Because of these conflicting results, there is a need for case-by-case assessment of drug accumulation to determine the relevance of genetic variants in *ABCB1*. An existing model of intracellular accumulation was used to assess the influence of *ABCB1* variants. Our experiments showed that the c.1199G>A, c.1236C>T, c.2677G>T and c.3435C>T variants have no effect on the ability of *ABCB1* to transport DRV in this model, similarly to what was observed for other substrates, such as rivaroxaban. While it could be argued that HEK293 cells are not a physiologically representative model, they feature a low basal expression level of *ABCB1*, ensuring all *ABCB1* activity in our model can be attributed to the transfection process [15,16], unlike other cell lines like Caco-2, that would be more representative of the digestive tract but would also introduce a bias due to a combination of native and transfection-induced expression. Further, this *in vitro* model is characterized by supraphysiological levels of *ABCB1* expression and these SNPs may exert slightly different effects with *in vivo* levels. In any event, variants in other influx and efflux transporters, as well as a range of genetic and non-genetic factors, could potentially explain the wide range of plasma and intracellular concentrations that have been reported for DRV. In conclusion, using a recombinant model of HEK293 cells, we showed that *ABCB1* variants do not appear to modulate the ability of this efflux pump to transport DRV, and as such, they do not alter the intracellular accumulation of this antiretroviral.

DATA AVAILABILITY

Data generated and analyzed during this study are available from the authors on reasonable request.

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ABBREVIATIONS

COB: Cobicistat

CSF: Cerebrospinal fluid

DRV: Darunavir

DRV-d9: Deuterated darunavir
EDTA: Ethylenediaminetetraacetate
FBS: Fetal bovine serum
FITC: Fluorescein isothiocyanate
HEK: Human embryonic kidney
LC-MS/MS: Liquid chromatography coupled with tandem mass spectrometry
MAF: Minor allele frequency
PBS: Phosphate buffer saline
PK: Pharmacokinetic
RTV: Ritonavir
SNP: Single nucleotide polymorphism

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AUTHOR CONTRIBUTIONS

GS, VH and LE designed the study. GS, HD and NP performed accumulation and flow cytometry experiments. GS, HD and KAD performed LC-MS/MS measurements. GS, HD, HEH and NP performed protein quantification, HEH performed cell viability experiments. All authors contributed to data analysis and visualization. GS wrote the first draft of the manuscript. All authors read and approved the final version of the manuscript.

ADDITIONAL INFORMATION

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Competing interests

The authors declare no competing interests.

2.3.4. Paper 3 summary

- Recombinant HEK293 cell lines were used to assess the effect of genetic variants in *ABCB1* on the intracellular accumulation of DRV.
- Neither the c.1199G>A polymorphism nor the haplotype defined by c.1236C>T, c.2677G>T and c.3435C>T had any effect on intracellular concentrations of DRV.

3. CONCLUSIONS AND PERSPECTIVES

The present work was focused on the PK of DRV, a widely used HIV protease inhibitor. Through a combination of *in vivo* studies and *in silico* modeling, supplemented with *in vitro* experiments, we explored the sources of its inter-individual variability and provided a quantitative model of its PK in a population of diverse patients. Since the results themselves were already extensively discussed in their respective papers, the following section will offer some general thoughts on this work and how it could be expanded upon.

The results from our clinical study show that genetic, pharmacological and physiological parameters only partly explain the variability in DRV PK. As mentioned in the discussion of **Paper 1**, the design of our study was limited by practical considerations, presented several issues inherent to this type of study (such as inaccuracies in drug intake and sampling time), and it was not powered toward any specific endpoint. Therefore, it cannot be ruled out that some of the covariates we studied actually do contribute to PK heterogeneity despite not being retained in the final model. Likewise, some of the associations we detected were fairly sensitive to the model and did not systematically turn up as significant, depending on the type of model used.

On the topic of covariate analysis, there are a few other points to consider. The SNP panel in our study was designed to encompass major proteins expected to play a role in the biotransformation and transport of DRV, but a wider screening approach could reveal additional information. Concerning CYP3A5, an isoform which has historically been neglected due to the low prevalence of expressors in Caucasian populations, and which may sometimes have been lumped with CYP3A4, it was previously shown by our research group that the *CYP3A5*3* variant was linked with altered DRV metabolism, although this effect was restricted to a subgroup of patients [145]. A similar effect, but for a larger population, was observed in our cohort, and therefore, *CYP3A5*3* was retained as a covariate in our model (**Paper 1**). And while the *CYP3A5*3* variant is certainly the most relevant, it cannot be excluded that other polymorphisms causing a reduction or loss of function in this gene might also be responsible for part of the variability observed in human subjects. Likewise, *CYP3A4* variants (which are of particular interest when CYP3A5 is non-functional) are not limited to *CYP3A4*22* (which had no discernible effect in our study); other notable variants include *CYP3A4*20*, a loss-of-function variant that is rare in Caucasians, but has a non-negligible allelic frequency in Hispanics [139]. As mentioned in the Introduction, there are also several transporters of the SLCO family that are responsible for DRV uptake, including SLCO1A2, SLCO1B1 and SLCO1B3. Although Moltó et al. previously investigated SNPs in several genes of this family as potential covariates, they only had access to the genotypes of a very restricted pool of subjects [142]. More appropriately powered studies are necessary to investigate this question. Still, many of these SNPs often have low allelic frequencies and thus, probably cannot completely explain the large PK variability that remains even after adjustment for several covariates. Further, both our clinical and *in vitro* studies failed to demonstrate a large impact of even the most relevant SNPs (in terms of functional effect and allelic frequency), so perhaps the answer lies beyond genetic polymorphisms in enzymatic and

transport pathways. Since AAG levels were associated with DRV PK both in our cohort and in previous studies, AAG phenotyping – although technically cumbersome – might also be an interesting piece of the puzzle. Indeed, AAG variants, both of a genetic and epigenetic nature (glycosylation status), have been described in the literature [180]. Measuring AAG concentrations remains much easier than phenotyping but it is hard to say if this simple measurement fully captures all AAG-related variability.

In the Introduction, we touched upon the topic of populations with particular requirements such as children, pregnant women and HIV-2 patients, to which we could also add patients with specific comorbidities and coinfections such as tuberculosis or hepatitis. These populations cannot be treated like standard adults and require further pharmacometric research before recommendations can be formulated (**Paper 1**).

Another limitation that was already discussed is that the very use of plasma concentrations is debatable. Using plasma concentrations for TDM has often become the default option, in part due to technical limitations (lack of sufficiently sensitive analytical methods and adequate extraction protocol, difficulty in obtaining the required biological matrix, e.g., CSF, PBMCs, tissue samples) and in part due to a lack of experimental data showing the usefulness of non-plasma measurements in clinical pharmacology. In the case of DRV, local concentrations at the site of action (lymphocytes) or in viral sanctuaries (CNS, genital tissue) appear promising as new avenues of research (section 1.2.3, p.23), especially to provide some insight into cases of treatment failure, once non-adherence, RAMs or alterations of plasma PK have been excluded as potential explanations. However, the usefulness of non-plasma data as routine biomarkers is currently limited due to the technical challenges that come with attempting to measure them, as well as the difficulty in interpreting them, whereas plasma concentrations are cheap, easily obtained and straightforward to understand. If intracellular concentrations (or concentrations in the CSF or genital fluids, for instance) prove to be useful biomarkers but their measurement remains too complicated for routine practice, it might still be possible to extrapolate them from plasma data once an appropriate PK model linking plasma and non-plasma data has been developed (though it cannot be guaranteed that the process would be so trivial, as multiple plasma measurements or additional covariates may be necessary to account for the highly nonlinear processes involved, combined with major inter-individual variability). This would allow clinicians to gain insight into biomarkers that are not readily available.

In our second paper, we proposed some sampling strategies that could be applied to DRV TDM or to future clinical studies. Beyond what has already been said in that paper, it is important to mention that TDM of plasma concentrations, even if it is simpler and more well-established than TDM using other biomarkers, is not so easy to perform in practice either, because it requires a longer stay of the patient in the hospital, multiple blood samples (perhaps requiring a catheter for easier access), and the analysis of the results is slightly more complex. TDM using multiple timepoints, despite being statistically superior to single-point TDM, is simply too bothersome in many cases, even using the C_0 - C_1 - C_4 strategy we proposed. Therefore, C_0 -based TDM probably remains the best option in terms of convenience. Rather than requiring patients to remain in the hospital for several hours, one option would be to

collect samples remotely using techniques such as dried blood spots (DBS), which enable measurement from a drop of blood collected by the patient themselves. DBS are an attractive alternative to traditional inpatient TDM due to being very convenient for patients. There is a growing number of studies comparing them with standard TDM and DBS are increasingly being used for certain drugs, for instance the immunosuppressants tacrolimus and mycophenolic acid [196,197]. Further, they can be used to monitor other biomarkers, including the viral load. The main obstacles to their implementation are patient training in sample collection, and the development of appropriate (pre-)analytical methods. Assuming the use of DBS is appropriately validated and evaluated through clinical trials, they could offer a new option for TDM. Still, in the context of multi-sample TDM, it may prove difficult to properly educate patients on how to best collect the samples, limiting the use of DBS.

Modeling-wise, a holistic approach involving simultaneous study of several coadministered ARVs may also prove very helpful in interpreting clinical data in HIV patients, which are almost always polymedicated. The main issue with this approach remains a technical one: even with sufficient clinical data to work with, simultaneous modeling of multiple drugs can prove difficult using currently available software tools and methodologies. Nevertheless, it seems like a logical progression to account for all ARVs and how they interact with one another, rather than consider isolated drugs.

Despite their wide range of applications, PK models sometimes fail to prove of much use, mostly because these methodologies are not yet widely accepted (and understood) by non-quantitative scientists and clinicians, or lack in applicability. Throughout this thesis, we have provided some examples of what pharmacometric tools can enable, with a focus on the topic of ART and particularly DRV-based therapy, but perhaps this could be further emphasized here with a broad discussion of TDM and what pharmacometric models can offer to the clinician. TDM has become an integral part of patient care in several fields of medicine, with tools ranging from analytical procedures to pharmacometric software. While the former are probably understood well enough (and used widely enough) by clinicians that they need no further explanation, the latter is still a developing art. One might wonder how pharmacometric models can be of practical use and what steps are necessary before they can be applied. As discussed throughout this work, the main applications of PK models to clinical practice would be the study of atypical patient profiles, the extrapolation of full PK profiles based on sparse samples to interpret TDM data or to assess the eligibility of the patient to alternative dosing regimens (based on individual PK and EC_{50} target), the extrapolation of non-plasma data (concentrations at the site of action or in sanctuary compartments), and the assessment of the efficacy of prophylactic regimens. All of these can be considered different ways to optimize DRV therapy (and ART in general).

Regarding the practical aspects of using PK models, the main prerequisite is a functional and accurate model, which in turn requires sufficient data to be collected. This part of the work has been described in great detail already and needs no further elaboration. But how can this type of model be used and how can the recommendations be applied to everyday practice? Through the process of Bayesian estimation, measured concentrations can be fed into an

existing model that is known to be a good representation of reality (i.e., that has been validated) to derive an individual's PK profile. Technically speaking, this is a straightforward process that can be completed rather quickly, provided there is an adequate workflow. Once the TDM data and any necessary covariates are available, the individual profile can be calculated based on the model. Simulations can be used to determine how this profile would vary under different scenarios (lower dose, lower frequency of administration, change in individual covariates, change in treatment adherence, etc.). But even the simple process of Bayesian estimation may be too cumbersome for everyday practice, as it would require copying the value returned by the analytical lab into a piece of pharmacometric software, convert the units or format the data in a specific way, execute the required series of operations, perhaps validate these results by checking a few elements (for instance, whether the software returns any error messages), then interpret the results based on the model, the literature, expert knowledge and data pertaining to that patient, and in turn formulate a practical recommendation, such as "Increase the dose by x mg" or "This patient is eligible for a reduction of the daily dose to 400 mg q24h". Fundamentally, this is no different than any other laboratory analysis, in that there are intermediate steps to obtain the desired answer, and a specific expertise is necessary to validate the results (or even multiple levels of expertise, to validate each step of the process). Of course, pharmacometric tools result in an additional and perhaps unwelcome step that requires yet another type of expertise and the development of a software workflow that allows the desired information to be easily manipulated, interpreted and archived. Therefore, if one wishes for these tools to become truly useful, it will be necessary to foster expertise in pharmacometrics, promote the use of quantitative methods and develop appropriate software tools and workflows for everyday practice. That is not to say that these things have not been done yet, but simply that the contribution of pharmacometrics is still growing and that it will take time before we see most clinicians request and interpret this type of data as naturally as they do the glomerular filtration rate. Of course, not all fields of medicine would benefit equally from pharmacometrics, but many therapeutic areas ranging from antibiotics to anticancer agents and many others pose challenges such as drugs with narrow therapeutic indices, high inter- and intra-individual variability in PK/PD, low clinical response rates for unknown reasons, etc. This is only scratching the surface of how complex biological problems can be solved using quantitative methods, but such a discussion would be beyond the scope of this thesis.

Regarding the results of our simulations of alternative regimens, it is important to once again question the validity of some of the PK targets. Three target concentration levels that are used throughout the literature were selected. While we showed in our simulations that the probability of target attainment decreased significantly at the population level (which, of course, says nothing about individual probability, which should be assessed through, e.g., Bayesian estimation) depending on the regimen and PK target, there is clinical evidence that seems to indicate that low DRV exposure is not a cause of virological failure in multi-drug regimens [119]. This has two potential implications: either some of the commonly defined PK targets are irrelevant to this question, or maintenance therapy simply does not necessarily require the same level of antiviral activity as initial suppression therapy. In other words, treatment success or failure cannot be easily predicted based on the PK profile of the

individual; but it may be, as mentioned earlier, that a holistic PK/PD approach would provide more information on individual drug contributions in ART. In the future, as concepts for new simplified drug regimens emerge (new drug combinations, new dose levels, new formulations, etc.), it might also be interesting to simulate their effect using population models, both to select patients that could benefit from them and to better understand how DRV and its boosters modulate their own PK at different dose levels. Indeed, as response to many ARVs (and drugs in general) is highly variable, TDM supplemented with PK modeling would allow clinicians to tailor the treatment to the individual, to determine whether a specific combination of drugs, dose level, frequency of administration or even indication (e.g., prophylaxis, patient with specific coinfections) is appropriate for the individual. Covariates might also prove useful in some cases, but the *a posteriori* information provided by TDM would usually trump *a priori* information. While treatment recommendations can be formulated at the population level, this would ignore individual patient traits and result in a significant loss of accuracy of those recommendations. True optimization means individualization, and therefore, there is a need for appropriate pharmacometric tools, as we have already discussed earlier in this section.

On the *in vitro* side of things, while our experiments clarified the absence of influence from four different *ABCB1* polymorphisms, covering most of the variability for this gene, this is but a small fraction of all possible SNPs we could have studied. Judging by our *in vivo* results, variants in *SLCO3A1* may be of interest, especially since there is currently no data showing that DRV is even a substrate of this ubiquitous transporter, or how *SLCO3A1* could alter PK processes. Further, our *in vitro* results are difficult to translate *in vivo*, as is often the case with this type of experiments. Not only were the expression levels of *ABCB1* supra-physiological (a design choice), but the question also remains of how *SLCO* transporters, as well as other efflux transporters such as *ABCC2*, interact with *ABCB1*. Co-transfection cellular models incorporating at least a few relevant transporters could help answer these questions.

There is also the question of whether DRV has any future as a therapeutic agent. While it was once a major part of the ARV arsenal and while it is still the first choice among PIs, it could also be said that its popularity is waning, in no small part thanks to the arrival of new drugs with more favorable toxicity and interaction profiles, in particular among INSTIs, which are now the cornerstone of initial ART in several international guidelines [21,22]. However, these same guidelines maintain DRV as an option for initial triple drug regimens. In addition, DRV retains a place in maintenance therapy, in particular as part of two-drug regimens, and in salvage therapy (in the event of virological failure), in particular for patients failing on INSTI-based regimens [22]. As mentioned in the Introduction, DRV also shows promise as a prophylactic agent, an area which is gaining increasing attention. On that note, the EACS 2020 recommendations for PEP include a triple ART of TDF (or TAF) + FTC + a third agent such as DRV/booster or RAL (first-line options); or TDF (or TAF) + FTC + INSTI (BIC or DTG) [21]. Dual therapy with TDF (or TAF) + FTC remains the only option for PrEP [21,22].

Long-acting injectable formulations of ARVs is yet another research area that shows great promise and that could create new opportunities for the use of DRV and ARVs in general.

Long-acting formulations aim to provide drug coverage over an extended period of time, potentially several weeks or even months, which would greatly reduce the drug burden for the patient and provide an extremely convenient way of maintaining viral suppression. For instance, a long-acting injectable form of rilpivirine and cabotegravir, to be delivered every four or every eight weeks, has been developed and has proven to be as effective as oral triple therapy [198]. Additionally, using tissue-targeted formulations, one could also more effectively reach compartments that act as viral sanctuaries and reduce off-target toxicity. For instance, a recent paper reports on the development of experimental nanoformulations based on hydrophobic ester prodrugs of DRV [199], although only animal data is available at the moment. One of the main limitations of injectables are related to the low acceptable volume of injection, which makes it difficult to use molecules with low aqueous solubility, such as DRV. The development of new formulations like these could improve the physico-chemical profile of DRV to make it a better candidate for injectable forms, and even compensate for some of its other downsides, such as the need for PK boosting. In addition to technical challenges, long-acting regimens also increase the risk of subtherapeutic drug levels due to their mode of administration, with infrequent dosing and a lack of control over drug delivery.

From a pharmacological point of view, the opportunities that these new formulations offer emphasize the importance of possessing a solid understanding of individual variations in drug exposure and how it relates to the processes of absorption, distribution and elimination, as well as knowledge of appropriate PK/PD targets and the risk of RAM emergence, in order to derive optimal drug doses and combinations to be used in these new formulations. The idea that patients could use an injectable form that would provide extended coverage compared to *per os* regimens is a very appealing one and could prove to be a major advance in HIV care. In some ways, this could be seen as an extension of the trend to combine multiple ARVs together, which is justified by the desire to simplify treatments as much as possible. While this is laudable goal, it is somewhat at odds with the idea of individualizing therapy, since combined formulations tend to reduce the spectrum of choices in terms of doses and drug combinations, rather than increase it. This may not be an issue for many ARVs due to their large therapeutic intervals, which would mean that the highest effective dose could be used in all patients with very little risk (DRV most certainly falls in this category). Therefore, optimization will likely strive to find the lowest universally effective dose and frequency of administration for each drug, as well as the optimal ARV combination. And as we discussed before, optimization could still be performed on an individual basis, if only by determining whether a specific individual could safely fit into a *predetermined* optimized regimen, rather than *what* regimen would be optimal for them in particular.

To conclude on the topic of future developments in ART, it is not uncommon for drugs to gain new indications (this is perhaps less likely for PIs than it would be for other pharmacological classes, especially antibiotics) or to become more popular after new ways to optimize their use are discovered, as has been the case for EFV, a topic which is discussed in our review in Annex 1 (p.127). It is difficult to say whether DRV will continue to be as widely used, but it is likely that treatment recommendations (for DRV and for ARVs in general) will continue to evolve as new data becomes available.

Until curative treatments or vaccines are discovered, the HIV epidemic will continue to be a major healthcare issue. Not only should we aim for wider access to diagnostic methods and ART, but also optimization of available therapeutic tools to reduce costs, prevent toxicity (especially in the long term), limit the potential for drug interactions, improve patient compliance, and deal with comorbidities and coinfections, all the while ensuring ART efficacy. Much research is also being dedicated to new therapeutic approaches such as PrEP and PEP, as well as new formulations (such as long-acting formulations and co-formulations of multiple ARVs). In conclusion, optimization of ART will most certainly continue to be a talking point in the years to come, and it is likely that pharmacometrics will have a significant role to play in this field of research.

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ANNEXES

Annex 1. Major drug-drug interactions for DRV therapy

Victim	Perpetrator	Interaction
DRV	Phenobarbital, phenytoin, rifampicin, St John's wort	Contraindicated due to potential for CYP induction by these substances.
DRV	Other RTV- or COB-boosted drugs	Avoid or combine unboosted DRV with boosted drugs.
Efavirenz*	DRV/RTV	DRV AUC ₁₂ reduced by 13% and efavirenz AUC ₂₄ increased by 21%. Use with caution and monitor drug levels.
	DRV/COB	Not recommended.
Nevirapine*	DRV/RTV	Nevirapine and DRV AUC increased by 27 and 24%, respectively. Appears safe.
	DRV/COB	Not recommended.
Etravirine*	DRV/RTV	Slightly increased levels of etravirine.
	DRV/COB	Not recommended.
Maraviroc	DRV/RTV, DRV/COB	Increase of maraviroc AUC by 305%, do not exceed 150 mg q12h.
Carbamazepine*	DRV/RTV	Carbamazepine AUC ₁₂ increased by 45%. Patient monitoring and dose titration advised if coadministration is necessary.
	DRV/COB	Contraindicated (no data).
Lamotrigine	DRV/RTV, DRV/COB	Unknown (possible decrease in lamotrigine levels). Close monitoring recommended.
Statins	DRV/RTV, DRV/COB	Increased statin exposure. Recommended to start atorvastatin/rosuvastatin at a low daily dose, titrate and do not exceed 20 mg atorvastatin. Pravastatin also appears safe. Simvastatin and lovastatin contraindicated due to risk of myotoxicity.
Clarithromycin	DRV/RTV, DRV/COB	Clarithromycin AUC ₁₂ increased by 57% with DRV/RTV. Increased incidence of dysgeusia. Monitor patients.
Azole antifungals*	DRV/RTV, DRV/COB	DRV AUC ₁₂ increased by 42% and ketoconazole AUC ₁₂ increased by 212%. Ketoconazole or itraconazole daily dose should not exceed 200 mg. Voriconazole should be avoided. Caution with other azole antifungals.
Oral contraceptives	DRV/RTV, DRV/COB	Decreased exposure to ethinylestradiol and norethisterone (14-44% lower AUC ₂₄). Additional/alternative contraceptive measures required.
Methadone	DRV/RTV	16% lower AUC ₂₄ for the (active) R-enantiomer of methadone. Monitoring advised.

Amiodarone, dronedarone, quinidine, lidocaine, bepridil	DRV/RTV, DRV/COB	Contraindicated due to risk of life-threatening cardiac arrhythmia.
Alfuzosin	DRV/RTV	Contraindicated due to risk of serious hypotension.
Astemizole, terfenadine	DRV/RTV	Contraindicated due to risk of life-threatening cardiac arrhythmia.
Cisapride	DRV/RTV, DRV/COB	Contraindicated due to risk of life-threatening cardiac arrhythmia.
Pimozide, quetiapine, sertindole, lurasidone	DRV/RTV, DRV/COB	Contraindicated due to risk of life-threatening cardiac arrhythmia.
Ergot derivatives	DRV/RTV, DRV/COB	Contraindicated due to risk of ergot toxicity.
Midazolam, triazolam	DRV/RTV, DRV/COB	Contraindicated due to risk of increased sedation and respiratory depression. Parenteral midazolam should only be used under close monitoring.
Direct oral anticoagulants	DRV/RTV, DRV/COB	Could increase anticoagulant concentrations and the risk of bleeding. Apixaban not recommended for patients using a low dose (2.5 mg). For higher values, reduce the dose. Betrixaban, dabigatran and edoxaban coadministration appear to be safe.
Ticagrelor	DRV/RTV, DRV/COB	Not recommended due to risk of bleeding.
Warfarine	DRV/RTV	Only under close monitoring due to risk of bleeding.
	DRV/COB	Not studied. Expected to differ from interaction with DRV/RTV.
Colchicine	DRV/RTV, DRV/COB	Careful monitoring and dose adjustment required due to increased colchicine exposure. Contraindicated in patients with renal or hepatic failure.
Tacrolimus, ciclosporine, sirolimus, everolimus	DRV/RTV DRV/COB	Therapeutic drug monitoring required. Ditto. Everolimus contraindicated.
HCV direct acting agents	DRV/RTV	Boceprevir and telaprevir: decreased exposure to both DRV and the HCV PIs. Simeprevir: AUC ₂₄ increased by 59%. Elbasvir and grazoprevir: contraindicated due to greatly majored levels (166-750%). Glecaprevir concentrations increased 5-fold, avoid coadministration.
	DRV/COB	No data.

AUC_{12/24}: Area under the curve over 12/24 hours, HCV: Hepatitis C Virus, *: Victim and perpetrator. Compiled from [14,57,99,101,181–185]. DDIs in this table have been selected due to the possibility of coadministration and have either been clinically demonstrated to have a major impact, or remain theoretical but are considered important due to their magnitude and the associated risk of treatment inefficacy or toxicity. Note that many DRV/RTV DDIs may apply to DRV/COB, but these have not always been separately evaluated.

Annex 2. CYP-mediated metabolism of DRV

Preclinical data shows DRV is a substrate for several CYP isoforms, including CYP2C19, CYP2D6, CYP3A4 and CYP3A5, the latter two constituting the most significant contributors to its biotransformation (Table 10). Human liver microsomes (HLMs) are a representative system of liver metabolism, although other systems, such as E. Coli-expressed CYP and Supersomes, were also used to investigate the contribution of individual CYP isoforms, with varying results [59]. As can be seen in Table 10, different CYP isoforms favor different metabolic pathways.

Table 10. In vitro metabolism of DRV – Preclinical data [59]

2.6.5.10.D Pharmacokinetics: Metabolism In Vitro-Darunavir

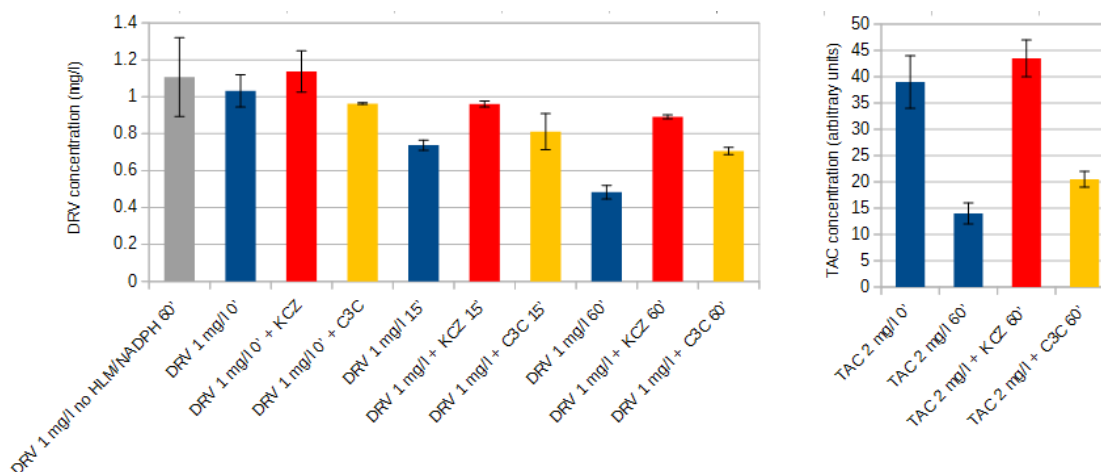
Test article: darunavir

Location in CTD		4.2.2.4						
Study No.		TMC114-NC202						
Type of Study: CYP reaction phenotyping- Effect of diagnostic CYP inhibitors on the metabolism of TMC114								
Method: Inhibition of the metabolism of TMC114 in human liver microsomes by diagnostic inhibitors was carried out with ¹⁴ C-TMC114 (3 ¹ M) for 20 minutes at a protein concentration of 0.5 mg/ml. The amounts of unchanged TMC114 and its metabolites (M6, M19 ¹ , M23 ¹ , M27, M28 and M29 ^{1,3}) were determined by radio-HPLC. The values represent the percentage of inhibition obtained for each inhibitor in comparison to a control incubate (without inhibitor). Each value represents mean of three observations.								
Results:								
Diagnostic Inhibitor	CYP P450 Form	Overall ^{3,4}	M6	M19 ¹	M23 ¹	M27 ²	% Inhibition of Metabolism ²	
Furafylline (10 µM)	CYP1A2	4.6	59.1	44.5	7.8	-25.6	-66.7	13.0
Coumarin (100 µM)	CYP2A6	-1.1	40.9	39.4	-2.8	-57.3	-61.1	13.5
Sulphaphenazole (10 µM)	CYP2C8/9/10	-2.5	27.3	34.6	3.6	-50.0	-75.0	-1.3
Quinidine (10 µM)	CYP2D6	-2.0	34.8	31.5	4.2	-17.1	-69.4	-7.6
4-methylpyrazole (20 µM)	CYP2E1	-14.7	31.8	36.6	12.2	-4.9	-138.9	-1.3
Ticlopidine (5 µM)	CYP2C19/D6	4.4	27.3	19.4	5.8	2.4	-51.5	11.7
Ketoconazole (1 µM)	CYP3A4	47.2	69.7	65.8	61.4	42.7	30.6	58.3
Troleandomycin (200 µM)	CYP3A4	79.4	100.0	93.5	96.1	100.0	100.0	93.5
Clarithromycin (15 µM)	CYP3A	32.4	60.6	34.2	32.2	31.7	25.0	37.0
Ritonavir (0.15 ¹ M)	CYP3A4	59.6	65.2	72.6	75.6	68.3	55.6	63.2
1-aminobenzotriazole (1000 µM)	CYP P450	-12.5 ⁵	100.0	99.3	99.2	100.0	100.0	97.4
Additional Information								
¹ : Major metabolites in human liver microsomes (> 5 % of the sample radioactivity)								
² : Calculated from control incubation (without inhibitor); higher the positive value and higher the extent of inhibition								
³ : Negative values indicates higher % product formation in test sample compared to the control. This was more prominent with the minor metabolites. For all qualitative purposes, all negative values were considered as no inhibition.								
⁴ : Overall % metabolism of TMC114 calculated from % drug that remained in the sample at the end of the incubation								
⁵ : Although complete inhibition of the metabolites was observed, there was an unusual peak observed in this sample. This was not observed any of the HLM samples.								

We also assessed the degradation of DRV in HLMs. In this experiment, DRV (1 mg/l) was incubated for up to 60 minutes in TRIS buffer (pH 7.5) at 37°C with HLMs pooled from 20 donors (20 mg/ml protein) and 1 mM NADPH, then the reaction was stopped with a mixture of acetonitrile, formic acid and clomipramine (internal standard). Concentrations and incubation times were optimized in preliminary tests. Experimental conditions were:

- TRIS buffer alone (light grey)
- HLMs/NADPH (blue)
- HLMs/NADPH and 2 µM ketoconazole (KCZ), an inhibitor of CYP3A4 and CYP3A5 [186] (red)
- HLMs/NADPH and 0.5 µM CYP3cide (C3C), a specific inhibitor of CYP3A4 [187] (yellow)
- Tacrolimus (TAC), used as positive control for CYP3A-mediated enzymatic degradation.

Figure 19. Contribution of CYP3A isoforms to the degradation of DRV in vitro



Data presented as mean $\pm$ SE or single value.

Degradation of DRV was primarily mediated by CYP3A, as evidenced by KCZ effectively blocking this pathway, while C3C only partially inhibited it, indicating some amount of degradation was mediated by the CYP3A5 isoform (Figure 19). Contrast these results with those obtained for TAC, showing predominantly CYP3A5-mediated degradation, as previously described in the literature [186].

Annex 3. Review of CYP pharmacogenetics for antiretrovirals

G. Stillemans, L. Belkhir, D.A. Hesselink, V. Haufroid and L. Elens, Pharmacogenetic associations with cytochrome P450 in antiretroviral therapy: what does the future hold?, Expert Opinion on Drug Metabolism & Toxicology 14 (2018), pp. 601–611 (Impact factor 2018: 3.721)

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REVIEW



Pharmacogenetic associations with cytochrome P450 in antiretroviral therapy: what does the future hold?

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ABSTRACT

Introduction: Several antiretroviral drugs used to treat infection with the human immunodeficiency virus (HIV) are substrates of enzymes belonging to the cytochrome P450 (CYP) superfamily, which are polymorphically expressed. It may therefore be useful to take into account the genetic variation in these enzymes to predict the likelihood of anti-HIV treatment success, toxicity and the potential for drug–drug interactions.

Areas covered: In this manuscript, the authors discuss the current state of knowledge regarding pharmacogenetic associations between CYP and all major antiretrovirals, as well as the importance of these associations.

Expert opinion: While many pharmacogenetic associations for CYP have been described in the literature, replication studies are sometimes lacking. The implementation of this knowledge in clinical practice also remains difficult. Further efforts are required both to expand this field of knowledge and to enable its use in everyday clinical practice.

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Antiretroviral; cytochrome P450; human immunodeficiency virus; non-nucleoside reverse transcriptase inhibitor; pharmacogenetics; pharmacogenomics; protease inhibitor

1. Introduction

With the advent of antiretroviral therapy (ART), treatment of patients infected with the human immunodeficiency virus (HIV) is now widely available, although an actual cure for the infection has yet to be discovered. ART aims at decreasing the viral load to undetectable levels and restoring the immune function of the host, thereby reducing HIV-related morbidity and mortality [1,2], as well as decreasing the risk of HIV transmission [3].

ART typically involves the combination of several drugs acting at different stages of the viral cycle in order to prevent the selection of drug-resistance mutations. Indeed, mutations in the viral genome are frequent, and inadequate choice of treatment, insufficient drug exposure or low adherence may lead to the selection of resistant strains. The drugs available include nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase strand transfer inhibitors (INSTIs), protease inhibitors (PIs), fusion inhibitors and CCR5 antagonists. For treatment-naïve patients, therapy usually combines a dual NRTI backbone with an NNRTI, INSTI or PI.

Some of these antiretrovirals are substrates of enzymes belonging to the cytochrome P450 (CYP) superfamily, which are responsible for the phase I metabolism of many marketed drugs. With CYP metabolism being a key determinant of drug exposure, knowledge of the major isoforms involved in the

biotransformation of a given drug, and characterization of the extent of this metabolism is critical to predict the likelihood of treatment success, toxicity and potential for drug–drug interactions (DDI), especially for a lifelong treatment such as ART. Suboptimal doses and subsequent underexposure can lead to anti-viral failure and selection of drug-resistance mutations, conditioning the choice of ART further down the line. Likewise, overexposure can lead to toxicity, non-adherence and possibly viral failure. This is a particular concern for antiretrovirals with a low genetic barrier (e.g. efavirenz (EFV), nevirapine (NVP), rilpivirine (RPV)), for which resistance mutations can emerge very quickly.

It is well known that genetic polymorphisms can alter the level of expression and/or intrinsic activity of enzymes, with the most prevalent form being single nucleotide polymorphisms (SNPs). Characterizing the functionality of these genetic variants and their impact on drug pharmacokinetics (PK) and pharmacodynamics (PD) is the goal of pharmacogenomics (or pharmacogenetics, as it would be called for single drug–gene interactions). The frequency of these SNPs can vary across ethnicities, which partly explains why race is sometimes found to influence clinical outcomes.

Such information could potentially be incorporated in new treatment algorithms: patients presenting a given polymorphism (or combination thereof) could be assigned to a therapy that maximizes their chance of success and/or minimizes the

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Article highlights

- Several antiretrovirals are known substrates of CYP.

1330 to be polymorphic. It has been extensively studied in the context of ART: its most commonly described decrease- or loss-of-function SNPs include c.516G>T (CYP2B6*9,

There is also much heterogeneity in *CYP3A5* expression. The most commonly described SNP, rs776746, defines the *CYP3A5**3 allele. The g.6986A>G substitution creates a cryptic splice site resulting in a truncated, inactive protein [9]. Allelic frequencies for this SNP are high, ranging from 0.2 in Africans to 0.65 in East Asians and 0.9 in Caucasians [10]. Other polymorphisms worth mentioning include *CYP3A5**6 (rs10264272) and *CYP3A5**7 (rs41303343). Considering the former variant, g.14690G>A leads to the formation of a cryptic splice site and skipping of exon 7, producing an inactive enzyme [9]. For the latter, a premature stop codon is generated by the insertion g.27131_27132insT.

Other, seldom discussed isoforms (at least in the context of ART) include *CYP1A2*, *CYP2D6*, and *CYP2C9*.

1.2. Literature search strategy

A systematic search was conducted in the MEDLINE database using relevant terms, such as 'cytochrome,' 'antiretroviral,' 'genotype,' 'polymorphism,' 'pharmacogenetic,' 'pharmacogenomic,' 'pharmacokinetic,' 'variant' and the names of the various drugs of interest. As mentioned in the introduction, we chose to focus on currently recommended antiretrovirals that are known substrates of CYP (i.e. EFV, NVP, RPV, atazanavir (ATV), darunavir (DRV), lopinavir (LPV) and elvitegravir). All articles written in English were screened for relevancy. Case reports, poster presentations, reviews, studies that did not include PK and/or PD analyses, small-scale studies, duplicate studies, and articles of poor quality (unclear rationale, inadequate clinical or statistical methodology) were discarded. When replication studies were abundant, our main selection criteria were: large sample size, methodological strength, new information, and/or reporting of new alleles. Elvitegravir was eventually excluded due to a lack of pharmacogenetic studies.

2. Review

2.1. Efavirenz

Thanks to its relatively low cost and once-daily dosing schedule, EFV is a widely used NNRTI in developing countries, while it is less favored in developed countries, in part due to a high rate of CNS side effects, including dizziness, insomnia, somnolence, abnormal dreams, anxiety and depression. These symptoms occur in up to two-thirds of patients [11,12] and can increase the odds of treatment non-adherence or discontinuation. However, they are most frequently reversible, resolving within a few weeks for some (but not all) patients. Initially, a dose of 600 mg once-daily was recommended. It is still the standard dose in most guidelines, while low-dose EFV (400 mg) is only found as an option in the WHO guidelines [13]. Based on clinical observations, it has been suggested to set the therapeutic index as plasma mid-dose concentrations of 1–4 mg/l (in treatment-naïve patients) [14–17]. It should be noted that while the link between plasma PK and CNS toxicity is rather well established, the same does not hold true for a PK/efficacy relationship.

EFV is primarily metabolized by *CYP2B6*, and to a lesser extent by *CYP3A4*, *CYP3A5*, *CYP1A2* and *CYP2A6* [18,19]. Its main metabolite, 8-hydroxy-EFV, can be further oxidized by

CYP2B6 into 8,14-hydroxy-EFV, while the formation of the 7-hydroxy metabolite is catalyzed by *CYP2A6* only. Formation of sulfate and glucuronide conjugates has also been described [20]. Of note is the ability of EFV to induce its own metabolism.

A wealth of evidence is available regarding the effect of the *CYP2B6**9 variant. This polymorphism leads to reduced metabolism and increased EFV plasma concentrations [21–67]. Some studies have also suggested that this genotype and the resulting increased exposure could be correlated to a higher incidence of CNS toxicity [21–25]. The effect of the genotype on exposure seems to be proportional to the number of variant alleles, being stronger for TT homozygotes than for GT heterozygotes.

Another commonly studied SNP that can result in increased plasma exposure is *CYP2B6**18 [23,29–31,33,51–62,68,69], although a few studies have failed to replicate this association [34,36,41].

While the magnitude of the effect is much smaller than for the previous polymorphisms, variant g.15582C>T could also play a role [29,36,54,64]. It is usually not a major predictor of exposure when other SNPs are already taken into account; however, it can aggravate a metabolic deficit.

Finally, even though it has been linked with lower levels of enzyme in the liver, variant *CYP2B6**5 does not appear to affect EFV PK [23,27,30,61,70].

Results from studies on the impact of any of these alleles on the probability of treatment success reveal a complex situation: the *CYP2B6* slow metabolizer phenotype could compensate for intermittent non-compliance by keeping plasma concentrations high, but the accumulation of the drug could also lead to an increased incidence of side effects (most notably CNS symptoms) and more treatment discontinuations – at least in the early months, while late treatment failure would be less likely in those individuals. This could explain why some studies have found a relationship between *CYP2B6* genotype and treatment end points, while others have not. The results could also differ due to the use of different definitions for treatment success/failure (such as different viral load thresholds), different follow-up periods and time frames (early initiation in naïve patients versus established therapy), etc. Turning to cerebrospinal fluid (CSF) concentrations, the slow metabolizer phenotype can lead to high CSF concentrations, but also a low rate of conversion to phase I metabolites (which could participate to the overall toxicity of the drug) – hence, paradoxically, slow and fast metabolizer status both could be risk factors for CNS toxicity.

Similarly, non-CNS side effects could be more frequent in some individuals. For instance, a study in healthy volunteers showed that repeated administration of EFV significantly lengthened the QT interval in *CYP2B6**6 homozygotes but not in heterozygous or wild-type homozygous subjects [61]. An increased risk of hepatotoxicity has also been found in *CYP2B6**9/*9 individuals [71].

Fewer studies are available for *CYP3A* variants. Neither the presence of the *CYP3A4* loss-of-function alleles *CYP3A4**1B [27,41,54,67,72] or *CYP3A4**22 [64], nor enzymatic activity (as assessed by 1'-hydroxymidazolam formation after administration of the parent drug) [72] seems to be significantly correlated with EFV exposure. Regarding *CYP3A5*, most studies have reported no association at all between exposure and the *CYP3A5**3

[25,27,29,34,36,41,54,60,61,67,68,71–73] or the *CYP3A5**6 and *CYP3A5**7 alleles [34,36,51,67,68,71]. Only one study described increased plasma concentrations of EFV in *CYP3A4**1B carriers and decreased concentrations in *CYP3A5**3 carriers (a rather paradoxical observation, which has never been replicated) [21]. Overall, the impact of *CYP3A* polymorphisms on EFV PK appears to be quite small, possibly because these isoforms are only involved in secondary metabolic pathways.

The same holds true for *CYP2A6*, for which polymorphisms (most often *CYP2A6**9B and *CYP2A6**17) have a negligible impact (or none at all) on EFV PK [31,34,36,38,41,43,51,54,55,58,61,67,68] or viral response [74]. However, in some instances, the composite genotype *CYP2A6**9/*17B has been found to be predictive of EFV PK, even when *CYP2B6* metabolizer status was already taken into account [52,64]. Information regarding the *CYP2A6* genotype of an individual may be of limited use in subjects with functional *CYP2B6*, whereas it could help further refine predictions in *CYP2B6* slow metabolizers.

To account for these numerous sources of genetic variability, some authors have proposed the use of multiple or composite genotypes (or haplotypes), most often involving *CYP2B6*. These would be weighted according to their individual relevance to EFV metabolism, for example, in population PK models involving multiple SNPs [60,75] or using risk scores based on the number of CYP decrease- or loss-of-function alleles to predict the probability of treatment success or discontinuation [76,77]. *CYP2B6* slow metabolizer status has also been shown to be protective against viral failure [78], while the risk of suicidality increased with the number of *CYP2B6*- and *CYP2A6*-variant alleles (a significant hazard ratio was found in Whites and Hispanics, but not Blacks) [79].

Based on these observations, *CYP2B6* genotype-based dose recommendations are conceivable. In a study conducted in Japanese patients, those with plasma concentrations above 6 mg/l and who harbored the *CYP2B6**6 or *26 haplotypes (the latter being a combination of the *CYP2B6**6 haplotype and c.499C>G) were assigned to a dose reduction protocol [80]. The dose was safely reduced from the standard, once-daily 600 mg to 400 mg or 200 mg while maintaining viral suppression. Therapy was then initiated at lower doses for a group of treatment-naïve patients possessing those alleles with similar results. An improvement of CNS symptoms was also noted, although the design of this study did not allow the authors to exclude the possibility of a placebo effect.

Although its protocol was not to adjust the dose based on genotype, but rather on plasma concentrations and the occurrence of CNS toxicity, yet another study demonstrated the importance of considering the c.516G>T polymorphism [81]. Most subjects whose dose was lowered to 400 mg possessed at least one variant allele, and all subjects with a 200 mg dose were TT homozygotes. This dose reduction greatly improved CNS symptoms and allowed for substantial cost savings.

Unfortunately, the design of some of these studies makes it difficult to conclude whether the resolution of symptoms was truly a result of dose adjustments. Regardless, it is important to reiterate that the toxicity of EFV, which has caused it to fall out of favor as a first-line treatment option in some countries, could be mitigated by using dose reduction approaches possibly guided by pharmacogenetics.

Last but not least, a cost-effectiveness analysis compared three different strategies: standard EFV therapy, universal dose reduction to 400 mg (as seen in the ENCORE1 trial) or genotype-based dosing [82]. The latter was found to be cost-effective. In contrast, based on the results from the pharmacogenetic subsection of the ENCORE1 trial, the authors concluded that while a dose of 400 mg was safe and could help reduce toxicity, genotype-guided dosing would be of limited use [64]. It could be argued that a universal dose reduction would be a more effective approach – at least in some population types (depending on the frequency of the various SNPs of interest). Caution should still be exercised; for instance, PK simulations using data from pregnant women showed a higher risk of suboptimal exposure or toxicity if the dose reduction was not guided by *CYP2B6* genotyping [83].

Thus, it appears that knowledge of *CYP2B6* genotype could prove useful in individualizing EFV therapy (Table 2). In particular, the role of *CYP2B6**9 has been demonstrated by numerous studies, including genome-wide association studies (GWAS). With loss-of-function alleles being more frequent in Africans compared to Caucasians, there is a need for population-specific guidelines (for both western and developing countries). However, the PK/PD and genotype/PD relationships of EFV have yet to be fully elucidated.

2.2. Nevirapine

NVP is the oldest NNRTI, and is still widely used worldwide. Side effects include potentially life-threatening skin reactions (Stevens–Johnson syndrome and toxic epidermal necrolysis) and hepatotoxicity. To reduce the risk of these side effects, it is recommended to proceed as follows: after a two-week run-in phase of 200 mg once-daily, the dosage can be increased to 200 mg twice-daily (or the patient may start taking NVP extended release 400 mg once-daily). The risk of hepatotoxicity is also higher when NVP is initiated in antiretroviral-naïve women and men with CD4 counts <250 cells/μl or <400 cells/μl, respectively. Therefore, NVP-based therapy is contraindicated in these patients [84,85]. It has been proposed to set the minimal effective plasma concentration of NVP to 3 mg/l (no clear upper boundary is currently defined) [86,87].

Like EFV, NVP is a substrate of *CYP2B6*, and much research has focused on the same SNPs for both drugs. In addition to *CYP2B6*, *CYP3A4* plays some role in the metabolism of NVP [88,89], as do *CYP3A5* and *CYP2C9* isoforms to a lesser extent. NVP is also an inducer of its own metabolism.

*CYP2B6**9 has been shown to increase the plasma levels of NVP in most [22,30,90–100], but not all studies [61,101]. The effect is usually apparent in *CYP2B6**9 homozygotes only.

Similarly, some studies have reported an association between allele *CYP2B6**18 and exposure [30,90,92–96,100], while others have not [61,91]. Interestingly, NVP exposure could sometimes be predicted using a combination of variants (*CYP2B6**6 homozygotes or *6/*18 heterozygotes versus other genotypes) that were not significant in univariate analysis [61].

Variant *CYP2B6**4 was not significant in any of the studies conducted so far [61,101,102], except for one, where a weak association was detected, albeit in univariate analysis only [90].

Table 2. Relevance of CYP polymorphisms to non-nucleoside reverse transcriptase inhibitor (NNRTI) therapy.

Allele	rs number	Nucleotide change	Clinical outcome	Level of evidence
CYP2A6*9	rs28399433	c.-47A>C	No effect on EFV PK	Low
CYP2A6*9B	rs8192726	g.1836G>T	No effect on EFV PK	Low
CYP2A6*17	rs28399454	c.1093G>A	Increased exposure to EFV	Low
			No effect on NVP PK	Low
CYP2B6*4	rs2279343	c.785A>G	Increased exposure to EFV	Low
			No effect on NVP PK	Low
CYP2B6*5	rs3211371	c.1459C>T	No effect on EFV or NVP PK	Low
CYP2B6*6		c.516G>T, c.785A>G	(cfr. CYP2B6*9 and CYP2B6*4)	Low
CYP2B6*9	rs3745274	c.516G>T	Increased exposure to EFV and NVP	High
			Increased risk of EFV CNS symptoms, fewer viral failures	Medium
			Increased risk of NVP cutaneous toxicity	Low
CYP2B6*15	rs4803419	c.1172T>A	Increased exposure to EFV	Medium
			No effect on NVP PK	Low
CYP2B6*18	rs28399499	c.983T>C	Increased exposure to EFV and NVP	High
			Increased risk of EFV CNS symptoms, fewer viral failures	Medium
			Increased risk of NVP cutaneous toxicity	Low
CYP2C19*2	rs4244285	c.681G>A	No effect on RPV PK	Low
CYP2C19*17	rs12248560	c.-806C>T	No effect on RPV PK	Low
CYP3A4*1B	rs2740574	c.-392A>G	No effect on EFV or NVP PK	Low
CYP3A4*22	rs35599367	g.20493C>T	No effect on EFV, NVP or RPV PK	Low
CYP3A5*3	rs776746	g.6986A>G	No effect on EFV or NVP PK	Medium
			No effect on RPV PK	Low
CYP3A5*6	rs10264272	g.14690G>A	No effect on EFV PK	Low
CYP3A5*7	rs76293380	g.27131_27132insT	No effect on EFV PK	Low

Level of evidence (low/medium/high) was assigned based on the amount and quality of available evidence. CNS: central nervous system; EFV: efavirenz; NVP: nevirapine; PK: pharmacokinetics; RPV: rilpivirine.

Variant *CYP2B6*5* has not been found to be predictive of NVP PK either [30,61,91,101,102].

Moving on to CYP3A, neither *CYP3A4*1B* [91,97,102], nor *CYP3A4*22* [96], nor *CYP3A5*3* [61,91,96,101,102] seems to be associated with NVP PK.

Toxicogenetic studies of NVP have mostly investigated SNPs related to the immune system, but also occasionally to phase I metabolism. A GWAS in individuals of various ethnicities found *CYP2B6*9* (as well as two non-coding SNPs in linkage disequilibrium with it) to be a significant predictor of adverse cutaneous reactions [103]. This finding was replicated in a candidate gene study in Caucasians [101], while another one in Africans was only able to demonstrate a trend toward association [104], and several others could not replicate these results. Other SNPs, including *CYP2B6*4* [105], *CYP2B6*5* [105], as well as *CYP3A4*1B* [104,105], *CYP3A5*3* [104,105] and *CYP3A5*6* [105], do not appear to be relevant to cutaneous toxicity. *CYP2B6*18*, however, was strongly associated with the risk of such side effects [104,106]. Unfortunately, the sensitivity and specificity of these markers may not be sufficient for a clinical application. As for hepatotoxicity, neither of the available GWAS has revealed any association with CYP polymorphisms [103,105].

The increased exposure in *CYP2B6* slow metabolizers (defined based on *CYP2B6*9* and *CYP2B6*18*) can also protect against viral failure in patients receiving standard doses [78]. Simulations based on population PK models have shown that an off-label dose of 400 mg once-daily (immediate release), rather than the recommended 200 mg twice-daily, could reduce NVP exposure to unacceptable levels [92,97]. However, safe exposure can be maintained in *CYP2B6* slow metabolizers with 400 mg once-daily, meaning genotyping for *CYP2B6*9* and *CYP2B6*18* could help in determining an appropriate dosing schedule (with the once-daily schedule potentially improving patient compliance). Other simulations suggest that standard dosing may actually result in subtherapeutic concentrations in a small proportion of patients with functional *CYP2B6* [90].

NVP is a good candidate for pharmacogenetics-guided therapy (Table 2). However, perhaps due to a lack of conclusive data, the available knowledge has not yet matured into any therapeutic recommendations. Initiating treatment only in subjects with normal *CYP2B6* metabolism as well as using reduced doses or once-daily dosing schedules in individuals harboring *CYP2B6* defective alleles could potentially improve treatment outcomes.

2.3. Rilpivirine

RPV is the latest NNRTI. It is a substrate of CYP3A4 and to a lesser extent of CYP2C19.

Only one pharmacogenetic study is currently available. None of the following SNPs were associated with drug clearance: *CYP2C19*2*, *CYP2C19*17*, *CYP3A4*22* or *CYP3A5*3* [107] (Table 2).

Further investigations are needed to assess the pharmacogenetics of RPV in Asian and African populations, as well as the impact of other SNPs.

2.4. Ritonavir, cobicistat

Several antiretrovirals, especially among PIs, are coadministered with a PK booster in order to increase exposure to the active molecule. The booster can be either ritonavir (RTV) (a PI with no activity at the dose used for boosting) or cobicistat (COB) (a structural derivative of RTV that is completely inactive against HIV). Both boosters are strong inhibitors of CYP3A4 and CYP3A5. RTV can also induce other CYP isoforms, such as CYP1A2, CYP2C9 and CYP2C19.

Pharmacogenetic studies have focused on the active antiretrovirals rather than the boosters, but it is important to consider the possibility of an indirect effect of SNPs on antiretroviral PK through RTV or COB PK. Additionally, boosters complicate the interpretation of results by introducing a powerful DDI (see further).

2.5. Atazanavir

ATV is a PI administered in a standard dose of 300 mg once-daily in combination with either 100 mg RTV or 150 mg COB. Unboosted ATV may also be used at 400 mg once-daily in stable patients. Drug-specific side effects include icterus, sometimes hepatitis or cholelithiasis, and rarely nephrolithiasis; while lipodystrophy is considered a class effect for PIs.

ATV is metabolized by CYP3A4 and CYP3A5, as well as UDP-glucuronosyl-transferases (UGTs); a small percentage is also excreted unchanged in the urine. It is an inhibitor of CYP3A4, though less so than RTV or COB.

Pharmacogenetic studies have investigated the effect of CYP decrease-of-function alleles on ATV PK, often in conjunction with SNPs in phase II enzymes and/or transport proteins. CYP3A5 non-expressor status (defined as homozygosity for loss-of-function alleles *CYP3A5*3*, *CYP3A5*6* or *CYP3A5*7*) was predictive of slower clearance in healthy volunteers receiving unboosted ATV, while this effect was no longer observed in all subjects in the RTV-boosting phase of the study [108].

This highlights an important (and sometimes unavoidable) drug interaction for PIs boosted with RTV or COB: inhibition of CYP could produce a slow metabolizer phenotype, regardless of the actual CYP genotype (a hypothesis that also applies to other boosted PIs, e.g. DRV and LPV).

In HIV patients, *CYP3A5*3* was associated with exposure in subjects receiving boosted ATV [109], but not in those receiving the unboosted drug [110]. The latter study also found a lower rate of virological failure in *CYP3A5* expressors, adding to the confusion.

The role of *CYP3A4*1B* is even less clear. One study found an association with higher drug exposure [108], while another could not link the genotype to either plasma or intracellular concentrations [111].

Lastly, a GWAS failed to detect any significant association between ATV exposure and polymorphisms in phase I drug metabolism pathways, casting doubt on the relevance of previous observations [112].

Thus, SNPs in CYP may not play a major role in ATV PK (Table 3). SNPs in UGT may be more relevant to individualization of therapy, as evidenced by guidelines produced by the Clinical Pharmacogenetics Implementation Consortium [113], although this is out of the scope of this review.

2.6. Darunavir

DRV is the most recently approved – and one the most frequently used – PI. Like several other drugs of this class, it is combined with a PK booster. The standard regimen is 800 mg DRV + 100 mg RTV (or 150 mg COB) once-daily, while 600 mg DRV + 100 mg RTV twice-daily is recommended in treatment-experienced patients. DRV has the advantage of being less toxic than some of the older PIs, although hepatotoxicity remains a potential issue.

DRV is a substrate of CYP3A4, with all of its metabolites exhibiting low activity [114,115].

In a population PK study of 60-predominantly Caucasian patients, none of the studied SNPs (*CYP3A5*3*, *CYP3A7* variants rs10211 and rs2257401; *CYP3A4*1B* and rare variants rs4987161 and rs4646437) were associated with DRV clearance [116].

Table 3. Relevance of CYP polymorphisms to protease inhibitor (PI) therapy.

Allele	rs number	Nucleotide change	Clinical outcome	Level of evidence
<i>CYP3A4*1B</i>	rs2740574	c.-392A>G	No effect on ATV, DRV, or LPV PK	Low
<i>CYP3A4*22</i>	rs35599367	g.20493C>T	Increased exposure to LPV	Low
<i>CYP3A5*3</i>	rs776746	g.6986A>G	No effect on LPV PK Increased exposure to DRV No effect on ATV PK	Medium Low Low
<i>CYP3A5*6</i>	rs10264272	g.14690G>A	No effect on ATV or LPV PK	Low
<i>CYP3A5*7</i>	rs76293380	g.27131_27132insT	No effect on ATV PK	Low

Level of evidence (low/medium/high) was assigned based on the amount and quality of available evidence. ATV: atazanavir; CNS: central nervous system; DRV: darunavir; LPV: lopinavir; PK: pharmacokinetics.

Meanwhile, a study conducted in 135 African and Caucasian patients demonstrated lower drug exposure for subjects harboring the variant *CYP3A5*1*, but only in patients receiving efavirine, a known CYP inducer, while no effect was discernible at the population level [117]. This highlights the importance of taking DDI into consideration, including for RTV- or COB-boosted antiretrovirals.

There is currently not enough data to make any genotype-based dose recommendations for DRV (Table 3). Studies of additional SNPs and of ethnically heterogeneous populations are required.

2.7. Lopinavir

LPV is a PI administered at 400 mg + 100 mg of RTV twice-daily. Drug-specific side effects include QT interval prolongation and an increased risk of myocardial infarction. It is metabolized by – and can inhibit – CYP3A isoforms.

The largest study to date investigated over 1500 polymorphisms and 115 genes in a Caucasian population [118]. LPV clearance was reduced by 18% in subjects harboring the variant rs6945984, an intergenic tag SNP in linkage disequilibrium with *CYP3A5*3* and *CYP3A7*2* (a gain-of-function allele). Oddly, the related functional SNPs were not significant at the chosen threshold. Also, another large-scale study could not demonstrate an association between rs6945984 and the risk of treatment discontinuation [76].

Studies of smaller scale were no more successful at demonstrating a clear relationship between LPV PK/PD and SNPs such as *CYP3A4*1B* [119,120], *CYP3A5*3* [119–123] and *CYP3A5*6* [119–121]. One study found that homozygosity for *CYP3A4*22* was associated with a major reduction in clearance [124]. The fact that *CYP3A4*22* homozygotes are quite rare could explain why this is an isolated finding.

As with other boosted PIs, CYP3A polymorphisms may play a role in the PK/PD variability of LPV, with RTV mitigating this effect, but there is currently too little evidence to make any recommendations (Table 3).

3. Conclusion

Several antiretroviral drugs are substrates of CYP enzymes, for which functional genetic polymorphisms exist. These polymorphisms can alter the PK of antiretrovirals, with an impact

on treatment efficacy, toxicity and potential for DDI. We summarized the main pharmacogenetic associations described in the literature and how they could shape therapeutic guidelines. In most cases, pharmacogenetic information has not yet led to specific dose recommendations.

4. Expert opinion

In order for theoretical knowledge to be of practical use, several hurdles must be overcome. First, this knowledge must be well established and rigorously validated, so that it can be trusted. Second, the individuals who can make use of it must be sensitized to the issue. Third, the knowledge must be incorporated in a framework that enables its use by the interested individuals. As far as pharmacogenetic associations in ART are concerned, all of these hurdles have yet to be fully overcome, although much progress has been made.

A good example of pharmacogenetic testing for antiretrovirals is the guidelines for the use of abacavir, which requires prior screening for HLA-B*5701. Patients carrying this allele are at an increased risk of presenting major hypersensitivity reactions; despite the relatively low occurrence of such reactions (5–8%), genotyping before treatment initiation has been shown to be cost-effective in Caucasian and Hispanic populations [125] and is now mentioned in the package insert [126]. However, such examples are few and far between, especially for CYP. One should remember that strong, monogenic associations are the easiest case to study. The reality is much more complex, and taking into account multiple loci and multiple SNPs may be required to understand how the components of an individual's genetic background influence each other, as one SNP may mitigate or potentiate another.

Another complication arises from DDI. Indeed, polypharmacy is the norm rather than the exception for HIV patients, both because of the need for multiple antiretrovirals and because of comorbidities. Examples of comedications with high CYP DDI potential that HIV patients may receive include tuberculosis therapy with rifampicin (a well-known CYP inducer), or CYP inhibitors such as azole antifungals (e.g. voriconazole and itraconazole) or Hepatitis C virus PIs (e.g. simeprevir and boceprevir). As we have mentioned in this review, many antiretrovirals also happen to be CYP inducers or inhibitors themselves, or are coadministered with PK boosters. Adding or removing the perpetrator drug could lead to unexpected results in patients whose true phenotype was previously hidden due to the DDI. Possible differences between RTV and new boosters like COB should also be investigated.

While various genetic polymorphisms have been described for CYP, associations for a given SNP–drug pair are not always consistent. These discrepancies can be explained by small study sample sizes and lack of representation of some ethnicities (precluding recruitment of a sufficient number of individuals carrying rare alleles), inappropriate study design (purely retrospective study, too lax or too restrictive inclusion/exclusion criteria, and low-quality PK data) and statistical analysis (candidate gene studies with no correction for multiple comparisons), different outcome definitions, or simply more stringent methodology for establishing an association. It should also be mentioned that some studies overlook possible gene–gene interactions or epigenetics, choosing to

focus solely on a handful of SNPs. Validation of available results on large cohorts would help consolidate our knowledge of how these SNPs can impact drug therapy. In addition to replication studies, experiments designed to acquire mechanistic insight into these polymorphisms and how they shape drug PK would be beneficial. The contribution of some metabolites of EFV (or NVP) to their toxicity also highlights the need to consider more than just the parent drug when it comes to pharmacogenetic associations. Finally, another area requiring greater attention is whether the impact of CYP polymorphisms on drug PK is also relevant to the long-term efficacy and safety, e.g. viral suppression and immune function recovery, emergence of resistance and biomarkers of HIV morbidity. Stronger levels of evidence will result in official guidelines or recommendations (in the form of drug label updates or as part of clinical decision support systems).

These recommendations will then have to be implemented in routine practice: a robust and cost-effective framework must be established so that pharmacogenetic information can be of use. Regarding the cost, genotyping should be compared to other strategies, such as TDM (although obviously, they can be seen as complementary). For instance, in resource-limited settings, TDM alone may be a cheaper alternative. This could change in the future as genotyping becomes more widely available and affordable. Pharmacoeconomic studies and simulations of multiple scenarios are required to weigh the benefits of the various treatments available and set strategies adapted to the needs and resources of each country (as well as the ethnic composition of the local population, which determines the allelic frequencies of various polymorphisms).

Unfortunately, a drug of interest and its corresponding CYP do not exist together in a vacuum; they are part of a larger system, composed of multiple CYP enzymes, other enzymes, influx and efflux transporters, nuclear receptors regulating biotransformation and transport of xenobiotics, PD targets (including viral genome and immune response pathways), comedications and various patient-specific characteristics, not to mention patient-, physician- and setting-related limitations (economical and practical). Simplistic recommendations such as those seen with weight-based dosing are no longer doable when one enters the realm of the so-called 'precision-based medicine' that pharmacogenetics can contribute to. Advanced mathematical models are necessary to handle this abundance of information. New methodologies (for example, population PK) will help in this regard. The development of more user-friendly tools, their incorporation in the clinical routine workflow, and the appropriate training of clinicians in the use of the software should be our next priorities.

One last caveat of genotype-tailored therapy is that appropriate pharmaceutical forms must be available; yet the increasing popularity of fixed-dose combinations for the treatment of HIV might limit opportunities for individualizing therapy.

In summary, pharmacogenetic studies have shed some light on the role of genetic polymorphisms in ART, but this knowledge must now be incorporated in decision-making systems. There is still much to be done if the field of pharmacogenomics is to see more than marginal use.

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Annex 4. Previously published population PK models for darunavir

	Vis et al 2006 [188]	Sekar et al 2008 [78]	Ter Heine et al 2009 [68]	Kakuda et al 2012 [189]	Moltó et al 2013 [142]	Arab-Alameddine et al 2014 [143]
n	580	335	11	376	75	105
Sampling	Rich/sparse (5082 observations)	Sparse (~2680 observations)	Rich (~121 observations)	Rich/sparse (~3000 observations)	Rich/sparse (705 observations)	Sparse (289 observations)
Disposition compartments	2		1		2	1
Absorption	First order		2 transition compartments		First order	First order
CL (l.h ⁻¹)	41.9		21.3		10.7	10.9
Q (l.h ⁻¹)	15				21.3	
V or V _c (l)	122		220		36.9	121.2
V _p (l)	84.3				1130	
K _a (h ⁻¹)	0.455				0.3	1.04
Tlag (h)						
MAT (h)			0.965			
f _u (%)						
AUC (ng.h.ml ⁻¹)		87854 (AUC ₂₄)		60642 (AUC ₁₂)		
C ₀ (mg.l ⁻¹)		2.041		3.624		
Covariates	• AAG on CL_{DRV} • Weight, Cr CL and sex statistically but not clinically significant	• AAG on CL_{DRV}		• Sex on AUC/C_{0 DRV} • Age on AUC/C_{0 DRV}	• AAG on V_{DRV} • Weight on CL_{RTV} • SLCO3A1 SNPs on CL/V_{DRV}	• Weight on CL_{RTV} • AUC_{RTV} on CL_{DRV}
Notes	• Dose range: 400-800 mg bid and 400-1200 mg q24h. • Comparison of formulations.	Bayesian estimation using the model from Vis et al 2006.	PBMC accumulation ratio: 1.32.	Bayesian estimation using the model from Vis et al 2006.		

	Brochot et al 2015 [39]	Dickinson et al 2015 [190]	Lê et al 2018 [71]	Daskapan et al 2018 [191]	Schalkwijk et al 2019 [192]	Dickinson et al 2019 [146]
n	102	51	15	198	85	716
Sampling	Rich (659 observations)	Rich (~500 observations)	Rich (~180 observations)	Sparse (198 observations)	Rich (2265 observations)	Sparse (1317 observations)
Disposition compartments		2	2	1	2	2
Absorption		First order, lagged	First order, lagged	First order	1 transition compartment	First order
CL (l.h⁻¹)	51.0	14.8	12.7	9.5	130 ■	14.6
Q (l.h⁻¹)	19.1	21.9	24.1		13 ■	30.4
V or V_c (l)	137	128	97.5	2.1 ■	8.3 ■	41.4
V_p (l)	254	420	609		134 ■	1130
K_a (h⁻¹)	0.528	1.01	1	1.04 ■		0.3
Lag (h)		0.53	0.42			
MAT (h)					4.3	
f_u (%)			7.6			
AUC (ng.h.ml⁻¹)			65563 (predicted AUC ₂₄)			55480-57420 ■ (predicted AUC ₂₄)
C₀ (mg.l⁻¹)						1.68-1.75 ■ (predicted)
Covariates		• Weight on V/CL_{DRV} • AUC_{RTV} on CL_{DRV} • Rifampicin on CL_{DRV}			• Linking CL_{DRV} and CL_{RTV} • AAG used to derive protein binding parameters.	
Notes	• Pooled analysis of data from DUET1/2, DELPHI, DIONE and ARIEL (72 children, 30 adults). • Using model from Vis et al 2006. • Simulate exposure from a variety of once-daily regimens in children aged 6-12.		• Dose reduction study, PK parameters are pre-reduction. • Measured total and free concentrations in blood and seminal plasma.	• Two models presented in article. Final model chosen by authors shown here. • F fixed to 0.82.	• Comparison of pregnant and post-partum women. • Measured total and free concentrations (in some subjects only). • Simultaneous DRV/RTV model.	• Simultaneous modeling of DRV, RTV, TFV and FTC. Fixed interaction model between DRV/RTV.

AAG: Alpha-1-acid glycoprotein, MAT: Mean absorption time, n: Number of subjects, Lag: Lag time.

Volumes of distribution and clearances are apparent parameters, unless noted otherwise. All parameters reported for model with no covariates, unless noted otherwise.

- Per unit of lean body weight.
- Fixed to the value calculated in [143].
- Parameters given for a 70 kg individual and using covariate relationships.
- Values for NRTI-sparing arm versus standard triple ART.