Year : 2010

Pharmacogenetics of antiviral treatment

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UNIL | Université de Lausanne
Faculté de biologie et de médecine

Institut de Microbiologie (IMUL)

Pharmacogenetics of Antiviral Treatment

THÈSE DE DOCTORAT ÈS SCIENCES DE LA VIE (PhD)

Présentée à la Faculté de Biologie et de Médecine de l'Université de Lausanne

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LAUSANNE

2010

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Ecole Doctorale

Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président	Monsieur	Prof.	François	**Spertini**
Directeur de thèse	Monsieur	Prof.	Amalio	**Telenti**
Experts	Monsieur	Prof.	Ulrich M.	**Zanger**
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le Conseil de Faculté autorise l'impression de la thèse de

Madame Julia Di Iulio

pharmacienne diplômée de la Confédération Helvétique

intitulée

Pharmacogenetics of Antiviral Treatment

Lausanne, le 10 décembre 2010

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. François Spertini

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Summary

In this thesis work the relevance of pharmacogenetics of antiviral treatment has been assessed by investigating, through three different approaches, the impact of host genetic variation on antiretroviral drug disposition (namely efavirenz and lopinavir) and on natural or treatment-induced clearance of hepatitis C virus.

The influence of host genetic variation on **efavirenz** and its primary metabolite plasma levels was assessed by **single candidate gene** approach, through comprehensive analysis of cytochrome P450 (CYP) 2A6 – involved in efavirenz accessory metabolic pathway. The study could demonstrate that *CYP2A6* genotype became increasingly relevant in the setting of limited CYP2B6 function – involved in efavirenz main metabolic pathway – and that individuals with both main and accessory metabolic pathways impaired were at higher risk for treatment discontinuation, overall emphasizing the predictive power of genotyping.

The influence of host genetic variation on **lopinavir** clearance was assessed by **large scale candidate gene** approach, through analysis of genes involved in the absorption, distribution, metabolism and elimination. The study identified four genetic variants in drug transporters and metabolizing enzymes that explained 5% of the interindividual variability in lopinavir clearance.

The influence of host genetic variation on **hepatitis C virus** (HCV) natural or treatment-induced clearance was assessed through **genome-wide association study** approach. This study identified an intergenic polymorphism, part of a linkage disequilibrium block encompassing the interferon-λ3 gene, as highly associated with treatment-induced and spontaneous HCV clearance. Resequencing and recombinant mapping lead to the identification of four potentially causal genetic variants. Finally, we could assess the net contribution of genetic variants in interferon-λ3 to clearance by controlling for viral diversity, gender and co-infection status in a single source infected cohort.

This thesis highlights the various genetic tools available to pharmacogenetic discovery (candidate gene, pathway or and genome-wide approaches), and the importance of resequencing for mapping of causal variants.

Résumé

Dans ce travail de thèse, l'importance de la pharmacogénétique des traitements antiviraux a été évaluée en déterminant, au moyen de trois différentes approches, l'impact de variations génétiques sur la pharmacocinétique de deux traitements antirétroviraux (à savoir l'efavirenz et le lopinavir) ainsi que sur la capacité de pouvoir éliminer le virus de l'hépatite C de façon naturelle ou suite à un traitement médicamenteux.

L'influence des variations génétiques sur les taux plasmatiques de **l'efavirenz** et de ses métabolites primaires a été évaluée par l'analyse d'**un seul gène candidat** : le cytochrome P450 (CYP) 2A6, impliqué dans une voie métabolique accessoire de l'efavirenz. Cette étude a permis de démontrer que le génotype du *CYP2A6* devient cliniquement déterminant en l'absence de fonction du CYP2B6, impliqué dans la voie métabolique principale, et que la perte simultanée des voies métaboliques principales et accessoires entraine une augmentation du risque d'interruption du traitement, soulignant la valeur prédictive du génotypage.

L'influence de la génétique sur la clairance du **lopinavir** a été évaluée par l'analyse **à grande échelle de gènes candidats**, à savoir les gènes potentiellement impliqués dans l'absorption, le métabolisme, la distribution et l'élimination d'un médicament. Cette étude a permis l'identification de 4 polymorphismes, dans des transporteurs et des enzymes métaboliques, associés à la clairance du lopinavir et expliquant 5% de la variabilité inter-individuelle de ce phénotype.

L'influence de la génétique sur la capacité d'éliminer le virus de **l'hépatite C**, de façon naturelle ou à la suite d'un traitement, a été évaluée par l'analyse du **génome entier**. Cette étude a permis l'identification d'un polymorphisme situé à proximité de l'interféron-λ3. Quatre variations génétiques potentiellement causales ont ensuite pu être identifiées par reséquencage. Finalement, la contribution nette de ce gène sur l'élimination du virus a pu être évaluée dans une cohorte infectée par une seule et même source, permettant ainsi de contrôler l'effet de la diversité virale, du genre et de la présence de co-infections.

Cette thèse a permis de mettre en évidence les diverses méthodes disponibles pour la recherche en pharmacogénétique, ainsi que l'importance du reséquencage pour l'identification de variations génétiques causales.

Abbreviations

ABC	ATP binding cassette transporter
ADME	Absorption-distribution-metabolism-elimination
AIDS	Acquired immune deficiency syndrome
ART	Antiretroviral therapy
bp	base pair
CNS	Central nervous system
CNV	Copy number variation
CYP	Cytochrome P450
DME	Drug metabolizing enzyme
DNA	Deoxyribonucleic acid
DOF	Decrease-of-function allele
DT	Drug transporter
EFV	Efavirenz
FDA	Food and Drug Administration
Gln	Glucuronide
GOF	Gain-of-function allele
GWAS	Genome wide association study
HAART	Highly active antiretroviral therapy
HCV	Hepatitis C virus
Het	Heterozygous
HIV	Human immunodeficiency virus
Hom	Homozygous
IFN	Interferon
IL28B	Interleukin 28B/Interferon-λ3
ISG	Interferon-stimulated gene
LD	Linkage disequilibrium
LOF	Loss-of-function allele
LPV(/r)	Lopinavir(/ritonavir)
MAF	Minor allelic frequency
N(t)RTI	Nucleoside (nucleotide) reverse transcriptase inhibitor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
OATP	Organic anion transporting polypeptide
OH	Hydroxy
P-gp	P-glycoprotein
PI	Protease inhibitor
PK	Pharmacokinetics
PD	Pharmacodynamics
QD	Quaquie die (Latin)
RNA	Ribonucleic acid
RTV	Ritonavir
SCCS	Swiss HCV Cohort Study
SHCS	Swiss HIV Cohort Study
SLC(O)	Solute carrier (organic anion) transporter
SNP	Single nucleotide polymorphism
TDM	Therapeutic drug monitoring
UGT	UDP-glucuronosyltransferase

Acknowledgements

This thesis would not have been feasible without the help of many people. First, I would like to thank my scientific mentor and thesis director, Prof. Amalio Telenti, for giving me the opportunity to work in his laboratory. He supported me throughout my whole thesis and his dedication to research was a permanent stimulation and motivation for me.

I would also like to thank all members of the jury committee for kindly accepting to be part of this thesis.

Many thanks to all the people I had the opportunity to collaborate with, and especially Dr Andri Rauch and Dr Laurent Decosterd for their advice and encouragements during these years.

I am also very grateful to all my colleagues and friends from the Institute of Microbiology (with a special thank to Dr Margalida Rotger and Dr Angela Ciuffi for their daily “coaching”) for their help and the great moments we have shared, making of these years an unforgettable scientific and social experience.

Finally, I would like to thank my family and all my friends for their constant support.

1. GENERAL INTRODUCTION

1.1. Genetics

The genetic code is part of a multifactorial interplay making of each individual a unique entity with specific phenotypes. Genetics has generated much interest for years, but has particularly exploded this last decades with the completion of the Human Genome Project (http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml [101]), the International HapMap Project (http://www.hapmap.org/ [102]) and the 1000 Genomes Project (http://www.1000genomes.org/page.php [103]). These databases together with the availability of new high throughput genotyping and sequencing technologies have made genetic analyses much more accessible.

The human genome is made of approximately 3 billion base pairs (bp) [1]. While ~99.9% of the DNA sequence is shared by any two unrelated individuals, the remaining 0.1%, or ~$3*10^6$ bp, is not [2, 3]. DNA sequence differences involve single nucleotide polymorphisms (SNPs), nucleotide insertions, deletions and inversions, as well as copy number variations (CNVs) and variable number of tandem repeats [2, 3]. These genetic variations may affect the DNA secondary structure, the mRNA transcription, stability and splicing, the amino acid sequence, etc., overall potentially altering the expression or the function of the encoded protein at the cellular level. At the organism level, there are two main issues where phenotypic traits might be influenced by genetic variants: the first being native phenotypic traits including characteristics such as blood type and eye color, and diseases or disorders such as hemophilia and albinism; and the second being phenotypic traits displayed in response to exogenous influences such as drugs, pathogens or food.

Understanding how genetic variations might influence the latter phenotypes, and more especially response to treatment (called “pharmacogenetics” by Friedrich Vogel since 1959) and disease susceptibility, is of great interest given the prospects of providing individualized drug therapy and personalized medicine for preventing or treating complex diseases [4].

For this purpose, three main approaches are currently being used:

1. **Single candidate gene studies**, consisting in analyzing one or more genes encoding proteins already identified as being involved in a given phenotype.
2. **Large scale candidate gene analyses**, consisting in analyzing genes encoding proteins potentially involved in a given pathway or phenotype, according to their described or supposed functions.
3. **Genome wide association studies (GWAS)**, consisting in screening the whole genome for genetic variations associated with a given phenotype, with no *a priori* hypothesis of the involved encoded proteins.

One essential genomic feature, largely decreasing the number of variants investigated in the two latter approaches, is that nearby genetic variants might be inherited together, forming haplotypes (**Figure 1**) [3]. This non-random occurrence of genetic variants on the same allele is measured by the degree of linkage disequilibrium (LD). The LD between two genetic variants can be defined by two parameters: r^2 and D'. R^2 is the square of the correlation coefficient for a given marker pair, it ranges from 0 (no correlation) to 1 (perfect correlation, implying that both genetic variants have the same allelic frequency and are always present together on the same allele; in this case the two variants are said to be in perfect linkage). D' is the normalized covariance for a given marker pair and can be seen as the probability of non-randomly finding the two genetic variants on the same allele. D' ranges from 0 (both genetic variant are independent) to 1 (complete dependency of one of the marker pair [the less frequent one] with the other one [more frequent]; in this case the two variants are said to be in perfect or complete linkage, depending whether $r^2=1$ or $r^2<1$) [5].

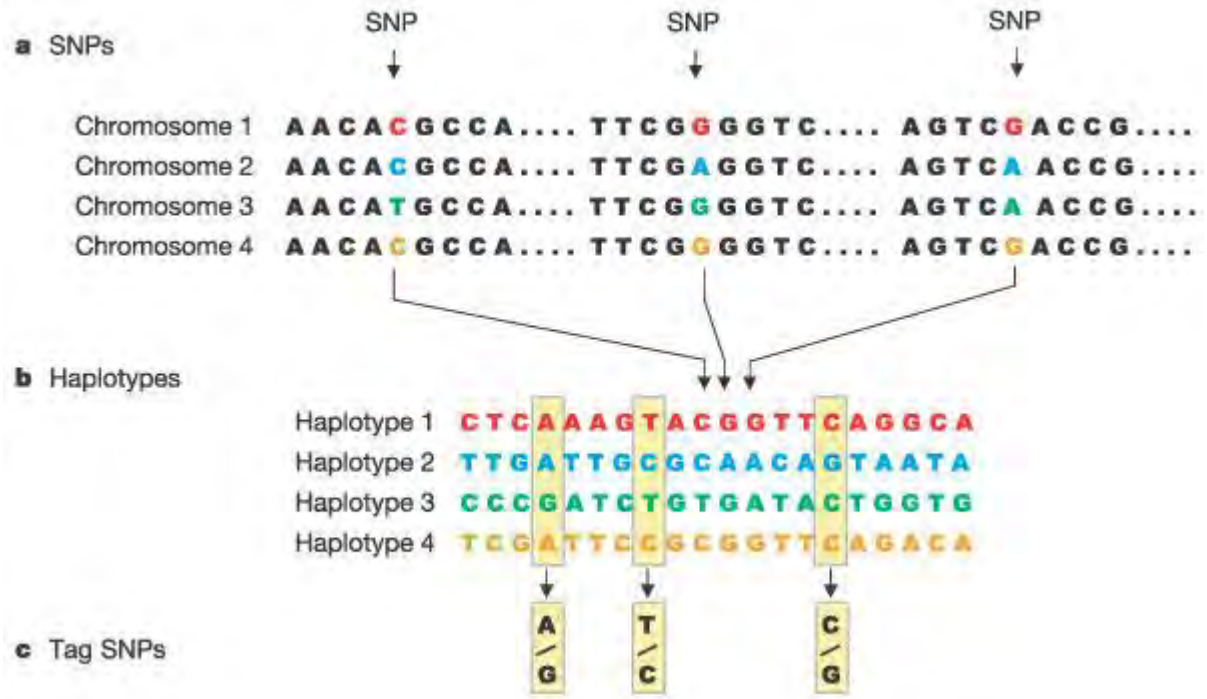


Figure 1: **SNPs, haplotypes and tag SNPs** [3].

a, SNPs. Shown is a short stretch of DNA from four versions of the same chromosome region in different people. Most of the DNA sequence is identical in these chromosomes, but three bases are shown where variation occurs.

b, Haplotypes. A haplotype is made up of a particular combination of alleles at nearby SNPs. Only the variable bases are shown, including the three SNPs that are shown in panel a.

c, Tag SNPs. Genotyping just the three tag SNPs out of the 20 SNPs is sufficient to identify these four haplotypes uniquely.

An approach consisting in the selective assessment of a specific SNP tagging a given haplotype (tag SNP), instead of performing a comprehensive analysis of all variants in the chromosomic region encompassing genetic variants in high LD (or LD block), is therefore used in large scale candidate gene studies and GWAS [3, 6, 7]. Typically, any untyped genetic variants should have a minimum r^2 threshold of 0.8 with a tag SNP [8], for further inference in a given haplotype [3, 6, 7].

In return, the SNPs identified in these studies as being associated with the studied phenotype (hit SNPs), are not necessarily the causal variants, but might rather tag them.

No standard method for the identification of the causal genetic variant responsible for the associated phenotype has yet been established. However, one strategy consists in resequencing the chromosomic region or the gene, in individuals with concordant and discordant genotype-phenotype. Concordant individuals carry the hit SNP and harbor the associated phenotype, or at the opposite neither carry the hit SNP nor harbor the associated phenotype. Discordant individuals carry the hit SNP but do not harbor the associated phenotype, or at the opposite do not carry the hit SNP but harbor the associated phenotype. Recombinant mapping of the genetic region in these individuals with extreme phenotype-genotype combinations should allow the identification of genetic variants tagged by the hit SNP. The candidate causal variants are expected to be as frequent as or more frequent than the hit SNP in concordant individuals and/or less frequent in discordant individuals.

The three different approaches above mentioned were used to investigate the two main issues addressed in this thesis work:

- The influence of host genetics on **antiretroviral agents'** pharmacokinetics
- The influence of host genetics on **hepatitis C virus** natural or treatment-induced clearance

1.2. Antiretroviral Therapy

Twenty-seven years after the human immunodeficiency virus (HIV) was discovered as the agent responsible of acquired immune deficiency syndrome (AIDS), 25 anti-HIV compounds have been approved for clinical use by the US Food and Drug Administration (FDA) [104]. These compounds fall into five categories according to the step they target in the virus life cycle (illustrated in **Figure 2** [9]):

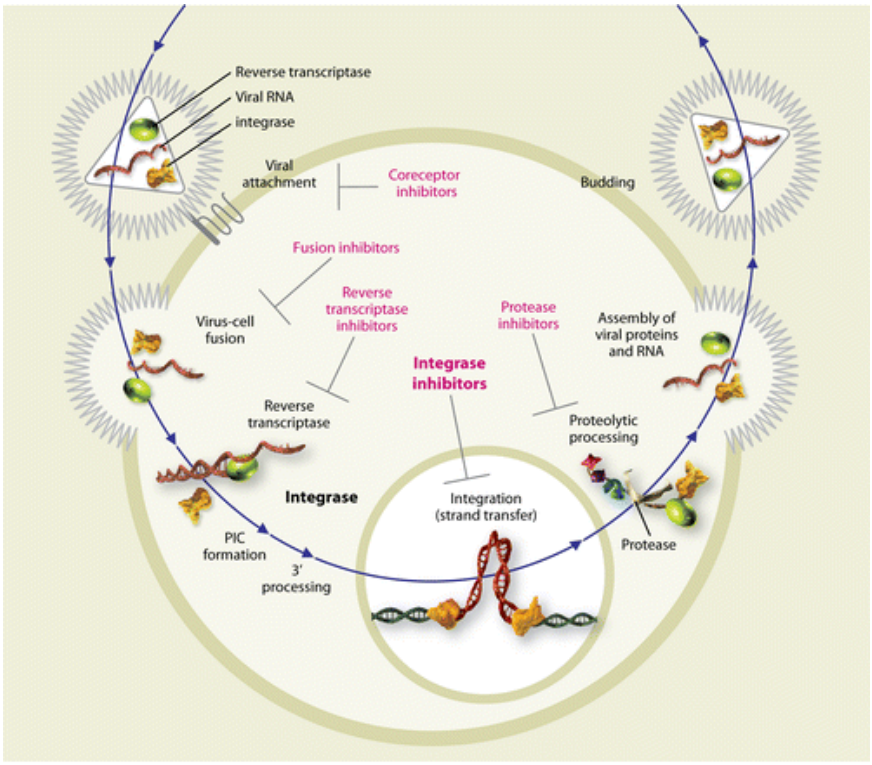
1. M (macrophage)-tropic and T (lymphocyte)-tropic HIV strains use respectively the co-receptors CC chemokine receptor 5 (CCR5) or CXC chemokine receptor 4 (CXCR4) to enter the target cells. The only **co-receptor inhibitor** approved at present, maraviroc, prevents viral entry by binding to the human CCR5 chemokine receptor [10-12].

2. Enfuvirtide, the only **fusion inhibitor** currently approved, is a polypeptide homologous to a segment of the viral envelope glycoprotein gp41. As a consequence of their coil-coil interaction, fusion of the virus particle with the outer cell membrane is blocked [10, 12].

3. The reverse transcriptase, a specific viral enzyme that retrotranscribes the viral single-stranded RNA genome to double-stranded proviral DNA, is the target for three classes of inhibitors: **nucleoside reverse transcriptase inhibitors** (NRTIs: zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir and emtricitabine), **nucleotide reverse transcriptase inhibitors** (NtRTIs: tenofovir) and **non-nucleoside reverse transcriptase inhibitors** (NNRTIs: nevirapine, delavirdine, efavirenz and etravirine). The NRTIs and NtRTIs are converted to their active form in the host cell by tri/di-phosphorylation respectively, acting as competitive inhibitors/alternate substrates of the normal deoxynucleoside triphosphate substrate, leading to the chain termination of the proviral DNA. The NNRTIs induce allosteric changes in the reverse transcriptase, assumed to disturb its normal functioning, by binding to a pocket located at a short distance from the catalytic site [10].

4. The viral integrase is required for each of the three sequence-specific events critical for integration of the HIV-1 proviral DNA into the host cell genome: assembly with the viral DNA, 3' endonucleolytic processing and strand transfer. The **integrase inhibitor** raltegravir elicits its antiviral effect by binding to the site of the integrase enzyme normally responsible for binding to human chromosomal DNA, therefore blocking the integration of the viral DNA [9, 11].

5. Finally, the **protease inhibitors** (saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanavir, fosamprenavir, tipranavir and darunavir) prevent the cleavage by the viral protease of the precursor viral polyprotein into smaller mature (both structural and functional) viral proteins. They act as competitive inhibitors by binding to the active site of the viral protease [10-12].



Hazuda D, et al. 2009.
Annu. Rev. Pharmacol. Toxicol. 49:377–94

Figure 2: the HIV-1 life cycle and antiretroviral agent targets. PIC: pre-integration complex [9].

1.2.1. Pharmacogenetics of Antiretroviral Agents

Availability of highly active antiretroviral therapy (HAART) has considerably decreased HIV morbidity and mortality, transforming HIV-infection from an inevitable fatal to a treatable chronic disease [13]. Drug tolerability is a key issue for treatments that must be taken lifelong. However, most of the antiretroviral drugs have been approved following an accelerated procedure, because of the advantageous benefice/risk ratio of their commercialization. This has limited the capacity to fully define some of the pharmacokinetic parameters and the profile of medium and long term toxicity. Moreover, given HIV propensity to replicate and mutate rapidly, antiretroviral therapy (ART) relies on the combination of multiple agents (at least three drugs belonging to minimum two classes) to prevent viral resistance. This may increase rates of toxicity, drug-drug interactions, diminished compliance and treatment failure, depicted by a median duration of initial regimens of only 1.6 years – half of the discontinuation events being related to toxicity [14].

The high interindividual variability in antiretroviral agents' pharmacokinetics and pharmacodynamics is multifactorial but host genetics is believed to play a significant role [15]. Pharmacogenetics of antiretroviral agents could thus help identifying the best tolerated combination of drugs when comparable efficacy in controlling HIV infection is expected, or guiding dose adaptation when toxicity or resistance emergence are anticipated due to undesired drug concentration [16].

Understanding the interindividual variability in antiretroviral drug pharmacokinetics is therefore crucial and might be achieved through the genetic assessment of encoded proteins involved in the drug **A**bsorption, **D**istribution, **M**etabolism and **E**limination (**ADME**) [17].

1.2.1.1. ADME Related Genes

There are two main categories of proteins implicated in ADME: drug metabolizing enzymes (DMEs) and drug transporters (DTs) [18].

Both classes can influence drug pharmacokinetics by two distinct ways. DMEs regulate the relative quantity of the parental compound and its metabolites [18], whereas DTs regulate the amount of drug passing through diverse membranes (gut lumen, enterocytes, hepatocytes, etc.) and are therefore implicated in the absorption, distribution and elimination steps [18].

1.2.1.1.1. Drug Metabolizing Enzymes

Drugs can undergo phase I (functionalisation) and/or phase II (conjugation) biotransformation rendering the compound more polar and hydrophilic to farther excretion [19]. It is estimated that 20-25% of all drug therapies are influenced by polymorphism in genes encoding DMEs to an extent that treatment outcome is affected [20, 21].

The most relevant DMEs in drug pharmacokinetics are the cytochrome P450 (CYP) proteins. CYP superfamily plays a critical role in metabolism of both endogenous and exogenous compounds. Isoenzymes of families CYP1, CYP2 and CYP3 are collectively responsible for most (>75%) phase I biotransformation of drugs and other xenobiotics in human liver [22, 23]. In addition, anti-HIV drugs are largely metabolized by CYP2 and CYP3 families (http://www.hiv-pharmacogenomics.org/ [105]; [24]). The human *CYP* genes are highly polymorphic. The different alleles are summarized at the Human *CYP* allele nomenclature committee home page (www.cypalleles.ki.se [106]), wherein the highest number of variant alleles are described for *CYP2D6* (78 alleles), *CYP2A6* (37), *CYP2C9* (34) and *CYP2B6* (29).

Because of such variability, the population can be classified into four phenotypes [25, 26]:

- Ultrarapid metabolizers, with more than 2 active copies of the gene
- Extensive metabolizers, carrying two functional copies of the gene
- Intermediate metabolizers, usually carrying 1 functional and 1 defective allele but may also carry 2 partially defective alleles
- Poor metabolizers, lacking functional enzyme due to defective/deleted genes

1.2.1.1.2. Drug Transporters

While drug passage across cell membranes can occur passively through simple diffusion, depending on the drug physicochemical properties, DTs are present on cell membrane to assist this step through active transport [27]. DTs are divided into two classes, uptake and efflux transporters, according to their location in the cell (basolateral/apical membrane) and/or their role. Both classes together regulate the systemic and intracellular drug levels by contributing, like phase I and phase II DMEs, to the detoxification system: uptake transporters help delivering the drug to the metabolizing system, whereas efflux transporters help delivering the compounds to the excretion systems [28]. There are two major transporter superfamilies, the solute carrier (SLC) transporters, which can generally transport substrates bidirectionally using electrochemical potential or ion gradient, and the ATP-binding cassette (ABC) transporters, which actively pump out substrates from intracellular compartment using ATP hydrolysis energy to cross the membrane against a concentration gradient [29].

The role of ABC and SLC transporters is relevant to the disposition of antiretroviral drug (reviewed in [30]), and is a determinant of effective treatment by enabling drug accumulation in cells that support viral replication.

It is only recently that DTs have been recognized as determinants of drug pharmacokinetics and dynamics, resulting in a relatively late emergence of DT

pharmacogenetic and genomic research as compared to DMEs. Although DT pharmacogenetics is on its early stage, it should be emphasized that drug pharmacokinetic properties are mainly the results of interacting activity of drug transporters and metabolizing enzymes, with the uptake clearance determining the amount of drug accessing to the metabolism machinery and the efflux clearance decreasing the load of the DMEs [28].

Despite high genetic diversity (http://pharmacogenetics.ucsf.edu/cgi-bin/Study.py [107]), no genetic variant leading to a complete loss of function of the encoded proteins have yet been described for transporters, resulting in more subtle phenotypes than seen for cytochrome P450 family [31]. Nevertheless, there is increasing evidence for the presence of clinically relevant polymorphisms in SLC transporter superfamily, notably in *SLCO1B1* (specifically expressed in the liver and considered to be of particular importance for hepatic drug disposition), as shown by the increased plasma concentration and enhanced risk of simvastatin-induced myopathy associated with a non-synonymous amino acid change (V174A) in the encoded protein [32]. On the other hand, although efflux transporters are recognized as playing a determinant role in intracellular drug accumulation, variability in genes encoding ABC transporters does not seem to affect drug response in a relevant and conclusive manner, potentially due to overlapping substrate specificity [28, 31].

Of note, serum binding proteins and transcription factors or modifiers that can either alter the expression of ADME genes or affect the biochemistry of ADME proteins (such as cytochrome P450 oxidoreductase and nuclear receptors) may also have a role in drug pharmacokinetics [26].

In this work, we have investigated the influence of genetic variants on two antiretroviral drugs, namely efavirenz and lopinavir.

1.2.2. Efavirenz

Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor approved for the treatment of HIV-1 infection, as a first line therapy, in combination with other antiretroviral drugs, in treatment naïve patients [33]. Its long half-life (40 to 55 hours after multiple-dose oral administration) allows a once daily administration (usually 600 mg) [33]. EFV is highly bound to plasma proteins (>99.5%, mainly to albumin) and has been shown to cross the blood-brain barrier [33]. It is extensively metabolized, principally by CYP isoenzymes to hydroxylated metabolites with subsequent glucuronidation [33]. Up to date eight metabolites have been reported (**Figure 3**) [34-36], of which three are primary metabolites (8-hydroxy-EFV [8-OH-EFV], 7-hydroxy-EFV [7-OH-EFV] and N-glucuronide-EFV [N-gln-EFV]) and six are secondary metabolites. CYP2B6 has been shown to be the main isoenzyme involved in EFV 8-hydroxylation, the major primary metabolic pathway [33, 36].

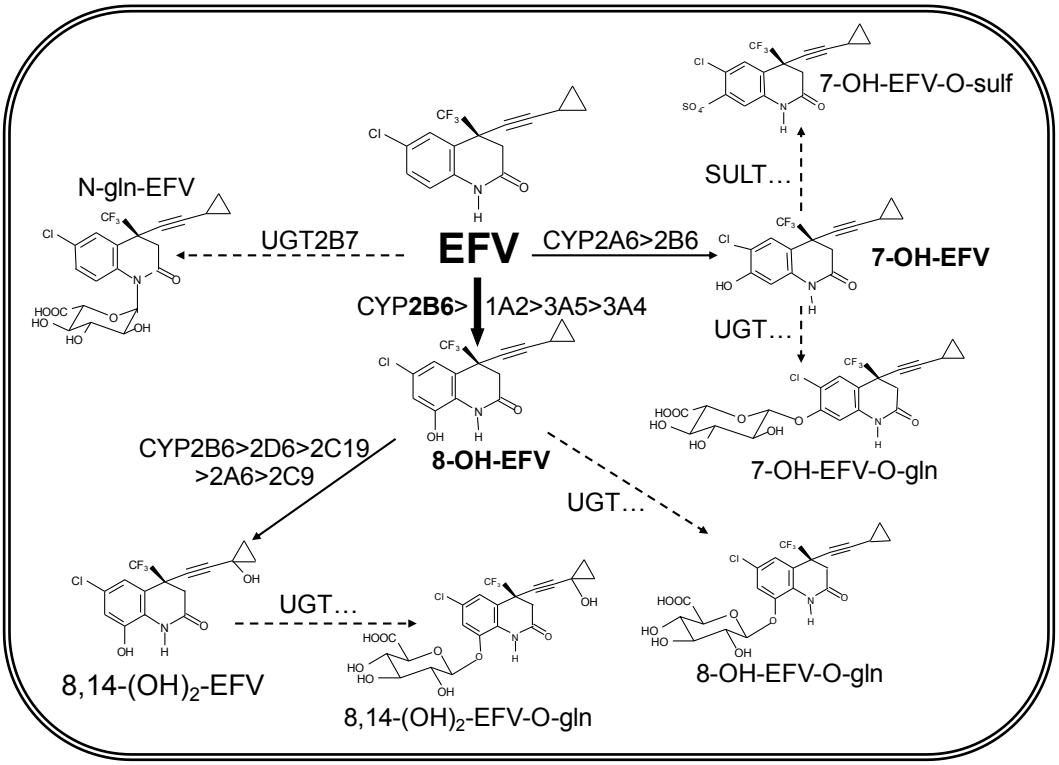


Figure 3: Efavirenz metabolic pathways [34-37]. CYP, cytochrome P450. UGT, UDP-glucuronosyl-transferase. SULT, sulfotransferase.

The most frequently reported adverse effect for EFV-containing therapy is neuropsychological toxicity that can affect up to 50% of individuals [38]. Central nervous system (CNS) disturbances range from dizziness to hallucinations, including frequent nightmares, impaired concentration, somnolence and insomnia [33]. Normally, adverse effects are mild, such as headache, dizziness, insomnia or fatigue; however, cases of severe toxicity such as psychosis with suicidal ideations, hallucinations, depression or manic episodes have also been described. Symptoms usually resolve within the first month of therapy but neuropsychological toxicity may also occur during long term therapy [33].

The high interindividual and low intraindividual variability in EFV plasma concentrations, as well as the potential relationship between treatment failure and CNS side effects with EFV plasma levels, support therapeutic drug monitoring (TDM) [39-41]. Factors involved in EFV interindividual variability have given rise to extensive research. Most of the data seem to suggest a trend toward higher plasma EFV concentrations in women than in men, which may be related to differences in body weight [15, 42]. Ethnicity has also been associated with EFV plasma levels, being generally higher in non-Caucasian individuals [43]. Finally, genetic variations in *CYP2B6* account for a large extent to the interindividual variability in EFV exposure [44-47], but the high remaining interindividual variability in individuals with impaired CYP2B6 is still unexplained, and we hypothesize that it might be attributed to other genes involved in EFV metabolism.

In this work, we have investigated EFV accessory metabolic pathway, through a **single candidate gene** approach, by a comprehensive analysis of *CYP2A6* which is known to be involved in the formation of 7-OH-EFV (**Figure 3**) [34].

1.2.3. Lopinavir

Lopinavir (LPV) is a protease inhibitor (PI) approved for the treatment of HIV-1 infection, as a first line therapy, in combination with other antiretroviral drugs, in treatment naïve and experienced adults and in children older than 6 months [48, 49]. Administered alone, LPV exhibits very low bioavailability, and is extensively metabolized by CY3A isoenzymes into 12 oxidative metabolites [50]. Co-administration of low sub-therapeutic doses of ritonavir (RTV), a PI with potent inhibiting activity against members of the CYP and ABC transporters family, dramatically increases LPV bioavailability and prevent its extensive metabolism [51], illustrated by 89% plasma radioactivity attributed to the parent compound after single dose administration of ^{14}C-LPV [49]. LPV is the first and only protease inhibitor to be solely found co-formulated with RTV, further referred as LPV/r (Kaletra®) [49]. This co-formulation allows reduced pill burden, by increasing LPV bioavailability and short half-life from ~1 hour to 4-6 hours after multiple-dose oral administration LPV/r, making once daily administration possible in treatment naïve individuals, whereas twice daily administration is still recommended in treatment experienced individuals [49].

At steady state, LPV is approximately 98-99% bound to plasma proteins, namely alpha-1-acid glycoprotein (AAG) and albumin, with a higher affinity for AAG [49], and has been shown in the co-formulated form to cross the blood-brain barrier [52].

LPV/r is generally well tolerated. The most frequently reported adverse effect is diarrhea that can affect up to 25% of individuals, nausea and vomiting. Like other members of the protease inhibitor class, LPV/r may however cause significant lipid elevations and fat redistribution. Less common adverse effects include allergic reaction, asthenia, malaise, headache, myalgias, arthralgia, myocardial infarction, seizures and lactic acidosis. Severe side effects of LPV/r are unusual [48, 49, 53].

The related relationship between LPV/r through plasma concentration with efficacy and toxicity (increased lipid elevation is expected above 8mg/L) [54, 55], together with high described interindividual variability and two different efficacy threshold ascribed according to the patient treatment history (1mg/L in treatment naïve individuals, and 4 to 5.7 mg/L in treatment experienced individuals) support TDM [56, 57]. Identification of factors contributing to the high interindividual variability in LPV/r pharmacokinetics are thus of prime importance, in the goal of reducing the proportion of individuals with drug concentration outside the therapeutic window. Up to date, the only factor related to LPV/r exposure is body weight, however, leaving a large remaining unexplained variability [57]. Little is known about pharmacogenetic determinant of LPV/r disposition. RTV boosting effect might in part explain the lack of determinant genetic factors identified. Moreover, at the opposite of the well described LPV metabolism, LPV transport has not been yet completely solved. LPV has consistently been shown *in vitro* to be a substrate for P-glycoprotein (P-gp, encoded by *ABCB1*) [58, 59], whereas its transport by multidrug resistance protein (MRP) 1 and 2 (encoded by the *ABCC1* and *2*) is controversial [58-60]. Recently LPV has also been reported to be a substrate of the organic anion transporting polypeptide (OATP) 1A2, 1B1 and 1B3 (respectively encoded by *SLCO1A2*, *1B1* and *1B3*) [61]. In addition, potential interaction between efflux transporters in the gastro-intestinal tract and CYP3A metabolizing enzymes may also be a source of variation associated with LPV/r absorption and distribution [63].

Overall, little is known about pharmacogenetic factors determinant for LPV/r pharmacokinetics; thus we performed in this work a **large scale candidate ADME gene analysis**, and then comprehensively analyzed the identified candidate gene(s) by resequencing and fine mapping strategy to identify potential causal variants.

1.3. Hepatitis C Virus Infection

Hepatitis C virus (HCV) infection is one of the major causes of chronic hepatitis, liver cirrhosis, hepatocellular carcinoma and liver-related morbidity and mortality. Worldwide, around 170 million people are chronically infected, and ~3% of the world population is thought to be or have been infected [64]. Although a wide range of innate and adaptive immune responses are induced upon acute infection with HCV, the virus might escape host immunologic detection and elimination, resulting in a chronic infection in most individuals [65], while only 20-50% acutely infected individuals are able to clear the virus [65-67]. Spontaneous HCV clearance and chronic infection are respectively characterized by the absence and presence of HCV RNA in the serum of HCV seropositive individuals [65]. The still limited therapeutic options and current lack of protective vaccine, mainly due to the multiple existing HCV genotypes and the protein antigenic property alterations caused by the virus constant mutations [68], turn HCV infection into a major public health problem.

Female gender, symptomatic acute hepatitis C, young age and non-african ethnicity correlate with lower rate of HCV chronicity, but the control of HCV is thought to be largely dependent on the effectiveness of host immune responses [65, 68].

The first host immune response activated by HCV particles is mediated through interferon production by infected hepatocytes [67]. This response is initiated by two pattern recognition receptors, toll like receptor 3 (TLR3) and retinoic acid-inducible gene I (RIG-I). After activation, both processes result in downstream signaling ultimately leading to synthesis of interferon β (IFN$-\beta$). Binding of IFN$-\beta$ to IFN-α/β receptors, in turn, activates signaling cascade resulting in the induction of interferon-stimulated genes (ISGs) [67].

ISG products activate viral-regulatory functions that limit HCV replication through processes that include disruption of viral RNA translation and inhibition of RNA synthesis [67, 69]. ISG expression not only causes an antiviral state but also amplifies the IFN response by stimulation of IFN-α synthesis [67, 69]. In addition to inducing ISG expression, IFN-α induces

the maturation of immune effector cells, stimulating the production of other proinflammatory cytokines and indirectly modulating the cell-mediated defenses and adaptive immunity to HCV [69]. Local IFN production in hepatic tissue is therefore likely to influence HCV replication and contribute to the resolution of acute HCV infection [69]. However, HCV is able to attenuate IFN signaling through multiple mechanisms, involving notably the viral protease complex NS3-4A, resulting in control of ISG expression or function [67, 69]. The virus constant mutations resulting in quasispecies formation affords significant adaptive potential for HCV to co-exist with the host immune response. Indeed, viral variants able to persist and resist IFN action may be selected under host immune pressure [69]. The flow-diagram shown in **Figure 4** provides a model of virus-host interactions and viral adaptation that form a foundation for chronic infection [69].

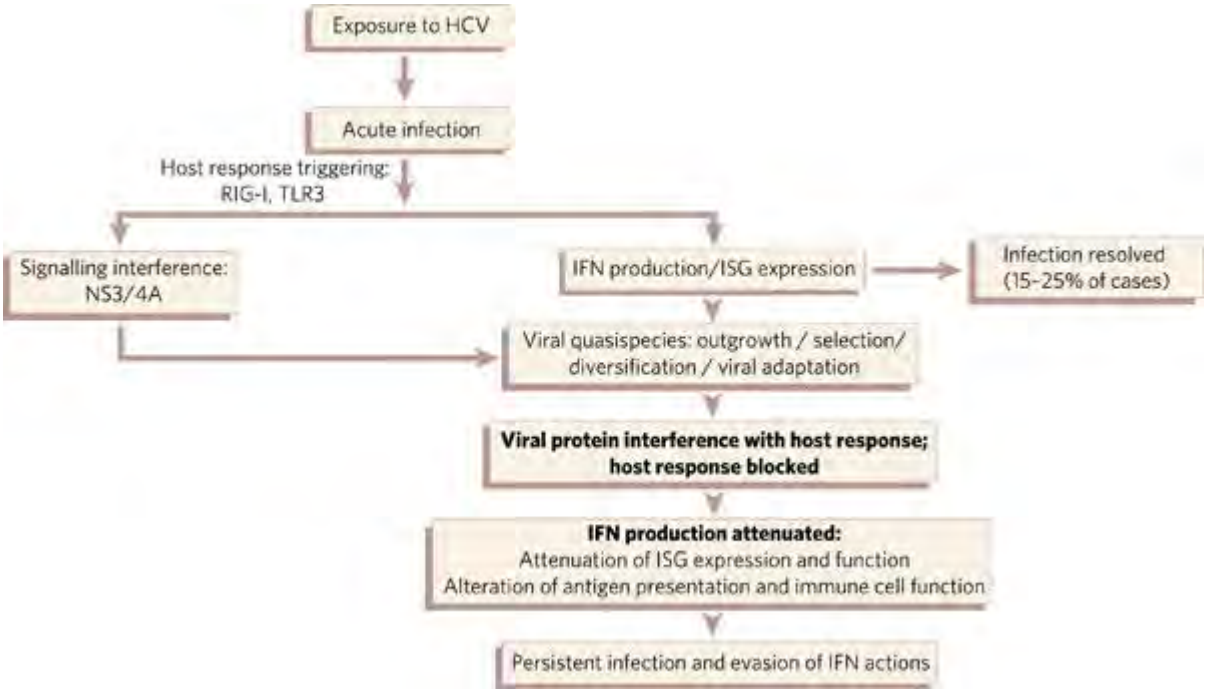


Figure 4: HCV infection outcome. Virus-host interactions within the host response to HCV infection define the outcome from acute exposure to HCV. RIG-I, retinoic acid-inducible gene I. TLR3, toll like receptor 3. IFN, interferon. ISG, interferon-stimulated gene [69].

1.3.1. Pharmacogenetics and Clearance of Hepatitis C Virus

The basis of spontaneous and treatment-induced HCV clearance phenotype is multifactorial but host genetics is believed to play a significant role, highlighted by single source HCV exposed cohorts sharing similar demographic characteristics while undergoing both outcomes (either spontaneous/treatment-induced HCV clearance or chronic infection) [70].

Identification of host genetic factors susceptible to influence natural or treatment-induced HCV infection outcome could therefore help to better understand the molecular pathogenesis of HCV infection. In addition, better understanding HCV-host interaction network is strongly needed and might help developing new preventive strategies and treatments, as the current standard regimen therapy consisting in pegylated IFN-α (peg-IFN-α) and ribavirin is not well tolerated, expensive, and inefficient in up to 50% cases [65].

On this purpose, as HCV is known to interact with innate and adaptive immune response, many candidate gene studies have been carried out (reviewed in [65]), including immune response genes, killer immunoglobulin-like receptors, human leukocyte antigens, chemokines, cytokines and lipoprotein receptors (see **Figure 5**, [65]). However, many studies were underpowered, or did not apply correction for population stratification or multiple comparisons, and candidates were not confirmed in validation cohorts.

As candidate gene studies failed to identify determinant host genetic factors involved in HCV clearance, larger studies including analysis of non-*a priori* involved genes such as GWAS are needed.

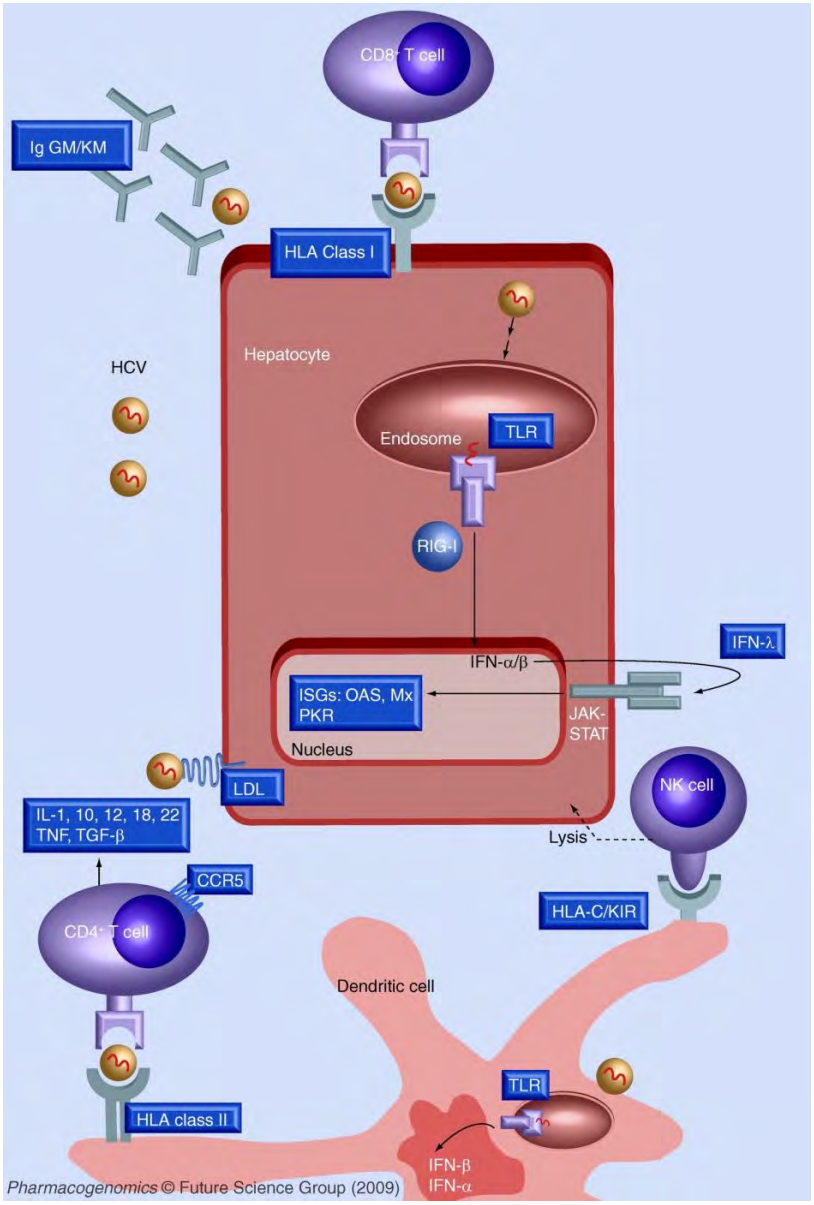


Figure 5: Host genes with published (boxes) polymorphisms associated with hepatitis C clearance. HLA, human leucocyte antigen. ISG, interferon-stimulated gene. KIR, killer immunoglobulin-like receptor. LDL, low-density lipoprotein. NK, Natural killer. OAS, 2'5'-oligoadenylate-synthetase-directed ribonuclease. PKR, Protein kinase R. TLR, Toll-like receptor.

In this work, we have therefore investigated the influence of host genetics on HCV infection outcome using a **GWAS** approach and then comprehensively analyzed the identified candidate gene(s) by resequencing and fine mapping strategy in order to identify potential causal variants.

2. AIMS

2.1. General Aims

The study strategy intends to increase, through 3 different approaches (single candidate gene, candidate pathway, GWAS), the current understanding of:

- toxicity and efficacy of antiviral treatment at patient and population level, in order to improve the long term tolerability and response.
- infectious disease susceptibility, in order to improve our understanding of the host-pathogen interactions in view of developing new preventive and treatment strategies.

2.2. Pharmacogenetics of Antiretroviral Agents

2.2.1. Efavirenz

The specific aims are:

- to investigate the contribution of EFV accessory metabolic pathway (7-hydroxylation, see **Figure 3**) on EFV and its primary metabolite plasma levels, through **single candidate gene** (*CYP2A6*) **analysis**. We expect that integration of EFV metabolite profile phenotypes will help at deciphering the multiple genetic and environmental influences affecting EFV disposition.
- to determine the clinical implementation of the results by making a population pharmacokinetics analysis.
- to assess the clinical relevance of changes in EFV metabolic pathways.
- to compare EFV individualized dose adjustment determined by therapeutic drug monitoring or predicted by genetic analyses.

2.2.2. Lopinavir

The specific aims are:

- to investigate the contribution of genes encoding proteins involved in ADME, or implicated in ADME protein regulation, on LPV/r clearance, by performing a **large scale candidate gene analysis**.
- to identify the causal variant(s) responsible for alteration in LPV/r clearance.
- to determine the clinical implementation of the results by making a population pharmacokinetics analysis.
- to assess the clinical relevance of the genetic variations associated with altered LPV/r clearance.

2.3. Pharmacogenetics and Clearance of Hepatitis C Virus

The specific aims are:

- to investigate the association between host genetics and hepatitis C virus treatment and infection outcome, through a **genome wide association study** approach.
- to identify the causal variant(s) responsible for increased susceptibility to hepatitis C virus spontaneous or treatment-induced clearance.
- to estimate the net contribution of host genetics on hepatitis C virus infection outcome, by analyzing a single source cohort allowing the control for cofactors such as viral genotype, gender and co-infection.

3. MATERIALS AND METHODS

3.1. General Feature – Nomenclature

Current Nomenclature is to use *italics* for genes and alleles – which are designated by the name of the gene followed by an asterisk and an Arabic number (with or without an upper-case Roman letter) – and roman type for the encoded protein. The base A in the initiation codon ATG is denoted +1 and the base before A is numbered –1. “g.” followed by the nucleotide number refers to genomic DNA and “c.” refers to coding DNA.

3.2. Pharmacogenetics of Antiretroviral Agents

3.2.1. Efavirenz

Materials and methods are described in the original articles

(Chapters 4.1.1.1., 4.1.1.2., 4.1.1.3. & 4.1.1.4.).

3.2.2. Lopinavir

Materials and methods are described in the original articles

(Chapters 4.1.2.1. & 4.1.2.2.).

3.3. Pharmacogenetics and Clearance of Hepatitis C Virus

Materials and methods are described in the original articles and manuscripts

(Chapters 4.2.1.1. & 4.2.1.2.).

4. RESULTS

4.1. Pharmacogenetics of Antiretroviral Agents

4.1.1. Efavirenz

4.1.1.1. Original article

In Vivo Analysis of Efavirenz Metabolism in Individuals with Impaired CYP2A6 Function

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Pharmacogenetics and Genomics 2009 April; 19(4):300-309

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Genetic analysis: **J. di Iulio**, M. Rotger

Drug and metabolite levels determination: A. Fayet, L. A. Decosterd, S. Colombo

Population pharmacokinetics: C. Csajka, M. Arab-Alameddine

Data interpretation: **J. di Iulio**, M. Rotger, A. Telenti

Sample contribution: M. Cavassini, H. Furrer, H. F. Günthard

Study supervision: A. Telenti, L. A. Decosterd

Drafting of the manuscript: **J. di Iulio**, A. Telenti

Critical revision of the manuscript for important intellectual content: all authors

Detailed personal contribution

I was the main person in charge of the project. I notably participated in the study concept and design, sample recruitment, data analyses and interpretation, drafting of the manuscript, as well as in the genetic characterization of individuals included in the study by performing comprehensive *CYP2A6* analysis, consisting in:

- *CYP2A6* exon-intron boundaries resequencing (N=23)
- Genotyping of 12 *CYP2A6* alleles (N=146)
- *CYP2A6* copy number variation assessment (N=169)

Comments on the article:

As it has been reported that CYP2A6 contributes to EFV metabolism *in vitro*, the aim of this study was to fully characterize *CYP2A6* genetic diversity (polymorphism, copy number variation, gene chimera). This information allowed the assessment of CYP2A6 role *in vivo* in EFV metabolism among individuals with different *CYP2B6* genetic background.

CYP2A6 functional variants were investigated in one hundred and sixty-nine HIV-infected individuals receiving EFV. Drug and metabolite concentrations were measured in plasma samples. Correlation between EFV and its primary metabolite plasma levels and *CYP2A6* genotype was assessed among individuals with known CYP2B6 functionality.

By investigating *CYP2A6* genotype, we identified a new allele (**34*). In addition, the results are consistent with the role of CYP2A6 in EFV metabolism as accessory isoenzyme. Its function becomes increasingly relevant in the setting of limited CYP2B6 function. Thus, the simultaneous occurrence of impaired function in CYP2B6 and CYP2A6 results in the redirection of EFV metabolism from hydroxylation to N-glucuronidation, which however does not compensate the loss of the other isoenzymes, leading to marked EFV accumulation.

300 Original article

In vivo analysis of efavirenz metabolism in individuals with impaired *CYP2A6* function

Julia di Iulio[a], Aurélie Fayet[b], Mona Arab-Alameddine[b], Margalida Rotger[a], Rubin Lubomirov[a], Matthias Cavassini[c], Hansjakob Furrer[e], Huldrych F. Günthard[f], Sara Colombo[a], Chantal Csajka[b,g], Chin B. Eap[d], Laurent A. Decosterd[b], Amalio Telenti[a] and the Swiss HIV Cohort Study

Introduction **The antiretroviral drug efavirenz (EFV) is extensively metabolized into three primary metabolites: 8-hydroxy-EFV, 7-hydroxy-EFV and *N*-glucuronide-EFV. There is a wide interindividual variability in EFV plasma exposure, explained to a great extent by cytochrome P450 2B6 (CYP2B6), the main isoenzyme responsible for EFV metabolism and involved in the major metabolic pathway (8-hydroxylation) and to a lesser extent in 7-hydroxylation. When CYP2B6 function is impaired, the relevance of CYP2A6, the main isoenzyme responsible for 7-hydroxylation may increase. We hypothesize that genetic variability in this gene may contribute to the particularly high, unexplained variability in EFV exposure in individuals with limited CYP2B6 function.**

Methods **This study characterized *CYP2A6* variation (14 alleles) in individuals (*N*=169) previously characterized for functional variants in *CYP2B6* (18 alleles). Plasma concentrations of EFV and its primary metabolites (8-hydroxy-EFV, 7-hydroxy-EFV and *N*-glucuronide-EFV) were measured in different genetic backgrounds *in vivo*.**

Results **The accessory metabolic pathway CYP2A6 has a critical role in limiting drug accumulation in individuals characterized as CYP2B6 slow metabolizers.**

Conclusion **Dual CYP2B6 and CYP2A6 slow metabolism occurs at significant frequency in various human populations, leading to extremely high EFV exposure. *Pharmacogenetics and Genomics* 19:300–309 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Pharmacogenetics and Genomics 2009, **19**:300–309

Keywords: efavirenz, metabolism, pharmacogenetics

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Received 9 November 2008 **Accepted** 5 January 2009

Introduction

The antiretroviral drug efavirenz (EFV) is primarily metabolized by the cytochrome P450 2B6 (CYP2B6) [1]. There is a high interindividual variability in plasma drug levels after drug administration at the usual dosage regimen of 600 mg/day [2]. This observation reflects the existence of slow and rapid metabolizer phenotypes associated with genetic variations in *CYP2B6* [3–7]. Although *CYP2B6* alleles explain to a large extent the observed interindividual variability in EFV exposure, there is a remaining variability, in particular among individuals with impaired CYP2B6 function [5]. This unexplained variability suggests the participation of other genetic and environmental influences in EFV elimination.

EFV is extensively metabolized into three primary metabolites; two of them are hydroxylated metabolites [8-hydroxy-EFV (8-OH-EFV) and 7-hydroxy-EFV (7-OH-EFV)] and the third is a glucuroconjugated product [*N*-glucuronide-EFV (*N*-gln-EFV)] (Fig. 1). 8-Hydroxylation is the main metabolic pathway (92%) and is essentially the result of CYP2B6 activity [1]. Recent *in vitro* data suggest that 7-hydroxylation is the second most important pathway (< 8%), mainly because of CYP2A6 activity and to a lesser extent CYP2B6 activity [8] (Fig. 1). *N*-gln-EFV has been identified in human plasma and urine although the pathway has not been characterized [9] (Fig. 1). Here we hypothesize that, in individuals with impaired CYP2B6 function, functional polymorphism in *CYP2A6* may be of clinical importance.

CYP2A6 has been shown to have large interindividual and interethnic variability in levels of expression and activity, and this is thought to be largely because of

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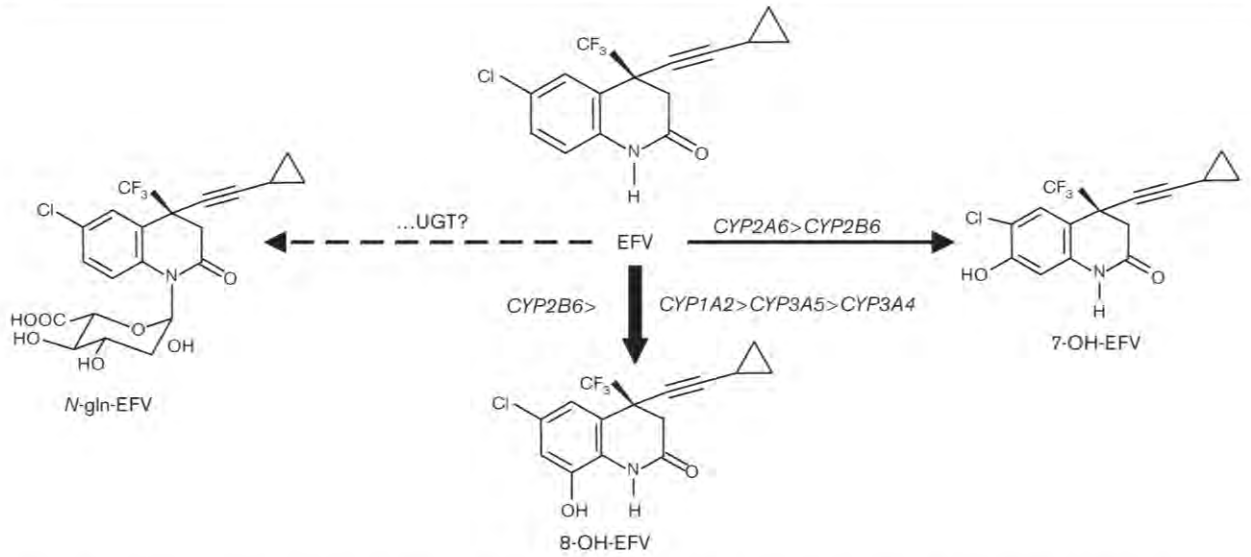
Fig. 1



Efavirenz (EFV) primary metabolic pathways. EFV is extensively metabolized into three primary metabolites: 8-hydroxy-EFV (8-OH-EFV), 7-hydroxy-EFV (7-OH-EFV) and *N*-glucuronide-EFV (*N*-gln-EFV).

genetic polymorphisms [10]. Variation in activity affects the metabolism of CYP2A6 substrates such as nicotine and coumarin.

In this study, we conducted detailed assessment of functional genetic variation in *CYP2A6* in a well-characterized population of HIV-infected individuals receiving EFV, and validated the observed genotypic/phenotypic associations by *in vivo* metabolite profiling.

Materials and methods

Study population

The study group includes 169 individuals extensively characterized for their *CYP2B6* genotype in a previous study [5], and described in Supplementary Table S1. The ethics committees of all participating centres approved the genetics project and participants gave written informed consent for genetic testing.

Population pharmacokinetics

A population pharmacokinetic analysis of EFV was fitted according to our previous study [11] to derive average population parameters and interpatient variability, and to estimate the influence of demographic factors in its elimination. EFV plasma levels were collected as a part of therapeutic drug monitoring protocol according to local treatment guidelines. EFV pharmacokinetics was characterized using a one-compartment model with first order absorption from the gastrointestinal tract and assigned an interindividual variability on oral clearance (CL), assuming a lognormal distribution with mean zero and variance Ω. The influence of demographic covariates (sex, ethnicity, age, body weight, height and other comedications such as antiretroviral drugs and few other medications) was analysed using linear relationships. A proportional error model with a mean of zero and a variance of σ^2 was used to describe intraindividual variability. Individual Bayesian estimates of CL were used to derive individual area under the curves (AUC=dose/CL) that were used for statistical analyses. The population pharmacokinetics analysis was performed with NONMEM (version VI, NM-TRAN version II, Icon Development Solutions, Ellicott City, Maryland, USA) using FOCE INTERACTION to fit the models. Model choice was based on the likelihood ratio test, and goodness-of-fit plots.

CYP2A6 genotyping

Analysis of *CYP2A6* genetic variation included extensive genotyping of known alleles, full resequencing for selected individuals for the identification of new alleles, and study of copy number variation. Genotyping targeted all alleles with known impact on expression or function in Caucasians, as well as the most common decrease-of-function (DOF)/loss-of-function (LOF) alleles found in Africans and Asians (Table 1, and references therein). We used TaqMan allelic discrimination (ABI prism 7000 sds software, Applied Biosystems, Foster City, California, USA) to investigate five alleles (*CYP2A6*2*, *CYP2A6*9*, *CYP2A6*13*, *CYP2A6*15*, *CYP2A6*17*). Alleles resulting from gene conversion (*CYP2A6*5*, *CYP2A6*7*, *CYP2A6*10*, *CYP2A6*12*, *CYP2A6*19*) and promoter

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Table 1 ***CYP2A6*** **alleles investigated**

Allele	Functional SNP (rs)	Proposed functional consequence	Reference
*CYP2A6*1H, CYP2A6*1J*	g.−745A>G (rs61663607)	DOF (<50% decreased activity *in vitro*)	[12]
*CYP2A6*1 × 2A–B*	Gene duplication	GOF (increased activity *in vivo*)	[13]
*CYP2A6*2*	g.1799T>A (rs56844942)	LOF (catalytically inactive)	[14]
*CYP2A6*4A–F*	GC with *CYP2A7* in intron 8	LOF (gene deletion)	[15,16]
*CYP2A6*5*	g.6582G>T (rs5031017)	LOF (catalytically inactive)	[17]
*CYP2A6*7, CYP2A6*10, CYP2A6*19*	g.6558T>C (rs5031016)	DOF (≥ 50% decreased activity *in vitro*)	[18]
*CYP2A6*9, CYP2A6*13, CYP2A6*15*	g.−48T>G (rs28399433)	DOF (≥ 50% decreased activity *in vivo/in vitro*)	[19]
*CYP2A6*12*	GC with *CYP2A7* in intron 2	DOF (≥ 50% decreased activity *in vivo/in vitro*)	[20]
*CYP2A6*17*	g.5065G>A (rs28399454)	DOF (≥ 50% decreased activity *in vivo/in vitro*)	[21]
*CYP2A6*34*	GC with *CYP2A7* in intron 4	LOF (partial gene deletion)	This study

DOF, decrease-of-function allele; GC, gene conversion; GOF, gain-of-function allele; LOF, loss-of-function allele; SNP, single nucleotide polymorphism.

alleles (*CYP2A6*1H*, *CYP2A6*1J*) were genotyped by sequencing. Primers and probes are shown in Supplementary Table S2.

On account of the working hypothesis that *CYP2A6* variation would be clinically relevant among individuals with impaired CYP2B6 metabolism, *CYP2A6* promoter region, and all nine exons and intron–exon boundaries (3775 bp) were fully resequenced in all individuals homozygous for a LOF of *CYP2B6* (n=23). Primers are shown in Supplementary Table S3.

Gene copy number was determined in all individuals, as gene deletions (*CYP2A6*4A–4F*) and gene duplications (*CYP2A6*1 × 2A–B*) have been described for this gene [13,22–25]. Real-time PCR was performed using the TaqMan ABI prism 7000 sds software (Applied Biosystems). For each analysis, standard curves (seven serial 1:2 dilutions of genomic DNA) were built for a reference gene (*β-globin*, two copies per diploid genome), and for *CYP2A6* in separate wells. *β-globin* primers and probe were published earlier [26] (Supplementary Table S2). *CYP2A6* primers and probe were obtained as assay-on-demand from Applied Biosystems (TaqMan Gene Expression Assays: HS0001002_cn) (Supplementary Table S2). The probe is located in exon 7, which is missing in the gene deletions described in the literature [22–24].

CYP2B6 genotyping

The details of *CYP2B6* genotyping have been presented earlier [5].

Nomenclature

CYP2A6 allele designation was performed on the basis of genotyping of known functional single nucleotide polymorphisms (SNPs), as well as by full resequencing. Novel alleles were designated in concordance with the CYP Allele Nomenclature Committee (*http://www.cypalleles.ki.se*). Position numbering refers to genomic DNA (indicated as g.) according to NT_011109.15 (base pair 1=A of ATG of *CYP2A6*). *CYP2B6* allele designation was established as before [5]. *CYP2A6* investigated alleles were reported as DOF alleles or LOF alleles, according to the known/proposed activity of the encoded protein. The DOF alleles were further broken down into two categories: DOF < 50% (less than 50% decrease in activity or expression) and DOF ≥ 50% (more or equal to 50% decrease in activity or expression) (Table 1).

In vivo metabolite analyses

Plasma concentrations of EFV, 8-OH-EFV, 7-OH-EFV and *N*-gln-EFV were determined by liquid chromatography coupled to triple quadrupole tandem mass spectrometry (LC-MS/MS; Thermo Scientific Corporation, San Jose, California, USA) after protein precipitation with acetonitrile using an adaptation of our previously reported method [27]. 8-OH-EFV-d_4 and 8-OH-EFV were obtained from Toronto Research Chemicals Inc. (North York, Canada). The m/z transition, collision energy (V) and tube lens used for 7-OH-EFV and 8-OH-EFV in the selected reaction monitoring mode were 330.0→ 257.9, 31 and 97, respectively. *N*-gln-EFV was analysed in the selected ion monitoring mode (m/z=490.0).

Chromatographic profiles and LC-MS spectra of EFV metabolites were compared with available data [1,9]. Quantitative analysis of EFV and 8-OH-EFV was performed with matrix-matched calibration samples using the internal standard method (EFV-d_4 and 8-OH-EFV-d_4, respectively), whereas 7-OH-EFV and *N*-gln-EFV levels were expressed in arbitrary units (a.u.) using signal peak areas. The lower limit of quantification is 50 ng/ml for EFV and 8-OH-EFV. Values were adjusted by dose intake for three individuals that were not receiving a 600 mg EFV regimen.

Results

Population pharmacokinetics

Three hundred and ninety-three EFV plasma concentrations provided by 169 HIV-infected individuals (range 0.1–59.4 µg/ml) were analysed. Average CL was 11.3 l/h with an interindividual variability of 65% (coefficients of variability %), volume of distribution (V) was 388 l and the absorption constant (ka) 0.62/h. Body weight was

retained as significant demographic factor, yielding a 70% increase in CL on body weight doubling; it accounted for the effect of height, age and sex and explained 3% of CL variability. Individual AUC values ranged between 15.4 and 436.0 μg × h/ml. Drug interactions were ruled out as significant contributor to EFV pharmacokinetics in this study, as the presence of ritonavir revealed no statistically significant effect on EFV exposure and only a few individuals were coadministered a nonantiretroviral CYP inducer (n=2) or inhibitor (n=4) drug.

CYP2A6 genotyping

Fifty-five (33%) individuals carried one *CYP2A6* DOF or LOF allele and 13 (8%) carried two DOF and/or LOF alleles (Table 2). Analysis of copy number identified three individuals heterozygous for a gene deletion and two individuals heterozygous for gene duplication (Fig. 2a). These two individuals with a *CYP2A6* gene duplication (expected to represent a gain-of-function allele) were included in the group of extensive CYP2A6 metabolizers.

CYP2A6 was fully resequenced in the 23 individuals homozygous for a LOF allele of *CYP2B6* (slow metabolizers). *CYP2A6* resequencing identified one polymorphism in the promoter region, 16 polymorphisms in exons and 25 polymorphisms in exon–intron boundaries. The promoter SNP g.−48T > G (*CYP2A6*9*, *CYP2A6*13*, *CYP2A6*15*), modifies the TATA box leading to a decreased expression of the gene [19,28,29]; five exonic SNPs are nonsynonymous, two of which are associated with a DOF (g.5065G > A [V365M], in *CYP2A6*17* [21]; g.6558T > C [I471T], in *CYP2A6*7*, *CYP2A6*10*, *CYP2A6*19* [18]) (Fig. 2b). In addition, resequencing identified an individual that carried a deletion of the first

Table 2 Frequency of *CYP2A6* genotypes in different ethnicities

Region	Allele(s)	SNP	All participants, % (n)	Caucasians, % (n)	Africans, % (n)	Hispanics, % (n)	Asians, % (n)
5′ UTR	*CYP2A6*1H*, *CYP2A6*1J*	−745A>G					
		AA	79.8 (134)	81.6 (115)	62.5 (10)	100.0 (7)	50.0 (2)
		AG	20.2 (34)	18.4 (26)	37.5 (6)	0.0 (0)	50.0 (2)
		GG	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
5′ UTR	*CYP2A6*9*, *CYP2A6*13*, *CYP2A6*15*	−48T>G					
		TT	83.9 (141)	81.6 (121)	62.5 (10)	85.7 (6)	100.0 (4)
		TG	15.5 (26)	18.4 (20)	31.3 (5)	14.3 (1)	0.0 (0)
		GG	0.6 (1)	0.0 (0)	6.2 (1)	0.0 (0)	0.0 (0)
Exon 3	*CYP2A6*2*	1799T>A					
		TT	96.4 (162)	96.5 (136)	100.0 (16)	85.7 (6)	100.0 (4)
		TA	3.6 (6)	3.5 (5)	0.0 (0)	14.3 (1)	0.0 (0)
		AA	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Exon 7	*CYP2A6*17*	5065G>A					
		GG	98.2 (166)	100.0 (141)	81.3 (13)	100.0 (7)	100.0 (5)
		GA	1.8 (3)	0.0 (0)	18.7 (3)	0.0 (0)	0.0 (0)
		AA	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Exon 9	*CYP2A6*7*, *CYP2A6*10*, *CYP2A6*19*	6558T>C					
		TT	98.2 (166)	99.3 (140)	100.0 (16)	100.0 (7)	60.0 (3)
		TC	1.8 (3)	0.7 (1)	0.0 (0)	0.0 (0)	40.0 (2)
		CC	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Exon 9	*CYP2A6*5*	6582A>G					
		AA	100.0 (169)	100 (141)	100.0 (16)	100.0 (7)	100.0 (5)
		AG	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
		GG	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Intron 2	*CYP2A6*12*	GC with *CYP2A7*					
		No GC	98.2 (165)	98.6 (139)	100.0 (16)	85.7 (6)	100.0 (5)
		Het GC	1.8 (3)	1.4 (2)	0.0 (0)	14.3 (1)	0.0 (0)
		Hom GC	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Intron 4	*CYP2A6*34*	GC with *CYP2A7*					
		No GC	99.4 (168)	100.0 (141)	100.0 (16)	100.0 (7)	80.0 (4)
		Het GC	0.6 (1)	0.0 (0)	0.0 (0)	0.0 (0)	20.0 (1)
		Hom GC	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Intron 8→3′UTR	*CYP2A6*4A–F* (deletion)	GC with *CYP2A7*					
		No GC	98.2 (166)	99.3 (140)	100.0 (16)	100.0 (7)	60.0 (3)
		Het GC	1.8 (3)	0.7 (1)	0.0 (0)	0.0 (0)	40.0 (2)
		Hom GC	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
Intron 8→3′UTR	*CYP2A6*1 × 2A–B* (duplication)	GC with *CYP2A7*					
		No GC	98.8 (167)	99.3 (140)	100.0 (16)	85.7 (6)	100.0 (5)
		Het GC	1.2 (2)	0.7 (1)	0.0 (0)	14.3 (1)	0.0 (0)
		Hom GC	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)

The individual genotype frequency was calculated based on the number of individuals for whom genotyping for the selected SNPs has been successfully performed (n).
SNP position is based on genomic (g.) DNA numbering (base pair 1 = A of ATG).
GC, gene conversion; Het, heterozygous; Hom, homozygous; SNP, single nucleotide polymorphism; UTR, untranslated region.

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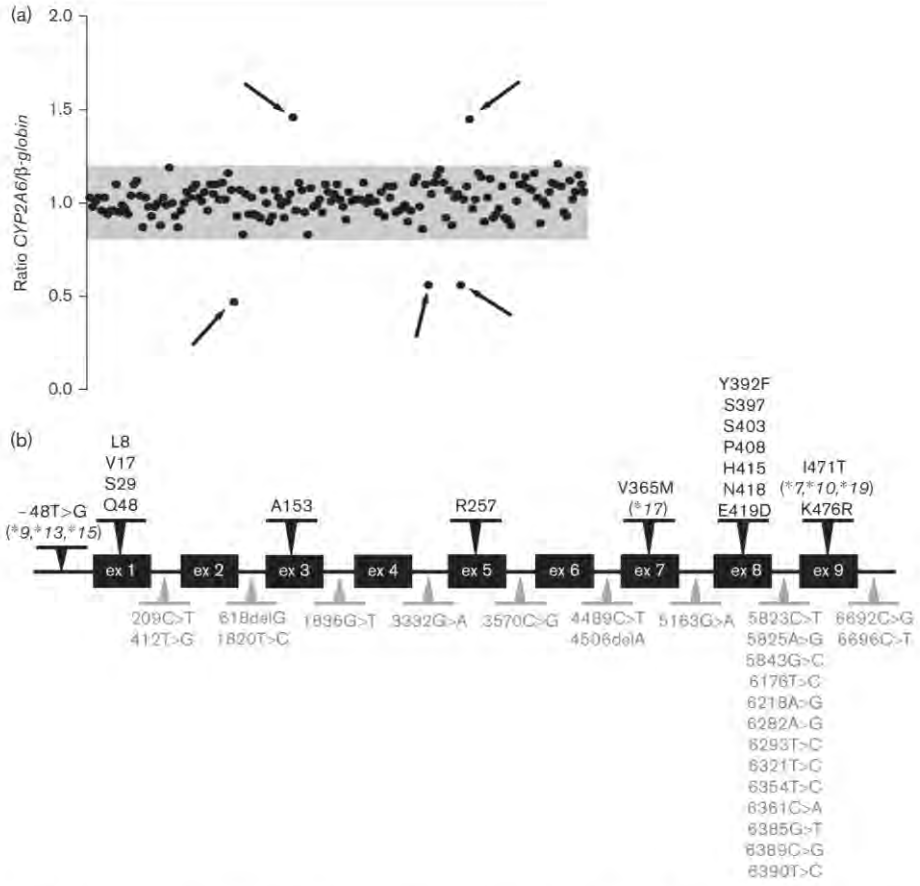
Fig. 2



CYP2A6 genotyping. (a) Gene copy number determination. Each dot represents one individual ($N=169$). The grey box represents the *CYP2A6/β-globin* ratio interval for individuals with two *CYP2A6* copies (ratio average value = 1.02, 95 percentile, 0.87–1.16). Black arrows indicate the individuals heterozygous for a gene deletion (*CYP2A6*4A – F*) (ratio 0.47/0.56/0.56, respectively, from left to right) and individuals heterozygous for a gene duplication (*CYP2A6*1 × 2A – B*) (ratio 1.47/1.45, respectively, from left to right). (b) Single nucleotide polymorphisms (SNPs) identified by resequencing *CYP2A6* in the individuals *CYP2B6* homozygous loss-of-function (LOF) ($n=23$). There is one decrease-of-function (DOF) SNP (−48T>G) in the promoter region (TATA box), 11 synonymous SNPs, three nonsynonymous SNPs and two nonsynonymous DOF SNPs (V365M, I471T) in the exons, and 25 SNPs in the intron–exon boundaries. In the introns and promoter region, SNP position is based on genomic DNA numbering. In the exons, SNP position is based on amino acid numbering. ex, exon.

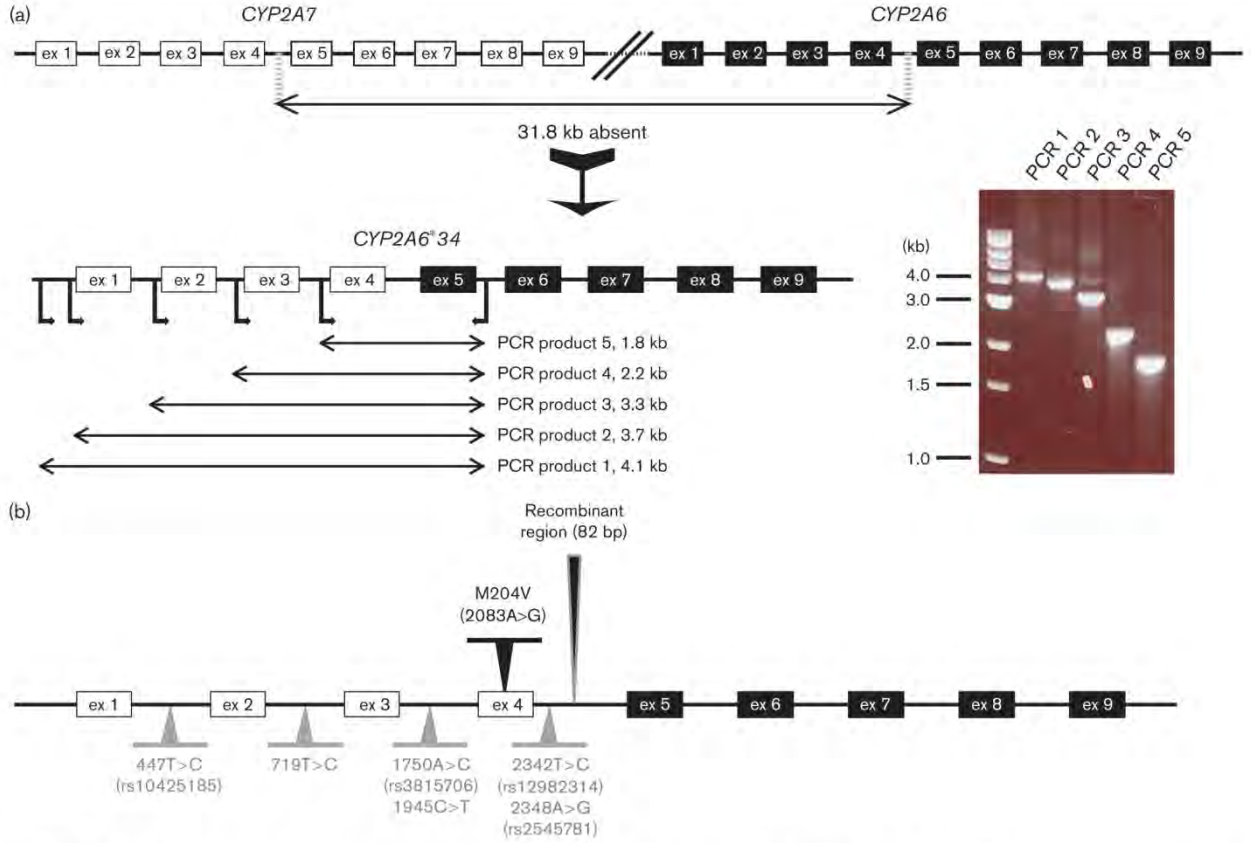
four exons of *CYP2A6*. This new partial gene deletion could not have been identified by gene copy number determination using a probe in exon 7. The new allele results from an unequal crossover in intron 4 between *CYP2A7* and *CYP2A6* (Fig. 3a and b). Primers used for the identification of the new allele are shown in Supplementary Table S4. Further analyses concluded that the patient carried both a known deletion (allele **4A*) on one chromosome, as well as the new allele (*CYP2A6*34*, GenBank accession: EU814898) on the second chromosome. The new allele has 10 amino acid substitutions characterizing the DOF allele *CYP2A6*12* [20] and substitutions R128L and S131A present in the LOF allele *CYP2A6*26* [24], as well as additional nonsynonymous variants. Thus, this partial gene deletion was considered a LOF allele.

Association of *CYP2A6* genotype and efavirenz plasma exposure

Without consideration for CYP2B6 function, *CYP2A6* allele functions seem to have a limited effect on EFV exposure (Fig. 4a). Individuals with reference alleles had a median log10 EFV AUC of 1.67 μg × h/ml, individuals carrying one DOF or LOF allele had a median log10 EFV AUC ranging from 1.64 to 1.68 μg × h/ml ($P=0.29$ vs. reference alleles), whereas individuals carrying two DOF and/or LOF alleles had a median log10 EFV AUC ranging from 1.75 to 2.99 μg × h/ml ($P=0.002$ vs. reference alleles).

On account of the working hypothesis that *CYP2A6* genotype would be clinically relevant among individuals with impaired CYP2B6 metabolism, data were stratified

Fig. 3



*CYP2A6*34* identification and characterization. (a) Identification of a new hybrid allele (*CYP2A6*34*). PCRs were run with forward primers specific to *CYP2A7* and reverse primer specific to *CYP2A6*. (b) Single nucleotide polymorphisms (SNPs) identified by resequencing all exons and intron–exon boundaries of *CYP2A6*34*. White boxes represent exons of *CYP2A7* origin; black boxes represent exons of *CYP2A6* origin. In the introns and promoter region, SNP position is based on genomic DNA numbering. In the exons, SNP position is based on amino acid numbering. ex, exon.

according to the number of *CYP2B6* functional alleles. As this stratification reduced the number of individuals per category, all *CYP2A6* DOF and LOF alleles were referred here as DOF/LOF alleles in order to increase the power (the raw results are shown in Supplementary Table S5). The contribution of *CYP2A6* alleles was particularly relevant among individuals CYP2B6 slow metabolizers. Here, individuals with common *CYP2A6* alleles presented lower median log10 EFV AUC values of 2.18 μg × h/ml, compared with individuals carrying one DOF/LOF allele (2.28 μg × h/ml), and individuals homozygous for a DOF/LOF allele (2.48 μg × h/ml), $P=0.06$ (Fig. 4b).

In vivo metabolite analyses

To support the genotyping results, we assessed the concentration of 8-OH-EFV, 7-OH-EFV and *N*-gln-EFV in different genetic backgrounds *in vivo*. For this analysis, we identified individuals ($n=48$) representative of the various genetic profiles (Supplementary Table S6). Chromatogram profiles of individuals with three different genetic backgrounds are shown in Fig. 5a–c.

CYP2B6 slow metabolizers, when compared with CYP2B6 extensive metabolizers, presented an 11% decrease in the median log10 8-OH-EFV concentration (ng/ml), a 6% decrease in the median log10 7-OH-EFV (a.u.) – consistent with a role of CYP2B6 in this pathway (Fig. 1) – and a 35% increase in the median log10 *N*-gln-EFV (a.u.). The limited decrease in 8-OH-EFV and 7-OH-EFV concentrations was the result of the significant increase of the parent compound. When results were expressed as ratios, we observed a 67% decrease in the median log10 8-OH-EFV/EFV ratio, a 33% decrease in the median log10 7-OH-EFV/EFV ratio and a 5% decrease in the *N*-gln-EFV/EFV ratio.

Carriers of one and two *CYP2A6* DOF/LOF alleles, when compared with CYP2A6 extensive metabolizers, were

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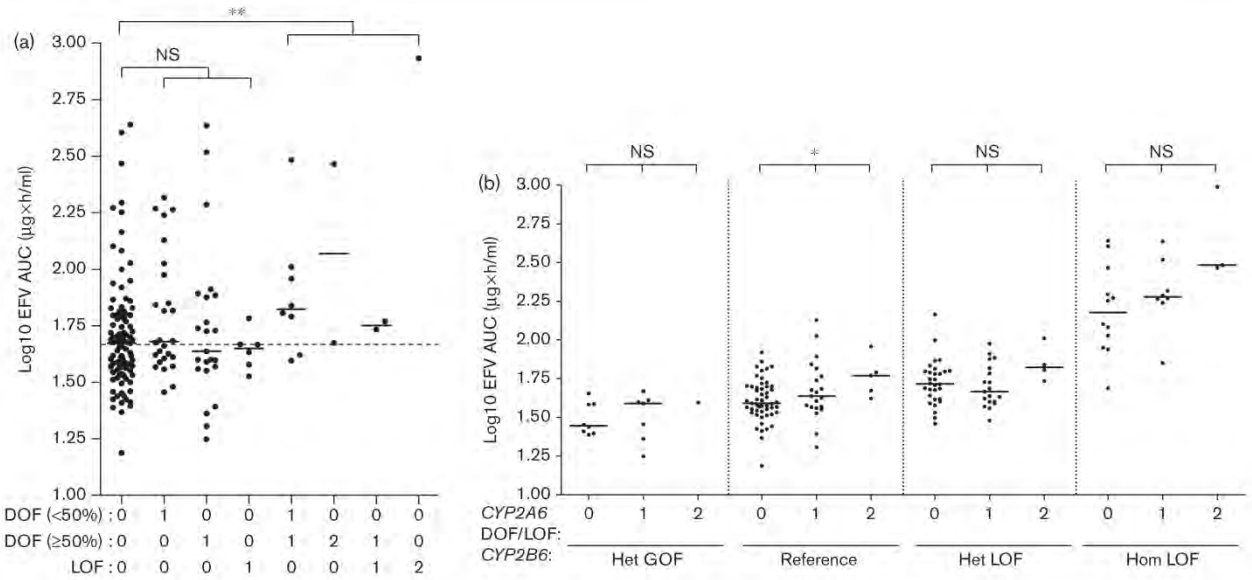
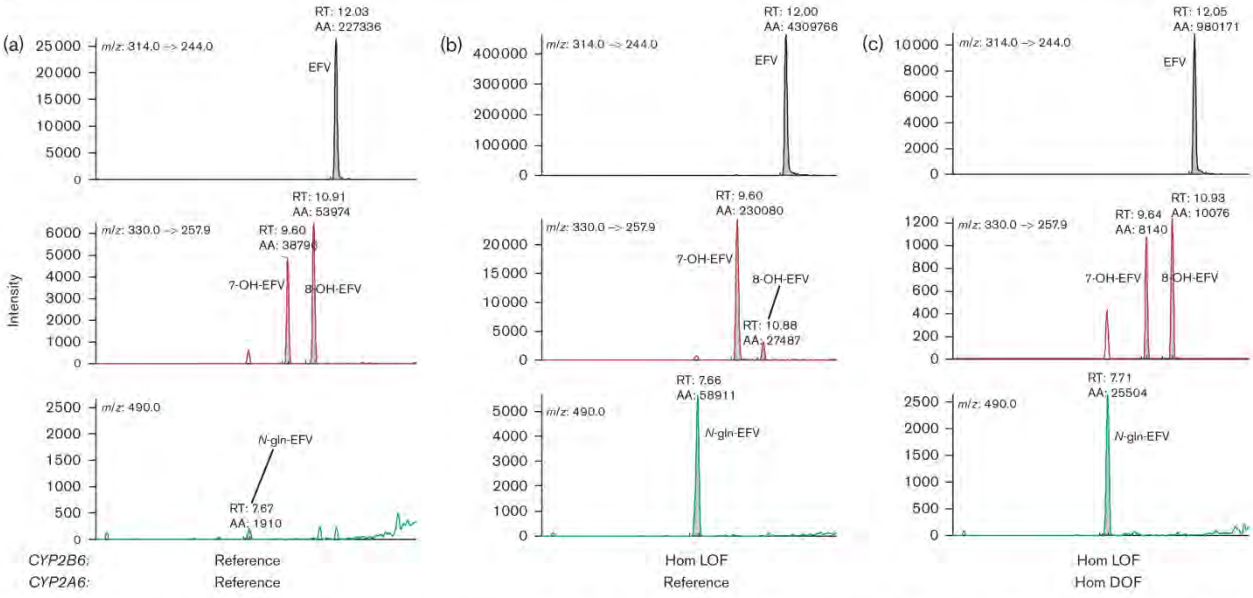
Fig. 4



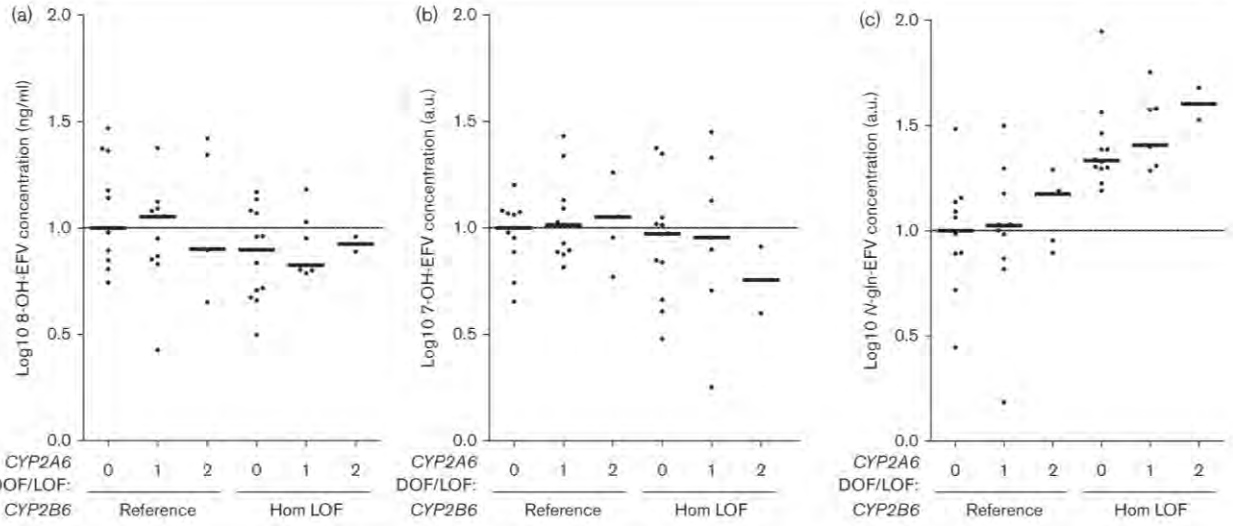
Association of *CYP2A6* genotype and efavirenz (EFV) plasma exposure. (a) Individuals were classified according to their number of *CYP2A6* decrease-of-function (DOF) $<50\%$, DOF $\geq 50\%$ and/or loss-of-function (LOF) alleles. (b) Data were stratified according to *CYP2B6* genotype and according to the number (0, 1 or 2) of *CYP2A6* DOF and/or LOF alleles, all referred to here as DOF/LOF because of the small number of individuals in each category. AUC, area under the curve; GOF, gain-of-function; Het, heterozygous; Hom, homozygous; NS, non significant; Reference, reference alleles. $*P<0.05$, $**P<0.005$.

Fig. 5



Efavirenz (EFV) metabolite profiling. Plasma concentrations of EFV, 7-hydroxy-EFV (7-OH-EFV), 8-hydroxy-EFV (8-OH-EFV) and *N*-glucuronide-EFV (*N*-gln-EFV) were determined by liquid chromatography coupled with triple quadrupole tandem mass spectrometry. Shown are chromatographic profiles of plasma samples from three individuals with representative genotypes. (a) Reference alleles for *CYP2B6* and *CYP2A6*. (b) Homozygous (Hom) loss-of-function (LOF) *CYP2B6* (**6/*6*), and reference alleles for *CYP2A6*. (c) Hom LOF *CYP2B6* (**6/*18*) and Hom decrease-of-function (DOF) *CYP2A6* (**9/*9*). Note that the scale varies for the different profiles. Reference, reference alleles; RT, retention time.

Fig. 6



EFV metabolite concentration in individuals with different *CYP2B6* and *CYP2A6* genetic profiles. EFV metabolite concentrations were stratified according to *CYP2B6* genotype (Reference or Hom LOF) and according to the number (0, 1 or 2) of *CYP2A6* DOF/LOF alleles. (a) 8-OH-EFV concentration (ng/ml). (b) 7-OH-EFV concentration (a.u.). (c) N-gln-EFV concentration (a.u.). The median log10 value for CYP2B6 and CYP2A6 extensive metabolizers is normalized to 1. Reference, reference alleles; DOF/LOF, decrease/loss-of-function; Hom, homozygous.

associated with a 1 and 6% decrease in the median log10 8-OH-EFV concentration (ng/ml), a 1% increase and a 2% decrease in the median log10 7-OH-EFV (a.u.) and a 0 and 3% decrease in the median log10 *N*-gln-EFV (a.u.), respectively.

Data were then stratified according to the number of *CYP2B6* functional alleles. The contribution of *CYP2A6* alleles was more relevant among *CYP2B6* slow metabolizers. In this group, carriers of one and two *CYP2A6* DOF/LOF alleles, when compared with *CYP2A6* extensive metabolizers, presented an 8% decrease and a 3% increase in the median log10 8-OH-EFV concentration (ng/ml), a 2 and 22% decrease in median log10 7-OH-EFV (a.u.) and a 6 and 20% increase in the median log10 *N*-gln-EFV (a.u.), respectively (Fig. 6a–c). These results are consistent with the role of CYP2A6 as an alternative pathway in EFV metabolism, and the role of *N*-glucuronidation in the setting of multiple DOF/LOF in main and accessory hydroxylating pathways.

Discussion

Detailed genetic analysis of EFV metabolic pathways allows a better understanding of the interindividual variability in EFV plasma exposure. It highlights the critical role of the accessory metabolic pathways in limiting drug accumulation in individuals characterized as CYP2B6 slow metabolizers.

We performed a comprehensive assessment of *CYP2A6* genetic variations because of the role of this isoenzyme in the *in vitro* formation of 7-OH-EFV [8]. Analysis included a large number of functional alleles associated with impaired CYP2A6 function, and the assessment of gene copy number. In addition, we identified a new functional genetic variant in this locus through gene resequencing that results from the recombination of *CYP2A7* and *CYP2A6*. Overall, 70 of 169 study participants carried one or more DOF/LOF alleles or copy number variants. The pharmacokinetic data confirmed to a large extent the reported or predicted functional effect of these variants.

Analysis of the primary EFV metabolites *in vivo* showed results consistent with the predicted balance between the main and accessory hydroxylating pathways. LOF *CYP2B6* alleles were associated with a decrease in EFV hydroxy metabolites as previously reported by *in vitro* studies [8], whereas increased levels of *N*-gln-EFV were observed indicating the redirection of the metabolism through this accessory pathway. Among CYP2B6 extensive metabolizers, *CYP2A6* DOF/LOF alleles did not modify EFV 7-hydroxylation because this pathway is still supported by CYP2B6 in the absence of CYP2A6 activity [8]. However, in the presence of impaired CYP2B6 function, *CYP2A6* DOF/LOF alleles were associated with a further decrease in 7-OH-EFV – consistent with the hypothesis that this alternative pathway becomes increasingly relevant in the context of impaired CYP2B6 function – and with a marked increase in *N*-gln-EFV. Analysis of *N*-gln-EFV is particularly reliable because it is

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not further metabolized in humans, and the concentration is not dependent on other isoenzyme genetic profiles [9].

This study is limited by the incomplete understanding of functional variations in other genes involved in EFV metabolism. We could not investigate variations of the gene(s) involved in *N*-glucuronidation because of lack of information on the UDP glucuronosyl transferase isoenzyme responsible of this step, and the large number of members in the family. Although CYP1A2 was reported to play a role in EFV metabolism, leading to 8-OH-EFV [1], report on functional alleles has been mostly limited to Asians (*http://www.cypalleles.ki.se* [30]). There is a paucity of functional alleles in *CYP3A4* despite extensive investigation by many groups over the years [31], and the low frequency of functional polymorphisms found in the coding regions cannot account for the variation observed [32]. In addition, a number of factors may affect CYP3A expression, tissue-specific splicing, variable control of gene transcription by endogenous and exogenous molecules and genetic variations in proteins that regulate CYP3A expression through nuclear hormone receptors [33].

This study indicates that the presence of multiple LOF alleles at both the main (CYP2B6) and accessory (CYP2A6) hydroxylation pathways results in extremely high EFV exposure. The clinical relevance of dual CYP2B6 and CYP2A6 slow metabolism is determined by the frequency of DOF/LOF alleles in various human populations. Indeed, the LOF allele *CYP2B6*6* can reach very high frequencies in the population (up to 26% in Caucasians, 47% in Africans and 18% in Asians) [5,34]. Similarly, the prevalence of *CYP2A6* alleles lacking or showing reduced enzymatic activity is elevated in the various populations (9% in Caucasians, 22% in Africans and up to 50% in Asians) ([35] and this study). Therefore, dual CYP2B6 and CYP2A6 slow metabolism will occur at significant frequency in various populations, leading to extremely high EFV exposure in a relevant proportion of individuals. A formal population pharmacokinetic–pharmacogenetic analysis of the data is presented in a separate publication [36].

The approach of identifying functional alleles in multiple metabolic pathways, in combination with metabolite assessment *in vivo* can be of general interest for the validation of *in vitro* studies for other drugs, and to complement pharmacological analysis during drug development.

Supplementary data

Supplementary data are available at *The Pharmacogenetics and Genomics Journal Online* (*www.pharmacogeneticsandgenomics.com*).

Acknowledgements

This study has been financed in the framework of the Swiss HIV Cohort Study, supported by the Swiss National Science Foundation (grants 3345CO-100935 and 324700-112655).

The members of the Swiss HIV Cohort Study are M. Battegay, E. Bernasconi, J. Böni, H.C. Bucher, Ph. Bürgisser, A. Calmy, S. Cattacin, M. Cavassini, R. Dubs, M. Egger, L. Elzi, M. Fischer, M. Flepp, A. Fontana, P. Francioli (President of the SHCS, Centre Hospitalier Universitaire Vaudois, CH-1011- Lausanne), H. Furrer (Chairman of the Clinical and Laboratory Committee), C. Fux, M. Gorgievski, H. Günthard (Chairman of the Scientific Board), H. Hirsch, B. Hirschel, I. Hösli, Ch. Kahlert, L. Kaiser, U. Karrer, C. Kind, Th. Klimkait, B. Ledergerber, G. Martinetti, B. Martinez, N. Müller, D. Nadal, M. Opravil, F. Paccaud, G. Pantaleo, A. Rauch, S. Regenass, M. Rickenbach (Head of Data Center), C. Rudin (Chairman of the Mother and Child Substudy), P. Schmid, D. Schultze, J. Schüpbach, R. Speck, P. Taffé, A. Telenti, A. Trkola, P. Vernazza, R. Weber, S. Yerly.

References

1 Ward BA, Gorski JC, Jones DR, Hall SD, Flockhart DA, Desta Z. The cytochrome P450 2B6 (CYP2B6) is the main catalyst of efavirenz primary and secondary metabolism: implication for HIV/AIDS therapy and utility of efavirenz as a substrate marker of CYP2B6 catalytic activity. *J Pharmacol Exp Ther* 2003; **306**:287–300.

2 Marzolini C, Telenti A, Decosterd LA, Greub G, Biollaz J, Buclin T. Efavirenz plasma levels can predict treatment failure and central nervous system side effects in HIV-1-infected patients. *AIDS (London)* 2001; **15**:71–75.

3 Rotger M, Colombo S, Furrer H, Bleiber G, Buclin T, Lee BL, *et al.* Influence of CYP2B6 polymorphism on plasma and intracellular concentrations and toxicity of efavirenz and nevirapine in HIV-infected patients. *Pharmacogenet Genomics* 2005; **15**:1–5.

4 Rotger M, Csajka C, Telenti A. Genetic, Ethnic, and Gender Differences in the Pharmacokinetics of Antiretroviral Agents. *Curr HIV/AIDS Rep* 2006; **3**:118–125.

5 Rotger M, Tegude H, Colombo S, Cavassini M, Furrer H, Decosterd L, *et al.* Predictive value of known and novel alleles of CYP2B6 for efavirenz plasma concentrations in HIV-infected individuals. *Clin Pharmacol Ther* 2007; **81**:557–566.

6 Tsuchiya K, Gatanaga H, Tachikawa N, Teruya K, Kikuchi Y, Yoshino M, *et al.* Homozygous CYP2B6*6 (Q172H and K262R) correlates with high plasma efavirenz concentrations in HIV-1 patients treated with standard efavirenz-containing regimens. *Biochem Biophys Res Commun* 2004; **319**:1322–1326.

7 Haas DW, Ribaudo HJ, Kim RB, Tierney C, Wilkinson GR, Gulick RM, *et al.* Pharmacogenetics of efavirenz and central nervous system side effects: an Adult AIDS Clinical Trials Group study. *AIDS (London)* 2004; **18**:2391–2400.

8 Desta Z, Saussele T, Ward B, Blievernicht J, Li L, Klein K, *et al.* Impact of CYP2B6 polymorphism on hepatic efavirenz metabolism in vitro. *Pharmacogenomics* 2007; **8**:547–558.

9 Mutlib AE, Chen H, Nemeth GA, Markwalder JA, Seitz SP, Gan LS, *et al.* Identification and characterization of efavirenz metabolites by liquid chromatography/mass spectrometry and high field NMR: species differences in the metabolism of efavirenz. *Drug Metab Dispos* 1999; **27**:1319–1333.

10 Xu C, Goodz S, Sellers EM, Tyndale RF. CYP2A6 genetic variation and potential consequences. *Adv Drug Deliv Rev* 2002; **54**:1245–1256.

11 Csajka C, Marzolini C, Fattinger K, Decosterd LA, Fellay J, Telenti A, *et al.* Population pharmacokinetics and effects of efavirenz in patients with human immunodeficiency virus infection. *Clin Pharmacol Ther* 2003; **73**:20–30.

12 Von Richter O, Pitarque M, Rodriguez-Antona C, Testa A, Mantovani R, Oscarson M, *et al.* Polymorphic NF-Y dependent regulation of human nicotine C-oxidase (CYP2A6). *Pharmacogenetics* 2004; **14**:369–379.

13 Fukami T, Nakajima M, Yamanaka H, Fukushima Y, McLeod HL, Yokoi T. A novel duplication type of CYP2A6 gene in African-American population. *Drug Metab Dispos* 2007; **35**:515–520.

14 Yamano S, Tatsuno J, Gonzalez FJ. The CYP2A3 gene product catalyzes coumarin 7-hydroxylation in human liver microsomes. *Biochemistry* 1990; **29**:1322–1329.

15 Nunoya K, Yokoi T, Takahashi Y, Kimura K, Kinoshita M, Kamataki T. Homologous unequal cross-over within the human CYP2A gene cluster as a mechanism for the deletion of the entire CYP2A6 gene associated with the poor metabolizer phenotype. *J Biochem (Tokyo)* 1999; **126**:402–407.

16 Nunoya KI, Yokoi T, Kimura K, Kainuma T, Satoh K, Kinoshita M, *et al.* A new CYP2A6 gene deletion responsible for the in vivo polymorphic metabolism of (+)-cis-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one hydrochloride in humans. *J Pharmacol Exp Ther* 1999; **289**:437–442.

17 Oscarson M, McLellan RA, Gullsten H, Agundez JA, Benitez J, Rautio A, *et al.* Identification and characterisation of novel polymorphisms in the CYP2A locus: implications for nicotine metabolism. *FEBS Lett* 1999; **460**:321–327.

18 Ariyoshi N, Sawamura Y, Kamataki T. A novel single nucleotide polymorphism altering stability and activity of CYP2a6. *Biochem Biophys Res Commun* 2001; **281**:810–814.

19 Pitarque M, von Richter O, Oke B, Berkkan H, Oscarson M, Ingelman-Sundberg M. Identification of a single nucleotide polymorphism in the TATA box of the CYP2A6 gene: impairment of its promoter activity. *Biochem Biophys Res Commun* 2001; **284**:455–460.

20 Oscarson M, McLellan RA, Asp V, Ledesma M, Bernal Ruiz ML, Sinues B, *et al.* Characterization of a novel CYP2A7/CYP2A6 hybrid allele (CYP2A6*12) that causes reduced CYP2A6 activity. *Hum Mutat* 2002; **20**:275–283.

21 Fukami T, Nakajima M, Yoshida R, Tsuchiya Y, Fujiki Y, Katoh M, *et al.* A novel polymorphism of human CYP2A6 gene CYP2A6*17 has an amino acid substitution (V365M) that decreases enzymatic activity in vitro and in vivo. *Clin Pharmacol Ther* 2004; **76**:519–527.

22 Oscarson M, McLellan RA, Gullsten H, Yue QY, Lang MA, Bernal ML, *et al.* Characterisation and PCR-based detection of a CYP2A6 gene deletion found at a high frequency in a Chinese population. *FEBS Lett* 1999; **448**:105–110.

23 Nunoya K, Yokoi T, Kimura K, Inoue K, Kodama T, Funayama M, *et al.* A new deleted allele in the human cytochrome P450 2A6 (CYP2A6) gene found in individuals showing poor metabolic capacity to coumarin and (+)-cis-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one hydrochloride (SM-12502). *Pharmacogenetics* 1998; **8**:239–249.

24 Mwenifumbo JC, Al Koudsi N, Ho MK, Zhou Q, Hoffmann EB, Sellers EM, *et al.* Novel and established CYP2A6 alleles impair in vivo nicotine metabolism in a population of Black African descent. *Hum Mutat* 2008; **29**:679–688.

25 Rao Y, Hoffmann E, Zia M, Bodin L, Zeman M, Sellers EM, *et al.* Duplications and defects in the CYP2A6 gene: identification, genotyping, and in vivo effects on smoking. *Mol Pharmacol* 2000; **58**:747–755.

26 Gonzalez E, Kulkarni H, Bolivar H, Mangano A, Sanchez R, Catano G, *et al.* The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. *Science* 2005; **307**:1434–1440.

27 Colombo S, Beguin A, Telenti A, Biollaz J, Buclin T, Rochat B, *et al.* Intracellular measurements of anti-HIV drugs indinavir, amprenavir, saquinavir, ritonavir, nelfinavir, lopinavir, atazanavir, efavirenz and nevirapine in peripheral blood mononuclear cells by liquid chromatography coupled to tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005; **819**:259–276.

28 Kiyotani K, Yamazaki H, Fujieda M, Iwano S, Matsumura K, Satarug S, *et al.* Decreased coumarin 7-hydroxylase activities and CYP2A6 expression levels in humans caused by genetic polymorphism in CYP2A6 promoter region (CYP2A6*9). *Pharmacogenetics* 2003; **13**:689–695.

29 Yoshida R, Nakajima M, Nishimura K, Tokudome S, Kwon JT, Yokoi T. Effects of polymorphism in promoter region of human CYP2A6 gene (CYP2A6*9) on expression level of messenger ribonucleic acid and enzymatic activity in vivo and in vitro. *Clin Pharmacol Ther* 2003; **74**:69–76.

30 Ingelman-Sundberg M, Sim SC, Gomez A, Rodriguez-Antona C. Influence of cytochrome P450 polymorphisms on drug therapies: pharmacogenetic, pharmacoepigenetic and clinical aspects. *Pharmacol Ther* 2007; **116**:496–526.

31 Burk O, Wojnowski L. Cytochrome P450 3A and their regulation. *Naunyn Schmiedebergs Arch Pharmacol* 2004; **369**:105–124.

32 Eiselt R, Domanski TL, Zibat A, Mueller R, Presecan-Siedel E, Hustert E, *et al.* Identification and functional characterization of eight CYP3A4 protein variants. *Pharmacogenetics* 2001; **11**:447–458.

33 Lamba JK, Lin YS, Schuetz EG, Thummel KE. Genetic contribution to variable human CYP3A-mediated metabolism. *Adv Drug Deliv Rev* 2002; **54**:1271–1294.

34 Klein K, Lang T, Saussele T, Barbosa-Sicard E, Schunck WH, Eichelbaum M, *et al.* Genetic variability of CYP2B6 in populations of African and Asian origin: allele frequencies, novel functional variants, and possible implications for anti-HIV therapy with efavirenz. *Pharmacogenet Genomics* 2005; **15**:861–873.

35 Nakajima M, Fukami T, Yamanaka H, Higashi E, Sakai H, Yoshida R, *et al.* Comprehensive evaluation of variability in nicotine metabolism and CYP2A6 polymorphic alleles in four ethnic populations. *Clin Pharmacol Ther* 2006; **80**:282–297.

36 Arab-Alameddine M, di Iulio J, Buclin T, Rotger M, Lubomirov R, Cavassini M, *et al.* Pharmacogenetic-based population pharmacokinetic analysis of efavirenz in HIV-1 infected individuals. *Clin Pharmacol Ther* [in press].

4.1.1.2. Original article

Pharmacogenetics-Based Population Pharmacokinetic Analysis of Efavirenz in HIV-1-Infected Individuals

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Clinical Pharmacology and Therapeutics 2009; 85(5): 485-494

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Drafting of the manuscript: M. Arab-Alameddine, C. Csajka, T. Buclin

Critical revision of the manuscript for important intellectual content: all authors

Detailed personal contribution

I was one of the main persons in charge of the project. I notably participated in the study concept and design, sample recruitment, data analyses and interpretation, as well as in the genetic characterization of individuals included in the study by performing:

1. Comprehensive *CYP2A6* characterization, including:
    - *CYP2A6* exon-intron boundaries resequencing (N=23)
    - Genotyping of 12 *CYP2A6* alleles (N=146)
    - *CYP2A6* copy number variation assessment (N=169)

2. *CYP3A* characterization, including:
    - Genotyping of 8 *CYP3A* alleles (N=169)

Comments on the article:

We demonstrated in our previous work the determinant impact of genetic variations in genes encoding enzymes involved in EFV accessory metabolic pathways.

In this study, we performed a population pharmacokinetic analysis using not only demographic and environmental influences as covariates, but also the genetic data of the enzymes involved in EFV main (CYP2B6) and accessory (CYP2A6 and CYP3A family) hydroxylating metabolic pathways, in order to better characterize the interplay between these different influences and quantify their contribution to the interindividual variability in EFV pharmacokinetics (PK).

We could demonstrate that while *CYP2B6* functional alleles accounted for the majority of EFV PK interindividual variability, genetic variations in EFV accessory metabolic pathways influenced EFV disposition as well. This study provides new insights in the understanding of the mechanisms of genetic influences and can be used to build up rational dosage guidance according to multiple genetic polymorphisms.

Thus, this study formalizes, in a population pharmacokinetics/pharmacogenetics analysis, the relevance of principal and accessory pathways to EFV metabolism.

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Besides CYP2B6, other polymorphic enzymes contribute to efavirenz (EFV) interindividual variability. This study was aimed at quantifying the impact of multiple alleles on EFV disposition. Plasma samples from 169 human immunodeficiency virus (HIV) patients characterized for *CYP2B6, CYP2A6*, and *CYP3A4/5* allelic diversity were used to build up a population pharmacokinetic model using NONMEM (non-linear mixed effects modeling), the aim being to seek a general approach combining genetic and demographic covariates. Average clearance (CL) was 11.3 l/h with a 65% interindividual variability that was explained largely by *CYP2B6* genetic variation (31%). *CYP2A6* and *CYP3A4* had a prominent influence on CL, mostly when *CYP2B6* was impaired. Pharmacogenetics fully accounted for ethnicity, leaving body weight as the only significant demographic factor influencing CL. Square roots of the numbers of functional alleles best described the influence of each gene, without interaction. Functional genetic variations in both principal and accessory metabolic pathways demonstrate a joint impact on EFV disposition. Therefore, dosage adjustment in accordance with the type of polymorphism (*CYP2B6, CYP2A6*, or *CYP3A4*) is required in order to maintain EFV within the therapeutic target levels.

Efavirenz (EFV), a non-nucleoside reverse transcriptase inhibitor, is widely used in combination with nucleoside inhibitors as first-line treatment of type I human immunodeficiency virus (HIV-1) infection. It is generally prescribed at a fixed dosage of 600 mg daily, despite the presence of a marked interindividual variability in tendency to produce elevated plasma drug concentration levels[1–3] that have been shown to be associated with central nervous system toxicity.[4–6]

EFV is metabolized primarily by *CYP2B6* and, to a lesser extent, by accessory pathways involving *CYP2A6*, *CYP3A4/3A5*, and uridine-glucuronyl-transferases.[7–9] Several studies have shown that *CYP2B6* is highly polymorphic and that genetic variations play an important part in EFV plasma concentration variability.[5,10–15] Genetic polymorphisms of *CYP3A4/3A5* have also been associated with higher EFV exposure,[6] but the influence of the *CYP2A6* polymorphism on EFV pharmacokinetics has not yet been characterized. Considering the increasing number of allelic variants that are being described and the resulting complexity of allele combinations that could influence EFV elimination, we conducted a population pharmacokinetic analysis in HIV-1-infected individuals fully characterized for *CYP2B6*, *CYP2A6*, and *CYP3A4/A5* genetic variations. Our main areas of focus were: (i) to assess the relative contributions of multiple functional alleles involved in EFV elimination along with other demographic or environmental factors, (ii) to characterize the nature of the relationship between individual allelic constitution and EFV disposition, and (iii) to explore models for gene–gene interactions that could lead to a better understanding of the interrelationships of specific enzymes involved in EFV elimination.

RESULTS

In total, 393 plasma samples were collected from 169 individuals. Concentration measurements ranged between 100 and 59,400 ng/ml. A one-compartment model with first-order absorption from the gastrointestinal tract fitted the data

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Received 28 August 2008; accepted 1 December 2008; advance online publication 18 February 2009. doi:10.1038/clpt.2008.271

appropriately. Average clearance (CL) was 11.3 l/h with an interindividual variability of 65%, the volume of distribution (V) was 388 l, and the absorption constant (k_a) was 0.62 h^{-1}. The assignment of interindividual variability on either V or k_a did not improve the fit (change in objective function (ΔOF) = 0.0).

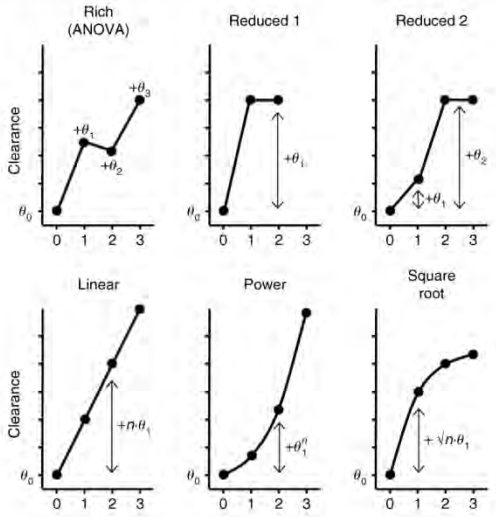

Figure 1 Various parameter models were tested to describe the level of oral clearance (*Y*-axis) as a function of the number of functional alleles of an enzyme (*X*-axis): 0 = Hom-LOF, 1 = Het-LOF, 2 = Hom-Ref, 3 = Het-GOF (a specific parameter for Het-GOF, is required only with *CYP2B6*). ANOVA, analysis of variance; GOF, gain of function; Het, heterozygous; Hom, homozygous; LOF, loss of function; Ref, reference allele.

Demographic analyses

Body weight and black ethnicity influenced CL, as did gender, age and, to a lesser extent, height. Co-medications were shown to have no significant influence on EFV pharmacokinetics. A multivariable combination of demographic factors revealed that body weight accounted for the effect of height, age, and gender while explaining 3% of CL variability, and it was the only demographic factor influencing CL besides black ethnicity, which remained statistically significant beyond body weight and reduced the variability in CL by another 3%.

Univariate genotype analyses

The influence of *CYP2B6*, *CYP2A6*, *CYP3A4/AA5* functional alleles on EFV CL was first tested in single-gene analyses, in which the allelic variants (Hom-LOF, Het-LOF, and Hom-Ref plus Het-GOF for *CYP2B6*) were entered into the model as covariates that partitioned individuals based on their genetic constitution.

Genetic variation of *CYP2B6* had by far the most salient impact on CL. Several competing models were tested, as depicted in Figure 1. The richest possible model, which assigned a separate fixed effect to each of the *CYP2B6* allelic variants (Eqs. 1/1a), markedly improved the fit and explained 31% of the 65% interindividual variability on CL. The average CL was 2.8 l/h in the Hom-LOF group and 10.8, 13.3, and 18.8 l/h in individuals carrying Het-LOF, Hom-Ref, and Het-GOF alleles, respectively. A series of reduced models showed CL to be statistically different among all *CYP2B6* groups (ΔOF = +9 and ΔOF = +10 for model reduced 1 and 2). Competing simplified models were tried to

Table 1 Functional alleles evaluated in the study and genotype-based activity score classification

Functional alleles					
Functional consequence	**CYP2B6 alleles**	**CYP2A6 alleles**	**CYP3A4 alleles**	**CYP3A5 alleles**	
Loss of function (LOF)	*11, *15, *28	*2, *4		*3, *6, *7, *10, *11	
Diminished function (DOF)	*6, *18, *27, *29	*1H, *1J, *5, *7, *9, *10, *12, *13, *15, *17, *19, *34	rs4646437 *1B	—	
>25%		*5, *7, *9, *10, *12, *13, *15, *17, *19, *34	—	—	
<25%		*1H, *1J	—	—	
Reference	*1, *2, *3, *5, *17	*1	*1	*1	
Gain of function (GOF)	*4, *22	*1X2	—	—	
Genotypes and activity score classification					
Score A	**Alleles (allele 1/allele 2)**	**Score B**	**Alleles (allele 1/allele 2)**	**Score C***	**Alleles (allele 1/allele 2)**
0	LOF/LOF LOF/DOF DOF/DOF	0	LOF/LOF	0	LOF/LOF
1	Ref/LOF Ref/DOF	0.25	LOF/DOF	0.25	LOF/DOF
2	Ref/Ref	0.5	Ref/LOF DOF/DOF	0.5	Ref/LOF
3	Ref/GOF	1	Ref/LOF	0.75	DOF/DOF
		1.5	Ref/DOF	1	Ref/DOF >25%
		2	Ref/Ref	1.5	Ref/DOF <25%
				2	Ref/Ref

DOF, diminished function; GOF, gain of function; LOF, loss of function; Ref, reference allele.

*Only for CYP2A6 alleles.

estimate CL as a function of the number of functional alleles, as defined by the activity score A (Table 1) and were compared with the richest model (Eqs. 1/1a); the use of a linear model (Eq. 2) or a power function model (Eq. 3) did not fit the data appropriately (ΔOF = +18.5 and +58.8, respectively). As interim explorations showed CL to be modestly reduced by ~25% in Het-LOF carriers but cut down by 75% in Hom-LOF individuals, a square root function model achieved the best fit using either an additive (Eq. 4) or a proportional model (ΔOF = −167.7). The recourse to alternative activity scores B for *CYP2B6*, allowing for the distinction between loss and decrease of function alleles, did not better characterize the genotype–phenotype relationships using Eq. 2, 3, or 4 (ΔOF >+8.2).

The assignment of *CYP2A6* allelic variants on CL using the richest model (Eqs. 1/1a) improved the fits (ΔOF = −7.9) and decreased by 1% the overall variability on CL. The average CL was 7.0, 10.8, and 12.1 l/h in Hom-LOF, Het-LOF, and Hom-Ref individuals, respectively. The difference in CL between Hom-Ref and Het-LOF individuals was not significant (ΔOF = −1.0). The description of the relationship between CL and the functional score, using either linear (Eq. 2) or power (Eq. 3) models, did not fit the data adequately when compared with the richest model (ΔOF >+6.8), whereas it was again best characterized using a square root function (Eq. 4) that provided a fit almost identical to that of the rich model (ΔOF = −7.8). Here too, the model integrating partial activity levels (scores B/C) did not improve data description (ΔOF = −0.4 for score B and −0.1 for score C).

The impact of *CYP3A4* on EFV CL was tested using two alleles associated with changes in function, *CYP3A4*1B* and *CYP3A4_* rs4646437. The rich model (Eqs. 1/1a) showed that both alleles influenced CL to a significant extent (ΔOF = −25.4 for *CYP3A4_* rs4646437, −10.4 for *CYP3A4*1B*). CL in Hom-LOF, Het-LOF, and Hom-Ref carriers were, respectively, 5.1, 10.3, and 11.9 l/h for *CYP3A4*1B* and 3.7, 10.7, and 12.3 l/h for *CYP3A4_* rs4646437 alleles. After inclusion of *CYP3A4*1B* and *CYP3A4_* rs4646437 alleles in the model, interindividual variability in CL dropped from 65 to 62 and 59%, respectively. The differences in CL between Hom-Ref and Het-LOF individuals were not significant, both for **1B* and for rs4646437 (ΔOF = −1.4 for *CYP3A4_* rs4646437 and −0.9 for *CYP3A4*1B*). When compared with the richest model, linear and power function models (Eqs. 2/3) did not fit the data adequately (ΔOF >+8.0); square root models (Eq. 4) described the data best (ΔOF = −10.3 for *CYP3A4*1B* and −24.8 for *CYP3A4_* rs4646437).

The influence of *CYP3A5* functional alleles on CL was shown to be small but significant, using the rich model (Eqs. 1/1a, ΔOF = −8.1), with a residual 64% interindividual variability. The difference in CL between individuals with Het-LOF allele and those with Hom-Ref allele was not significant (ΔOF = −0.0). None of the above models (Eqs. 2/3) could characterize the relationship between CL and *CYP3A5* allele variants better than the square root model could (Eq. 4, ΔOF = −8.2).

The effect of black ethnicity remained a statistically influencing covariate on CL in addition to genetic variation, causing an additional 25–40% decrease in CL when associated with functional alleles (ΔOF compared to *CYP2B6* = −8.1, *CYP2A6* = −9.9, *CYP3A4*1B* = −4.0, and *CYP3A5* = −7.8), except for *CYP3A4_* rs4646437 (ΔOF = −0.4), which was present in most of the black individuals, thus limiting the power to detect any association.

Gene–gene interaction analyses

The joint influence of functional alleles on EFV CL was first tested through the conjunction of *CYP2B6* with each of the other *CYP* alleles in dual-gene models, to finally build up the model including all genetic variables having influence on CL. The richest model (Eq. 5) characterizing the joint influence of *CYP2B6* and *CYP2A6*, using a fixed effect parameter for each allelic combination, suggested an additional contribution of *CYP2A6* in EFV elimination (ΔOF = −20 as compared to the final model for *CYP2B6*, Eq. 4). Competitive models were developed based on a functional score A (Table 1) using square root function models. The models evaluating the contribution of *CYP2A6* variation, whether on each *CYP2B6* genotypic group separately (Eq. 6) or through the use of a single parameter estimate across all *CYP2B6* allelic variants (Eq. 7), fitted the data with similar adequacy (ΔOF >−16), resulting in an absolute increase in CL of 1.2 and 1.7 l/h in *CYP2A6* Het-LOF and Hom-Ref individuals, respectively, as compared to Hom-LOF carriers. The contribution of *CYP2A6* functional alleles was more prominent in *CYP2B6* Hom-LOF carriers, in whom the relative change in CL was estimated to be 44% per active allele as compared to an 11% change in Hom-Ref individuals. The joint gene influence could be further described by simply adding *CYP2B6* and *CYP2A6* square root functions (Eq. 8, ΔOF = −12). This model was not considered statistically different from previous models (Eqs. 6/7), considering the reduced degrees of freedom and the loss of fit from the richest possible model (Eq. 5, ΔOF = +8). The introduction of a single interaction factor (Eq. 9) to evaluate some hyper- or hypo-additive trend did not produce any significant change (ΔOF = +3).

The same paradigm was successfully applied to characterize the other gene–gene interactions. The rich model (Eq. 5) characterizing the joint influence of *CYP2B6* with the *CYP3A4*1B* or *CYP3A4* rs4646437 alleles suggested an additive effect of both genes on EFV CL (ΔOF = −14 for **1B*, −25 for rs4646437). Reduced models integrating square root functions of *CYP3A4* activity scores on *CYP2B6* stratified by allelic variation (Eq. 6) described the data appropriately (ΔOF = −13 for **1B*, −18 for rs_4646437), and no deterioration of the fit was observed when allowing a single parameter estimate (Eq. 7) for the effect of *CYP3A4* across all *CYP2B6* allelic variants (ΔOF = +1.0 for **1B*, 0.0 for rs_4646437 as compared to Eq. 6). The additive contribution of *CYP3A4* on CL was 1.1 and 1.5 l/h in **1B* Het-LOF and Hom-Ref carriers, respectively, and 1.4 and 1.9 l/h in rs_4646437 Het-LOF and Hom-Ref carriers, respectively. CL increased by 40 (**1B*) and 48% (rs_4646437) per active allele of *CYP2B6* and *CYP3A4* as compared to the respective Hom-LOF. Further characterization of the joint contribution of *CYP2B6* and *CYP3A4* on EFV CL, using a mere addition of square root functions (Eq. 8), fitted the data appropriately, the loss of fit in comparison with previous models (Eqs. 5/6/7) being not significant (ΔOF = −9.0

Table 2 Summary of the key models used to examine the influence of demographic and genetic covariates on EFV clearance

Step 1	Demographic model	Model	θ_0	θ_1	θ_2	θ_3	ΔOF	*P*
	Body weight (BW)	$CL = \theta_0 \cdot (1 + \theta_1 \cdot BW)$	11.5	1.2			−25.6	**
	Height	$CL = \theta_0 \cdot (1 + \theta_1 \cdot Hgt)$	11.2	2.3			−4.2	*
	Age	$CL = \theta_0 \cdot (1 + \theta_1 \cdot age)$	12	0.7			−5.7	*
	Sex (M = 0, F = 1)	$CL = \theta_0 \cdot (1 + \theta_1 \cdot sex)$	12	0.2			−4.1	*
	Race	$CL = \theta_0(1 - q) + \theta_1 \cdot q$						
	Black (q = 1) vs. others (q = 0)		11.8	6.25			−13.2	**
	White (q = 1) vs. others (q = 0)		12	7.1			−7.7	**
	Hispanic (q = 1) vs. others (q = 0)		15.1	11.2			−0.9	NS
	Asian (q = 1) vs. others (q = 0)		11.3	9.8			−0.13	NS
	Other ARV							
	Ritonavir (RTV)	$\theta_0 \cdot (1 + \theta_1 \cdot RTV)$	11.3	0.01			0.2	NS
	Zidovudine (AZT)	$\theta_0 \cdot (1 + \theta_1 \cdot AZT)$	10.4	0.12			−1.1	NS
	Lamivudine	$\theta_0 \cdot (1 + \theta_1 \cdot 3TC)$	10.6	0.09			−0.6	NS
	NRTI in general	$\theta_0 \cdot (1 + \theta_1 \cdot NRTI)$	8.6	0.3			−1.8	NS
	PI in general	$\theta_0 \cdot (1 + \theta_1 \cdot PI)$	11.3	0.01			0.6	NS
Step 2	**Genotype-variant analysis**	**Model**	**CL_0**	**θ_1**	**θ_2**	**θ_3**	**ΔOF**	***P***
CYP2B6								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref, I_3: GOF	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2 + \theta_3 I_3$	2.8	10.8	13.3	18.8	−171	**
Reduced 1	I_1: Het-LOF or Hom-Ref, I_3: GOF	$CL = CL_0 + \theta_1 I_1 + \theta_3 I_3$	2.8	12.2	—	18.9	−162	**,[a]
Reduced 2	I_1: Het-LOF, I_2: Hom-Ref or GOF	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2$	2.8	10.8	14.2		−161	**,[a]
Eq. 2	n = 0, 1, 2, 3	$CL = CL_0 + \theta_1 \cdot n$	3.11	5.99			−152	**,[a]
Eq. 3		$CL = CL_0 + \theta_1^n$	5.03	2.9			−76	**,[a]
Eq. 4		$CL = CL_0 + \theta_1 \cdot \sqrt{n}$	2.8	7.8			−168	NS[a]
CYP2A6								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2$	7.0	10.8	12.1		−7.9	*
Reduced 1	I_1: Het-LOF or Hom-Ref	$CL = CL_0 + \theta_1 I_1$	7.0	11.7			−6.9	NS[a]
Eq. 2	n = 0, 1, 2	$CL = CL_0 + \theta_1 \cdot n$	7.75	2.31			−6.8	NS[a]
Eq. 3		$CL = CL_0 + \theta_1^n$	7.8	2.11			−5.3	NS[a]
Eq. 4		$CL = CL_0 + \theta_1 \cdot \sqrt{n}$	7.0	3.63			−7.8	NS[a]
CYP3A4 rs4646437								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2$	3.7	10.7	12.3		−25.4	**
Reduced 1 Eq. 1	I_1: Het-LOF or Hom-Ref	$CL = CL_0 + \theta_1 I_1$	3.7	11.9			−24	NS[a]
Eq. 2	n = 0, 1, 2	$CL = CL_0 + \theta_1 \cdot n$	4.84	4.06			−20	*,[a]
Eq. 3		$CL = CL_0 + \theta_1^n$	6.2	2.5			−12.9	*,[a]
Eq. 4		$CL = CL_0 + \theta_1 \cdot \sqrt{n}$	3.89	6.1			−24.8	NS[a]
*CYP3A4*1B*								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-Ref	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2$	5.1	10.3	11.9		−10.4	**
Reduced 1 Eq. 1	I_1: Het-LOF or Hom-Ref	$CL = CL_0 + \theta_1 I_1$	5.1	11.7			−9.5	NS[a]
Eq. 2	n = 0, 1, 2	$CL = CL_0 + \theta_1 \cdot n$	6	3.6			−9.0	NS[a]
Eq. 3		$CL = CL_0 + \theta_1^n$	6.4	2.3			−7.0	NS[a]
Eq. 4		$CL = CL_0 + \theta_1 \cdot \sqrt{n}$	5.2	4.8			−10.4	NS[a]
CYP3A5								
Rich: Eq. 1	I_1: Het-LOF, I_2: Hom-LOF	$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2$	4.4	10.3	11.8		−8.4	**
Reduced 1 Eq. 1	I_1: Het-LOF or Hom-LOF	$CL = CL_0 + \theta_1 I_1$	4.4	11.5			−7.4	NS[a]
Eq. 2	n = 0, 1, 2	$CL = CL_0 + \theta_1 \cdot n$	6.15	2.96			−6.4	NS[a]
Eq. 3		$CL = CL_0 + \theta_1^n$	7.0	2.22			−4.8	*,[a]
Eq. 4		$CL = CL_0 + \theta_1 \cdot \sqrt{n}$	4.7	5.2			−8.1	NS[a]

Table 2 Continued on next page

Table 2 (Continued)

Step 3	Gene–gene interaction analysis	*CYP* contribution	CL_0	θ_1	θ_2	θ_3	θ_4	ΔOF[a]
Eq. 5[b]	*CYP2B6/CYP2A6*	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.8	2.59 7.42 9.74 14.6	3.15 11.6 11.9 19.6	10.9 14.3 18.8		−16
Eq. 6	*CYP2B6/CYP2A6*	*CYP2B6* $CYP2A6 \cdot \sqrt{q}$	1.75	8.15 0.95	2.42	9.21 3.44	19.3 2.49	−17
Eq. 7	*CYP2B6/CYP2A6*	*CYP2B6* $CYP2A6 \cdot \sqrt{q}$		2.02 1.21	7.7	9.45	13.6	−16
Eq. 8	*CYP2B6/CYP2A6*	$CYP2B6 \cdot \sqrt{p}$ $CYP2A6 \cdot \sqrt{q}$	1.5	7.7 1.2				−12
Eq. 5[b]	*CYP2B6/CYP3A4* _rs4646437	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.66	2.46 10.5 13.3 16.3	3.9 11 12.3 17.0	10.6 13.5 19.3		−25
Eq. 6	*CYP2B6/CYP3A4* _rs4646437	*CYP2B6* $CYP3A4\_rs \cdot \sqrt{r}$		1.6 1.4	10.7 0.1	11.2 1.6	15.3 2.6	−22
Eq. 7	*CYP2B6/CYP3A4* _rs4646437	*CYP2B6* $CYP3A4\_rs \cdot \sqrt{r}$		1.62 1.36	8.99	11.5	16.9	−22
Eq. 8	*CYP2B6/CYP3A4* _rs4646437	$CYP2B6 \cdot \sqrt{p}$ $CYP3A4\_rs \cdot \sqrt{r}$	1.62	7.3 1.34				−18
Eq. 5[b]	*CYP2B6/CYP3A4*1B*	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.69	2.33 10.6 13.4 16.4	3.23 10.3 11.7 17.2	10.8 13.6 19.4		−14
Eq. 6	*CYP2B6/CYP3A4*1B*	*CYP2B6* $CYP3A4*1B \cdot \sqrt{s}$		1.64 1.1	10.7 0.4	11.2 1.2	15.3 2.7	−13
Eq. 7	*CYP2B6/CYP3A4*1B*	*CYP2B6* $CYP3A4*1B \cdot \sqrt{s}$		1.62 1.1	8.99	11.5	16.9	−12
Eq. 8	*CYP2B6/CYP3A4*1B*	$CYP2B6 \cdot \sqrt{p}$ $CYP3A4*1B \cdot \sqrt{s}$	1.65	7.6 1.1				−9
Eq. 5[b]	*CYP2B6/CYP3A5*	$CL = CL_0 + \theta_1 I_{01} + \theta_2 I_{02} + \theta_1 I_{10} + \theta_2 I_{11} + \theta_3 I_{12} + \theta_1 I_{20} + \theta_2 I_{21} + \theta_3 I_{22} + \theta_1 I_{30} + \theta_2 I_{31} + \theta_3 I_{32}$	1.45	2.22 6.0 13.4 16.5	3.25 11.6 11.9 17.0	10.6 13.6 19.2		−16
Eq. 6	*CYP2B6/CYP3A5*	*CYP2B6* $CYP3A5 \cdot \sqrt{t}$		1.38 1.1	10.7 0.1	11 1.8	15 2.4	−14
Eq. 7	*CYP2B6/CYP3A5*	*CYP2B6* $CYP3A5 \cdot \sqrt{t}$		1.4 1.22	9.1	11.7	17.1	−13
Eq. 8	*CYP2B6/CYP3A5*	$CYP2B6 \cdot \sqrt{p}$ $CYP3A5 \cdot \sqrt{t}$	1.4	7.6 1.22				−10

ΔOF, difference in the objective function, compared to the final model structural model; ΔOF[c], difference in the objective function compared to the model including CYP2B6 solely; ARV, antiretroviral medication; NRTI, non-nucleoside/nucleotide reverse transcriptase inhibitors; *n*, *p*, *q*, *r*, *s*, numbers of functional alleles (0 = Hom-LOF, 1 = Het-LOF, 2 = Hom-Ref, 3 = Het-GOF); NS, not statistically significant; PI, protease inhibitors.

[a]Differences in objective function compared to the rich model (Eq. 1). [b]I_{xy} represents the number of functional alleles for the x/y cytochromes. For clarity, θ have been numbered from 1 to 4. *$P < 0.05$, **$P < 0.01$.

for **1B*, −18 for rs_4646437). No factor accounting for more than an additive interaction was observed (ΔOF = 0.0).

The joint assignment of *CYP2B6* and *CYP3A5* on CL also improved the fit as compared to *CYP2B6* alone (Eq. 5, ΔOF = −16). The successive nesting of models using functional scores successfully followed the paradigm previously described for other *CYPs*. The influence of *CYP3A5* on *CYP2B6* was appropriately described using Eqs. 6 or 7 (ΔOF >−13) or as a square root additive model (Eq. 8, ΔOF >−10). Again, an interaction between the two genes was not significant (ΔOF = 0.0).

A final joint model characterizing the cumulative influence of all the genetic variants on EFV CL was tested on the basis of the two-by-two combinations of genetic effects. The richest model that integrated the effects of all CYP alleles, using a generalization of Eqs. 6 and 7, improved the fit (ΔOF >−29 as compared to *CYP2B6* alone), but the impact of *CYP3A4*1B* and *CYP3A5* did not remain significant (ΔOF = 0.0 as compared to the rich model without those two alleles). The final model employed a single parameter estimate to quantify the influence of each of the alleles, *CYP2B6*, *CYP2A6*, and *CYP3A4* _rs4646437, using additive square root functions, which fitted the data appropriately (Eq. 10, ΔOF = −24). All models specified with proportional rather than additive effects gave very similar results (ΔOF <−0.5). In addition to genetic influences on CL, the influence of body weight remained the only significant demographic covariate (ΔOF = 19.2), while any ethnic influence vanished completely.

The final model estimated an average CL of 1.3 l/h in individuals carrying Hom-LOF *CYP2B6*, *CYP2A6*, and *CYP3A4_*rs4646437, which increased by 7.3, 0.7, and 1.03 l/h for the first active allele of those genes, respectively, and by another 0.41 times those factors for the second allele (where $0.41 = \sqrt{2} - 1$), with an additional 70% increase in CL associated with doubling of body weight. A summary of the model building procedure is presented in Table 2 and the final population estimates in Table 3. A plot of the model-predicted concentration profile stratified by *CYP2B6* is shown in Figure 2. Figure 3 represents the individual *post hoc* CL estimates with population average predictions for the different allelic combinations encountered.

Based on our final model, simulations show that, with the standard regimen of 600 mg of EFV daily, average trough concentrations are 1.19 (90% prediction interval: 0.6–2.35), 1.6 (0.8–2.5), and 8.1 mg/l (4.5–14.5) in *CYP2B6* Hom-Ref, Het-LOF, and Hom-LOF carriers, respectively, and 0.5 mg/l (0.2–1.0) in *CYP2B6* Het-GOF carriers. Taking into account the interindividual variability leads to the suggestion that most *CYP2B6* Hom-LOF individuals will exhibit concentrations exceeding the 1–4 mg/l range that is generally considered acceptable, and most individuals carrying a Het-GOF will have concentrations <1 mg/l. Predicted concentrations of 2.7 mg/ml (1.5–4.9) would be expected at a dosage regimen of 200 mg/day in *CYP2B6* Hom-LOF carriers. However, individuals having Hom-LOF of *CYP2A6/CYP3A4_* rs3434367 and *CYP2B6* will be exposed to considerably higher drug levels, yielding average predicted concentrations of 6.2 mg/ml (3.5–11.0) with a regimen of 200 mg/day.

Table 3 Final population pharmacokinetic parameter estimates of EFV

	Population mean		Interindividual variability	
Parameter	Estimate	SE (%)[a]	Estimate (%)[b]	SE (%)[c]
CL/F (l/h)[d]	1.3	18	27.8	43.8
θ_{2B6}[e]	7.3	7		
θ_{2A6}[e]	0.7	36		
$\theta_{3A4\_rs4646437}$[e]	1.03	27		
θ_{BW}[f]	0.7	42		
V_d/F (l)	332	16		
k_a (h^{-1})	0.6	38		
σ (CV%)[g]	30.8	44.2		

CL/F, mean apparent clearance; F, bioavailability; k_a, mean absorption rate constant; V_d/F, mean apparent volume of distribution.

[a]SEs of the estimates (SEE), defined as SE/estimate and expressed as percentages. [b]Estimate of variability is expressed as coefficient of variation (CV) (%). [c]SEs of the CV, taken as $\sqrt{(SE/estimate)}$ and expressed as percentage. [d]CL value in patients with Hom-LOF for *CYP2B6*, *CYP2A6*, and *CYP3A4_*rs4646437. [e]Contribution of *CYP2B6*, *CYP2A6*, and *CYP3A4_*rs4646437 to efavirenz (EFV) CL multiplied by $\sqrt{n}$ where $n = 0, 1, 2, 3$ for *CYP2A6*, $n = 0, 1, 2$ for *CYP2A6* and *CYP3A4_*rs4646437 (see text). [f]Relative influence of body weight on EFV clearance (see text). [g]Residual intrapatient variability, expressed as a CV (%).

DISCUSSION

This study enabled us to characterize and quantify for the first time the conjugated effects of major and minor metabolic pathways and their genetic variants on EFV pharmacokinetics in a population of HIV-1-infected patients. The average CL and variability of EFV plasma concentrations are in the range of values reported previously.[1–3] Among nongenetic covariates, we observed an impact of body weight on CL, as reported by others.[16–20] In our study, no differences could be observed between data from male and female subjects, but conflicting results exist in the literature.[20–23] The well-known influence of black ethnicity, which has been associated with *CYP2B6* and *CYP3A4* heterogeneity,[24,25] could

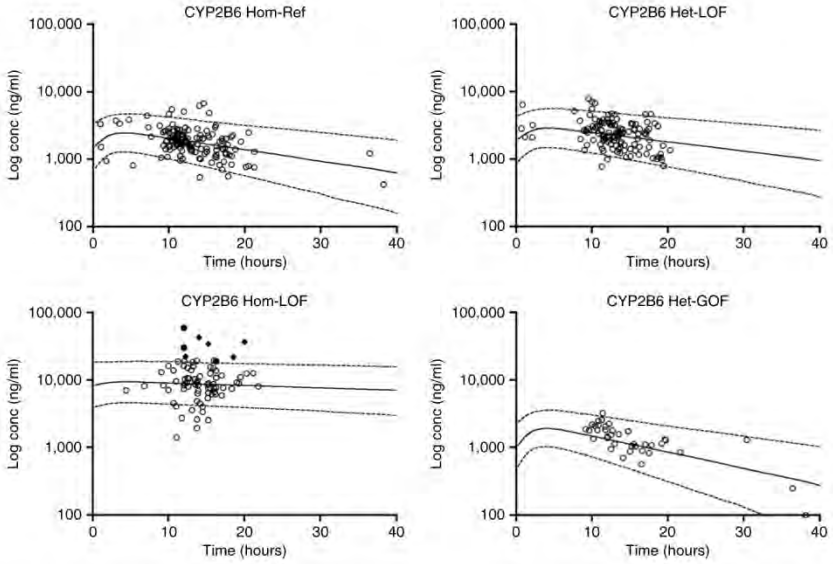

Figure 2 Efavirenz (EFV) plasma concentrations ($n = 393$) in 169 type I human immunodeficiency virus (HIV-1) individuals (open circles) in relation to *CYP2B6* polymorphism. Population predictions of the corresponding genotype are represented by black lines, and the 90% prediction interval is shown by gray dotted lines. Left lower panel: filled circles represent concentrations in individuals who are Hom-LOF for CYP2B6, CYP2A6, and CYP3A4_rs46464337, and filled diamonds represent concentrations in individuals who are Hom-LOF for CYP2B6 and Het-LOF for CYP3A4_rs4646437. Het, heterozygous; Hom, homozygous; LOF, loss of function.

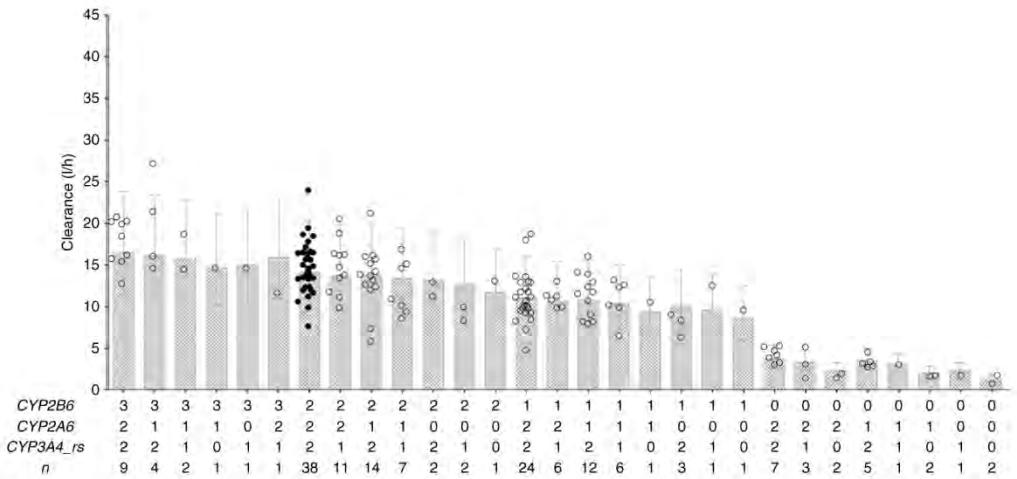

Figure 3 Individual predicted Bayesian clearances (open circles, the black circles correspond to individuals with reference alleles for all three cytochromes) and average predicted clearances (bars) with 90% population prediction interval for each *CYP2B6, CYP2A6*, and *CYP3A4* _rs4646437 allelic combination (according to score A categorization: 0 Hom-LOF/DOF, 1 Het-LOF/DOF, 2 Hom-Ref, and 3 Het-GOF; see text); *n* = number of individuals carrying the allelic combination (*n* = 0 for the eight combinations that are not represented). DOF, diminished function; GOF, gain of function; Het, heterozygous; Hom, homozygous; LOF, loss of function; Ref, reference allele.

be mostly explained by the joint influence of *CYP2B6*, *CYP2A6*, and *CYP3A4* variations, whereas its influence remained discernible when these *CYP* alleles were considered separately. Given the limited number of Hispanic ($n = 6$) and Asian ($n = 5$) individuals in our study, no clear effect on EFV levels could be attributed to these ethnic groups, although an influence has been reported previously.[1,3,10,20] Among genetic covariates, *CYP2B6* allelic variation accounted for most of the interindividual differences in EFV CL. The *CYP2A6* and *CYP3A4/A5* accessory pathways appeared to influence EFV elimination independent of *CYP2B6*. Among those, the overall impact of *CYP3A4*_ rs4646437 was the largest; it accounted for 6% of CL variability. The unexpected lower CL in *CYP3A5* Hom-Ref carriers as compared to Hom-LOF carriers can be explained by the linked Hom/Het-LOF of *CYP2B6* and *CYP3A4*_ rs4343437 observed in most individuals. This hypothesis is further confirmed by the lack of effect of *CYP3A5* when the influence of all cytochromes is assessed in a joint analysis. When information on *CYP2B6* was included along with *CYP2A6* and *CYP3A4* in joint analyses, an additive effect of accessory pathways was still present, both on fully functional *CYP2B6* and in the presence of reduced-function or gain-of-function alleles. However, the compensation ensured by these *CYP* alleles was small (1–2 l/h) and therefore more discernible on *CYP2B6* Hom-LOF. An evaluation of *CYP* genetic variations according to an activity score, as recently proposed by Gaedigk *et al.*[26] for *CYP2D6*, enabled us to quantify genetic influences of all alleles based on the same paradigm. A model of remarkable parsimony, which included only one parameter per allele and required no interaction term, could capture the nonlinear relationship between CL and the different genotypic groups. The use of such square root relationships in gene–dose effects was not described for other *CYP* isoenzymes, in particular for the noninducible CYP2D6,[24] which tend more toward linear effects.[27] This phenomenon suggests adaptive mechanisms that might be explained by the upregulation of extensive metabolizer alleles in response to an increase in concentration, possibly through the activation of nuclear receptors.[28,29] It is noteworthy that a similar pattern has already been reported for *CYP2B6*, not only in relation to EFV[30] but also in relation to S-methadone.[31] The use of different functional score classifications to implement partial activity levels failed to improve the model when compared with the traditional classification. The mechanisms by which allelic variants express a loss/diminished function could therefore not be translated in a "semiquantitative gene–dose system," as described by Steimer *et al.*[32] for amitriptyline and nortriptyline.

The cumulative influence of *CYP2B6*, *CYP2A6*, and *CYP3A4* _rs4646437 allelic variants on CL implies a critical 90% decrease of EFV elimination in triple-Hom-LOF individuals, in whom CL appeared reduced to 1.3 l/h as compared to the estimated 12.9 l/h in triple-Hom-Ref individuals. Of note, the only individual who exhibited extremely high EFV concentrations[33] was found to be triple-Hom-LOF; this emphasizes the importance of accessory pathways in EFV elimination. The interindividual variability in CL dropped from 65 to 27% in the final model, with *CYP2B6* genetic variants accounting for 31% and another 7% being explained by *CYP2A6, CYP3A4*_ rs4646437, and body weight variations. The remaining variability might be attributed to adherence issues[34] or to variation in uridine-glucuronyltransferase metabolic pathways.

In conclusion, functional alleles of *CYP2B6* accounted for the major part of EFV interindividual variability. Genetic variations in EFV accessory metabolic pathways demonstrated their importance in EFV pharmacokinetics in addition to *CYP2B6*, in particular in individuals with limited *CYP2B6* function. The dosage is required to be reduced to 200 mg/day of EFV in individuals with impaired *CYP2B6* function so as to ensure drug levels within the therapeutic range. The expression of pharmacogenetic influences on EFV elimination could be characterized using a single common paradigm across all *CYPs*. This paradigm involves the addition of the contribution of each enzymatic pathway proportional to the square root of the number of

Table 4 Demographic and genetic characteristic of the population study

Characteristics	Value	% Of study population
Sex (no.)		
Men	124	73
Women	45	27
Age (years)		
Median (range)	47 (30–73)	—
Body weight (kg)		
Median (range)	77.5 (44–101)	—
Height (cm)		
Median (range)	179 (153–193)	—
Ethnicity (no.)		
White	142	83
Black	16	10
Hispanic	6	4
Asian	5	3
PIs (no.)		
Ritonavir	20	13
Saquinavir	4	3
Amprenavir	0	0
Lopinavir	15	9
Atazanavir	18	11
NRTIs (no.)		
Lamivudine	116	72
Stavudine	15	9
Didanosine	29	18
Abacavir	0	0
Tenofovir	29	18
Emtricitabine	4	2
Zidovudine	81	50
Entry inhibitors (no.)		
Enfuvirtide	4	2
CYP P450 inducers (no.)	2	1
CYP P450 inhibitors (no.)	4	2
CYP2B6 *genetic polymorphism (no.)*		
Hom-Ref	75	44
Het-LOF	53	33
Hom-LOF	23	14
Het-GOF	16	9
Het-LOF/Het-GOF[a]	2	1
CYP2A6 *genetic polymorphism (no.)*		
Hom-Ref	99	61
Het-LOF	55	30
Hom-LOF	13	8
Het-GOF	2	1
CYP3A4 *genetic polymorphism (no.)*		
Hom-Ref	138	82
Het-LOF *1B	24	14
Hom-LOF *1B	7	4
CYP3A4 *genetic polymorphism (no.)*		
Hom-Ref	118	70
Het-LOF_rs4646437	41	24
Hom-LOF_rs4646437	10	6
CY3A5 *genetic polymorphism (no.)*		
Hom-Ref	5	3
Het-LOF	30	18
Hom-LOF	134	79

GOF, gain of function; Het, heterozygous; Hom, homozygous; LOF, loss of function; NNRTIs, non-nucleoside reverse transcriptase inhibitors; NRTIs, nucleoside reverse transcriptase inhibitors; PIs, protease inhibitors; Ref, reference allele.

[a]Two individuals are LOF/GOF for *CYP2B6* and were considered to be Het-LOF.

functional alleles. Such a model appropriately predicts average CL for various allele combinations, and its mechanistic explanation warrants further investigation.

METHODS

Study population. A total of 169 HIV-1-infected individuals from the Swiss HIV Cohort Study were characterized with respect to *CYP2B6*, *CYP2A6*, and *CYP3A4/A5* genetic variations. EFV drug levels were measured during routine therapeutic drug monitoring according to local treatment guidelines. All participants gave their informed consent for genetic testing. A median of 1 concentration sample per individual (range 1–23) was collected between 0.6 and 38 h after the last drug intake under steady-state conditions.

Method of analysis. Blood samples (5 ml) were collected into lithium heparin or EDTA-K Monovette syringes (Sarstedt, Nümbrecht, Germany). Plasma was isolated by centrifugation, inactivated for virus at 60 °C for 60 min, and stored at −20 °C until analysis. Plasma EFV levels were determined by liquid chromatography coupled with tandem mass spectrometry in accordance with a validated method. The calibration curves were found to be linear up to 10 μg/ml, with a lower limit of quantification of 0.1 μg/ml.

Nomenclature and functional score. Alleles are designated in concordance with the *CYP* Allele Nomenclature Committee (http://www.cypalleles.ki.se). Proposed functional consequences for EFV pharmacokinetics have been reported for *CYP2B6*,[15] *CYP2A6*, and *CYP3A4/A5*.[35] The study participants were categorized into genotypic groups on the basis of the number of functional alleles (Table 1). The simplest scoring scheme (score A) assigned values of 2, 0, and 1 to the fully functional reference (Hom-Ref), homozygous (Hom-LOF), and heterozygous (Het-LOF) diminished/loss of function, respectively, and a value of 3 to *CYP2B6* gain-of-function alleles (Het-GOF). Two individuals having a single-gene duplication of *CYP2A6* were assimilated to the Hom-Ref group because they constituted only a small number. The classification was refined to distinguish between individuals with *CYP2B6* and *CYP2A6* loss/diminished function alleles, to reflect the predicted level of activity from *in vitro* studies (Table 1, scores B/C).

Pharmacokinetic structural model. EFV pharmacokinetics were characterized using a one-compartment model, as assessed previously.[2] Since EFV is administered only orally, CL and *V* represent apparent values.

Covariate model. The analyses of the covariate effects on CL were divided into three main sections for assessing: (i) the influence of demographic variables and concomitant medications, (ii) the impact of *CYP2B6*, *CYP2A6*, *CYP3A4*, and *CYP3A5* alleles based on univariate analyses, and (iii) the joint effect of *CYP2B6* with *CYP2A6*, *CYP3A4*, and *CYP3A5* alleles in multivariate analyses.

Demographic analyses. The typical value of CL was modeled to depend linearly on a covariate X (body weight, centered on the mean; categorical covariates coded as 0/1) as shown in the equation: $CL = \theta_a \cdot (1 + \theta_b \cdot X)$, where θ_a is the average estimate and θ_b is the relative deviation (positive or negative) from average attributed to the covariate X. The available demographic covariates were sex, ethnicity, age, body weight, and height. Only a few co-medications were recorded, and these were principally other antiretroviral drugs and known *CYP* inducers or inhibitors (Table 4).

Univariate genotype. In these analyses, each genotype was entered solo into the model. Several models relating CL with functional scores were tested using different methods (Figure 1) and compared with the richest possible model, which assigned a separate fixed effect to each score level as follows:

$$CL = CL_0 + \theta_1 I_1 + \theta_2 I_2 + \theta_3 I_3 \quad (1)$$

$$CL = CL_0 \cdot (1 + \theta_1 I_1) \cdot (1 + \theta_2 I_2) \cdot (1 + \theta_3 I_3) \quad (1a)$$

where CL_0 is the typical value of CL in Hom-LOF individuals (Hom-Ref for *CYP3A5*), I_i is an indicator variable that takes the value of 1 if an individual carries the ith genotypic score (i.e., I_1: Het-LOF, I_2: Hom-Ref, and I_3: Het-GOF) and 0 otherwise, and θ_i is the absolute or fractional (Eqs. 1/1a) change in CL relative to the Hom-LOF group. The impact of functional alleles on EFV CL was further explored to distinguish the difference between the genotypic groups, using two reduced models in which the same genotyping group was assigned to Hom-Ref and Het-LOF or to Hom-Ref and Het-GOF carriers (reduced 1 and 2, Figure 1). Competing models attempted to account for gene effect as a function of the number of functional alleles (Table 1, score A), using linear and power relationships with either additive or proportional (data not shown) impact, using the following models:

$$CL = CL_0 + \theta_1 \cdot n \quad (2)$$

$$CL = CL_0 + \theta_1^n \quad (3)$$

$$CL = CL_0 + \theta_1 \sqrt{n} \quad (4)$$

where $n = 1$, 2, or 3 represents the functional score, and θ_1 the average contribution per active allele above that of Hom-LOF CL (CL_0). The alternative activity scores B/C for *CYP2B6* and *CYP2A6* were explored using parameter models in Eqs. 2, 3, or 4 and compared with an extension of the rich model (Eq. 1).

Gene–gene interaction analyses. The joint influence of functional alleles on EFV CL was first tested using pairwise conjunction of *CYP2B6* with each of the other *CYP* alleles, so as to finally build up a model including all genetic variants that have an influence. The investigation of the joint influence of *CYP2B6* and *CYP2A6* alleles is shown as an example. The richest model that served as reference for the evaluation of reduced competing models was:

$$CL = CL_0 + \theta_{01} I_{01} + \theta_{02} I_{02} + \theta_{10} I_{10} + \theta_{11} I_{11} + \theta_{12} I_{12} + \theta_{20} I_{20} + \theta_{21} I_{21} + \theta_{22} I_{22} + \theta_{30} I_{30} + \theta_{31} I_{31} + \theta_{32} I_{32} \quad (5)$$

where CL_0 is the Hom-LOF CL for both genes and I_{ij} is an indicator variable that takes the value of 1 for the *CYP2B6* ith/*CYP2A6* jth genotype carrier and is 0 otherwise, and each θ_{ij} estimates the absolute change in CL among the different genotypic groups. The same model was parameterized for relative changes (data not shown). The following competing models were evaluated:

$$CL = CL_0 + \theta_{0\_0} \cdot \sqrt{q} + \left(\theta_1 I_1 + \theta_{1\_1} \cdot \sqrt{q}\right) + \left(\theta_2 I_2 + \theta_{2\_2} \cdot \sqrt{q}\right) + \left(\theta_3 I_3 + \theta_{3\_3} \cdot \sqrt{q}\right) \quad (6)$$

$$CL = CL_0 + (\theta_1 I_1 + \theta_2 I_2 + \theta_3 I_3) + \theta_4 \cdot \sqrt{q} \quad (7)$$

$$CL = CL_0 + \theta_1 \cdot \sqrt{p} + \theta_2 \cdot \sqrt{q} \quad (8)$$

$$CL = CL_0 + \theta_1 \cdot \sqrt{p} + \theta_2 \cdot \sqrt{q} + \theta_3 \cdot (p \cdot q) \quad (9)$$

where p indicates the functional score for *CYP2B6* and q the score for *CYP2A6*. In Eq. 6, the contribution of *CYP2A6* (θ_{0_0},...,θ_{3_3}) is investigated on *CYP2B6* stratified by genotypic groups; in Eq. 7 the influence of *CYP2A6* is characterized using a single fixed-effect parameter θ_4 across all *CYP2B6* genotypes; and in Eq. 8 square root functions are integrated for both genes. Finally, an interaction term was allowed so as to further check for some nonadditive interaction between *CYP* variants (Eq. 9). All these models used either additive or proportional (data not shown) effects.

All significant allelic groups were integrated into a final model, wherein the contribution of each genotypic group was estimated using a generalization of Eqs. 6–8 to be finally formulated using the following additive or proportional (data not shown) relationships:

$$CL = CL_0 + \theta_1 \cdot \sqrt{p} + \theta_2 \cdot \sqrt{q} + \theta_3 \cdot \sqrt{r} + \theta_4 \cdot \sqrt{s} + \theta_5 \cdot \sqrt{t} \quad (10)$$

where CL_0 is the Hom-LOF CL for all genes and θ_i values estimate the absolute or fractional change in CL as a function of score A for different combinations of *CYP2B6* (p), *CYP2A6* (q), *CYP3A4* _rs4646437 (r), *CYP3A4*1B* (s), and *CYP3A5* (t) alleles.

Variance model. The individual CL values were modeled assuming a log-normal distribution (mean zero and variance Ω). A proportional error model (mean zero and variance σ^2) was used the description of intraindividual variability.

Parameter estimation and selection. NONMEM (version VI; NM-TRAN, version II, GloboMax, Hanover, MD) was used with FOCE INTERACTION to fit the models.[36] As goodness-of-fit statistics, NONMEM uses the objective function, which is approximately equal to minus twice the logarithm of the maximum likelihood. The likelihood ratio test, based on the reduction in objective function (ΔOF), was used to carry out comparisons between any two models. A ΔOF (−2 log likelihood, approximate χ^2 distribution) of 3.84, 5.99, and 7.81 points for 1, 2, or 3 additional parameters, respectively, was used for determining statistical significance ($P < 0.05$) of the difference between two models. The reliability of the results was checked on diagnostic goodness-of-fit plots, along with the measure of the SEs. The identification of potential outlier values resulting from compliance issues or inadequacy in self-reporting information was explored using a sensitivity analysis. One individual had an EFV concentration of 59,400 ng/ml, and this value was at first excluded to prevent single outlier effect but was integrated at the end. Except for this, all data were considered reliable. Simulations based on the final pharmacokinetic estimates were performed with NONMEM using 1,000 individuals to calculate the 90% prediction intervals. The concentrations encompassing the range from 5th to 95th percentile at each time point were retrieved in order to construct the intervals. Further simulations were performed for a series of genotype combinations in 1,000 individuals undergoing various dosage regimens (200, 400, 600, and 800 mg), so as to suggest the dosages that would ensure trough levels falling within the 1–4 mg/ml therapeutic interval in 90% of the individuals. The figures were generated using GraphPad Prism (version 4.00 for Windows; GraphPad Software, San Diego, CA, http://www.graphpad.com).

ACKNOWLEDGMENTS
M.A.-A. contributed to the pharmacokinetic modeling and J.D.I. to the genetic testing.

CONFLICT OF INTEREST
The authors declared no conflict of interest.

1. Barrett, J.S., Joshi, A.S., Chai, M., Ludden, T.M., Fiske, W.D. & Pieniaszek, H.J. Jr. Population pharmacokinetic meta-analysis with efavirenz. *Int. J. Clin. Pharmacol. Ther.* **40**, 507–519 (2002).
2. Csajka, C. *et al.* Population pharmacokinetics and effects of efavirenz in patients with human immunodeficiency virus infection. *Clin. Pharmacol. Ther.* **73**, 20–30 (2003).
3. Kappelhoff, B.S. *et al.* Population pharmacokinetics of efavirenz in an unselected cohort of HIV-1-infected individuals. *Clin. Pharmacokinet.* **44**, 849–861 (2005).
4. Marzolini, C., Telenti, A., Decosterd, L.A., Greub, G., Biollaz, J. & Buclin, T. Efavirenz plasma levels can predict treatment failure and central nervous system side effects in HIV-1-infected patients. *AIDS* **15**, 71–75 (2001).
5. Rotger, M. *et al.* Influence of CYP2B6 polymorphism on plasma and intracellular concentrations and toxicity of efavirenz and nevirapine in HIV-infected patients. *Pharmacogenet. Genomics* **15**, 1–5 (2005).
6. Haas, D.W. *et al.* Pharmacogenetics of efavirenz and central nervous system side effects: an Adult AIDS Clinical Trials Group study. *AIDS* **18**, 2391–2400 (2004).
7. Desta, Z. *et al.* Impact of CYP2B6 polymorphism on hepatic efavirenz metabolism in vitro. *Pharmacogenomics* **8**, 547–558 (2007).
8. Ward, B.A., Gorski, J.C., Jones, D.R., Hall, S.D., Flockhart, D.A. & Desta, Z. The cytochrome P450 2B6 (CYP2B6) is the main catalyst of efavirenz primary and secondary metabolism: implication for HIV/AIDS therapy and utility of efavirenz as a substrate marker of CYP2B6 catalytic activity. *J. Pharmacol. Exp. Ther.* **306**, 287–300 (2003).
9. Mutlib, A.E. *et al.* Identification and characterization of efavirenz metabolites by liquid chromatography/mass spectrometry and high field NMR: species differences in the metabolism of efavirenz. *Drug Metab. Dispos.* **27**, 1319–1333 (1999).
10. Cressey, T.R. & Lallemant, M. Pharmacogenetics of antiretroviral drugs for the treatment of HIV-infected patients: an update. *Infect. Genet. Evol.* **7**, 333–342 (2007).
11. Klein, K. *et al.* Genetic variability of CYP2B6 in populations of African and Asian origin: allele frequencies, novel functional variants, and possible implications for anti-HIV therapy with efavirenz. *Pharmacogenet. Genomics* **15**, 861–873 (2005).
12. Wyen, C. *et al.* Impact of CYP2B6 983T>C polymorphism on non-nucleoside reverse transcriptase inhibitor plasma concentrations in HIV-infected patients. *J. Antimicrob. Chemother.* **61**, 914–918 (2008).
13. Wang, J. *et al.* Identification of a novel specific CYP2B6 allele in Africans causing impaired metabolism of the HIV drug efavirenz. *Pharmacogenet. Genomics* **16**, 191–198 (2006).
14. Tsuchiya, K. *et al.* Homozygous CYP2B6 *6 (Q172H and K262R) correlates with high plasma efavirenz concentrations in HIV-1 patients treated with standard efavirenz-containing regimens. *Biochem. Biophys. Res. Commun.* **319**, 1322–1326 (2004).
15. Rotger, M. *et al.* Predictive value of known and novel alleles of CYP2B6 for efavirenz plasma concentrations in HIV-infected individuals. *Clin. Pharmacol. Ther.* **81**, 557–566 (2007).
16. Ofotokun, I., Chuck, S.K. & Hitti, J.E. Antiretroviral pharmacokinetic profile: a review of sex differences. *Gend. Med.* **4**, 106–119 (2007).
17. Hitti, J. *et al.* Sex and weight as covariates in the pharmacokinetics of efavirenz, indinavir, and nelfinavir. Program and Abstract of the 11th Conference on Retroviruses and Opportunistic Infections, San Francisco, CA, 8–11 February 2004. Abstract no. 604.
18. López-Cortés, L.F. *et al.* Pharmacokinetic interactions between efavirenz and rifampicin in HIV-infected patients with tuberculosis. *Clin. Pharmacokinet.* **41**, 681–690 (2002).
19. Matteelli, A. *et al.* Multiple-dose pharmacokinetics of efavirenz with and without the use of rifampicin in HIV-positive patients. *Curr. HIV Res.* **5**, 349–353 (2007).
20. Pfister, M. *et al.* Population pharmacokinetics and pharmacodynamics of efavirenz, nelfinavir, and indinavir: Adult AIDS Clinical Trial Group Study 398. *Antimicrob. Agents Chemother.* **47**, 130–137 (2003).
21. Burger, D. *et al.* Interpatient variability in the pharmacokinetics of the HIV non-nucleoside reverse transcriptase inhibitor efavirenz: the effect of gender, race, and CYP2B6 polymorphism. *Br. J. Clin. Pharmacol.* **61**, 148–154 (2006).
22. Rotger, M., Csajka, C. & Telenti, A. Genetic, ethnic, and gender differences in the pharmacokinetics of antiretroviral agents. *Curr. HIV/AIDS Rep.* **3**, 118–125 (2006).
23. Lamba, V. *et al.* Hepatic CYP2B6 expression: gender and ethnic differences and relationship to CYP2B6 genotype and CAR (constitutive androstane receptor) expression. *J. Pharmacol. Exp. Ther.* **307**, 906–922 (2003).
24. Ingelman-Sundberg, M., Sim, S.C., Gomez, A. & Rodriguez-Antona, C. Influence of cytochrome P450 polymorphisms on drug therapies: pharmacogenetic, pharmacoepigenetic and clinical aspects. *Pharmacol. Ther.* **116**, 496–526 (2007).
25. Lamba, J.K. *et al.* Common allelic variants of cytochrome P4503A4 and their prevalence in different populations. *Pharmacogenetics* **12**, 121–132 (2002).
26. Gaedigk, A., Simon, S.D., Pearce, R.E., Bradford, L.D., Kennedy, M.J. & Leeder, J.S. The CYP2D6 activity score: translating genotype information into a qualitative measure of phenotype. *Clin. Pharmacol. Ther.* **83**, 234–242 (2008).
27. Honda, M. *et al.* Multiple regression analysis of pharmacogenetic variability of carvedilol disposition in 54 healthy Japanese volunteers. *Biol. Pharm. Bull.* **29**, 772–778 (2006).
28. Faucette, S.R. *et al.* Relative activation of human pregnane X receptor versus constitutive androstane receptor defines distinct classes of CYP2B6 and CYP3A4 inducers. *J. Pharmacol. Exp. Ther.* **320**, 72–80 (2007).
29. Itoh, M. *et al.* Induction of human CYP2A6 is mediated by the pregnane X receptor with peroxisome proliferator-activated receptor-gamma coactivator 1alpha. *J. Pharmacol. Exp. Ther.* **319**, 693–702 (2006).
30. Nyakutira, C. *et al.* High prevalence of the CYP2B6 516G-->T(*6) variant and effect on the population pharmacokinetics of efavirenz in HIV/AIDS outpatients in Zimbabwe. *Eur. J. Clin. Pharmacol.* **64**, 357–365 (2008).
31. Crettol, S. *et al.* Methadone enantiomer plasma levels, CYP2B6, CYP2C19, and CYP2C9 genotypes, and response to treatment. *Clin. Pharmacol. Ther.* **78**, 593–604 (2005).
32. Steimer, W. *et al.* Allele-specific change of concentration and functional gene dose for the prediction of steady-state serum concentrations of amitriptyline and nortriptyline in CYP2C19 and CYP2D6 extensive and intermediate metabolizers. *Clin. Chem.* **50**, 1623–1633 (2004).
33. Hasse, B., Günthard, H.F., Bleiber, G. & Krause, M. Efavirenz intoxication due to slow hepatic metabolism. *Clin. Infect. Dis.* **40**, e22–e23 (2005).
34. Molto, J. *et al.* Variability in non-nucleoside reverse transcriptase and protease inhibitors concentrations among HIV-infected adults in routine clinical practice. *Br. J. Clin. Pharmacol.* **63**, 715–721 (2007).
35. Di Lulio, J. *et al. In-vivo* analysis of efavirenz metabolism in individuals with impaired CYP2A6 function. *Pharmacogenet. Genomics* (in press).
36. Beal, S.L., Sheiner, L.B. & Boeckmann, A.J. (eds.). NONMEM Users Guides, (1989–2006) (Icon Development Solutions, Ellicott City, MD, 2008).

4.1.1.3. Original article

Association of Pharmacogenetic Markers with Premature Discontinuation of first-line anti-HIV Therapy: an Observational Cohort Sstudy

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Journal of Infectious Diseases 2010; in press

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Study concept and design: A. Telenti, L. Lubomirov, S. Colombo, B. Ledergerber

Genetic analysis: **J. di Iulio**, R. Martinez

Sample collection: S. Colombo

Data analysis: R. Lubomirov, B. Ledergerber, S. Colombo

Clinical data contribution: M. Cavassini, B. Hirschel, E. Bernasconi, L. Elzi, P. Vernazza, H. Furrer, H. F. Gunthard

Study supervision: A. Telenti

Drafting of the manuscript: R. Lubomirov, S. Colombo, A. Telenti

Critical revision of the manuscript for important intellectual content: all authors

Detailed personal contribution

I participated in the sample recruitment and DNA extraction, data interpretation, as well as in the genetic characterization of individuals included in the study by genotyping 5 variants that could not be analysed in the array (for technical reason) or that failed quality control:

- rs28399433 (*CYP2A6*) in individuals under efavirenz containing regimen (N=272)
- rs4646437 (*CYP3A4*) in individuals under efavirenz containing regimen (N=272)
- rs7412 (*APOE*) in individuals under lopinavir containing regimen (N=184)
- rs429358 (*APOE*) in individuals under lopinavir containing regimen (N=184)
- rs8175347 (*UGT1A1*) in individuals under atazanavir containing regimen (N=121)

Comments on the article:

Given the high prevalence of treatment discontinuation in individuals under antiretroviral therapy, it is of importance to identify predictors susceptible to guide treatment combination or dosage.

We retrospectively determined if genetic variations influencing antiretroviral drug pharmacokinetics or pharmacodynamics would be associated with drug tolerance at the population level, assessed through the phenotype of treatment discontinuation.

Individuals under tenofovir, abacavir, **efavirenz**, lopinavir/ritonavir or atazanavir/ritonavir – containing regimen were included in the study.

We could demonstrate that individuals under efavirenz-containing regimen with impaired function of the main (CYP2B6) and accessory (CYP2A6 and/or CYP3A4) metabolic pathways discontinued their treatment significantly more often (P=0.008), and that toxicity was the main reason for discontinuation. This study demonstrates that genetic analysis might help predict the best drug dosage or combination for efavirenz containing regimen, but further prospective analyses are still needed to confirm these results.

Journal of Infectious Diseases Advance Access published December 9, 2010

MAJOR ARTICLE

Association of Pharmacogenetic Markers with Premature Discontinuation of First-line Anti-HIV Therapy: An Observational Cohort Study

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Background. Poor tolerance and adverse drug reactions are main reasons for discontinuation of antiretroviral therapy (ART). Identifying predictors of ART discontinuation is a priority in HIV care.

Methods. A genetic association study in an observational cohort to evaluate the association of pharmacogenetic markers with time to treatment discontinuation during the first year of ART. Analysis included 577 treatment-naive individuals initiating tenofovir ($n = 500$) or abacavir ($n = 77$), with efavirenz ($n = 272$), lopinavir/ritonavir ($n = 184$), or atazanavir/ritonavir ($n = 121$). Genotyping included 23 genetic markers in 15 genes associated with toxicity or pharmacokinetics of the study medication. Rates of ART discontinuation between groups with and without genetic risk markers were assessed by survival analysis using Cox regression models.

Results. During the first year of ART, 190 individuals (33%) stopped 1 or more drugs. For efavirenz and atazanavir, individuals with genetic risk markers experienced higher discontinuation rates than individuals without (71.15% vs 28.10%, and 62.5% vs 14.6%, respectively). The efavirenz discontinuation hazard ratio (HR) was 3.14 (95% confidence interval (CI): 1.35–7.33, $P = .008$). The atazanavir discontinuation HR was 9.13 (95% CI: 3.38–24.69, $P < .0001$).

Conclusions. Several pharmacogenetic markers identify individuals at risk for early treatment discontinuation. These markers should be considered for validation in the clinical setting.

Pharmacogenetic research has focused on understanding the mechanisms of adverse drug reactions (ADR) and on finding biomarkers that identify people at risk, with the aims of decreasing the number of adverse drug reactions and increasing drug efficacy. In human immunodeficiency virus (HIV) therapeutics, pharmacogenetics has been pursued because of the prevalence of toxicity [1, 2], the long-term nature of treatment, and the complexity inherent in a multidrug therapy that could benefit from predictive tools to identify the drug combination most likely to be tolerated and effective. Treatment decisions at the initiation of a first-line antiretroviral therapy (ART) in treatment-naive individuals are mostly based on CD4 T cell counts, viremia, symptoms, comorbidities, and analysis of primary drug resistance. Unfortunately, up to 45% of individuals discontinue or change treatment during the first year of ART [3, 4], frequently due to poor treatment tolerance [4–6].

For abacavir, a randomized prospective trial confirmed the value of genetic assessment before prescription to avoid hypersensitivity drug reactions [7]. Similar

Received 13 June 2010; accepted 30 August 2010.

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Potential conflicts of interest: None reported.

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The Journal of Infectious Diseases 2010;1–12

1537-6613/2010/02-0001$15.00
DOI: 10.1093/infdis/jiq043

information regarding the potential for a pharmacogenetic-driven intervention does not exist for other ART drugs. However, there is an increasing body of literature on genes and genetic markers that may relate to toxicity and or to pharmacokinetics of the drug. This includes extensive data on the pharmacokinetics/genetics of efavirenz, with some indication that drug levels may associate with central nervous system toxicity (*CYP2B6*, *CYP2A6*, and *CYP3A4*) [8–11]. Data from genome-wide association studies on dyslipidemia in the general population predict ritonavir-boosted protease inhibitor–associated dyslipidemia (*APOA5*, *CET*, *DOCK7*, *GCKR*, *LPL*, and *TRIB1*) [12, 13], and there are also genetic predictors of lopinavir drug levels [14, 15]. Atazanavir-associated hyperbilirubinemia is associated with alleles of *UGT1A1* [16], and has been reported in association with alleles of *ABCB1 (MDR1)* [17] and *NR1I2* (*PXR*) [18]. There is early information on the genetic basis of tenofovir tubulopathy (*ABCC2*, *ABBC4*) [19, 20].

Most of the above genetic markers are convincingly associated with an intermediate phenotype (ie, laboratory abnormality), which may or may not translate into symptoms and signs that lead to clinical actions such as treatment discontinuation. We hypothesized that if any of the markers presented above contribute to intolerance or toxicity, they would predict treatment modification or discontinuation. Therefore, this study aims to evaluate the association of recognized and proposed genetic predictors of toxicity or elevated drug levels with time to treatment discontinuation during the first year of first-line ART. As the study is retrospective, and the genotype was obtained a posteriori, it can be considered an unbiased assessment of genetic association with treatment discontinuation.

METHODS

Study Design and Population

In the Swiss HIV Cohort Study (SHCS, http://www.shcs.ch) database, clinical and laboratory information were collected prospectively on standardized questionnaires through a semi-annual structured interview and a set of prespecified biological analyses (The Swiss HIV Cohort Study [21]). Eligible study participants were all ART naive, starting one of the study medications (n = 688) between January 2004 and December 2007, and had at least 2 regular follow-up visits within 18 months after treatment initiation. Study ART included tenofovir or abacavir, 3TC or FTC, associated with lopinavir or atazanavir boosted with ritonavir (r) or efavirenz. Participant selection process was performed with Stata (version 10, StataCorp LP). The SHCS Genetics Project was approved by the ethics committees of all participating centers, and participants gave written informed consent for genetic testing. As this laboratory has been active in the description of associations of genetic markers with efavirenz pharmacokinetics [9–11, 22], lopinavir/r lipid metabolism and pharmacokinetics [13, 23], and atazanavir hyperbilirubinemia [16], we identified patients that could have been included in such previous studies. Only 1 of 272 individuals receiving efavirenz, 20 of 184 individuals receiving lopinavir/r, and none of 121 individuals receiving atazanavir had contributed to previous studies in this laboratory.

We defined a discontinuation episode as the first event of stop of each individual drug, whether the event included discontinuation of 1 or more drugs in the combination ART. At the time of treatment discontinuation, SHCS physicians are requested to fill in limited information that codes the reason for discontinuation. Codes are available for the following categories: treatment failure (virological), drug-associated toxicity (which includes coding for dyslipidemia, hypersensitivity reaction, gastrointestinal, liver, central nervous system, and kidney toxicity, and other toxicities), patient decision, physician decision, and other. Efavirenz dose reduction and the introduction of lipid-lowering agents in patients taking lopinavir/r were also considered as study events, and were coded as drug-related treatment modifications.

Genotyping

On the basis of existing knowledge [24] and recent publications [13–15, 18, 20], 23 genetic markers in 15 genes were included in the study (Table 1). Markers were genotyped using the Veracode technology (Illumina) with the exception of the *UGT1A1* promoter variants (number of TA repeats in the TATA box associated with hyperbilirubinemia), which were analyzed by sequencing.

Genetic Risk and Scores

For each drug, we assessed the contribution of each independent allele (Table 1). When supported by the literature, we also assessed their joint contribution in a genetic score. Genetic scores were established a priori based on literature by using an additive model [25] (Table 1).

Data Analyses

Rates of ART discontinuation were compared between individuals with and without genetic risk markers using Cox proportional hazards regression models. The nongenetic predictors included: age (stratified in 3 categories: $<$40, 40–49, and $>$50 years), body weight (stratified in 4 categories: $<$60, 60–69, 70–79, and $>$80 kg), sex, ethnicity (Caucasian, African, Asian, Hispanic, and other), CD4 T cell count (in 100-cell strata) and viral RNA concentration ($\log_{10}$ transformed) at the time of starting ART (baseline), transmission risk group (men who have sex with men [MSM], heterosexual, blood, injection drug user [IDU], other, and unknown), year of starting ART (2004, 2005, 2006, and 2007), ART regimen, center, and pregnancy status. Markers with potential prognostic value after univariable analyses were included in a multivariable Cox model for association with the ART treatment discontinuation during the 1-year follow-up period. The proportional hazards assumptions were assessed for all models using Schoenfeld residuals. We used Stata software, version 11 (StataCorp LP) for analyses.

Table 1. Genes, Genetic Variants, and Genetic Scores. Panel A: 23 genetic variants were selected on the basis of literature, and association with an intermediate pharmacokinetic or toxicity phenotype. Panel B: description of genetic risk scores. The numbers represent the count of variant alleles. Scores for efavirenz and lopinavir/r have been previously described. The atazanavir score has been explored post hoc

A.

Drug	Gene	Genetic variant (rs number)[a]	Genetic variant change	Minor allele (nomenclature)	Expected minor allele effect	Reference
TDF	*ABCC2*	rs2273697	G>A	A	Risk	[19]
	ABCC2	rs717620	C>T	T	Protective	[19, 20]
	ABCC4	rs899494	C>T	T	Risk	[19]
EFV	*CYP2A6*	rs28399433	T>G	G (**9*)	Risk	[10, 11]
	CYP2B6	rs3745274	G>T	T (**6*)	Risk	[8, 9, 22]
		rs35303484	A>G	G (**11*)	Risk	
		rs35979566	T>A	A (**15*)	Risk	
		rs28399499	T>C	C (**18*)	Risk	
	CYP3A4	rs4646437	C>T	T	Risk	[10, 11]
LPV	*APOA5*	rs3135506	C>G	G	Risk	[12, 13]
		rs662799	T>C	C	Risk	
	CETP	rs708272	G>A	A	Protective	
	DOCK7	rs1748195	C>G	G	Protective	
	GCKR	rs780094	C>T	T	Risk	
	LPL	rs6586891	A>C	C	Risk	
		rs328	C>G	G	Protective	
	TRIB1	rs17321515	A>G	G	Protective	
	ABCC2	rs717620	C>T	T	Protective	[14]
	CYP3A	rs6945984	T>C	C	Risk	[14]
	SLCO1B1	rs11045819	C>A	A (**4*)	Protective	[14]
		rs4149056	T>C	C (**5*)	Risk	[14, 15]
ATV	*ABCB1*	rs1045642	C>T	T	Risk	[17]
	NR1I2	rs 2472677	T>C	C	Protective	[18]
	UGT1A1	rs8175347	$A(TA)_6TAA > A(TA)_{5,\ 7}$ or $_8TAA$	$(TA)_7$ (**28*)	Risk	[16]
				$(TA)_5$ (**36*)	Protective	
				$(TA)_8$ (**37*)	Risk	

B.

Efavirenz		*CYP2B6/2A6/3A4* six-group genetic score					
Genes	Genetic variant	Score 1	Score 2	Score 3	Score 4	Score 5	Score 6
CYP2B6	rs3745274 (**6*) rs35303484 (**11*) rs35979566 (**15*) rs28399499 (**18*)	0	0	1	1	2	2
CYP2A6	rs28399433 (**9*)	0	1 to 4	0	1 to 4	0	1 to 4
CYP3A4	rs4646437						

Lopinavir		Hypertriglyceridemia additive genetic score[b,c]		
Genes	Genetic variant	Score 1	Score 2	Score 3
	Risk variant	-4 to -2	-1 to 0	+1 to +4
APOA5	rs3135506			
	rs662799			
GCKR	rs780094			
LPL	rs6586891			
	Protective variant			
DOCK7	rs1748195			
TRIB1	rs17321515			
CETP	rs708272			

LPL	rs328								
		Pharmacokinetic genetic score[d]							
		Score -2			Score 0			Score +2	
SLCO1B1	rs11045819 (**4*)	2			0 or 1			0	
	rs4149056 (**5*)	0			<2			⩾2	
ABCC2	rs717620,								
CYP3A	rs6945984								
Atazanavir		*UGT1A1/ABCB1* additive genetic score							
Genes	Genetic variant	Score 0	Score 1		Score 2			Score 3	Score 4
UGT1A1	rs8175347 (**28, *37*)	0	0	1	1	0	2	1	2	2
ABCB1	rs1045642	0	1	0	1	2	0	2	1	2

NOTE. *ABCB1*: ATP-binding cassette, subfamily B (MDR/TAP), member 1; *ABCC2* and *4*: ATP-binding cassette subfamily C members 2 and 4; *APOA5*: apolipoprotein A5; *ATV*: atazanavir; *CETP*: cholesteryl ester transfer protein; *CYP2A6, 2B6,* and *3A4*: Cytochrome P-450 2A6, 2B6, and 3A4; *DOCK7*: dedicator of cytokinesis 7; *EFV*: efavirenz; *GCKR*: glucokinase (hexokinase 4) regulator; *HCP5*: HLA complex P5; *LPL*: lipoprotein lipase; *LPV*: lopinavir/r; *NR1I2*: nuclear receptor subfamily 1, group I, member 2; *SLCO1B1*: solute carrier organic anion transporter family, member 1B1; *TDF*: tenofovir; *TRIB1*: tribbles homolog 1 (Drosophila); *UGT1A1*: UDP glucuronosyltransferase 1 family polypeptide A1.

[a] The rs numbers are the accession numbers in the National Center for Biotechnology Information single-nucleotide polymorphism database, dbSNP.

[b] Described previously by Arab-Alameddine et al. in the study of efavirenz plasma levels [11].

[c] Described previously by Rotger et al. The numbers represents the score values resulting from adding and subtracting the number of variant alleles of the risk and protective genetic variants, respectively [13].

[d] Described previously by Lubomirov et al. in the study of lopinavir plasma levels; patients with Score -2 have low plasma levels, and with Score +2 have high plasma levels compared to the Score 0 [14].

RESULTS

Demographic and Genetic Characteristics of Participants

Of a total of 668 patients starting ART with the study medication (ie, tenofovir, efavirenz, lopinavir/r, or atazanavir), 577 individuals were successfully genotyped and included in the analysis. Reasons for exclusion included an incomplete genetic consent ($n = 5$), unavailable biological material ($n = 7$), unsuccessful genotyping ($n = 2$), and samples that were not included because the standard size of the genotyping array was reached ($n = 77$). The 91 individuals not included in the analysis were comparable in age, sex, and ethnicity to those included, but more likely to receive efavirenz (86% vs 47%) because such samples were preferentially excluded once the array capacity was reached. Most of the participants were Caucasian (80%) and men (73%). ART regimen included tenofovir/emtricitabine or lamivudine ($n = 500$) or abacavir/emtricitabine or lamivudine ($n = 77$), and efavirenz ($n = 272$) or lopinavir/r ($n = 184$) or atazanavir boosted with ritonavir ($n = 121$). Age was median 44 years (interquartile range [IQR] 37–50 years). The presumed mode of HIV transmission was men who had sex with men (44.4%), heterosexual (40.6%), injection drug users (11.4%), and unknown/other (3.6%). Baseline (ART start) CD4+ T cell count was median 209 cells/μL (IQR: 125–282 cells/μL), and HIV viral load was median 4.9 log copies/mL (IQR: 4.4–5.4 log copies/mL). The exact time span of the study was from 5 January 2004 (earliest ART initiation) to 4 November 2008 (last registered follow-up). The median follow-up time (interval between ART initiation date and last registered follow-up date) was 618 days (IQR: 463–914 days). A total of 53 (9%) participants were lost for follow-up during the first year after ART initiation, and were censored. Distribution of alleles and genotypes were comparable for the populations receiving or not receiving the drug concerned by each specific genetic variant, with the exception of *CYP2B6* rs28399499, a genetic variant only present in Africans. A total of 190 (33%) individuals discontinued ART; in 117 instances, this concerned a single drug; in 62 instances, two or more components of the regimen; and 11 individuals stopped 2 or more drugs at different times.

Genetic Risk and Discontinuation of Tenofovir

Among 500 patients receiving tenofovir, 70 (14%) discontinued treatment in the first year. The various genetic variants evaluated did not present a statistically significant association with rates of treatment discontinuation (Table 2). In the first year, individuals carrying 1 (CT) or 2 (TT) risk alleles of *ABCC4* rs899494 discontinued tenofovir with cumulative rates of 19% and 7%, respectively, compared with 13% among individuals without the genetic risk allele (CC): adjusted hazard ratio (HR_a) (CT) 1.60 (95% CI: 0.95–2.70), $P_a = .080$; and HR_a (TT) 0.38 (95% CI: 0.05–2.85), $P_a = .345$, respectively (Figure 1A). Individuals carrying 1 (GA) or 2 (AA) risk alleles of *ABCC2* rs2273697 variant discontinued tenofovir with cumulative rates of 12.26% and 28.60%, respectively, compared with 14.23% among individuals without the genetic risk allele (GG): HR_a (GA) 0.77 (95% CI: 0.44–1.36), $P_a = .371$; and HR_a (AA) 1.39, (95% CI: 0.58–3.40), $P_a = .473$, respectively (Figure 1B). We did not observe differences in discontinuation rates associated with

Table 2. Detailed Results From the Cox Regression Models for Each Drug

Drug	SNP/score	Genotype/score group	Adjusted hazard ratio (95% confidence interval)	Adjusted *P* value	Cumulative discontinuation rate (%)
Tenofovir	*ABCC4* rs899494	CC (0)	1 (Reference)	—	12.66
		CT (1)	1.60 (0.95–2.70)	*.080*	19.00
		TT (2)	0.38 (0.05–2.85)	*.345*	6.70
	ABCC2 rs2273697	GG (0)	1 (Reference)	—	14.23
		GA (1)	0.77 (0.44–1.36)	*.371*	12.26
		AA (2)	1.39 (0.58–3.40)	*.473*	28.60
	ABCC2 rs717620	CC (0)	1 (Reference)	—	15.30
		CT (1)	0.85 (0.48–1.52)	*.582*	11.80
		TT (2)	1.15 (0.34–3.87)	*.815*	13.04
Efavirenz	*CYP2B6/2A6/3A4* score	Score 1	1 (Reference)	—	32.30
		Score 2	0.85 (0.43–1.70)	*.648*	28.90
		Score 3	0.57 (0.28–1.17)	*.125*	22.00
		Score 4	0.61 (0.27–1.38)	*.234*	29.90
		Score 5	0.86 (0.24–3.10)	*.820*	23.00
		Score 6	2.10 (0.70–6.01)	*.185*	71.15
	CYP2B6/2A6/3A4 score (dichotomized)	Score 1–5	1 (Reference)	—	28.10
		Score 6	3.14 (1.35–7.33)	***.008***	71.15
Lopinavir	Hypertriglyceridemia additive score	Score 1	1 (Reference)	—	30.00
		Score 2	0.99 (0.52–1.88)	*.967*	35.90
		Score 3	1.27 (0.61–2.61)	*.523*	36.40
	Pharmacokinetic score	Score -2	1 (Reference)	—	20.00
		Score 0	2.03 (0.26–15.87)	*.499*	34.00
		Score +2	2.15 (0.27–17.27)	*.473*	36.00
Atazanavir	*UGT1A1* rs8175347	**1/*1*	1 (Reference)	—	14.60
		**1/*28, *37*	1.97 (0.77–5.03)	*.158*	23.80
		**28/*28, *37*	9.13 (3.38–24.69)	***<.0001***	62.50
	NR1I2 rs2472677	TT (0)	1 (Reference)	—	21.80
		TC (1)	1.42 (0.61–3.26)	*.413*	28.90
		CC (2)	1.1 (0.32–3.67)	*.905*	22.30
	ABCB1 rs1045642	CC (0)	1 (Reference)	—	21.30
		CT (1)	1.54 (0.55–4.32)	*.416*	22.50
		TT (2)	2.33 (0.84–6.50)	*.105*	34.80
	UGT1A1/ABCB1 additive score	Score 0+1	1 (Reference)	—	14.61
		Score 2	2.53 (0.95–6.70)	*.062*	29.16
		Score 3	5.44 (1.86–15.90)	***.002***	39.40
		Score 4	7.22 (1.90–27.50)	***.004***	57.14

NOTE. SNP: single-nucleotide polymorphism.

The nongenetic covariables evaluated and included when appropriate included body weight, sex, ethnicity, CD4 T cell count and viral RNA concentration, transmission category, year of starting ART, treatment regimen, clinical center, and pregnancy status.

ABCC2 rs717620 genotypes (Figure 1C). The final Cox analysis identified body weight as significant covariable. Individuals with body weight lower than 60 kg or between 60 and 69 kg had higher risk of discontinuation (HR_a = 2.71, 95% CI: 1.14–6.44, P_a = .024; and HR_a = 2.43, 95% CI: 1.13–5.26, P_a = .024, respectively) compared with the reference group (70–79 kg).

Genetic Risk and Discontinuation of Efavirenz

Among 272 patients receiving efavirenz, 81 discontinued treatment in the first year with a cumulative rate of 30%. For 3 individuals, the event was a dose reduction rather than full discontinuation; 2 of the 3 had neuropsychological toxicity as the stated reason for dose reduction. The various genetic variants and scores were associated with different rates of treatment discontinuation (Table 2). Consistent with the known pharmacokinetic associations, the data indicated that loss of *CYP2B6* function (homozygocity, decrease/loss of function alleles) with a concomitant decrease of function in accessory metabolic pathways (decrease/loss of function alleles of *CYP2A6* and/or *CYP3A4*) was associated with a higher

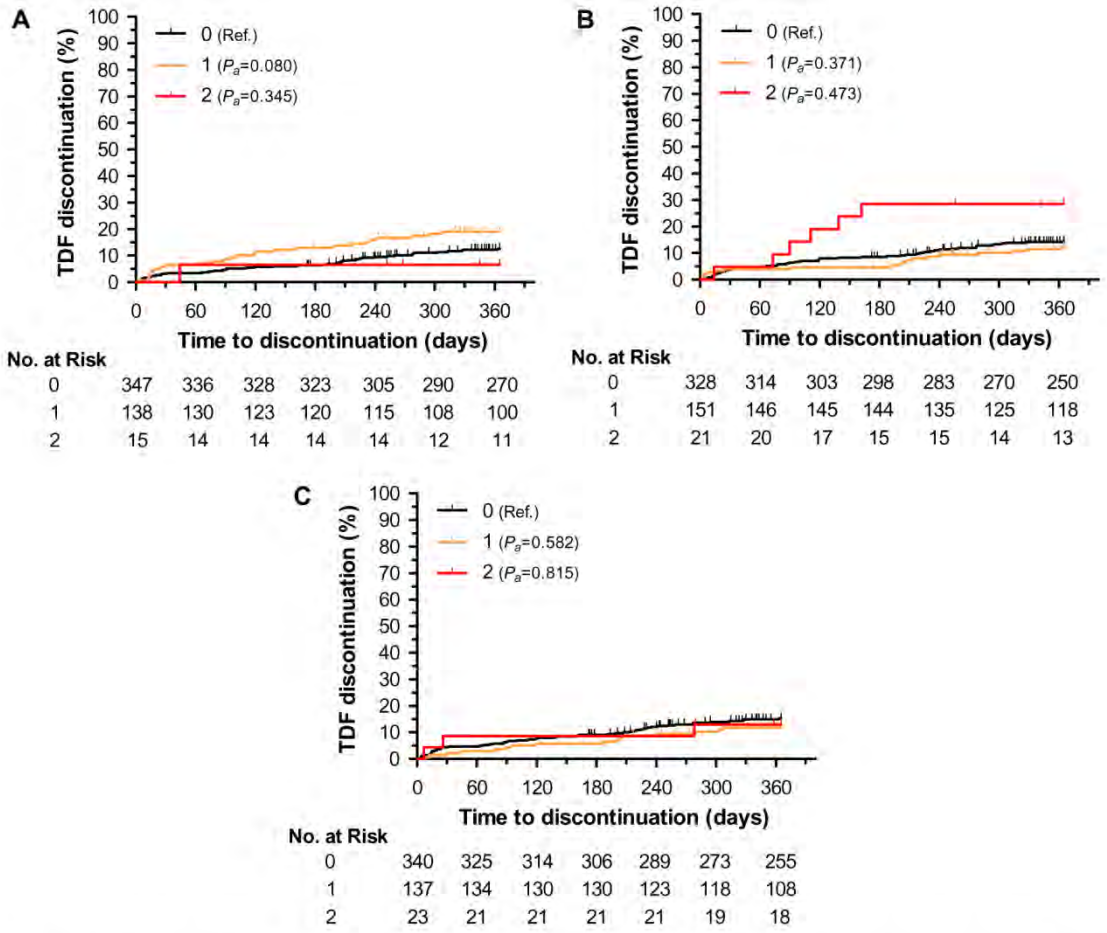

Figure 1. Genetic risk of tenofovir discontinuation at 1 year. Cumulative rates of discontinuation for 500 participants stratified by *ABCC4* and *ABCC2* genetic variants: (*A*) by *ABCC4* rs899494 genotypes CC (0), CT (1), and TT (2); (*B*) by *ABCC2* rs2273697 genotypes GG (0), GA (1), and AA (2); and (*C*) by *ABCC2* rs717620 genotypes CC (0), CT (1), and TT (2). P_a, covariate adjusted *P* value, estimated from Cox regression models.
NOTE. CC: individuals without the genetic risk allele *ABCC4* rs899494; CT: individuals carrying 1 risk allele *ABCC4* rs899494; TT: individuals carrying 2 risk alleles *ABCC4* rs899494; GG: individuals without genetic risk allele *ABCC2* rs2273697; GA: individuals carrying 1 genetic risk allele *ABCC2* rs2273697; AA: individuals carrying 2 genetic risk alleles *ABCC2* rs2273697.

risk of discontinuation (Figure 2). Individuals with the highest genetic risk score (score 6) discontinued efavirenz more frequently than individuals with lower genetic risk scores, with cumulative rates of 71.15% vs 28.10% (HR_a = 3.14, 95% CI: 1.35–7.33, P_a = .008) (Table 2). Among covariates, sex showed an independent statistically significant effect on efavirenz discontinuation, with women showing a higher risk (HR_a = 3.08, 95% CI: 1.62–5.85, P_a = .001), consistent with published estimates [26–28].

Genetic Risk and Discontinuation of Lopinavir

Among 184 patients receiving lopinavir, 60 discontinued treatment in the first year, with a cumulative rate of 34%. Eight genetic variants associated with hypertriglyceridemia [12, 13] were evaluated individually and in unweighted additive scores. Only *CETP* rs708272 G>A genotypes displayed when assessed in isolation, assuming a dominant genetic model (GG vs GA + AA), a marginally significant protection from early treatment discontinuation (HR_a = 0.60, 95% CI: 0.35–1.01, P_a = .056). The unweighted 3-level additive score (Table 1) did not show an association of genetic variants with early treatment discontinuation (Figure 3A and Table 2).

Separately, we assessed the contribution of 4 genetic variants in 3 genes that are associated with lopinavir/r pharmacokinetics [14]. None of the 4 single-nucleotide polymorphisms (SNPs) were individually associated with treatment discontinuation. A 3-level genetic score (Table 1) did not identify a significant statistical association with treatment discontinuation, although we observed low rates of treatment discontinuation among the rare individuals homozygous for *SLCO1B1*4* (rs11045819), a gain-of-function variant associated with low lopinavir/r levels (Figure 3B). No other covariates were associated with lopinavir/r discontinuation at 1 year.

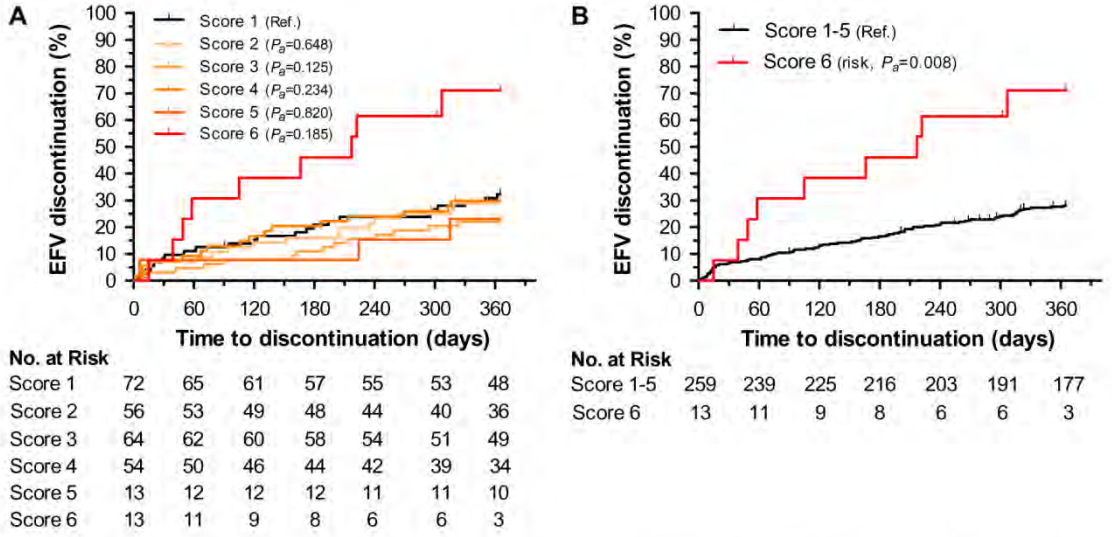

Figure 2. Genetic risk of efavirenz discontinuation at 1 year. Cumulative rates of discontinuation for 272 participants stratified by *CYP2B6, CYP2A6,* and *CYP3A4* genetic variants by six-group genetic score (*A*) and by dichotomized genetic score. P_a, covariate adjusted *P* value (*B*).

Genetic Risk and Discontinuation of Atazanavir

Among 121 patients receiving atazanavir boosted with ritonavir, 30 discontinued treatment in the first year with a cumulative rate of 25%. Homozygocity of decreased function *UGT1A1* alleles (**28/*28* or **28/*37*) was associated with risk of treatment discontinuation (HR_a = 9.13, 95% CI: 3.38–24.69, $P_a < .0001$) (Figure 4A and Table 2). There was a statistically nonsignificant increase in risk associated with the carrier state (HR_a = 1.97, 95% CI: 0.77–5.03, P_a =.158). First-year cumulative rates of treatment discontinuation were 62.5% for homozygous, 23.8% for heterozygous, and 14.6% for noncarrier individuals. No other covariates were associated with atazanavir discontinuation at 1 year. We explored whether differences in rates of discontinuation across centers (varying from 0% to 47%) could reflect center-specific policy and threshold of response to the specific toxicity. For the 4 centers prescribing atazanavir to more than 10 individuals, there was a statistically significant correlation between discontinuation rates and frequency of *UGT1A1*28* homozygocity (r^2 = 0.858, P =.048).

Two other studies have described the association of variants of *NR1I2* and *ABCB1* genes to atazanavir pharmacokinetics and/or hyperbilirubinemia [17, 18]. We did not observe an association of *NR1I2* variant and treatment discontinuation rates (Figure 4B). Variant rs1045642 G>A of *ABCB1* was associated with different rates of treatment discontinuation (Figure 4C). Homozygous individuals had an HR_a of 2.33 (95% CI: 0.84–6.50), although the results did not reach statistical significance (P_a =.105). The inclusion of *ABCB1* variant in an additive *UGT1A1/ABCB1* genetic

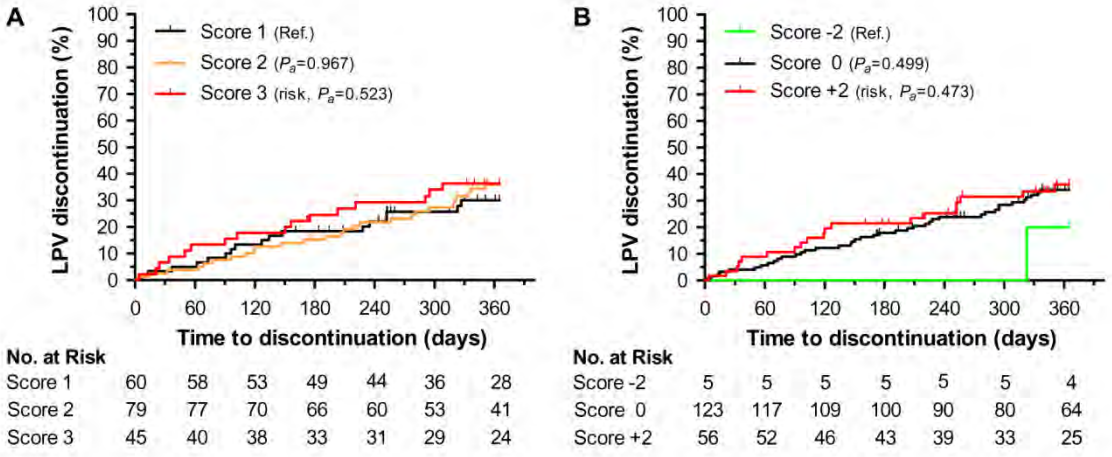

Figure 3. Genetic risk of lopinavir discontinuation at 1 year. Cumulative rates of discontinuation for 184 participants stratified by hypertriglyceridemia (*A*) and pharmacokinetic (*B*) genetic scores. P_a, covariate adjusted *P* value.

score (Table 1) stratified the risk of discontinuation conferred by *UGT1A1* variants (Figure 4D). In the joint analysis, the cumulative discontinuation rate ranged from 29% to 57% across genetic risk scores. However, these *UGT1A1/ABCB1* genetic score–containing Cox regression model did not reduce the Akaike information criterion compared with the model containing only *UGT1A1* (262.93 vs 257.40, respectively).

Early Discontinuation

Eleven (2%) of the participants discontinued treatment within the first week—the earliest discontinuation was on day 2. Six patients discontinued tenofovir, none were homozygous for the risk allele of *ABCC2* rs2273697. Four patients discontinued efavirenz; none had a genetic risk score of 6. Three patients discontinued lopinavir/r; 1 had a pharmacokinetic genetic risk score of +2, and 2 had a dyslipidemia genetic risk score of 3. Two patients discontinued atazanavir; both were homozygous for the risk allele of *UGT1A1* rs8175347.

Evaluation of Coded Reasons for Discontinuation

During routine care, the SHCS physician indicates a coded reason for discontinuation at the time of treatment stop or modification. Retrospective assessment of the codes for the overall study identified the following categories as main reasons: drug-associated toxicity (15%), physician decision (8%), and patient decision (6%), which are in accordance with previously reported reasons for treatment change [4]. The reported reasons for treatment discontinuation are presented for each drug in Table 3. Based on the data presented in the previous sections, we used a score of 6 to define efavirenz genetic risk. For atazanavir, we defined risk by the simpler *UGT1A1*-based risk (promoter allele **28/28* or **28/*37*) based on the Akaike information

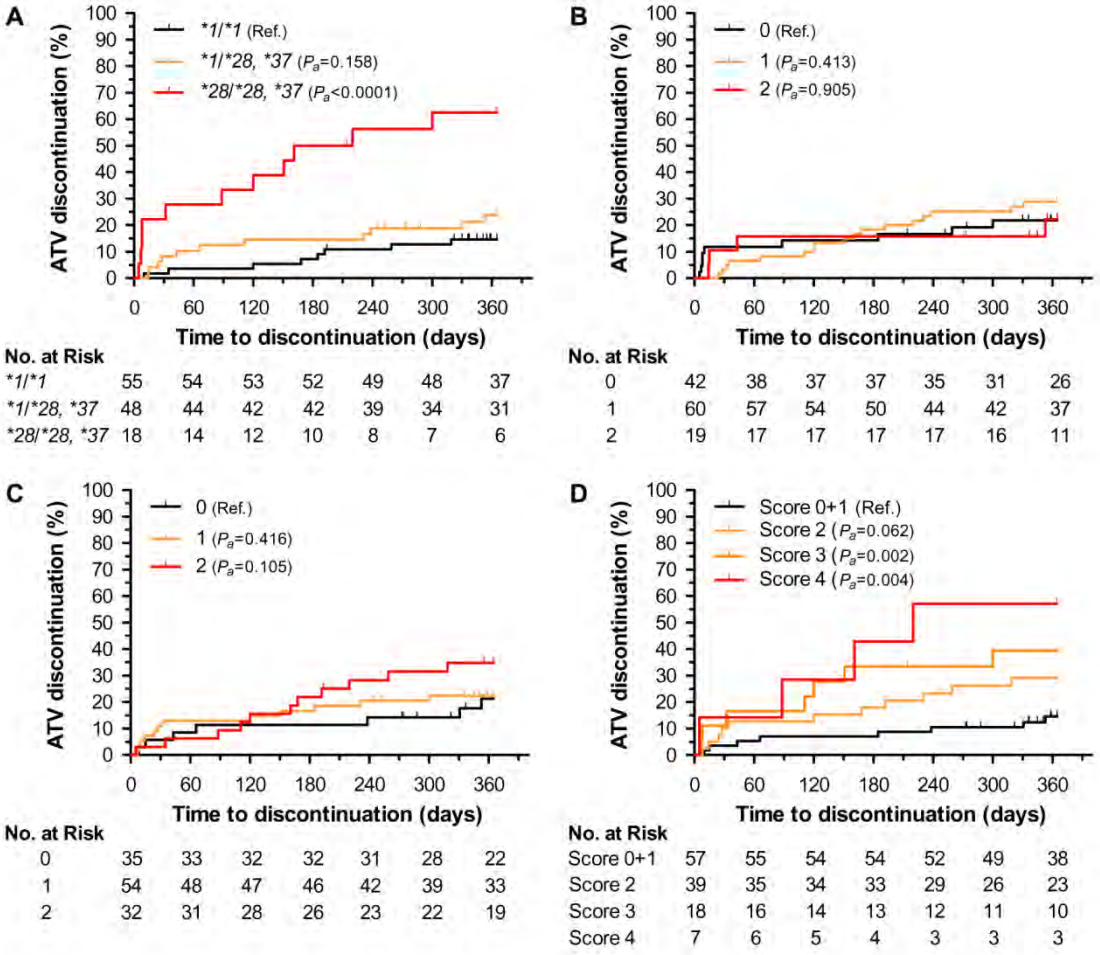

Figure 4. Genetic risk of atazanavir discontinuation at 1 year. Cumulative rates of discontinuation for 121 participants stratified by *UGT1A1*, *NR1I2*, and *ABCB1* genetic variants: (*A*) by *UGT1A1* rs8175347 genotypes **1/*1*, **1/*28* or **37*, and **28/*28* or **37*; (*B*) by *NR1I2* rs2472677 genotypes TT (0), CT (1), and CC (2); (*C*) by *ABCB1* rs1045642 genotypes CC (0), CT (1), and TT (2); (*D*) by *UGT1A1/ABCB1* additive genetic score. P_a, covariate adjusted *P* value. **NOTE.** TT: individuals carrying 2 risk alleles *ABCC4* rs899494; CT: individuals carrying 1 risk allele *ABCC4* rs899494; CC: individuals without the genetic risk allele *ABCC4* rs899494.

Table 3. Reasons for Drug Discontinuation According to the Coding Provided by the Treating Physician.

	Tenofovir[a]			Efavirenz[b]			Lopinavir/r[c]			Atazanavir[d]		
Reason for drug discontinuation	**Population with genetic risk, *n* = 21 Discontinuation events (%)**	**Population without genetic risk, *n* = 479 Discontinuation events (%)**	***P*[e]**	**Population with genetic risk, *n* = 13 Discontinuation events (%)**	**Population without genetic risk, *n* = 259 Discontinuation events (%)**	***P*[e]**	**Population with HTG genetic risk, *n* = 45 Discontinuation events (%)**	**Population without HTG genetic risk, *n* = 139 Discontinuation events (%)**	***P*[e]**	**Population with genetic risk, *n* = 18 Discontinuation events (%)**	**Population without genetic risk, *n* = 103 Discontinuation events (%)**	***P*[e]**
Virological treatment failure	0 (0.00)	6 (1.25)	*1*	0 (0.00)	15 (5.79)	*1*	0 (0.00)	0 (0.00)	*1*	0 (0.00)	0 (0.00)	*1*
Drug associated toxicity	3 (14.29)	22 (4.59)	*.081*	8 (61.54)[f]	32 (12.36)	***<.0001***	6 (13.33)	12 (8.63)	*.389*	6 (33.33)	7 (6.80)	***.004***
Patient decision	1 (4.76)	22 (4.59)	*1*	1 (7.69)	8 (3.09)	*.361*	5 (11.11)	11 (7.91)	*.545*	2 (11.11)	6 (5.83)	*.339*
Physician decision	0 (0.00)	12 (2.51)	*1*	0 (0.00)	12 (4.63)	*1*	5 (11.11)	20 (14.39)	*.803*	2 (11.11)	5 (4.85)	*.279*
Other	2 (9.52)	2 (0.42)	***.009***	0 (0.00)	5 (1.93)	*1*	0 (0.00)	1 (0.72)	*1*	1 (5.56)	1 (0.97)	*.274*

NOTE. Based on the data presented in the text, we used score 6 to define efavirenz genetic risk. For atazanavir, we defined risk by the simpler *UGT1A1*-based risk (promoter allele **28/28* or **28/*37*) based on the Akaike information criterion. Because there is no defined genetic risk for treatment discontinuation of tenofovir, homozygocity *ABCC2* rs2273697 was further explored for the analysis of reasons for treatment discontinuation. For lopinavir/r, we considered the genetic risk for dyslipidemia as surrogate for the analysis of reasons for treatment discontinuation.

[a] Population with (AA or GA) and without (GG) genetic risk of tenofovir discontinuation according to the *ABCC2* rs2273697 variant genotypes.

[b] Population with (score 6) and without (scores 1 to 5) genetic risk of efavirenz discontinuation according to the *CYP2B6/2A6/3A* genetic score.

[c] Population with (score 3) and without (scores 1 and 2) genetic risk of lopinavir/r discontinuation according to the risk of hypertriglyceridemia (HTG) additive genetic score.

[d] Population with (**28/*28* or **28/*37*) and without (**1/*1*, **1/*28*, or **1/*36*) genetic risk of atazanavir discontinuation according to the *UGT1A1* rs8175347 variant genotypes.

[e] Fisher exact test *P* value.

[f] Includes 3 instances of treatment dose modification.

criterion. There is no defined genetic risk for treatment discontinuation for tenofovir or lopinavir/r. For tenofovir, we explored whether the trend observed for *ABCC2* rs2273697 was further supported in the clinical analysis. For lopinavir/r, we considered the genetic risk for dyslipidemia as surrogate for the genetic risk of treatment discontinuation. The code "drug-associated toxicity" was invoked in 62% of instances when efavirenz was discontinued among individuals with a genetic risk versus 12% in those without ($P < .0001$). The code "drug-associated toxicity" was invoked in 33% of instances when atazanavir was discontinued among individuals with a genetic risk versus 7% in those without ($P = .004$).

DISCUSSION

This study tested the hypothesis that biological consequences of genetic variants will, if significant or severe, lead to treatment discontinuation, a well defined clinical outcome. Among 23 genetic variants included in the study, those in *CYP2B6*, *2A6*, and *3A4* previously associated with plasma levels of efavirenz, and those in *UGT1A1* previously associated with atazanavir-induced hyperbilirubinemia were also associated with early treatment discontinuation. The retrospective study was not designed to assess other clinical endpoints such as specific toxicities; however, assessment of the codes used in routine care to indicate the reasons for treatment discontinuation convincingly associated genetic risk to toxicity for those 2 drugs.

Thus, the study suggests that assessment of the genetic markers could lead to improved prescription of atazanavir and efavirenz. Unconjugated hyperbilirubinemia, the main adverse effect of atazanavir, is widely considered as irrelevant in clinical care [29]. However, despite the reversibility and the absence of clinical consequences of hyperbilirubinemia, 2 of 3 individuals homozygous for the *UGT1A1* promoter variant discontinued atazanavir in the present study. Differences in rates of discontinuation across centers reflected the frequency of homozygous individuals receiving the drug. Overall, 10% of Caucasians carry this genetic risk, but its frequency varies across ethnic groups [30].

The basis of treatment discontinuation of efavirenz is more complex. While the genetic determinants of efavirenz blood levels are by now well understood [10, 11], there is no consensus as to whether high drug levels are associated with neuropsychotoxicity, or on the usefulness of therapeutic drug monitoring and dose adjustment [31]. These considerations notwithstanding, the study suggests that treatment discontinuation correlates with decreased metabolism of efavirenz, and that the loss of the primary metabolic pathway, *CYP2B6*, is necessary but not sufficient [32] for an increased rate in efavirenz discontinuation. Extremely high plasma drug levels of efavirenz results from loss of both primary (*CYP2B6*) and accessory (*CYP3A4* and/or *CYP2A6*) metabolic pathways [10]. Consistent with such pharmacokinetic data, individuals that have loss of accessory metabolisms in the context of homozygous loss of function of *CYP2B6* present the highest risk of treatment discontinuation. As pointed out for atazanavir, these data question the interest to initiate efavirenz in individuals with the genetic risk if two-thirds of them will subsequently discontinue the drug.

The study included tenofovir despite the paucity of genetic data associated with toxicity for this drug. We analyzed the genetic variants as proposed by Izzedine et al. [19] in a study of 13 individuals with tubular dysfunction on tenofovir, and by Rodriguez-Novoa et al. [20] in a study of 115 HIV-infected patients of whom 19 had tubular dysfunction. The current study suggests a possible association of rs2273697 G>A variant in *ABCC2* with higher rates of drug discontinuation. The SHCS database did not allow for detailed analysis of the reasons for discontinuation in these individuals; in particular, for renal function (see below). Thus, the data on rs2273697 G>A should be considered preliminary; additional discovery efforts on the genetics of tenofovir are needed.

The case of lopinavir is a pertinent example of how a well-documented association of genetic variants and a laboratory phenotype (dyslipidemia) with potential long-term consequences—cardiovascular disease—may not translate into short-term clinical decisions. We did not document an association between the proposed risk score and treatment discontinuation or introduction of a lipid-lowering agent. However, the most appropriate clinical endpoint here would have been the actual laboratory data (reaching clinically relevant cut-offs of dyslipidemia) [12]. The patient carrying the genetic risk could be prescribed an alternative drug on that basis. Prediction of dyslipidemia will improve with the increasing availability of genetic markers [13, 33].

This study did not include abacavir hypersensitivity because clinical testing for *HLA*B5701* was introduced during the study period. We did, however, notice that the allelic frequency for the risk marker was lower in patients that received abacavir than among those that did not (data not shown)—confirming that the patients were increasingly screened before treatment initiation. Another relevant observation of the present study is that although genetic predisposition to toxicity might lead, through poor adherence, to virological treatment failure, there were few ($n = 15$) instances coded as virological treatment failure, and none among participants with the genetic risk marker. The current analysis did not consider drug adherence; its inclusion would have been informative.

This study should be considered as a pilot analysis that can provide data for power calculation, and support further discovery efforts toward identification of additional genetic markers. Thereafter, a prospective clinical trial should ideally formalize the analysis, and provide the basis for measuring the cost effectiveness of this approach [34].

Funding

This work has been partially financed within the framework of the SHCS funded by the Swiss National science foundation (grant number 33CSC0-108787 and 324730-12493), by the SHCS research foundation, and by the SHCS project number 548.

Acknowledgments

We thank the patients for participating in the Swiss HIV Cohort study, all the physicians and study nurses of the centers for excellent patient care, and the data center for data management.

The members of the Swiss HIV Cohort Study (SHCS) are M. Battegay, E. Bernasconi, J. Böni, H.C. Bucher, P. Bürgisser, A. Calmy, S. Cattacin, M. Cavassini, R. Dubs, M. Egger, L. Elzi, M. Fischer, M. Flepp, A. Fontana, P. Francioli (President of the SHCS), H. Furrer (Chairman of the Clinical and Laboratory Committee), C.A. Fux, M. Gorgievski, H.F. Günthard (Chairman of the Scientific Board), H.H. Hirsch, B. Hirschel, I. Hösli, C. Kahlert, L. Kaiser, U. Karrer, C. Kind, T. Klimkait, B. Ledergerber, G. Martinetti, N. Müller, D. Nadal, F. Paccaud, G. Pantaleo, A. Rauch, S. Regenass, M. Rickenbach (Head of Data Center), C. Rudin (Chairman of the Mother & Child Substudy), P. Schmid, D. Schultze, J. Schüpbach, R. Speck, B.M. de Tejada, P. Taffé, A. Telenti, A. Trkola, P. Vernazza, R. Weber, and S. Yerly.

Author Contributions: ICMJE criteria for authorship read and met: R.L., S.C., and A.T. Agree with the manuscript's results and conclusions: R.L., S.C., J.d.I., R.M., B.L., M.C., B.H., E.B., L.E., P.V., H.F., H.F.G., and A.T. Designed the experiments/the study: R.L., S.C., B.L., and A.T. Analyzed the data: R.L., B.L., and S.C. Collected data/did experiments for the study: S.C., J.d.I., and R.M. Contributed clinical data: M.C., B.H., E.B., L.E., P.V., H.F., and H.F.G. Wrote the first draft of the paper: R.L., S.C., and A.T. Contributed to the writing of the paper: all authors.

References

1. Fellay J, Boubaker K, Ledergerber B, et al. Prevalence of adverse events associated with potent antiretroviral treatment: Swiss HIV Cohort Study. Lancet **2001**; 358:1322–7.
2. Keiser O, Fellay J, Opravil M, et al. Adverse events to antiretrovirals in the Swiss HIV Cohort Study: effect on mortality and treatment modification. Antivir Ther **2007**; 12:1157–64.
3. Vo TT, Ledergerber B, Keiser O, et al. Durability and outcome of initial antiretroviral treatments received during 2000–2005 by patients in the Swiss HIV Cohort Study. J Infect Dis **2008**; 197:1685–94.
4. Elzi L, Marzolini C, Furrer H, et al. Treatment modification in human immunodeficiency virus-infected individuals starting combination antiretroviral therapy between 2005 and 2008. Arch Intern Med **2010**; 170:57–65.
5. d'Arminio Monforte A, Lepri AC, Rezza G, et al. Insights into the reasons for discontinuation of the first highly active antiretroviral therapy (HAART) regimen in a cohort of antiretroviral naive patients. I.CO.N.A. Study Group. Italian Cohort of Antiretroviral-Naive Patients. AIDS **2000**; 14:499–507.
6. Sabin CA, Smith CJ, Delpech V, et al. The associations between age and the development of laboratory abnormalities and treatment discontinuation for reasons other than virological failure in the first year of highly active antiretroviral therapy. HIV Med **2009**; 10:35–43.
7. Mallal S, Phillips E, Carosi G, et al. HLA-B*5701 screening for hypersensitivity to abacavir. N Engl J Med **2008**; 358:568–79.
8. Haas DW, Ribaudo HJ, Kim RB, et al. Pharmacogenetics of efavirenz and central nervous system side effects: an Adult AIDS Clinical Trials Group study. AIDS **2004**; 18:2391–400.
9. Rotger M, Colombo S, Furrer H, et al. Influence of CYP2B6 polymorphism on plasma and intracellular concentrations and toxicity of efavirenz and nevirapine in HIV-infected patients. Pharmacogenet Genomics **2005**; 15:1–5.
10. di Iulio J, Fayet A, Arab-Alameddine M, et al. In vivo analysis of efavirenz metabolism in individuals with impaired *CYP2A6* function. Pharmacogenet Genomics **2009**; 19:300–9.
11. Arab-Alameddine M, di Iulio J, Buclin T, et al. Pharmacogenetics-based population pharmacokinetic analysis of efavirenz in HIV-1-infected individuals. Clin Pharmacol Ther **2009**; 85:485–94.
12. Arnedo M, Taffe P, Sahli R, et al. Contribution of 20 single-nucleotide polymorphisms of 13 genes to dyslipidemia associated with antiretroviral therapy. Pharmacogenet Genomics **2007**; 17:755–64.
13. Rotger M, Bayard C, Taffe P, et al. Contribution of genome-wide significant single-nucleotide polymorphisms and antiretroviral therapy to dyslipidemia in HIV-infected individuals: a longitudinal study. Circ Cardiovasc Genet **2009**; 2:621–8.
14. Lubomirov R, di Iulio J, Fayet A, et al. ADME pharmacogenetics: investigation of the pharmacokinetics of the antiretroviral agent lopinavir coformulated with ritonavir. Pharmacogenet Genomics **2010**; 20:217–30.
15. Hartkoorn RC, Kwan WS, Shallcross V, et al. HIV protease inhibitors are substrates for *OATP1A2*, *OATP1B1* and *OATP1B3* and lopinavir plasma concentrations are influenced by *SLCO1B1* polymorphisms. Pharmacogenet Genomics **2010**; 20:112–20.
16. Rotger M, Taffe P, Bleiber G, et al. Gilbert syndrome and the development of antiretroviral therapy–associated hyperbilirubinemia. J Infect Dis **2005**; 192:1381–6.
17. Rodriguez-Novoa S, Martin-Carbonero L, Barreiro P, et al. Genetic factors influencing atazanavir plasma concentrations and the risk of severe hyperbilirubinemia. AIDS **2007**; 21:41–6.
18. Siccardi M, D'Avolio A, Baietto L, et al. Association of a single-nucleotide polymorphism in the pregnane X receptor (*PXR63396 C–>T*) with reduced concentrations of unboosted atazanavir. Clin Infect Dis **2008**; 47:1222–5.
19. Izzedine H, Hulot JS, Villard E, et al. Association between *ABCC2* gene haplotypes tenofovir-induced proximal tubulopathy. J Infect Dis **2006**; 194:1481–91.
20. Rodriguez-Novoa S, Labarga P, Soriano V, et al. Predictors of kidney tubular dysfunction in HIV-infected patients treated with tenofovir: a pharmacogenetic study. Clin Infect Dis **2009**; 48:e108–16.
21. The Swiss HIV Cohort Study. Cohort profile: the Swiss HIV cohort study. Int J Epidemiol. **2010**; 39:1179–89.
22. Rotger M, Tegude H, Colombo S, et al. Predictive value of known and novel alleles of *CYP2B6* for efavirenz plasma concentrations in HIV-infected individuals. Clin Pharmacol Ther **2007**; 81: 557–66.
23. Tarr PE, Taffe P, Bleiber G, et al. Modeling the influence of *APOC3*, *APOE*, *TNF* polymorphisms on the risk of antiretroviral therapy–associated lipid disorders. J Infect Dis **2005**; 191:1419–26.
24. Telenti A, Zanger UM. Pharmacogenetics of anti-HIV drugs. Annu Rev Pharmacol Toxicol **2008**; 48:227–56.
25. Kathiresan S, Melander O, Anevski D, et al. Polymorphisms associated with cholesterol and risk of cardiovascular events. N Engl J Med **2008**; 358:1240–9.
26. Spire B, Carrieri P, Garzot MA, L'henaff M, Obadia Y. Factors associated with efavirenz discontinuation in a large community-based sample of patients. AIDS Care **2004**; 16:558–64.
27. Domingo P, Suarez-Lozano I, Torres F, et al. First-line antiretroviral therapy with efavirenz or lopinavir/ritonavir plus two nucleoside analogues: The SUSKA study, a nonrandomized comparison from the VACH cohort. J Antimicrob Chemother **2008**; 61:1348–58.
28. Robison LS, Westfall AO, Mugavero MJ, et al. Short-term discontinuation of HAART regimens more common in vulnerable patient populations. AIDS Res Hum Retroviruses **2008**; 24:1347–55.
29. Nettles RE, Child MJ, Bertz RJ, Schnittman S. Gilbert syndrome and the development of antiretroviral therapy–associated hyperbilirubinemia: genetic screening is unnecessary. J Infect Dis **2006**; 193:1611–2.

30. Innocenti F, Grimsley C, Das S, et al. Haplotype structure of the UDP-glucuronosyltransferase 1A1 promoter in different ethnic groups. Pharmacogenetics **2002**; 12:725–33.
31. Rotger M, Telenti A. Optimizing efavirenz treatment: *CYP2B6* genotyping or therapeutic drug monitoring? Eur J Clin Pharmacol **2008**; 64:335–6.
32. Powers V, Ward J, Gompels M. *CYP2B6 G516T* genotyping in a UK cohort of HIV-positive patients: polymorphism frequency and influence on efavirenz discontinuation. HIV Med **2009**; 10:520–3.
33. Manolio TA. Cohort studies and the genetics of complex disease. Nat Genet **2009**; 41:5–6.
34. Telenti A. Time (again) for a randomized trial of pharmacogenetics of antiretroviral therapy. Pharmacogenomics **2009**; 10:515–6.

4.1.1.4. Original article

Successful Efavirenz Dose Reduction Led by Therapeutic Drug Monitoring

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Antiviral Therapy 2011; in press

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Efavirenz plasma levels determination: A. Fayet

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Critical revision of the manuscript for important intellectual content: all authors

Detailed personal contribution

I participated in the study concept and design, sample recruitment and DNA extraction, data analyses and interpretation, as well as in the genetic characterization of individuals included in the study by performing:

1. Genetic characterization of individuals with genetic consent selected for efavirenz dose reduction (N=12):
    - Comprehensive *CYP2B6* characterization including exon-intron boundaries resequencing and copy number variation assessment
    - Comprehensive *CYP2A6* characterization including exon-intron boundaries resequencing and copy number variation assessment
    - Genotyping of 2 *CYP3A4* variatiants (rs2740574 [*CYP3A4*1B*] and rs4646437)

2. Genetic characterization of individuals not selected for efavirenz dose reduction (N=59):
    - Genotyping of rs3745274 (*CYP2B6*6*)

Comments on the article:

Individuals receiving an efavirenz-containing regimen depict a wide interindividual variability in EFV exposure. In this study we investigated whether a therapeutic drug monitoring (TDM) based algorithm could help determining the dose reduction needed (either 400mg QD or 200mg QD) in individuals with high plasma levels, to fall into the therapeutic window (1000-4000 ng/mL). We then compared the dosage reduction estimated through the TDM-based algorithm with the one predicted by genetic analysis.

Individuals with EFV plasma concentration between the 75th and the 95th percentile received a reduced dose of 400mg EFV QD, whereas individuals with concentration above the 95th percentile received a dose of 200mg EFV QD.

The genetic dose reduction prediction was based on the hypothesis that all individuals without impaired CYP2B6 function would not require a dose reduction. Individuals with impaired CYP2B6 function and reference alleles for *CYP2A6* and *CYP3A4* would need a one unit dose reduction (=400mg EFV). Finally, individuals with impaired CYP2B6 function **and** impaired CYP2A6 and/or CYP3A4 would need a two unit dose reduction (=200mg EFV).

The genotyping results could predict nearly the same dose reduction than the TDM-based algorithm. There were only three discrepant individuals: two individuals remained with high EFV plasma concentration after a TDM-guided one unit dose reduction, whereas genotyping would have predicted a two unit dose reduction. One individual achieved the lowest EFV plasma concentration in the studied population after a TDM-guided two unit dose reduction, whereas genotyping would have predicted a one unit dose reduction.

This study emphasizes the predictive power of genotyping, but further prospective analyses are still needed to confirm these results.

Antiviral Therapy 2011; 16: in press (doi: 10.3851/IMP1742)

Original article

Successful efavirenz dose reduction guided by therapeutic drug monitoring

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Background: There are potential benefits to individualizing dosage in patients treated with efavirenz (EFV). We tested a simplified algorithm based on a Bayesian pharmacokinetic approach for guiding dose reduction in patients with EFV concentrations above the 75th percentile (P75) with documented virological efficacy.
Methods: We designed a prospective, open-label, multicentre study. All consenting participants with EFV concentrations above P75 on standard dosage were included in a dose-reduction cycle. Primary end point was the number of patients who reached plasma concentrations within target (1,000–4,000 ng/ml) after, at most, two cycles of dose reduction at 3 and 6 months. *CYP2B6* genetic characterization was performed.
Results: Seventy-two patients were screened and 13 fulfilled selection criteria. These patients, with undetectable viraemia on a stable 600 mg EFV-based regimen, had a median (interquartile range) EFV plasma level of 8,112 ng/ml (5,993–10,278) at baseline; 38% (between P75 and P95) qualified for a 400 mg EFV dose, and 62% (above P95) qualified for a 200 mg EFV dose. After one to two dose-reduction cycles, all patients reached targets for EFV plasma concentration at 24 weeks. The predictive dose reduction based on genetic profile differed from dose reduction according to therapeutic drug monitoring (TDM) in three patients. All patients maintained viral suppression at 6 months.
Conclusions: A standardized TDM-guided EFV dose-reduction strategy over a 24-week period was successful, safe and yielded EFV plasma concentrations within the recommended therapeutic range. In addition to improving neuropsychiatric tolerability, EFV dose reduction has the potential to substantially decrease treatment cost.

Introduction

Therapeutic drug monitoring (TDM) of efavirenz (EFV) identifies high or low concentrations which might be responsible for increased toxicity or for decreased efficacy [1]. In April 2003, the first version of a guideline to optimize TDM in HIV clinical care was presented. An update has been published in 2006 and recommends using TDM in selected patients, such as those with impaired renal or hepatic function, pregnancy or suspected drug–drug interaction, for example [2].

Investigations regarding the benefit of TDM in unselected populations have yielded conflicting results [3–6] because of the lack of standardized criteria in measurement of antiretroviral plasma concentrations, or in defining thresholds for dose change. A recent Cochrane systematic review concluded that there is a lack of support for routine use of TDM, but also underscored the lack of data to identify selected populations that might benefit from its use [7].

Prospective studies have demonstrated the relationship between EFV plasma concentration and clinical efficacy and toxicity, even in long-term users [1,8–10]. Moreover, EFV is metabolized mainly by cytochrome P450 (CYP) 2B6, and its concentration is associated with *CYP2B6* variation [11]. A recent study has tested the feasibility of a *CYP2B6* genotype-based dose reduction of EFV, with less central nervous system

symptoms in 10 out of 14 patients who received a reduced dosage [12]. More recently, the result of the ATHENA cohort demonstrated that TDM-guided EFV dose reduction in patients with high plasma concentrations did not compromise virological efficacy and led to a decreased risk of treatment discontinuation [13]. In addition to benefits in terms of tolerability, dose reduction can also influence individual treatment cost. Minimum effective dose can vary with interindividual differences in body weight, pharmacogenetic make-up and other factors [14]. Dose recommendations have often been derived from clinical trials that were not designed to establish minimum effective doses for individual patients. For example, EFV has been licensed by the US Food and Drug Administration in 1998 at a fixed 600 mg once-daily dose. A Phase II trial (DMP 005) showed no difference in efficacy between 200, 400 and 600 mg once-daily doses, when combined with zidovudine and lamivudine [15,16].

We aimed to test a simplified algorithm for dose reduction in patients with documented virological efficacy, treated by a stable EFV 600 mg once daily based regimen while presenting with elevated plasma concentrations. We used a standardized algorithm, based on a Bayesian population pharmacokinetic model developed by our group [17], to reduce doses in patients with plasma EFV concentrations above percentile 75 (P75). We hypothesized that dosage individualization was feasible and safe.

Methods

Patients

Patients were recruited at two University Hospitals (Service of Infectious Diseases, Lausanne University Hospital CHUV, Lausanne and HIV Unit, Geneva University Hospital, Geneva, Switzerland) from November 2006 to March 2008. All patients were on a stable EFV regimen (600 mg once daily), with a viral load <40 copies for ≥3 months. An EFV plasma concentration found above P75 at screening was confirmed on baseline, and the patients had to sign an informed consent form.

The protocol was approved by the Human Research Ethics Committees of Lausanne and Geneva University Hospitals. The study was conducted in accordance with the ethical principles laid out in the Declaration of Helsinki (1996) and Good Clinical Practice guidelines (Consolidated guidelines [E6] issued by the International Conference on Harmonization in May 1996).

Study design

This study was a prospective open-label multicentre trial on patients screened with an EFV plasma concentration above P75, measured between 8 to 24 h post-dose. Blood samples were taken at least 8 h after the last EFV dose intake, to ensure that sampling was taken during the elimination phase of the drug. All patients fitting the inclusion criteria underwent a dose reduction according to a standardized scheme based on a Bayesian approach (Figure 1). This approach evaluates the most likely contribution of intra- and interindividual variability in EFV kinetics, and adjusts the dosage specifically with respect to interindividual variability. Drug dosage was modified according to the protocol on week 2, after the confirmation that the EFV plasma concentration at baseline remained above P75. Patients with EFV concentration between P75 and P95 received EFV 400 mg once daily (two 200 mg tablets), whereas those with concentrations above P95 received EFV 200 mg once daily (one tablet). Plasma concentrations were then checked again on week 6. If EFV concentration was still above target, a second cycle of dose reduction was to be performed. EFV plasma concentrations were then monitored on weeks 10 and 24 for all patients. The protocol did not allow for more than two cycles of drug dosage adjustment. The primary end point was the number of patients who reached a plasma concentration within target (1,000–4,000 ng/ml) after at least one cycle (and maximum two cycles) of dose reduction at 6 months, according to the provided algorithm. The percentage of patients remaining with undetectable viral load at 3 and 6 months was also determined as a secondary end point.

Other laboratory measurements were also performed at baseline and at weeks 10 and 24: viral load, $CD4^+$ T-cell count, serum alanine aminotransferase, total cholesterol, triglycerides, high-density lipoprotein, low-density lipoprotein, glucose, creatinine, full blood cell count measurements and a pregnancy test (if required).

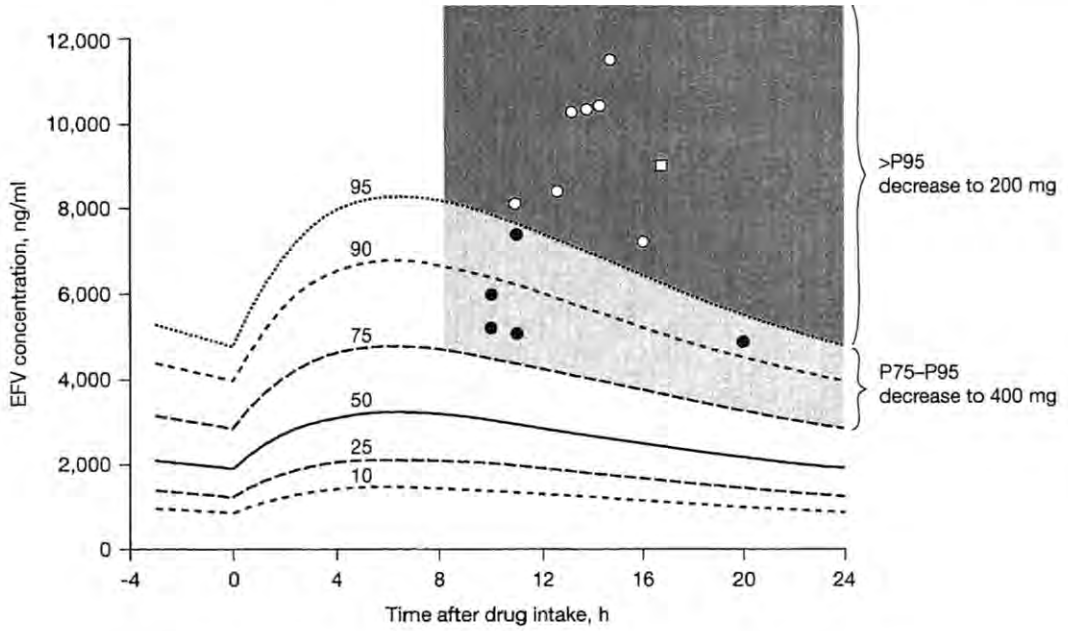
All patients completed questionnaires at baseline, week 10 and week 24. All questionnaires were administered by a trained study nurse. Symptoms of depression, anxiety and stress were assessed with the Depression Anxiety and Stress Scale (DASS) [18]. This scale was chosen for its high internal consistency, temporal stability and stable factor structure [19]. The DASS scale distinguishes among normal, mild, moderate, severe and extremely severe degrees of depression, anxiety or stress. Sleep quality was measured using Groningen Sleep Quality Score (GSQS) [20].

Finally, patients were genetically characterized for *CYP 2B6*, *2A6* and *3A4* [21], in order to document the influence of polymorphisms on EFV concentrations and the prediction of dose reduction (see *Genetic analyses* section).

Plasma concentrations

EFV total plasma concentrations were determined by HPLC coupled to tandem mass spectrometry

Figure 1. Percentile curves for EFV dose adjustment based on a Bayesian approach for patients receiving 600 mg/day



The grey area represents efavirenz (EFV) plasma concentrations for which a dose reduction was performed. Circles and squares represent EFV plasma concentrations at baseline for patients included in the study (n=13). White circles represent patients above percentile 95 (P95), qualifying for a 200 mg dose; the white square represents the patient above P95 who needed two cycles of dose reduction; grey circles represent patients between percentile 75 (P75) and P95, qualifying for a 400 mg dose.

(LC-MS/MS) after protein precipitation with acetonitrile (MeCN) using an adaptation of our previously reported methods [22,23].

EFV pure substance, kindly provided by Merck Sharp & Dohme-Chibret AG (Glattbrugg, Switzerland), was used to prepare calibration and quality control samples. The internal standard used was EFV-d_4, kindly provided by Toronto Research Chemicals Inc. (North York, ON, Canada).

We used selected transitions and collision energy previously reported [22], with some minor modifications: the *m/z* transition, collision energy (V) and tube lens used were 314.0→244.0, 29 and 60 for EFV, and 318.0→248.0, 27 and 37 for EFV-d_4. The range of calibration curves was established up to 10,000 ng/ml, with a lower limit of quantification of 250 ng/ml. The laboratory participates in an international external quality assurance program for the analysis of concentrations of antiretroviral drugs (Association for Quality Assessment in TDM and Clinical Toxicology [KKGT], The Hague, the Netherlands). In the last three external quality control rounds, the deviations from target quality control values were always within 1 to 6%.

Adherence

Adherence to EFV was assessed by using an electronic pill container, the Medication Event Monitoring System (MEMS®; AARDEX, Zug, Switzerland) [24], which allowed us to assess longitudinally the patient's drug-dosing history (that is, date and exact time of each opening of the pill container), and to compare it with the prescribed drug-dosing regimen and drug plasma level. Electronic data were combined with pill count and patients' interviews. EFV adherence was defined as the percentage of days with correct dosing according to physician's prescription.

Genetic analyses

CYP2B6 genetic characterization was carried out for all patients. When CYP2B6 function was impaired, additional characterization of the accessory pathways CYP2A6 and 3A4 was performed [11,25–27].

In order to comprehensively characterize CYP2B6 function in patients with plasma concentrations above P75, all nine *CYP2B6* exons and intron–exon boundaries (4299 bp) were fully resequenced. Primers and conditions were previously published [11,28]. On account of the working hypothesis that *CYP2A6* and *CYP3A4*

decrease or loss-of-function (LOF) alleles would be clinically relevant among patients with impaired CYP2B6 metabolism [21,27], *CYP2A6* and *CYP3A4* were characterized in patients homozygous for an LOF allele of *CYP2B6*. Given the large number of functional alleles associated with CYP2A6 LOF and their high prevalence, *CYP2A6* characterization included full resequencing (promoter region, all nine exons and intron–exons boundaries [4357 bp]), gene conversion and gene copy number assessment, as described [21]. Given the paucity of functional alleles associated with CYP3A4 LOF, despite extensive investigation by many groups over the years [29], only the two most frequent and potentially functional alleles/variants *CYP3A4*1B* [30,31] and *CYP3A4* rs4646437 [32] were genotyped by TaqMan allelic discrimination. Primers and probes were described elsewhere [33] or obtained by assay (TaqMan® SNP Genotyping Assay: C_32306227 10) on demand at Applied Biosystems (Darmstadt, Germany), respectively, for *CYP3A4*1B* and *CYP3A4* rs4646437 genotyping. Genotyping results did not interfere with the decision process of lowering the EFV dosage and were submitted to a post-hoc analysis; however, we hypothesized that all patients included in this study should be homozygous for an LOF allele of *CYP2B6*, as they were chosen to undergo EFV dose reduction according to TDM data. In order to compare the predictive effectiveness of the genotype in comparison to TDM, we built the following prediction model: patients with impaired CYP2B6 metabolism, but no LOF in the accessory pathways (CYP2A6 and CYP3A4), were expected to need a one unit EFV dose reduction (resulting in a 400 mg once-daily regimen). Patients with impaired CYP2B6 metabolism and carrying one or more LOF alleles in *CYP2A6* and/or *CYP3A4* were expected to need a two unit EFV dose reduction (resulting in a 200 mg once-daily regimen).

Table 1. Patients' baseline characteristics

Characteristic	*n* (%)	Median (IQR)
Patients	13 (100)	-
From Geneva	7 (53.9)	-
From Lausanne	6 (46.1)	-
Male	8 (61.5)	-
Age, years	-	45 (35–50)
Weight, kg	-	68 (62–75)
Height, cm	-	171 (160–181)
HIV disease severity at treatment initiation		
CDC score A	6 (46.2)	-
CDC score B	2 (15.4)	-
CDC score C	5 (38.4)	-
HIV duration, months	-	79.7 (37.0–108.2)
Ethnicity		
Caucasian	7 (53.8)	-
Black	4 (30.8)	-
Latin American	2 (15.4)	-
Treatment		
Efavirenz	13 (100)	51.2 (16.7–79.3)[a]
Abacavir	7 (53.8)	16.2 (5.7–71.2)[a]
Lamivudine	8 (61.5)	30.9 (10.2–62.0)[a]
Emtricitabine	5 (38.5)	18.3 (13.1–20.4)[a]
Tenofovir	6 (46.1)	20.8 (18.3–37.2)[a]
Efavirenz	13 (100)	8,112 (5,993–10,278)[b]
Between P75 and P95	5 (38.5)	5,206 (5,076–5,993)[b]
Above P95	8 (61.5)	9,644 (8,335–10,348)[b]

[a]Median (interquartile range [IQR]) treatment duration expressed in months.
[b]Median (IQR) efavirenz concentration expressed in ng/ml. P75, percentile 75; P95, percentile 95.

Statistical analyses

Differences in EFV plasma concentrations, in scores for DASS questionnaires, and in safety parameters between screening and/or baseline visits and week 24, were analysed with a non-parametric Wilcoxon matched-pairs test with a significance threshold of 5%. The quantitative score for GSQS questionnaire was collapsed into a binary variable: undisturbed to moderately disturbed sleep (score ≤ 5) versus very disturbed sleep (score >5), and analysed using a χ^2 test with a threshold of 5%. Statistical analyses were performed using STATA Release 10.0 (Stata Statistical Software; Stata Corporation, College Station, TX, USA).

Results

Patients

Out of 72 participants screened for the study, 15 (21%) were candidates for EFV dosage adjustment at baseline. Two subjects decided to stop the study at baseline and were excluded from the final analysis. One withdrew consent, the other had incident tuberculosis; thus, 13 patients were included in the study at baseline (Table 1): 62% were male, median age was 45 years; seven were Caucasian from Western Europe, four were born in Africa and two were of Hispanic/Latin origin. All had undetectable viraemia and were exposed to EFV for 4.3 years (median) at baseline. Additionally, they were receiving either the combination of emtricitabine and tenofovir (n=5), abacavir and lamivudine (n=7), or tenofovir plus lamivudine (n=1). Six patients were also on non-HIV medications such as anti-inflammatory drugs (ibuprofen, n=2), lipid-lowering drugs (n=2), angiotensin-converting enzyme inhibitors (n=2) or neuropsychiatric drugs (escitalopram and benzodiazepine, n=1).

Dose adaptation

Screening and baseline samples were drawn between 9.25 h and 22.3 h after last dose intake. At baseline

five patients (38%) had EFV concentrations between P75 and P95, qualifying for a 400 mg dose (Figure 1). As a result, the median (interquartile range [IQR]) EFV plasma concentration decreased from 5,206 (5,076–5,993) ng/ml at baseline to 3,021 (2,937–3,261) ng/ml at week 6 (P=0.043). Eight patients (62%) had concentrations above P95 and underwent a dose reduction directly from 600 mg once daily to 200 mg once daily (Figure 1). In this group, median (IQR) EFV concentrations decreased from 9,644 (8,335–10,348) ng/ml to 2,483 (2,111–2,861) ng/ml (P=0.012). For one patient, dose reduction was erroneously carried out at 400 mg once daily instead of 200 mg once daily on the first cycle, and required a second cycle of dose reduction to reach the recommended therapeutic interval.

After dose reduction, EFV drug concentrations remained above the trough threshold of 1,000 ng/ml, recommended by the US Food and Drug Administration, in all patients (Figure 2). Moreover, all EFV plasma concentrations (median, range) remained below 4,000 ng/ml on week 10 (2,826 ng/ml, 1,571–3760) and on week 24 (2,707 ng/ml, 1,604–3,940).

Interestingly, the reduction in concentration after dosage adjustment was greater than the dosage reduction itself, both in the patients who changed to 400 mg once daily (41% versus 33%) and in those who changed to 200 mg once daily (76% versus 67%); this was not an effect of sampling time differences.

Virological status

At the beginning of the study, all patients had a viral load <40 copies/ml by protocol. On weeks 12 and 24, all patients except one remained undetectable. One patient had an isolated blip on week 24 (54 copies/ml), but viral load was again below the detection level (<40 copies) on subsequent monitoring 12 months after study interruption without dosage change. This patient had been adjusted to an EFV dose of 400 mg once daily.

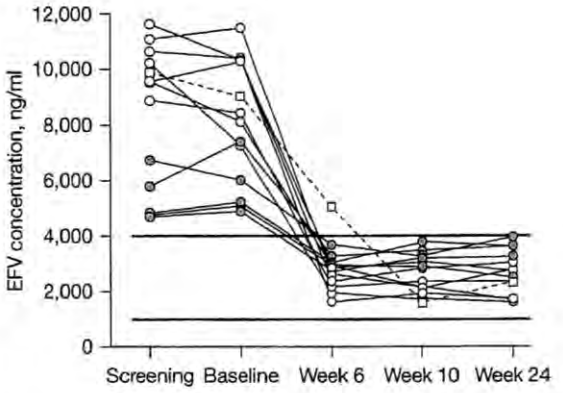
Safety

We observed a significant reduction in anxiety scores (P=0.036) and a trend in lower stress scores (P=0.077) in patients after dose reduction on week 24, as measured by the DASS questionnaire (Table 2). We also recorded a small but significant increase in $CD4^+$ T-cell count between study start and termination. By contrast, no change in sleep quality or length was identified. During the study period, one serious adverse event was declared; the patient experienced acute renal insufficiency and rhabdomyolysis that was thought to be related to cocaine consumption and not to the study drug.

Adherence

Eleven patients, out of the thirteen included, used the electronic pill-container. One patient refused the electronic pill-container and another one often prepared EFV doses in advance, thus invalidating the adherence data, which were discarded from the analysis. The median (IQR) adherence to EFV was 99% (98–100) in the 11 included participants. Out of a total of 1,771 monitored days, there were only 31 (1.7%) days without any EFV drug intake, whereas 15 (0.8%) days were reported with more than one EFV intake.

Figure 2. EFV concentrations before and after dose reduction in patients during the study



Data is from 13 patients. The area within the horizontal lines represents the target efavirenz (EFV) concentration (1,000–4,000 ng/ml) [8]. White circles represent patients qualifying for a 200 mg dose; the white square with dotted line represents the patient who needed two cycles of dose reduction; grey circles represent patients qualifying for a 400 mg dose.

Genetic analyses

Twelve patients were characterized for *CYP2B6*, *2A6* and *3A4*. No genetic consent could be obtained for one patient. All patients were homozygous for a *CYP2B6* LOF allele, except one patient who was heterozygous for an uncharacterized *CYP2B6* allele. As we could not determine whether this allele led to an LOF, we included this patient for the further assessment of the accessory pathways CYP2A6 and CYP3A4. Three patients did not carry any *CYP2A6* or *CYP3A4* LOF, and were therefore expected to need a one unit EFV dose reduction (resulting in a 400 mg once-daily regimen). Nine patients had at least one *CYP2A6* and/or *CYP3A4* LOF allele, and were therefore expected to need a two unit EFV dose reduction (resulting in a 200 mg once-daily regimen). The results are shown in Table 3. When comparing the predictive EFV dose reduction according to either TDM or genotype, all but three patients were classified in the same category and would have received a similar EFV dosage.

Table 2. Safety and questionnaire results at baseline and week 24

Characteristic	Baseline	Week 24	*P*-value
DASS questionnaire			
Stress score	4 (2–14)	0 (0–6)	0.077
Anxiety score	6 (2–10)	0 (0–4)	0.036
Depression score	2 (0–8)	0 (0–10)	0.788
GSQS			0.125
Undisturbed to disturbed sleep (GSQS score ≤5), *n* (%)	9 (69.2)	10 (76.9)	–
Very disturbed sleep (GSQS score <5), *n* (%)	4 (30.8)	3 (23.1)	–
Safety			
ALT, U/l	28 (23–39)	32 (21–44)	0.972
Total cholesterol, mmol/l	5.9 (5.3–6.5)	5.4 (5.2–6.4)	0.100
Triglycerides, mmol/l	1.2 (0.9–1.8)	1.4 (1.1–2.0)	0.208
HDL, mmol/l	1.8 (1.7–2.1)	1.7 (1.4–1.9)	0.162
LDL, mmol/l	3.2 (2.8–3.9)	3.1 (2.7–3.7)	0.402
Glucose, mmol/l	5.5 (5.1–6.1)	5.3 (5.0–5.7)	0.382
Creatinin, µmol/l	74 (64–80)	74 (63–82)	0.700
White blood cells, G/l	6.1 (5.1–7.2)	5.7 (5.1–7.3)	0.552
Haemoglobin, g/l	142 (123–150)	144 (124–148)	0.159
Platelets, G/l	214 (200–255)	219 (185–270)	0.196
$CD4^+$ T-cells, cells/mm^3	513 (448–591)	570 (513–609)	0.023
HIV viral load, copies/ml	40 (40–40)	40 (20–40)	0.263

Data presented as median (interquartile range) unless otherwise indicated. ALT, alanine aminotransferase; DASS, Depression, Anxiety and Stress Scale; GSQS, Groningen Sleep Quality Score; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table 3. Effective and predictive dose reduction according to TDM and genotype, respectively

	Genotype			Dose reduction		Interpretation of efavirenz concentration, percentile	
Patient	*CYP2B6*	*CYP2A6*	*CYP3A4*	Effective (TDM)	Predictive (genotype)	Baseline	Week 24
1	2LOF	1LOF	0LOF	200 mg	200 mg	>P95	P25
2	2LOF	1LOF	2LOF	200 mg	200 mg	>P95	P25–P50
3[c]	2LOF	0LOF	2LOF	200 mg	200 mg	>P95	P25–P50
4	2LOF	0LOF	0LOF	200 mg	400 mg[b]	>P95	P25–P50
5	2LOF	1LOF	0LOF	200 mg	200 mg	>P95	P50–P75
6	2LOF	0LOF	2LOF	200 mg	200 mg	>P95	P25–P50
7	2LOF	1LOF	2LOF	200 mg	200 mg	>P95	P50–P75
8	2LOF	2LOF	2LOF	200 mg	200 mg	>P95	P50–P75
9	2LOF	0LOF	0LOF	400 mg	400 mg	P75–P95	P50–P75
10	1 or 2LOF	0LOF	0LOF	400 mg	400 mg	P75–P95	P25–P50
11	2LOF	1LOF	0LOF	400 mg	200 mg[b]	P75–P95	P50–P75
12	2LOF	0LOF	2LOF	400 mg	200 mg[b]	P75–P95	P50–P75
13[c]	–	–	–	400 mg	–	P75–P95	P50–P75

[a]Patient who needed two cycles of dose reduction due to an error. [b]Predictive dose reduction according to genotype different from effective dose reduction according to therapeutc drug monitoring (TDM). [c]Patient not genotyped. LOF, decrease or loss-of-function; P25, percentile 25; P50, percentile 50; P75, percentile 75; P95, percentile 95.

Discussion

Our study indicates that TDM-guided EFV dose reduction is successful and safe, without any negative effect on virological outcome at follow-up. The use of a standardized Bayesian algorithm enabled correct dose reduction to target therapeutic range (1,000–4,000 ng/ml), without reaching subtherapeutic concentrations. Adherence to EFV was monitored electronically in order to ensure high level drug intake.

There are several reports suggesting that EFV could be used at reduced dose. Firstly, dose recommendations have often been derived from clinical trials that were not designed to establish minimum effective

doses in individual patients. Phase II dose-ranging studies did not show differences in virological efficacy among 137 treatment-naive patients randomized to 24 weeks of treatment with zidovudine and lamivudine plus EFV at doses of 200 mg, 400 mg or 600 mg once daily, or matching placebo. There was no difference in HIV RNA suppression rates between the three doses of EFV and these efficacy results were sustained to week 24 [15,16]. Secondly, a study of 180 individuals reduced 49 patients with high plasma drug concentrations from 600 mg to 400 mg without reporting virological risks with this strategy [13]. Our study shows that with a clear algorithm built up on population pharmacokinetic data to derive a simple percentile-based strategy, drug reduction is feasible without jeopardizing efficacy.

Reducing drug exposure has been associated with a decrease in adverse events and toxicity symptoms, although neuropsychological toxicity may persist longer because of long-term therapy [8,34–37]. In patients initiating an EFV-based regimen (n=108), stepped-dose versus full dose EFV proved to be of similar efficiency in a randomised trial [38]. In the present study, patients were not selected for adverse events; we observed, nonetheless, a significant reduction in anxiety scores and a trend in lower stress scores in patients on reduced dosing. No change in sleep quality or length, as measured by a standardized questionnaire, was identified; however, on study completion, all patients without exception chose to remain on the reduced dose, despite the intake of one additional pill for those receiving 400 mg once daily (that is, two 200 mg pills), compared to the standard treatment (that is, one 600 mg pill).

When considering the lifelong treatment, the reduction in drug exposure might translate into a decreased number of side effects.

The effect on overall drug costs can also be relevant; 600 mg pills cost 499 USD/month in Switzerland, whereas the cost of the 400 mg daily dose is 299 USD/month, and the cost of 200 mg once daily is 166 USD/month. We have assumed two TDM assessments (278 USD) and a comprehensive genetic analysis (200 USD). The economic analysis reveals a cost saving of the 400 mg dose reduction of 1,857 USD per year per patient, and of 3,453 USD per year per patient for a 200 mg dose reduction. The Swiss HIV Cohort Study (SHCS) comprises more than 80% of all HIV-infected patients followed-up in Switzerland: if 15% of the patients on EFV-based regimen had a dose reduction to 400 mg, and 10% to 200 mg according to TDM, the overall saving could be as high as 1,178,101 USD per year. EFV is not the only drug where cost could be decreased by lowering drug dosage; studies are ongoing with lamivudine, lopinavir/r and zidovudine, for example [39].

Drug dosage reduction could have been predicted with nearly similar precision using genetic testing, instead of TDM, although the post-hoc nature of the *CYP2B6* genetic characterization and the fact that no full genetic characterization was provided for EFV-tolerant patients not included in the study are potential limitations. The relative benefit of using one or the other strategy is unclear, considering that predictive dose reduction based on patient genetic profile differed from dose reduction according TDM in our study for three patients only. One patient underwent an EFV dose reduction to 200 mg/day lower than predicted by the genotyping results (400 mg/day), and reached the lowest EFV concentration on week 24 in the study. Two patients underwent an EFV dose reduction to 400 mg/day despite genotyping prediction (to 200 mg/day), and reached the highest EFV plasma levels on week 24. Thus, it is conceivable that all three patients would have reached the recommended target therapeutic range through genotyping. Still, further studies are needed to confirm the predictive potential of genetic testing, and, more specifically, to compare strategies to better identify the patients most likely to benefit from drug dose reduction.

We recognize some limitations to our study; firstly the limited sample size does not allow generalization of our findings to different population groups. Secondly, there was no randomization, and all patients benefited from a drug dose reduction. Thirdly, per protocol, patients had a maximum of two cycles of dose reduction to reach plasma concentrations within target (1,000–4,000 ng/ml). In our experience, all patients but one fell into the drug plasma concentration target after the first cycle of dose reduction. For patients who reached the upper part of the therapeutic target range (that is, 3,000–4,000 ng/ml), there is no evidence of a clinical benefit to be expected from a second dose reduction.

The observation of larger than proportional reductions in concentration after dose reduction might indicate some non-linearity in EFV clearance, as well as non-specific regression to the mean, justifying our Bayesian approach to EFV TDM, and further non-captured influences (for example diet or interacting comedications).

In conclusion, TDM-guided dose reduction in this and in other studies [12,13] appears safe and should be considered in patients with high EFV concentrations; EFV dose reduction also prevents toxicities and minimizes treatment cost. This is also of particular interest for resource limited settings as WHO recently issued revised recommendations suggesting that EFV be the preferred regimen.

Acknowledgements

The authors would like to thank Dr Andrew Hill, Pharmacology Research Laboratories, Liverpool

University, Liverpool, UK and Dr Alain Nguyen, Geneva University Hospital, Geneva, Switzerland.

The study was funded, in part, by educational grants from Geneva University Hospital. The SHCS is financed in the framework of the Swiss HIV Cohort Study, supported by the Swiss National Science Foundation.

The Swiss National Science Foundation (Grant numbers 324700-112655 and 32430-124943) has supported, in part, the salary of AFM and JdI. LAD has received a REQUIP Grant number 326000-121314/1 from the Swiss National Science Foundation for the acquisition of new LC-MS/MS instrumentation.

These data have been presented, in part, at the *10th International Workshop on Clinical Pharmacology of HIV Therapy*, 15–17 April 2009, Amsterdam, the Netherlands (abstract 47), and at the *5th IAS Conference on HIV Pathogenesis, Treatment and Prevention*, 19–22 July 2009, Cape Town, South Africa (abstract CDB076).

Disclosure statement

The authors declare no competing interests.

References

1. Gutiérrez F, Navarro A, Padilla S, *et al.* Prediction of neuropsychiatric adverse events associated with long term efavirenz therapy, using plasma drug level monitoring. *Clin Infect Dis* 2005; **41**:1648–1653.
2. La Porte CJL, Back DJ, Blatschke T, *et al.* Updated guideline to perform Therapeutic Drug Monitoring for antiretroviral agents. *Review Antivir Ther* 2006; 3:3–14.
3. Bossi P, Peytavin G, Ait-Mohand H, *et al.* GENOPHAR: a randomized study of plasma drug measurements in association with genotypic resistance testing and expert advice to optimize therapy in patients failing antiretroviral therapy. *HIV Med* 2004; 5:352–359.
4. Burger D, Hugen P, Reiss P, *et al.* Therapeutic Drug Monitoring of nelfinavir and indinavir in treatment-naive HIV-1-infected individuals. *AIDS* 2003; **17**:1157–1165.
5. Clevenbergh P, Garraffo R, Durant J, *et al.* PharmAdapt: a randomized prospective study to evaluate the benefit of therapeutic monitoring of protease inhibitors: 12 week results. *AIDS* 2002; **16**:2311–2315.
6. Fletcher CV, Anderson PL, Kakuda TN, *et al.* Concentration-controlled compared with conventional antiretroviral therapy for HIV infection. *AIDS* 2002; **16**:551–560.
7. Kredo T, Van der Walt JS, Siegfried N, Cohen K. Therapeutic drug monitoring of antiretrovirals for people with HIV. *Cochrane Database Syst Rev* 2009; **8**:CD007268.
8. Marzolini C, Telenti A, Decosterd LA, *et al.* Efavirenz plasma levels can predict treatment failure and central nervous system side effects in HIV-1-infected patients. *AIDS* 2001; **15**:71–75.
9. Gallego L, Barreiro P, Del Rio R, *et al.* Analyzing sleep abnormalities in HIV-infected patients treated with efavirenz. *Clin Infect Dis* 2004; **38**:430–432.
10. Rihs TA, Begley K, Smith DE, *et al.* Efavirenz and chronic neuropsychiatric symptoms: a cross-sectional case control study. *HIV Med* 2006; 7:544–548.
11. Rotger M, Tegude H, Colombo S, *et al.* Predictive value of known and novel alleles of *CYP2B6* for efavirenz plasma concentrations in HIV-infected individuals. *Clin Pharmacol Ther* 2007; **81**:557–566.
12. Gatanaga H, Hayashida T, Tsuchiya K, *et al.* Successful efavirenz dose reduction in HIV type 1-infected individuals with cytochrome P450 2B6 *6 and *26. *Clin Infect Dis* 2007; **45**:1230–1237.
13. van Luin M, Gras L, Richter C, *et al.* Efavirenz dose reduction is safe in patients with high plasma concentrations and may prevent efavirenz discontinuations. *J Acquir Immune Defic Syndr* 2009; **52**:240–245.
14. De Smet PAGM, Kramers C, Britten N. Can drug regimens be adapted to patients or vice versa? *Lancet* 2007; **370**:813–814.
15. Hicks C, Hass D, Seekins D, *et al.* A Phase II, double-blind, placebo-controlled, dose ranging study to assess the antiretroviral activity and safety of DMP 266 (efavirenz, SUSTIVA) in combination with open-label zidovudine (ZDV) with lamivudine (3TC). *5th Conference on Retroviruses and Opportunistic Infections.* 1–5 February 1998, Chicago, IL, USA. Abstract 698.
16. Mayers D, Jemsek J, Eyster E, Tashima K, Thompson M, Ruiz N. A double-blind, placebo-controlled study to assess the safety, tolerability and antiretroviral activity of efavirenz (EFV, Sustiva, DMP 266) in combination with open-label zidovudine (ZDV) and lamivudine (3TC) in HIV-1 infected patients (DMP 266-004). The Efavirenz Clinical Team. *12th International Conference on AIDS.* 28 June–3 July 1998, Geneva, Switzerland. Abstract 22340.
17. Csajka C, Marzolini C, Fattinger K, *et al.* Population pharmacokinetics and effects of efavirenz in patients with human immunodeficiency virus infection. *Clin Pharmacol Ther* 2003; **73**:20–30.
18. Psychology Foundation of Australia. Depression Anxiety Stress Scales (DASS). (Updated 30 May 2010. Accessed January 2008.) Available from http://www2.psy.unsw.edu.au/groups/dass/
19. Brown TA, Chorpita BF, Korotitsch W, Barlow DH. Psychometric properties of the Depression Anxiety Stress Scales (DASS) in clinical samples. *Behav Res Ther* 1997; **35**:79–89.
20. Meijman TF, de Vries-Griever AH, de Vries G. *The evaluation of the Groningen Sleep Quality Scale.* Heymans Bulletin 88-13-EX. Groningen: University of Groningen 1988.
21. di Iulio J, Fayet A, Arab-Alameddine M, *et al. In vivo* analysis of efavirenz metabolism in individuals with impaired CYP2A6 function. *Pharmacogenet Genomics* 2009; **19**:300–309.
22. Colombo S, Béguin A, Telenti A, *et al.* Intracellular measurements of anti-HIV drugs indinavir, amprenavir, saquinavir, ritonavir, nelfinavir, lopinavir, atazanavir, efavirenz and nevirapine in peripheral blood mononuclear cells by liquid chromatography coupled to tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005; **819**:259–276.
23. Fayet A, Béguin A, Martinez de Tejada B, *et al.* Determination of unbound antiretroviral drug concentrations by a modified ultrafiltration method reveals high variability in the free fraction. *Ther Drug Monit* 2008; **30**:511–522.
24. Aardex Group. MEMS™ electronic pill-containers (Accessed January 2008.) Available from www.aardexgroup.com/aardex_index.php?group=aardex
25. Rotger M, Telenti A. Optimizing efavirenz treatment: CYP2D6 genotyping or therapeutic drug monitoring. *Eur J Clin Pharmacol* 2008; **64**:335–336.
26. di Iulio J, Rotger M, Lubomirov R, Decosterd LA, Eap CB, Telenti A. Genetic variation in accessory pathways is associated with extreme efavirenz exposure in individuals with impaired CYP2A6 function. *Conference on Retroviruses and Opportunistic Infections.* 3–6 February 2008, Boston, MA, USA. Abstract 133.
27. Arab-Alameddine M, di Iulio J, Buclin T, *et al.* Pharmacogenetic-based population pharmacokinetic analysis of efavirenz in HIV-1 infected individuals. *Clin Pharmacol Ther* 2009; **85**:485–494.

28. Lang T, Klein K, Richter T, *et al.* Multiple novel nonsynonymous *CYP2B6* gene polymorphisms in Caucasians: demonstration of phenotypic null alleles. *J Pharmacol Exp Ther* 2004; **311**:34–43.

29. Burk O, Wojnowski L. Cytochrome P450 3A and their regulation. *Naunyn Schmiedebergs Arch Pharmacol* 2004; **369**:105–124.

30. Wandel C, Witte JS, Hall JM, Stein CM, Wood AJ, Wilkinson GR. CYP3A activity in African American and European American men: population differences and functional effect of the CYP3A4*1B5′-promoter region polymorphism. *Clin Pharmacol Ther* 2000; **68**:82–91.

31. Rodríguez-Antona C, Sayi JG, Gustafsson LL, Bertilsson L, Ingelman-Sundberg M. Phenotype–genotype variability in the human *CYP3A* locus as assessed by the probe drug quinine and analyses of variant *CYP3A4* alleles. *Biochem Biophys Res Commun* 2005; **338**:299–305.

32. Schirmer M, Rosenberger A, Klein K, *et al.* Sex-dependent genetic markers of CYP3A4 expression and activity in human liver microsomes. *Pharmacogenomics* 2007; **8**:443–453.

33. Crettol S, Déglon JJ, Besson J, *et al.* ABCB1 and cytochrome P450 genotypes and phenotypes: influence on methadone plasma levels and response to treatment. *Clin Pharmacol Ther* 2006; **80**:668–681.

34. Clifford DB, Evans S, Yang Y, *et al.* Impact of efavirenz on neuropsychological performance and symptoms in HIV-infected individuals. *Ann Intern Med* 2005; **143**:714–721.

35. Fumaz CR, Munoaz-Moreno JA, Molto J, *et al.* Long-term neuropsychiatric disorders on efavirenz-based approaches: quality of life, psychologic issues, and adherence. *J Acquir Immune Defic Syndr* 2005; **38**:560–565.

36. Lochet P, Peyrière H, Lotthé A, Mauboussin JM, Delmas B, Reynes J. Long-term assessment of neuropsychiatric adverse reactions associated with efavirenz. *HIV Med* 2003; **4**:62–66.

37. Kappelhoff BS, Can Leth F, Robinson PA, *et al.* Are adverse events of nevirapine and efavirenz related to plasma concentrations? *Antivir Ther* 2005; **10**:489–498.

38. Gutiérrez-Valencia A, Viciana P, Palacios R, *et al.* Stepped-dose versus full-dose efavirenz for HIV infection and neuropsychiatric adverse events: a randomized trial. *Ann Intern Med* 2009; **151**:149–156.

39. ENCORE Trial. (Updated 5 January 2011. Accessed February 2010.) Available from http://www.clinicaltrials.gov/ct2/show/NCT01011413?term=efavirenz&rank=31

Accepted 15 July 2010; published online 2 March 2011

4.1.2. Lopinavir

4.1.2.1. Original article

ADME Pharmacogenetics – Investigation of the Pharmacokinetics of the Antiretroviral Agent Lopinavir

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Pharmacogenetics and Genomics 2010 April; 20(4):217-230

Author contributions

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Resequencing and recombinant mapping: **J. di Iulio**, A. Telenti

Population pharmacokinetics: C. Csajka

Sample collection: S. Colombo

Data analysis: R. Lubomirov

Clinical data contribution: H. Furrer, P. Vernazza, A. Calmy, M. Cavassini

Study supervision: A. Telenti, C. Csajka

Drafting of the manuscript: R. Lubomirov, A. Telenti

Critical revision of the manuscript for important intellectual content: all authors

Detailed personal contribution

I contributed, with A. Telenti, in the establishment of a new approach to identify candidate causal variants responsible for the phenotype associated with a putatively non-functional hit SNP. This fine mapping strategy consisted in:

- *SLCO1B1* promoter, exon-intron boundaries and 3'UTR resequencing (N=24)
- Haplotype inference and phylogenetic classification
- Data analysis and interpretation for candidate causal variants identification

I also participated in the sample recruitment and DNA extraction as well as in the genetic characterization of individuals included in the study, in both discovery and replication steps:

1. Discovery step (N=207):
    - Genotyping of 5 putative/known functional variants that were not included in the array (rs2306283 & rs4149015 in *SLCO1B1*; rs35199625, rs2306168 & rs12422149 in *SLCO2B1*)

2. Replication step (N=148) included:
    - genotyping of 6 hit SNPs identified in the discovery step (rs4149056 & rs4149032 in *SLCO1B1*; rs10841795 in *SLCO1A2*; rs1077858 in *SLCO2B1*; rs717620 in *ABCC2* and rs6945984 in *CYP3A* locus)
    - genotyping of the 3 candidate causal variants (rs17328763, rs11045819 & rs11045891) tagged by rs4149032, identified through fine mapping strategy

Comments on the article:

Individuals receiving a lopinavir/ritonavir (LPV/r) containing regimen show a wide interindividual variability in LPV/r exposure. We aimed at identifying genetic factors influencing LPV/r pharmacokinetics parameters. On this purpose we investigated genes encoding proteins involved in drug absorption, distribution, metabolism and elimination (ADME).

One hit SNP, leading to a nonsynonymous amino acid change (V174A) in *SLCO1B1* (encoding a solute carrier transporter) reached study-wide significance and was associated with low LPV/r clearance. A second SNP in *SLCO1B1* (located in intron 2, with *a priori* no functional effect) was associated with the opposite phenotype, although it did not reach study-wide significance. We therefore investigated by resequencing and recombinant mapping strategy whether it was tagging a functional SNP in *SLCO1B1*. A potential causal variant, leading to a nonsynonymous amino acid change (P155T) and reported to modify substrate specificity, was identified. This SNP, together with two other candidate SNPs that did not reach study-wide significance in the first round analysis were tested in a replication cohort. Overall, we could confirm the effect of 4 SNPs on LPV/r clearance.

A pharmacogenetics-based population pharmacokinetic analysis of LPV/r was then completed, and the genetic variants identified in this study could explain 5% of LPV/r clearance variability.

Original article 217

ADME pharmacogenetics: investigation of the pharmacokinetics of the antiretroviral agent lopinavir coformulated with ritonavir

Rubin Lubomirov[a], Julia di Iulio[a], Aurélie Fayet[c], Sara Colombo[a], Raquel Martinez[a], Catia Marzolini[d], Hansjakob Furrer[e], Pietro Vernazza[f], Alexandra Calmy[g], Matthias Cavassini[b], Bruno Ledergerber[j], Katharina Rentsch[k], Patrick Descombes[h], Thierry Buclin[c], Laurent A. Decosterd[c], Chantal Csajka[c,i] and Amalio Telenti[a]; the Swiss HIV Cohort Study

Background **An ADME (absorption, distribution, metabolism and excretion) – pharmacogenetics association study may identify functional variants relevant to the pharmacokinetics of lopinavir coformulated with ritonavir (LPV/r), a first-line anti-HIV agent.**

Methods **An extensive search of literature and web resources helped select ADME genes and single nucleotide polymorphisms (SNPs, functional and HapMap tagging SNPs) with a proven or potentially relevant role in LPV/r pharmacokinetics. The study followed a two-stage design. Stage 1 (discovery) considered a Caucasian population ($n=638$) receiving LPV/r, where we selected 117 individuals with low LPV clearance (cases) and 90 individuals with high clearance (controls). Genotyping was performed by a 1536-SNP customized GoldenGate Illumina BeadArray. Stage 2 (confirmation) represented a replication study of candidate SNPs from the stage 1 in 148 individuals receiving LPV/r. The analysis led to formal population pharmacokinetic–pharmacogenetic modeling of demographic, environmental and candidate SNP effects.**

Results **One thousand three hundred and eighty SNPs were successfully genotyped. Nine SNPs prioritized by the stage 1 analysis were brought to replication. Stage 2 confirmed the contribution of two functional SNPs in *SLCO1B1*, one functional SNP in *ABCC2* and a tag SNP of the *CYP3A* locus in addition to body weight effect and ritonavir coadministration. According to the population pharmacokinetic–pharmacogenetic model, genetic variants explained 5% of LPV variability. Individuals homozygous rs11045819 (*SLCO1B1*4*) had a clearance of 12.6 l/h, compared with 5.4 l/h in the reference group, and 3.9 l/h in individuals with two or more variant alleles of rs4149056 (*SLCO1B1*5*), rs717620 (*ABCC2*) or rs6945984 (*CYP3A*). A subanalysis confirmed that although a significant part of the variance in LPV clearance was attributed to fluctuation in ritonavir levels, genetic variants had an additional effect on LPV clearance.**

Conclusion **The two-stage strategy successfully identified genetic variants affecting LPV/r pharmacokinetics. Such a general approach of ADME pharmacogenetics should be generalized to other drugs. *Pharmacogenetics and Genomics* 20:217–230 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Pharmacogenetics and Genomics 2010, **20**:217–230

Keywords: absorption, distribution, metabolism and excretion, lopinavir, pharmacogenetics, population pharmacokinetics

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Received 3 July 2009 **Accepted** 21 December 2009

Introduction

The HIV-1 protease inhibitor lopinavir (LPV) is used in first-line antiretroviral therapy of HIV-infected patients [1]. LPV has a low oral bioavailability owing to an extensive first-pass metabolism mediated by the cytochrome P450 (CYP) 3A isoenzymes [2] and the cellular efflux transporter P-glycoprotein (*ABCB1*) [3]. Coformulation with low-dose ritonavir (RTV, Kaletra, Abbott Laboratories, North Chicago, Illinois, USA) increases LPV exposure by inhibiting intestinal and hepatic CYP3A-mediated and P-glycoprotein-mediated metabolism and transport, respectively [4].

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DOI: 10.1097/FPC.0b013e328336eee4

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LPV coformulated with RTV, referred here in as LPV/r, is characterized by a large pharmacokinetic inter-individual variability [5,6]. Part of this variability is explained by body weight, age, sex, orsomucoid (α1-acid glycoprotein) plasma levels, drug–drug interactions, liver disease, pregnancy or by poor adherence [6–11]. It has been postulated that the remaining part of this variability could be explained by host genetic factors.

Previous pharmacogenetic studies on LPV/r pharmacokinetics have focused on candidate genes thought to be involved in drug metabolism and transport [12–17]. These studies investigated a limited number of genetic variants in four genes (*ABCB1*, *CYP3A5*, *CYP2D6* and *CYP2B6*), and found no association with LPV plasma levels. Low sample size, insufficient study power and the coformulation with RTV could explain those negative results.

A shift in paradigm emerges from the availability of data on common human variation through HapMap [18], the wealth of data on less common genetic polymorphism with functional effect, and the implementation of high-throughput genotyping technologies. The aim of this study is to identify novel associations of functional and putative functional variants in genes encoding for absorption, distribution, metabolism and excretion (ADME) proteins and LPV/r pharmacokinetics through a strategy involving an exploratory case–control association study and a confirmatory replication study.

Materials and methods

Study design and population

This study was conducted within the framework of the Swiss HIV Cohort Study (*www.shcs.ch*). The ethics committees of all participating centers approved the genetics project and participants gave written informed consent for genetic testing. HIV-infected Caucasian patients with LPV plasma levels measured during therapeutic drug monitoring (TDM) were included in the study. We used a two-stage study design with discovery and replication steps. The stage 1 discovery population included 638 patients with LPV TDM, recruited between January 2000 and April 2007, from which we selected cases and controls (see below). The stage 2 replication population included 148 consecutive patients that underwent LPV TDM between May 2007 and November 2008. Individuals presenting undetectable plasma drug levels and detectable viral load – suggestive of nonadherence – were excluded from the study.

Drug levels

Blood samples (5 ml) were collected into lithium heparin or EDTA-K Monovette syringes (Sarstedt, Nümbrecht, Germany). Plasma was isolated by centrifugation, inactivated for viruses at 60°C for 60 min, and stored at −20°C until analysis. Total plasma LPV and RTV concentrations were determined by high-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) after protein precipitation with acetonitrile using an adaptation of our previously reported methods [19,20]. The internal standard used was lopinavir-d_8 (LPV-d_8), kindly provided by TRC (Toronto Research Chemicals Inc., North York, Canada), at a 5 µg/ml concentration in methanol/H_2O (50 : 50). LPV and RTV pure substances, kindly provided by Abbott (Abbott Park, Illinois, USA), were used to prepare calibration and quality control samples.

The high-performance liquid chromatography system involved a Rheos 2200 binary pump (Flux Instruments, Basel, Switzerland) equipped with an online degasser and a temperature-controlled 324 vial autosampler maintained at +10°C (CTC Analytics AG, Zwingen, Switzerland). The chromatographic system was coupled to a triple-stage quadrupole mass spectrometer (TSQ Quantum Ion Max) from Thermo Electron Corporation (Waltham, Massachusetts, USA) equipped with an electrospray ionization interface and operated with the Excalibur 1.1 software (Thermo Electron Corporation, San Jose, California, USA). Chromatographic separations were carried out on an Atlantis dC18 column (2.1 × 50 mm, 3 µm) (Waters, Milford, Massachusetts, USA). Solution A consisted of 2 mmol/l ammonium acetate containing 0.1% formic acid. Solution B was acetonitrile with 0.1% formic acid. The mobile phase was delivered at 0.3 ml/min according to the following gradient elution program: 0–1 min, 2% solution B → 30% B at 3.2 min → 100% B at 10 min. The washing/reequilibration step included an intensive rinsing (10.1–15 min with 100% of B at 0.5 ml/min) followed by the initial solvent composition (2% solution B: 15.1–17.5 min at 0.4 ml/min, and 18–20 min at 0.3 ml/min). LPV and RTV quantification were performed using the selected reaction monitoring in the positive mode. We used selected transitions and collision energy previously reported for Protease Inhibitors [19], with some minor modifications: the *m/z* transition, collision energy (V) and tube lens used are 629.3→447.3, 17 and 77 for LPV, 721.4→596.2, 24 and 91 for RTV and 637.3→447.1, 16 and 90 for LPV-d_8. The range of calibration curves was established up to 20 µg/ml, with a lower limit of quantification of 0.1 µg/ml. The laboratory participates to an international external quality assurance program for the analysis of concentrations of antiretroviral drugs (KKGT, Stichting Kwaliteitsbewaking Klinische Geneesmiddelanalyse en Toxicologie, Association for Quality Assessment in TDM and clinical Toxicology, The Hague, The Netherlands). In the last three external quality control rounds, the deviations from target quality control values were always comprised within 0–6%.

Population pharmacokinetic models

Basic model

A population pharmacokinetic (Pop-PK) analysis of LPV/r was performed to assess average population parameters

and interpatient variability, and to estimate the influence of demographic and environmental factors on its elimination. LPV kinetics was determined by fitting one-compartment and two-compartment models with linear absorption and elimination from the gastrointestinal tract. Interindividual variability was assumed to be lognormally distributed with variance Ω. A combined proportional and additive error model with a mean of zero and a variance of σ^2 was used to describe the intraindividual (residual) variability.

Covariate models

Potentially influential covariates were incorporated sequentially into the pharmacokinetic model. The typical value of apparent clearance (CL) was modeled to depend linearly on a covariate X (such as body weight, centered on the mean; categorical covariates being coded as indicator variables into 0 or 1) as shown by the equation: $CL = \theta_a \cdot (1 + \theta_b \cdot X)$, where θ_a is the average estimate and θ_b is the relative deviation (positive or negative) of the average attributed to the covariate X. The available demographic covariates were sex, age, body weight, bilirubine levels and comedications such as antiretroviral drugs and additional medications with inducer or inhibitor capacity.

Replication analysis

A Pop-PK-pharmacogenetic analysis of the replication dataset (see below) was conducted through the same procedure as for the discovery data set, to validate LPV/r population model while integrating genotyping results. Analyses of the covariate effects on CL were divided into three main sections for assessment of: (i) the influence of demographic variables and concomitant medications as described earlier, (ii) the impact of candidate single nucleotide polymorphisms (SNPs) based on univariate analyses and (iii) the joint effect of influent SNPs in multivariate analyses (see below).

To discriminate between the influence of RTV levels and genetic polymorphisms on LPV kinetics, a subanalysis was conducted in 96 patients for whom RTV levels were obtained. The disposition of RTV was first characterized using the same basic, covariate (demographic, comedications and candidate SNPs) procedure as described for LPV. Individual Bayesian estimates of clearance (CL_{ind}), volume of distribution (V_{dind}), absorption constants (ka_{ind}) and area under curve ($AUC_{0-12 \text{ or } 24}$) (defined as dose/$CL_{ind}$) were derived from the final model incorporating covariates that best described RTV plasma levels.

The influence of RTV on LPV kinetics was first evaluated by means of a maximum effect function of RTV $AUC_{0-12 \text{ or } 24}$ using $CL/F = \theta_{CL/F} \times \frac{1-AUC}{AUC_{50}+AUC}$, where $\theta_{CL/F}$ is LPV average clearance in absence of RTV and AUC_{50} is the RTV AUC at half of maximal effect. The inhibition of LPV clearance by RTV was then modeled using a direct concentration-dependent relationship of the form $CL/F = \theta_{CL/F} \times 1 - \frac{I_{max} \cdot C_{RTV}}{IC_{50}+C_{RTV}}$, where now C_{RTV} is RTV plasma concentration at each time point, I_{max} is the maximum inhibitory effect of RTV (fixed to 1) and IC_{50} is the C_{RTV} producing 50% of the I_{max}. Owing to minimization difficulties while estimating both LPV and RTV kinetics simultaneously as a consequence of sparse sampling, individual Bayesian estimates of RTV kinetic parameters were used to derive RTV concentration–time curves and no further models could be tested. We used a score classification (see below) to assess the impact of genetic polymorphism in addition to RTV influence.

Model selection and estimation

The Pop-PK analysis was performed with NONMEM (version VI, NM-TRAN version II, Icon Development Solutions, Ellicott City, Maryland, USA) using FOCE INTERACTION to fit the models. Modeling decisions were based on the goodness-of-fit criterion, which is the change in the objective function (OF, which approximates a χ^2 distribution), regarded as statistically significant ($P < 0.05$) if it exceeds 3.8 for one additional parameter. Other criterions for model discrimination were goodness-of-fit plots, visual predictive checks and standard error of the estimates. A simulation based on the final pharmacokinetic estimates was performed with NONMEM using 1000 individuals to calculate 90% prediction intervals. The concentrations encompassing the 10th and 90th percentile at each time point were retrieved to construct the prediction intervals. The figures were generated with GraphPad Prism (Version 4.0 for Windows, GraphPad Software, San Diego, California, USA).

Study phenotype

On the basis of Pop-PK analysis, we used the LPV interpatient variability on clearance (η_{CL}) as a phenotype, considering that this parameter would best reflect the remaining unexplained variance in LPV elimination that could be accounted for by genetic variations. Histogram plots and the 'probit' distribution of η_{CL} values were used to identify the subpopulations of low and high CL outliers (i.e. patients with high and low LPV plasma levels, respectively) as cases and controls, respectively.

Genotyping and resequencing

DNA was extracted from peripheral blood mononuclear cells using the QIAamp DNA Mini Kit (Quiagen, Hilden, Germany) following the manufacturer's instructions. Genotyping was performed by a 1536-plex customized GoldenGate assay on an Illumina BeadArray station we designed for pharmacogenetic studies of ADME pathways of main antiretroviral drugs [21]. The array uses SNPs representing common genetic variation enriched by variants with proven or predicted functional effect in 115 genes relevant for antiretroviral drugs ADME pathways in Caucasians. The tagging SNPs (tSNPs) were

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selected based on HapMap Phase II data (release 21, *www.hapmap.org*) using Tagger software [22] to capture variation of polymorphisms with minor allelic frequency greater than 5% in the HapMap Caucasian population with mean maximum pairwise R^2 between tSNPs and not genotyped SNPs of 0.80. The tSNPs were selected to cover the genome regions based on positions of RefSeq genes' longest transcript in the HapMap Genome Browser (*www.hapmap.org*) adding 10 kb to the 5′-UTR region. As direct typing of causal variants is more powerful in association studies [23,24], we enriched the set of markers selected to genotyping with proven functional polymorphisms obtained from literature [21]. The selected genome regions were also screened for SNPs with inferred putative functional effect using FASTSNP with a cutoff score value of at least 3 (discriminates low from medium-high prediction of functionality) [25]. Only proven and putative functional polymorphisms described in Caucasians were included in the array. The 115 ADME genes included in the study are shown in the Supplementary Table S1, Supplemental digital content 1, *http://links.lww.com/FPC/A106*. The complete list of the 1536 polymorphisms with proven or putative functional effect and tSNPs included in the ADME array are shown in the Supplementary Table S2, Supplemental digital content 2, *http://links.lww.com/FPC/A107*.

In the replication study the candidate SNPs identified through the ADME array analysis were genotyped using commercially available TaqMan allelic discrimination assays (ABI prism 7000 SDS software, Applied Biosystems, Foster City, California, USA, see Supplementary Table S3, Supplemental digital content 3, *http://links.lww.com/FPC/A108*). Only the rs11045819 was genotyped by direct sequencing using the same primers used for *SLCO1B1* exon 5 amplification (see Supplementary Table S4, Supplemental digital content 4, *http://links.lww.com/FPC/A109*).

Resequencing was implemented in the follow-up of the most relevant candidate regions. This approach aims primarily to identify causal variants, such as rare and unknown SNPs in a tagged region [26]. The candidate *SLCO1B1* gene region was resequenced by targeting the promoter region (up to −1524 bp), and all 15 exons, intron–exon boundaries and the 3′UTR region (10 198 bp) in selected cases and control individuals with extreme LPV η_{CL} values. Primers are shown in Supplementary Table S4, Supplemental digital content 4, *http://links.lww.com/FPC/A109*. *SLCO1B1* gene haplotypes including all the identified SNPs for individual samples were constructed using PHASE, version 2.1 (University of Washington, Seattle, Washington, USA) [27,28]. The haplotype phylogenetic tree was performed with MEGA 3.1 software [29] using maximum parsimony and minimum evolution methods.

The associations in the discovery population were assessed by comparing allelic frequencies between cases and controls using PLINK [30]. Genotyping quality control was ensured by (i) including cases and controls in the same array plates, (ii) including duplicated samples in all array plates, (iii) removing SNPs that failed in more than 2% of samples, (iv) removing SNPs that departed from Hardy–Weinberg equilibrium ($P < 0.001$) [31] and (v) keeping genotyping blind with regard to the phenotyping status.

Genotype groups and genetic score

Study participants were categorized into genotypic groups according to the number of functional alleles assuming both alleles as contributing equally to the overall activity. The simplest scoring scheme assigned a value of '0' to the fully functional reference alleles (Hom Ref), and '1' and '2' to heterozygous (Het variant) and homozygous (Hom variant) variants in the gene, respectively.

In univariate analyses, genotype was entered into the model as a covariate using either a rich expression (eq. 1) or competing reduced expressions to test for a recessive- (eq. 2) or dominant (eq. 3) behavior of the gene:

$$CL = CL_0 I_0 + CL_1 I_1 + CL_2 I_2 \quad (1)$$

$$CL = CL_0 I_{0\_1} + CL_2 I_2 \quad (2)$$

$$CL = CL_0 I_0 + CL_1 I_{1\_2} \quad (3)$$

where CL_i, $i = 0,1,2$ are the typical value of clearance in the different genotypic groups and I_i, $i = 1,\ldots, n$, is an indicator variable that takes the value of 1 if a subject carries i variant alleles and 0 otherwise (0: Hom Ref, 1: Het variant, 2: Hom variant). A single indicator variable was assigned to Hom Ref or Het variants (I_{0_1}) and to Het or Hom variants (I_{1_2}) to test for a recessive or dominant effect of the gene, respectively.

The joint contribution of influent SNPs on LPV CL was first tested using pairwise conjunctions of SNPs, to finally build up the model including all genetic variants affecting LPV CL. The interaction between rs4149056 in *SLCO1B1* (*SLCO1B1*5*) and rs4149032 in SLCO1B1 alleles is developed as an example. As three score levels describe the functional activity of rs4149056 in *SLCO1B1* (*SLCO1B1*5*) and rs4149032, the richest possible model characterizing their joint impact on CL is the following one:

$$CL = CL_0 I_{00} + CL_1 I_{01} + CL_2 I_{02} + CL_3 I_{10} + CL_4 I_{11} + CL_5 I_{12} + CL_6 I_{20} + CL_7 I_{21} + CL_8 I_{22} \quad (4)$$

where I_{ij} is an indicator variable that takes the value of 1 if the individual carries the rs4149056 in *SLCO1B1* (*SLCO1B1*5*) ith genotype (Hom Ref, Het or Hom variant) along with the rs4149032 in *SLCO1B1* jth genotype and is '0' otherwise, and CL_k estimates CL among the different genotypic groups. Successive reduced models

were tested to merge genetic groups with CL not different from the Hom Ref group while identifying those causing a definite decrease or increase in LPV CL. The latter groups were entered in the final analysis based on semi-mechanistic considerations, and their joint influence was tested using either additive or proportional models:

$$CL = CL_{pop} + \theta_1 I_1 + \theta_2 I_2 \ldots + \theta_n I_n \quad (5)$$

$$CL = CL_{pop} \times (1 + \theta_1 I_1) \times (1 + \theta_2 I_2) \ldots \times (1 + \theta_n I_n) \quad (6)$$

where CL_{pop} is the average CL in the population, I_i, $i = 1,\ldots, n$, indicates now the genotypic group showing an effect on LPV CL, which takes the value of 1 if a subject carries the genotype and it is 0 otherwise, and n is the number of SNPs introduced in the final model. Eventually, an interaction term was introduced to further check whether some nonadditive interactions remained to be specified between the effects of distinct genotypic variants (p and q), which was formulated as follows:

$$CL = CL_{pop} + \theta_1 I_1 + \theta_2 I_2 \ldots + \theta_n I_n + \theta_i I_p I_q \quad (7)$$

where θ_i estimates some effect beyond simple additivity of SNPs, which was allowed to be either positive or negative.

A similar procedure was conducted with the genetic score (see below, replication analysis) using a simplification of the rich model (eq. 4), which allowed the estimation of CL for the genetic groups. Successive models were compared, resulting in a final reduced model that enabled the most parsimonious characterization of genetic groups associated with statistically different LPV CL levels compared to the reference group.

Results

Performance of the ADME-pathway array

On the basis of literature and database searches, 1896 SNPs were preselected to be genotyped on a 1536-plex Golden Gate Illumina BeadArray station. Among these, 19% obtained low (< 0.60) Illumina genotype design scores and were excluded from the array. From the retained 1536 SNPs, 10% failed quality control criteria for genotyping (see Materials and methods). Finally, 1380 SNPs (73%) were interrogated successfully, of which 50 were found monomorphic in this dataset. The genotyping success rate was comparable across functional, putative functional and tSNPs (Table 1).

Population pharmacokinetic modeling-discovery dataset

In total, 1472 plasma samples were collected from the 638 individuals included in the population analysis. The characteristics of the population are presented in Table 2. Concomitant anti-HIV drugs and other known cytochrome P450 inducers or inhibitors are shown in the Supplementary Table S5, Supplemental digital content 5, *http://links.lww.com/FPC/A110*. Concentration values ranged between 25 and 30 775 ng/ml. A one-compartment model with first order absorption from the gastrointestinal tract was found to describe the data appropriately and no improvement of the fit was added using a two-compartment model ($\Delta OF = 0$). The assignment of an interindividual variability term on V_d besides CL improved the fit ($\Delta OF = -64$, $P < 0.01$) as well as the introduction of a covariance between CL and V_d ($\Delta OF = -7.7$, $P < 0.05$). Allowing for interindividual variability on the absorption rate (k_a) improved the fit as well, but its estimate was very high owing to a limited number of data points during the absorption phase ($\Delta OF = -7.0$, $P < 0.05$). A combined error model for the residual variability fitted the data at best. Among the tested demographic variables, body weight influenced CL significantly ($\Delta OF = -6.3$, $P < 0.05$) and indicated a 60% increase in LPV CL for a doubling in body weight. LPV CL was 20% ($\Delta OF = -3.9$, $P < 0.05$) lower in female than in male patients but this parameter was correlated to body weight and therefore not kept in the model; no influence of neither age ($\Delta OF = 0.0$) nor bilirubin levels ($\Delta OF = 0.0$) could be detected. The influence of comedications on CL revealed a marked influence of amprenavir ($\Delta OF = -52.3$, $P < 0.01$) that suggested a 48% increase in LPV elimination in presence of this drug. Similarly and independently from amprenavir, the presence of a nonnucleotide analog reverse transcriptase inhibitor significantly increased LPV CL by 14% ($\Delta OF = -17.3$, $P < 0.01$). No other demographic or environmental covariates seemed to influence LPV to a significant or clinically relevant extent. The final

Table 1 Array genotyping capacity and performance

	Preselected		Included in array		Successfully genotyped	
SNP type	Number	Successful inclusion in array (%)	Number	Successful genotyping (%)	Number	Final array coverage (%)
PR-FP	167	79	132	88	116	69
PU-FP	382	75	288	92	265	69
tSNP	1347	83	1116	90	999	74
All SNPs	1896	81	1536	90	1380	73

PR-FP, proven functional polymorphisms; PU-FP, putative functional polymorphisms; tSNP, tagging single nucleotide polymorphism.

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Table 2 Characteristics of participants in the stage 1 (discovery) and stage 2 (replication) analyses

Characteristic	Discovery total (n=638)	Cases (n=117)[c]	Controls (n=90)[c]	Replication total (n=148)	RTV (n=96)[d]
Age (years)					
Median (range)	45 (19–74)	45.5 (26–74)	46 (21–64)	45 (19–80)	44.5 (22–68)
Sex, n (%)					
Men	484 (76)	96 (79)	71 (77)	113 (76)	72 (75)
Women	154 (24)	25 (21)	21 (23)	35 (24)	24 (25)
Body weight (kg)[a]					
Median (range)	69 (35–130)	67 (35–115)	69.5 (46–99)	68.5 (37–126)	68 (41–126)
Height (cm)[a]					
Median (range)	174 (145–195)	174 (154–193)	175 (156–195)	175 (148–193)	176 (148–193)
CD4 (cells/μl)[a]					
Median (range)	321 (15–1194)	289 (15–920)	307 (34–1029)	381 (15–1175)	394 (28–1073)
HIV-RNA <50 copies/ml[a]					
No (%)	239 (52.4)	39 (50.0)	29 (44.0)	75 (61.0)	38 (51%)
LPV/RTV daily dose (mg)[b]					
Low dose (<800/200 mg), n (%)	37 (2.51)	47 (12.95)*	1 (0.25)*	27 (11.07)**	10 (5.55)**
Standard dose (800/200 mg) n (%)	950 (64.54)	223 (61.43)	264 (64.86)	164 (67.21)	127 (70.56)
High dose (>800/200 mg), n (%)	485 (32.95)	93 (25.62)	142 (34.89)	53 (21.72)	43 (23.89)
Application scheme, n (%)[b]					
/12 h	1432 (97)	347 (96)	406 (99.75)	227 (93)	166 (92)
/24 h	40 (3)	16 (4)***	1 (0.25)***	17 (7)[†]	14 (8)[‡]
Formulation, n (%)[b]					
Capsules	1079 (73)	275 (76)	327 (80)	–	–
Tablets	393 (27)	88 (24)[‡]	80 (20)[‡]	244 (100%)	180 (100%)
Plasma levels (n)	1472	363	407	244	180

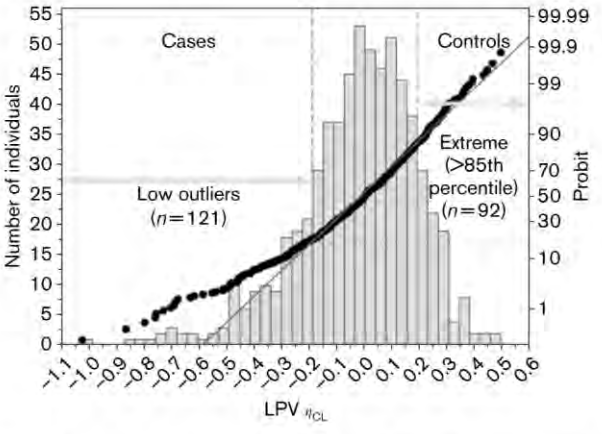
LPV, lopinavir; RTV, ritonavir.
[a]Only the measurements obtained close to the blood measurement (3 months before to 3 months after for body weight and height, and 1 month before to 3 months after for CD4 cells count and HIV-RNA).
[b]The numbers represent the daily dose, application scheme and formulation of each plasma level [the number of plasma levels exceed the number of individuals because there are several measurements per individual controls included in the stage 1 (discovery) after the exclusion of four cases and two controls with unavailable DNA].
[c]The number of cases and controls included in the stage 1 (discovery) after the exclusion of four cases and two controls with unavailable DNA.
[d]Individuals with coformulated ritonavir plasma levels available.
*P<0.0001 (Fisher's exact test).
**P>0.05 (Fisher's exact test).
***P<0.0001 (Fisher's exact test).
[†]P>0.05 (Fisher's exact test).
[‡]P>0.05 (Fisher's exact test).

estimated average CL was 4.32 l/h (CV 32%), V_d was 73.7 l (45%) and k_a 0.3/h (160%). CL increased to 6.4 and to 7.2 l/h in the presence of amprenavir and of a nonnucleotide analog reverse transcriptase inhibitor, respectively. Post-hoc individual estimates of η_{CL} were obtained from the final model for the selection of patients to be genotyped.

Phenotypic profile: discovery dataset

The histogram and probit analysis of the LPV η_{CL} in the discovery population (n = 638) indicated a deviation from normality at the lower end of the distribution (below −0.192, corresponding to percentile 19; Fig. 1). We selected those 121 individuals as 'cases'. In the absence of deviation from normality at the upper end of the distribution, we selected 92 individuals with LPV η_{CL} values above percentile 85 (> 0.180) as 'controls'. The mean and standard deviation of LPV clearance in the selected cases and controls were 3.4 ± 0.8 and 6.1 ± 1.0 l/h, respectively. DNA was available in 117 cases and 90 controls. The characteristics of the selected population are presented in Table 2.

Fig. 1



Distribution of the lopinavir (LPV) η_{CL} values. Frequency histogram of interpatient variability on LPV clearance (η_{CL}) and the corresponding probits in the discovery population (n=638). The 121 individuals with clearly departure from the expected normal distribution (grey line) were selected as cases and the 92 individuals with values above the percentile 85 were selected as controls.

Stage 1 genetic association analysis of the discovery population

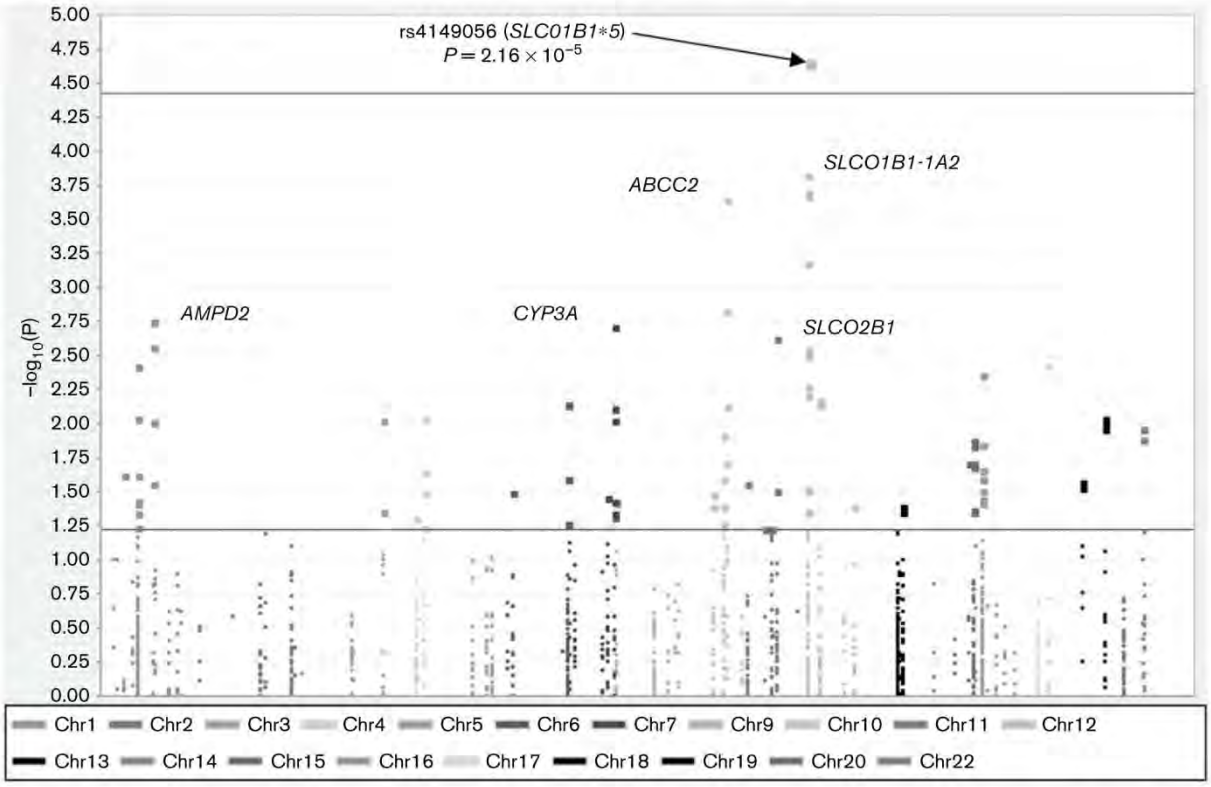
The individual allelic association analysis, performed with the 1330 successfully genotyped SNPs after excluding 50 monomorphic SNPs, identified rs4149056 as study-wide significant hit ($P = 2.16 \times 10^{-5}$) (Fig. 2). This non-synonymous SNP (c.521T > C, p.V174A) is located in the exon 5 of *SLCO1B1* and is associated with decreased uptake transport of the *SLCO1B1* substrates [32–34]. The variant allele rs4149056_C, described as *SLCO1B1*5*, by Tirona *et al.* [35], was most frequent among cases than controls of the discovery population (odds ratio = 3.90; 95% confidence interval: 2.01–7.57).

The analysis of linkage disequilibrium (LD, HapMap-Caucasian data) in the *SLCO1B1-1A2* locus using rs4149056 (*SLCO1B1*5*) and the other 51 SNPs genotyped in this gene cluster defined several LD blocks. This allowed identification of two potential association signals independent from rs4149056 (*SLCO1B1*5*) (Supplementary Figure S1, Supplemental digital content 6, *http://links.lww.com/FPC/A111*). One LD block included rs4149032 ($P = 2.01 \times 10^{-4}$), a tSNP located in the intron 2 of the *SLCO1B1*. A second LD block included rs10841795 ($P = 5.27 \times 10^{-3}$), a nonsynonymous SNP (p.I13T) located in the exon 3 of *SLCO1A2*, associated earlier with an increase in transport activity [36]. These independent signals were retained for the replication study. The complete listing of association *P* values for the 1330 successfully genotyped SNPs is included as Supplementary Table S6, Supplemental digital content 7, *http://links.lww.com/FPC/A112*.

Resequencing of the *SLCO1B1* region

To understand the role of the independent association signal identified by the tSNP rs4149032 in *SLCO1B1*, we selected 24 individuals who were homozygous for the minor allele of rs4149032. Twelve individuals were selected among cases and 12 among controls. This approach would allow the identification of the causal variant through the recombination analysis and possible haplotypes.

Fig. 2



Manhattan plot of results from the association study in stage 1. Shown are the 1330 single nucleotide polymorphisms (SNPs) analyzed in 20 chromosomes. The top line indicates the study-wide cut-off for significance, corresponding to Bonferroni correction of $P = 3.76 \times 10^{-5}$. The bottom line corresponds to the value at which the observed *P* values diverge from the null expectation. Only one SNP, in a solute carrier transporter, reached study-wide significance; other candidate loci are indicated. The *y* axis presents the negative log of the association *P* value. Complete list of the *P* values of the 1330 polymorphisms included in the association analysis in the discovery population is shown in the Supplementary Table S6.

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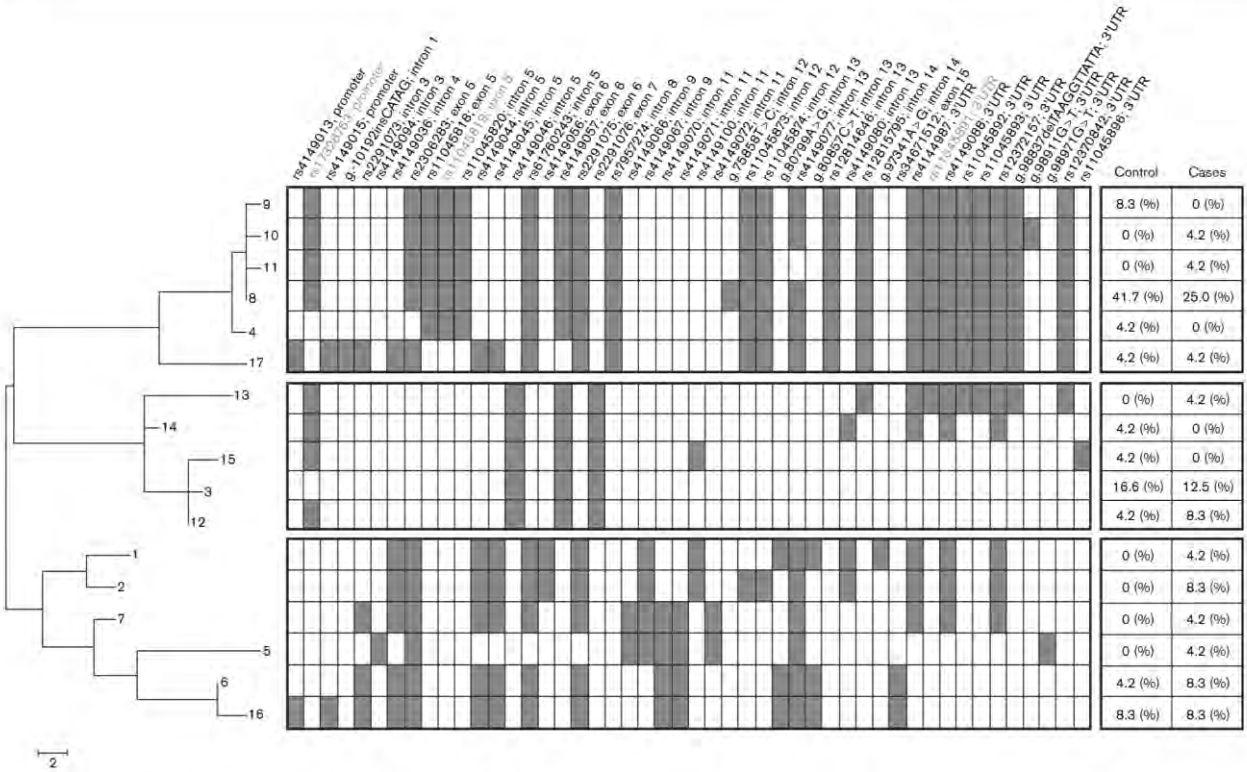
SLCO1B1 resequencing identified three polymorphisms in the promoter region, seven polymorphisms in exons, 28 polymorphisms in the exon–intron boundaries and 11 polymorphisms in the 3′UTR (Supplementary Figure S2, Supplemental digital content 8, *http://links.lww.com/FPC/A113*, and Table S7, Supplemental digital content 9, *http://links.lww.com/FPC/A114*). The 49 SNPs were submitted to haplotype analysis. Two haplotype families presented a differential association with LPV η_{CL} (Fig. 3) and led to the identification of three possible causal SNPs in LD with rs4149032: c.463C > A, rs11045819 ($R^2 = 0.412$, $D' = 1$) associated with nonsynonymous change P155T (*SLCO1B1*4*) in exon 3; g.–11939T > C, rs17328763 ($R^2 = 0.366$, $D' = 0.814$) in the promoter region, and g.98064A > C, rs11045891 ($R^2 = 0.487$, $D' = 0.938$) in the 3′UTR of the gene. All three candidate SNPs were brought forward to the replication stage.

To complete analysis, we also genotyped in the full discovery population two additional *SLCO1B1* SNPs with proven functional effect that failed to be included in the array: rs2306283 (c.388A > G, p.N130D *SLCO1B1*1b*) and rs4149015 (g.–11186G > A). They did not reach nominally significant *P* values.

Stage 2 replication study

The design of two-stage genome-association or large-scale association analyses invites the testing and validation of SNPs that remained significant after correction for multiple testing, as well as the selection of candidates from the pool of low *P* values that is enriched for false negatives [37]. Nine SNPs were selected for the replication study. Six were located in the *SLCO1B1-1A2* locus: rs4149056 as study-wide significant hit ($P = 2.16 \times 10^{-5}$), the independent signals rs4149032 ($P = 2.01 \times 10^{-4}$) and rs10841795 ($P = 5.27 \times 10^{-3}$) and three SNPs from the resequencing analysis: rs11045819, rs17328763 and rs11045891. Moreover, three additional SNPs from the stage 1 discovery analysis were retained for replication despite the fact that they had not reached study-wide significance. These SNPs presented low *P* values and were located in genes of potential interest; rs717620 in *ABCC2* ($P = 2.20 \times 10^{-4}$), earlier associated with decreased promoter activity and mRNA expression [38];

Fig. 3



Haplotypes identified through resequencing of *SCLO1B1*. The left panel presents the clustering of 17 haplotypes built on the basis of 49 single nucleotide polymorphisms (SNPs) identified through resequencing. The right panel depicts the SNPs differentially present across the three main families of haplotypes. Three SNPs were selected for further analysis based on function, location and pattern of linkage disequilibrium.

rs1077858, a tSNP in *SLCO2B1* ($P = 2.36 \times 10^{-3}$); and rs6945984, a tSNP in the *CYP3A* cluster ($P = 1.95 \times 10^{-3}$, Supplementary Figure S3, Supplemental digital content 10, *http://links.lww.com/FPC/A115*).

Population pharmacokinetic–pharmacogenetic modeling-replication data set

The replication data set consisted in 244 LPV levels collected in 148 HIV patients, whose blood levels ranged from 100 to 31 773 ng/ml. The data could be appropriately fitted using the same structural and variability model derived from the discovery analysis, with the exception of k_a that could not be precisely estimated and was therefore fixed to 0.3/h. Again, body weight significantly influenced LPV CL ($\Delta OF = -6.3$, $P < 0.05$). The influence of comedication was small in this data set and could confirm neither the influence of amprenavir nor of NNRTI detected in the discovery analysis owing to the limited number of patients exposed to these drugs. One individual showed a very high LPV concentration (> 30 000 ng/ml) at a single time point. We conducted a sensitivity analysis where the data concerning this patient was removed from the final model to check for a single outlier effect.

The subset of patients with LPV and RTV levels consisted of 96 patients contributing 180 plasma levels. A one-compartment model with first order absorption from the gastrointestinal tract was found to describe RTV concentration data appropriately. As the absorption rate k_a could not be precisely estimated, it was fixed to the literature [9]. An interindividual variability on V_d besides CL improved the fit ($\Delta OF = -15$, $P < 0.01$). The use of an additive residual model fitted the data better than the proportional one ($\Delta OF > -19.2$, $P < 0.01$), but no further improvement was obtained using a combined additive and proportional model ($\Delta OF = 0$). Among the tested demographic variables, body weight influenced CL significantly ($\Delta OF = -3.9$, $P = 0.05$) and indicated a doubling of RTV CL after a doubling in body weight. No other demographic or environmental covariates seemed to influence RTV levels to a significant or clinically relevant extent in this subset of patients. The population prediction of RTV plasma concentration versus time is shown in Supplementary Figure S4, Supplemental digital content 11, *http://links.lww.com/FPC/A116*. Among the 9 SNPs tested, *ABCC2* rs717620 improved the fit ($\Delta OF < -17.9$, $P < 0.01$). CL was reduced by 35% in Het LOF carriers and by 75% in Hom LOF individuals and explained 14% of the overall variability in CL (Supplementary Figure S5, Supplemental digital content 12, *http://links.lww.com/FPC/A117*). The influence of body weight did not remain significant after inclusion of the SNP effect and was not retained in the final model ($\Delta OF < -1.5$). The final estimates for RTV CL are 31.3, 18.9 and 7.3 l/h in Hom Ref, Het variant and Hom variant carriers (CV 54%), respectively, V_d 46.1 l and k_a 0.17/h.

The influence of RTV exposure ($AUC_{0-12 \text{ or } 24}$) on LPV CL slightly improved the fit ($\Delta OF = -4.4$ $P = 0.035$) and yielded an AUC_{50} of 1310 ng h/l. The inhibition of LPV clearance was however best described by a maximum effect model, with an IC_{50} of 374 ng/ml. This model described LPV data at best ($\Delta OF = -36$ $P < 0.01$) and reduced the variance in LPV elimination by 26%.

Genotype univariate analysis

The influence of the nine candidate alleles on LPV CL was first tested in single-SNP analyses, in which the allelic variants (Hom and Het variants, Ref alleles) were entered into the model as covariates that partitioned individuals based on their genetic background. At that stage, RTV levels were not introduced as a covariate in the model. Four variants had a statistically significant association with LPV CL (Table 3). The univariate analysis also served to propose a possible model of heritability – dominant (*CYP3A* rs6945984) and recessive (*SCLO1B1* rs11045819 and rs4149032, *ABCC2* rs717620). The explanation of interindividual variability in CL by the different SNPs taken individually was modest (1% on average). The joint impact of rs11045819 (*SLCO1B1*4*) with the tSNP rs4149032 in *SLCO1B1* did not provide any better

Table 3 Average clearance estimates for nine SNPs assessed in the replication analysis (univariate analysis)

Gene	dbSNP rs #	Hom Ref (0) mean CL (*n*)	RSE (%)	Het variant (1) mean CL (*n*)	RSE (%)	Hom variant (2) mean CL (*n*)	RSE (%)	ΔOF	*P* value[a]
SLCO1B1	rs4149056	5.6 (108)	7.8	4.8 (36)	10.1	4.4 (4)	12.7	−2.7	0.09 (0) vs. (1.2)
	rs4149032	4.9 (53)	7.6	5.2 (66)	10.1	6.3 (29)	12.6	−4.1	0.04 (0.1) vs. 2
	rs11045819	5.1 (96)	7.3	5.4 (46)	9.3	11.0 (6)	23	−5.1	0.025 (0.1) vs. (2)
	rs11045891	5.2 (87)	8.3	5.2 (53)	9.1	7.2 (8)	30	−1.9	NS
	rs17328763	5.0 (89)	7.4	5.5 (52)	10.2	8.3 (7)	26.7	−2.8	NS
SLCO1A2	rs10841795	5.2 (101)	8.0	5.4 (43)	10.1	5.8 (4)	19.3	−0.17	NS
ABCC2	rs717620	5.5 (93)	8.7	5.1 (49)	8.7	3.0 (6)	19.5	−3.9	0.05 (0.1) vs. 2
CYP3A	rs6945984	5.5 (122)	7.5	4.4 (21)	11.9	4.2 (5)	18.1	−3.9	0.05 (0) vs. (1.2)
SLCO2B1	rs1077858	5.6 (65)	9.7	5.0 (60)	8.7	5.2 (23)	10.4	−1.7	NS

The discrimination between models was based using either a rich model (eq. 1) or competing reduced models (eqs. 2 and 3) where population average clearance was estimated in each of the genotypic group (see text).

CL, clearance; Het, heterozygote; Hom, homozygote; *n*, number of individuals; NS, statistically not significant; ΔOF, change in the objective function; Ref, reference alleles; RSE, relative standard error of the estimates, defined as SE/estimate and expressed as percentage; SNP, single nucleotide polymorphism.

[a]Statistical difference between genetic groups.

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description of the data than rs11045819 (*SLCO1B1*4*) alone ($\Delta OF = -1$ $P = 0.3$). This confirmed the LD between these two SNPs, and suggested that rs4149032 was likely tagging the functional variant rs11045819 (*SLCO 1B1*4*). Only the latter was kept for further analyses.

Genotype score

Four SNPs were thus brought into joint analysis: *SCLO1B1* rs11045819 and rs4149056, *ABCC2* rs717620, and *CYP3A* rs6945984. We retained rs4149056 *SCLO 1B1*5* because, although it could not be formally replicated because of low allelic frequency, it was declared study-wide significant in the stage 1 discovery step. A genetic score was compiled using *SLCO1B1* rs4149056 (*SLCO1B1*5*, decrease of function), *ABCC2* rs717620 (decrease of function), *CYP3A* cluster rs6945984 (associated with decrease of function) and rs11045819 in *SLCO1B1* (*SLCO1B1*4*) (with known gain of function and altered substrate specificity), assuming additive genetic influences. In this score, a value of −2 was attributed to rs11045819 (*SLCO1B1*4*) Hom variant, −1 to *SLCO1B1*4* Het variant, 0 for Hom Ref alleles, 1, 2 and 3 for, 1, 2 or 3 variant alleles in any of *SLCO1B1* rs4149056 (*SLCO1B1*5*), *ABCC2* rs717620 or rs6945984, respectively in the *CYP3A* cluster.

The combination of selected SNPs and genotypes according to this scoring system indicated additive influences of all SNPs on LPV CL ($\Delta OF = -10.4$, $P < 0.01$). No difference between an additive or proportional contribution of the SNPs could be detected ($\Delta OF = 0.2$, $P = 0.7$). The sensitivity analysis indicated very similar results in gene effects with or without the inclusion of the outlier individual. The relative decrease in CL associated with *ABCC2* rs717620, *CYP3A* cluster rs6945984, and *SLCO1B1*5* rs4149056 was, respectively, 38% (−2.2 l/h), 18% (−1 l/h) and 15% (−1 l/h). *SLCO1B1*4* rs11045819 induced a doubling of LPV CL (95%, +5.26 l/h). No further interaction besides a simple addition of genetic influences could be detected ($\Delta OF > -0.1$, $P > 0.1$). As the combination of the four SNPs described the data best, the use of a simplified additive genetic score was tested and improved the fit (ΔOF −17.9, $P < 0.01$ compared with the baseline model). This model partitioned individuals into three groups: one group with score −2 (CL = 12.6 l/h), one group with scores 2 or 3 (CL = 3.7 l/h) and the remaining individuals with scores −1, 0, and 1 (CL = 5.5 l/h). The overall genetic impact of the four SNPs involved in LPV elimination explained 5% of the overall variability, in addition to 1% explained by body weight.

In the subanalysis of 96 patients with RTV levels, the influence of genetic polymorphisms remained statistically significant ($\Delta OF = -4.7$, $P = 0.03$) in presence of RTV, yielding an IC_{50} of 508 ng/ml. CL was 16.5 l/h in individual with rs11045819 (*SLCO1B1*4*) Hom variant, 7.4 l/h in carriers of 2 or 3 variants in *SLCO1B1* rs4149056 (*SLCO1B1*5*), *ABCC2* rs717620 or rs6945984 in the *CYP3A* cluster and 8.7 l/h in the remaining individual with Het variant or Ref alleles for the four SNPs (Supplementary Figure S5, Supplemental digital content 12, *http://links.lww.com/FPC/A117*). Genetic polymorphism explained an additional 2% in the variance of LPV clearance.

Table 4 Final population pharmacokinetic parameters and interindividual variability in the stage 2 replication data set

	Population mean		Interpatient variability[a]	
Parameter	Estimate	RSE[b] (%)	Estimate (%)	RSE[c] (%)
CL/F (l/h)	5.72	8	36	64
V_d/F (l)	70.8	29	134	56
k_a (per hour)	0.3[d]	–	–	
θ_{BW}	0.593	48	–	
$\theta_{rs11045819}$	0.954	55	–	
$\theta_{rs717620}$	0.384	45		
$\theta_{rs6945984}$	0.180	48		
$\theta_{rs4149056}$	0.151	45		
σ_{prop} (CV %)[e]	26.3%	41[c]		
σ_{add} (SD ng/ml)[f]	2150	22		

Final Model: $CL_i = CL \times (1 - \theta_{rs717620}) \times (1 - \theta_{rs6945984}) \times (1 - \theta_{rs4149056}) \times (1 + \theta_{rs11045819}) \times (1 + \theta_{BW} \times (BW - meanBW)/meanBW)$

BW, body weight; CL/F, mean apparent clearance; k_a, mean absorption rate constant; RSE, relative standard error; V_d/F, mean apparent volume of distribution.

[a]Estimates of variability expressed as coefficient of variation (CV %).

[b]Relative standard error of the estimates, defined as standard error/estimate and expressed as percentage.

[c]Relative standard error of the coefficient of variations, taken as $\sqrt{s.e_{estimate}}/estimate$, expressed as a percentage.

[d]Fixed to this value because could not be estimated precisely.

[e]Residual variability in the plasma concentrations associated with the proportional error term, expressed as a coefficient of variation (CV %).

[f]Residual variability in the plasma concentrations associated with the additive error term, expressed as a standard deviation (SD).

The final population estimates and variability are presented in Table 4. The final population pharmacokinetic model of the subanalysis of LPV–RTV inhibition along with the population final estimates of RTV is presented in Table 5. The population predicted and individual LPV CL values according to the genetic score are depicted in Fig. 4. The population prediction of LPV plasma concentration versus time with 90% prediction interval in association to the three genetic score groups is shown in Supplementary Figure S6, Supplemental digital content 13, *http://links.lww.com/FPC/A118*.

Discussion

This study illustrates an ADME pharmacogenetic approach to the assessment of both common variants and less common polymorphisms contributing to the pharmacokinetics of a drug. The study drug, LPV/r is a first-line antiretroviral agent characterized by large interindividual variability in plasma levels. Using a two-stage design, the study identified and validated four genetic variants that contribute to LPV CL. They implicate the solute carrier *SCLO1B1* [organic anion transporting polypeptide (OATP1B1)], as well as ABCC2 [multidrug resistance-related protein

Table 5 Final estimates of RTV and LPV/RTV models in the subanalysis of the stage 2 replication data set ($n=96$)

	Population mean		Interpatient variability[a]	
Parameter	Estimate	RSE[b] (%)	Estimate (%)	RSE[c] (%)
Ritonavir				
CL/F rs717620 Ref (l/h)	31.30	12	54	64
CL/F rs717620 Het variant (l/h)	18.90	14		
CL/F rs717620 Hom variant (l/h)	7.30	22		
V_d/F (l)	46.10	23	121	56
k_a (per hour)	0.17[d]	–	89	
σ_{add} (SD ng/ml)[e]	159	7		
Lopinavir				
CL/F score group −2	16.50	22	24	69
CL/F score group −1,0,1	8.70	11		
CL/F score group 2,3	7.41	9		
V_d/F (l)	63	3	99	34
k_a (per hour)	0.30[d]	–		
IC_{50} (ng/ml)	508	7		
σ_{prop} (SD ng/ml)[e]	2473			

CL/F, mean apparent clearance according to genetic scoring where the score group −2 represents rs11045819 (*SLCO1B1*4*) Hom variant carriers, the score group −1,0,1 represents *SLCO1B1*4* Het variant −1 carriers, for Hom Ref allele 0 carriers in any of the four SNPs included, and Het variant carriers in any of the three SNPs (rs4149056, rs717620, and rs6945984), finally the score group 2,3 represents the carriers of 2 or 3 variant alleles in any of *SLCO1B1* rs4149056 (*SLCO1B1*5*), *ABCC2* rs717620 or rs6945984 in the *CYP3A* cluster variants; LPV, lopinavir; IC_{50}, RTV (ritonavir) concentration producing 50% of maximal inhibition; k_a, mean absorption rate constant; RSE, relative standard error; V_d/F, mean apparent volume of distribution; SNP, single nucleotide polymorphism.

[a]Estimates of variability expressed as coefficient of variation (%).

[b]RSE of the estimates, defined as standard error/estimate and expressed as percentage.

[c]RSE of the coefficient of variations, taken as $\sqrt{\text{s.e}_{\text{estimate}}/\text{estimate}}$, expressed as a percentage.

[d]Fixed to value from the discovery dataset.

[e]Residual variability in the plasma concentrations, expressed as a standard deviation (SD).

(MRP2)] in the transport of the drug. It also supports a role for the *CYP3A* region even under conditions of suppression by the coformulated agent RTV.

The basic concept underlying the development of the dedicated 1536-SNP ADME array was the need to bridge common variations – as contributed by tagging SNPs from HapMap – to more rare functional variations known to be of particular relevance to metabolizing enzyme genes, and possibly to transporter genes [21,24]. The results validate the underlying concept for the development of the array: two of the initially identified variants are functional – *SCLO1B1*5* rs4149056 and *ABCC2* rs717620. In addition, the identification of rs4149032, a tSNP in *SCLO1B1*, led through resequencing to the identification of the likely causal variant, *SLCO1B1*4* rs11045819 that results in P155T, an amino acid change in the substrate recognition domain of the transporter. Finally, rs6945984 is an intergenic tSNP placed in a LD block that includes *CYP3A4*, *3A7* and *3A5*; here, we did not pursue towards localization of the possible causal variant. The comprehensive analysis provided by the array led to successful genotyping of 73% of the intended positions. This level of coverage reflects the technical limits to include all candidate SNPs in the array, and the failure to successfully generate genotype data on all included SNPs. In addition, coverage may have been limited by incomplete knowledge on unreported rare variants.

The identification of two functional SNPs in *SLCO1B1* that are associated with opposite effects on LPV CL is a strong indication of the relevance of this transporter. LPV has been recently reported to be a substrate of SLCO1B1 by Hartkoorn *et al.* [39]. *SLCO1B1* encodes OATP1B1, an uptake transporter of the OATP superfamily, which is localized to the basolateral membrane of hepatocytes and facilitates the entry of drug into the cells before metabolism and transporter-mediated excretion into the bile [33,34]. Therefore, variants in *SLCO1B1* such as rs4149056, earlier associated with impaired transport activity owing to cell surface trafficking defects [32,35], can affect the pharmacokinetics of drugs extensively metabolized by CYP enzymes such as LPV, thus explaining increased plasma concentrations. This is consistent with our observation that rs4149056 was more frequent among the cases with high LPV plasma levels as compared with controls in the discovery population. It is also in agreement with published observations showing increased plasma exposure to statins, antihypertensive and antitumoral drugs in carriers of this variant [33,34,40,41]. In this study, individuals homozygous *SLCO1B1*5* had a 15% reduction in LPV clearance. Less is known on rs11045819, that represents the basis of *SLCO1B1*4*. The effect of this variant on transport activity has been investigated only in two studies [35,42]. The uptake of estrone sulfate and estradiol 17β-D-glucuronide by *SLCO1B1*4* or by *SLCO1B1*1a* in transiently transfected HeLa cells was not affected [35]. However, Michalski *et al.* [42] described that in stably transfected MDCKII cells expressing *SLCO1B1*4*, the transport of cholyltaurine was abolished and the uptake of estradiol 17β-D-glucuronide was significantly reduced, whereas the uptake of bromosulfophthalein was not significantly different from *SLCO1B1*1a*. rs11045819 showed a profound gain of transport activity for LPV, suggesting that the associated amino acid at position 155 in the second extracellular loop of OATP1B1 is critical for substrate recognition as evidenced by in-vitro data associating this variant with different transport kinetics [42]. In this study, individuals homozygous *SLCO1B1*4* have a 95% increase in LPV clearance.

LPV is also reported to be substrate [3,43] and RTV to be substrate [44] and inducer [45] of ABCC2. *ABCC2* gene encodes MRP2, an efflux transporter of the MRP family. MRP2 expression has been localized to the canalicular membranes of hepatocytes and apical membranes of intestinal enterocytes. Genetic variation such as rs717620 has been associated with decreased promoter activity,

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mRNA expression and duodenal protein content [38,46]. It is therefore expected to result in increased plasma levels of drug substrates, as a result of decreased expression of MRP2 and consequently decreased biliary efflux of the compound. This variant has been implicated in changes in the pharmacokinetic parameters of irinotecan [47], mycophenolic acid [48], methotrexate [49], indinavir [50] and tenofovir [51]. In this study, individuals homozygous for the *ABCC2* variant have a 40% reduction in LPV clearance.

We were surprised by the consistency of support for the relevance of variants in the *CYP3A* region in the metabolism of LPV, despite the inhibitory action of RTV on CYP3A4/5. The intergenic rs6945984 was retained during the first screen and in the replication. The position of this particular tSNP in the recombination block would suggest a role for variants in LD, such as those that define *CYP3A7*2* (gain of function, T409R, *P* value in the discovery set of 0.0077), and *CYP3A5*3* (loss of function through splicing defect, *P* value in the discovery set of 0.036). Other possible causal variants did not reach nominal significance in the discovery set.

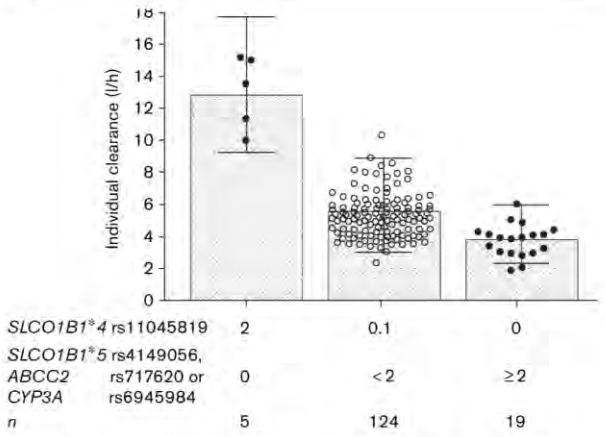
We assessed the contribution of the four variants identified through the two-stage procedure of discovery-replication using a population pharmacokinetic approach. Pharmacokinetic estimates and variability were in good agreement with the values reported earlier [9,52].

Among other covariates that significantly influenced LPV CL, body weight was correlated with an increase in CL, is in agreement with earlier observations [6,53]. The increase in LPV elimination in presence of amprenavir results from the additive effect on PXR (pregnane × receptor) activation, a nuclear receptor regulating the transcription of various CYP/drug transporters [54], and is consistent with clinical observations showing a decrease in both protease inhibitors levels when coadministered [55].

Fluctuations in RTV levels have been shown to have a major impact on LPV kinetics and variability [52]. Our results are in line with the reported reversible inhibition of LPV clearance by RTV, with an IC_{50} close to reported values from in-vitro and clinical studies, and associated with a marked reduction of the variance in LPV elimination [9]. The slight increase in IC_{50} in the final model reflects the fact that the contribution of RTV levels is reduced by genetic variations. Conditional analysis of the data identified an independent influence of *ABCC2* rs717620 on RTV kinetics. Thus, the influence of this variant on LPV kinetics can be both direct, and indirect effect by modulating RTV levels.

Genetic variants explained not more than 5% of LPV overall clearance, mainly because they affected only a minority of individuals. However, the absolute magnitude of effect was clinically relevant for those individuals that

Fig. 4



Influence of a genetic score on lopinavir clearance. Circles, individual predicted Bayesian LPV clearances (open circles correspond to individuals within score reference group). *Bars*, average predicted clearances with 90% population prediction interval for each 4 SNPs score group combinations; 0, Hom-common allele; 1 Het-rare allele; 2 Hom-rare allele; <2, less than two rare alleles or ≥ 2, two or more rare alleles in combination for any of the three rs4149056, rs717620 or rs6945984 polymorphisms; *n*, number of individuals carrying the allelic combination.

carried two or more of the identified variants: 3% of individuals were homozygous *SCLO1B1*4* (gain of function) and had an average clearance of 12.6 l/h, compared with 5.5 l/h in the reference population and 3.7 l/h in the 13% of individuals with multiple variants of *SCLO1B1*, *CYP3A* and *ABCC2* associated with decreased function (Fig. 4). These genetic effects seemed to act additively, in line with the absence of self induction or of other regulatory mechanisms able to introduce nonlinearities in the model (e.g. in the case of efavirenz pharmacogenetics [56]). In analysis including the influence of RTV levels, the role of genetic variants of *SCLO1B1*, in particular the **4* allele remained as independent contributors to LPV kinetics. The study addressed a population that contributed data from TDM. The nonadherence to treatment can introduce significant noise in the analysis. However, the two-stage replication served to validate the identification of the various SNPs despite this limitation. It is also expected that the genotypes would distribute following Mendelian randomization across adherent and nonadherent individuals.

This study is relevant for the SNPs and genes identified, as much as for the apparent absence of major common variants explaining large components of the variance. Thus, this study illustrates the current paradigm in genome-wide and large-scale association analyses, where multiple common genetic variations have limited contribution to the study phenotype in the population [26]. In conclusion, this study reinforces the interest of extending pharmacogenetic research efforts to drugs

associated with significant interindividual pharmacokinetic variability. It validates a heuristic approach that complements arrays based on common human genetic variation with a complete set of rare candidate functional variants, following a discovery/replication cycle. It also leads to quantify the remaining unexplained and still large amount of variability in LPV clearance that remains to be assessed.

Acknowledgements

This study is supported by the Swiss National Science Foundation, the Swiss HIV Cohort study, and by the SHCS research foundation. The population pharmacokinetic analyses were performed in part at the Vital-IT, center for high-performance computing of the Swiss Institute of Bioinformatics. The authors thank Dr Marta Valle for her advice on LPV/RTV modeling. The members of the Swiss HIV Cohort Study (SHCS) are Battegay M, Bernasconi E, Böni J, Bucher HC, Bürgisser P, Calmy A, Cattacin S, Cavassini M, Dubs R, Egger M, Elzi L, Fischer M, Flepp M, Fontana A, Francioli P (President of the SHCS), Furrer H (Chairman of the Clinical and Laboratory Committee), Fux CA, Gorgievski M, Günthard HF (Chairman of the Scientific Board), Hirsch HH, Hirschel B, Hösli I, Kahlert C, Kaiser L, Karrer U, Kind C, Klimkait T, Ledergerber B, Martinetti G, Müller N, Nadal D, Paccaud F, Pantaleo G, Rauch A, Regenass S, Rickenbach M (Head of Data Center), Rudin C (Chairman of the Mother and Child Substudy), Schmid P, Schultze D, Schüpbach J, Speck R, de Tejada BM, Taffé P, Telenti A, Trkola A, Vernazza P, Weber R, Yerly S.

References

1 Hammer SM, Eron JJ Jr, Reiss P, Schooley RT, Thompson MA, Walmsley S, *et al.* Antiretroviral treatment of adult HIV infection: 2008 recommendations of the International AIDS Society-USA panel. *JAMA* 2008; **300**:555–570.

2 Kumar GN, Jayanti V, Lee RD, Whittern DN, Uchic J, Thomas S, *et al.* In vitro metabolism of the HIV-1 protease inhibitor ABT-378: species comparison and metabolite identification. *Drug Metab Dispos* 1999; **27**:86–91.

3 Agarwal S, Pal D, Mitra AK. Both P-gp and MRP2 mediate transport of lopinavir, a protease inhibitor. *Int J Pharm* 2007; **339**:139–147.

4 King JR, Wynn H, Brundage R, Acosta EP. Pharmacokinetic enhancement of protease inhibitor therapy. *Clin Pharmacokinet* 2004; **43**:291–310.

5 Molto J, Blanco A, Miranda C, Miranda J, Puig J, Valle M, *et al.* Variability in non-nucleoside reverse transcriptase and protease inhibitor concentrations among HIV-infected adults in routine clinical practice. *Br J Clin Pharmacol* 2006; **62**:560–566.

6 Van der Leur MR, Burger DM, la Porte CJ, Koopmans PP. A retrospective TDM database analysis of interpatient variability in the pharmacokinetics of lopinavir in HIV-infected adults. *Ther Drug Monit* 2006; **28**:650–653.

7 Colombo S, Buclin T, Decosterd LA, Telenti A, Furrer H, Lee BL, *et al.* Orosomucoid (alpha1-acid glycoprotein) plasma concentration and genetic variants: effects on human immunodeficiency virus protease inhibitor clearance and cellular accumulation. *Clin Pharmacol Ther* 2006; **80**:307–318.

8 Jullien V, Urien S, Hirt D, Delaugerre C, Rey E, Teglas JP, *et al.* Population analysis of weight-, age-, and sex-related differences in the pharmacokinetics of lopinavir in children from birth to 18 years. *Antimicrob Agents Chemother* 2006; **50**:3548–3555.

9 Molto J, Barbanoj MJ, Miranda C, Blanco A, Santos JR, Negredo E, *et al.* Simultaneous population pharmacokinetic model for lopinavir and ritonavir in HIV-infected adults. *Clin Pharmacokinet* 2008; **47**:681–692.

10 Peng JZ, Pulido F, Causemaker SJ, Li J, Lorenzo A, Cepeda C, *et al.* Pharmacokinetics of lopinavir/ritonavir in HIV/hepatitis C virus-coinfected subjects with hepatic impairment. *J Clin Pharmacol* 2006; **46**:265–274.

11 Stek AM, Mirochnick M, Capparelli E, Best BM, Hu C, Burchett SK, *et al.* Reduced lopinavir exposure during pregnancy. *AIDS* 2006; **20**:1931–1939.

12 Estrela RC, Santoro AB, Barroso PF, Tuyama M, Suarez-Kurtz G. CYP3A5 genotype has no impact on plasma trough concentrations of lopinavir and ritonavir in HIV-infected subjects. *Clin Pharmacol Ther* 2008; **84**:205–207.

13 Estrela RC, Ribeiro FS, Barroso PF, Tuyama M, Gregorio SP, Dias-Neto E, *et al.* ABCB1 polymorphisms and the concentrations of lopinavir and ritonavir in blood, semen and saliva of HIV-infected men under antiretroviral therapy. *Pharmacogenomics* 2009; **10**:311–318.

14 Gupta SK, Rosenkranz SL, Cramer YS, Koletar SL, Szczech LA, Amorosa V, *et al.* The pharmacokinetics and pharmacogenomics of efavirenz and lopinavir/ritonavir in HIV-infected persons requiring hemodialysis. *AIDS* 2008; **22**:1919–1927.

15 Ma Q, Brazeau D, Zingman BS, Reichman RC, Fischl MA, Gripshover BM, *et al.* Multidrug resistance 1 polymorphisms and trough concentrations of atazanavir and lopinavir in patients with HIV. *Pharmacogenomics* 2007; **8**:227–235.

16 Winzer R, Langmann P, Zilly M, Tollmann F, Schubert J, Klinker H, *et al.* No influence of the P-glycoprotein genotype (MDR1 C3435T) on plasma levels of lopinavir and efavirenz during antiretroviral treatment. *Eur J Med Res* 2003; **8**:531–534.

17 Wyen C, Fuhr U, Frank D, Aarnoutse RE, Klaassen T, Lazar A, *et al.* Effect of an antiretroviral regimen containing ritonavir boosted lopinavir on intestinal and hepatic CYP3A, CYP2D6 and P-glycoprotein in HIV-infected patients. *Clin Pharmacol Ther* 2008; **84**:75–82.

18 Frazer KA, Ballinger DG, Cox DR, Hinds DA, Stuve LL, Gibbs RA, *et al.* A second generation human haplotype map of over 3.1 million SNPs. *Nature* 2007; **449**:851–861.

19 Colombo S, Beguin A, Telenti A, Biollaz J, Buclin T, Rochat B, *et al.* Intracellular measurements of anti-HIV drugs indinavir, amprenavir, saquinavir, ritonavir, nelfinavir, lopinavir, atazanavir, efavirenz and nevirapine in peripheral blood mononuclear cells by liquid chromatography coupled to tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005; **819**:259–276.

20 Fayet A, Beguin A, de Tejada BM, Colombo S, Cavassini M, Gerber S, *et al.* Determination of unbound antiretroviral drug concentrations by a modified ultrafiltration method reveals high variability in the free fraction. *Ther Drug Monit* 2008; **30**:511–522.

21 Lubomirov R, Csajka C, Telenti A. ADME pathway approach for pharmacogenetic studies of anti-HIV therapy. *Pharmacogenomics* 2007; **8**:623–633.

22 De Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D. Efficiency and power in genetic association studies. *Nat Genet* 2005; **37**:1217–1223.

23 Eberle MA, Ng PC, Kuhn K, Zhou L, Peiffer DA, Galver L, *et al.* Power to detect risk alleles using genome-wide tag SNP panels. *PLoS Genet* 2007; **3**:1827–1837.

24 Peters EJ, McLeod HL. Ability of whole-genome SNP arrays to capture 'must have' pharmacogenomic variants. *Pharmacogenomics* 2008; **9**:1573–1577.

25 Yuan HY, Chiou JJ, Tseng WH, Liu CH, Liu CK, Lin YJ, *et al.* FASTSNP: an always up-to-date and extendable service for SNP function analysis and prioritization. *Nucleic Acids Res* 2006; **34**:W635–W641.

26 Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, Hunter DJ, *et al.* Finding the missing heritability of complex diseases. *Nature* 2009; **461**:747–753.

27 Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet* 2001; **68**:978–989.

28 Stephens M, Donnelly P. A comparison of bayesian methods for haplotype reconstruction from population genotype data. *Am J Hum Genet* 2003; **73**:1162–1169.

29 Kumar S, Nei M, Dudley J, Tamura K. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* 2008; **9**:299–306.

30 Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007; **81**:559–575.

31 Balding DJ. A tutorial on statistical methods for population association studies. *Nat Rev Genet* 2006; **7**:781–791.

32 Kameyama Y, Yamashita K, Kobayashi K, Hosokawa M, Chiba K. Functional characterization of SLCO1B1 (OATP-C) variants, SLCO1B1*5, SLCO1B1*15 and SLCO1B1*15 + C1007G, by using transient expression

systems of HeLa and HEK293 cells. *Pharmacogenet Genomics* 2005; **15**:513–522.
33 Konig J, Seithel A, Gradhand U, Fromm MF. Pharmacogenomics of human OATP transporters. *Naunyn Schmiedebergs Arch Pharmacol* 2006; **372**:432–443.
34 Niemi M. Role of OATP transporters in the disposition of drugs. *Pharmacogenomics* 2007; **8**:787–802.
35 Tirona RG, Leake BF, Merino G, Kim RB. Polymorphisms in OATP-C: identification of multiple allelic variants associated with altered transport activity among European- and African-Americans. *J Biol Chem* 2001; **276**:35669–35675.
36 Badagnani I, Castro RA, Taylor TR, Brett CM, Huang CC, Stryke D, *et al.* Interaction of methotrexate with organic-anion transporting polypeptide 1A2 and its genetic variants. *J Pharmacol Exp Ther* 2006; **318**:521–529.
37 Pahl R, Schafer H, Muller HH. Optimal multistage designs–a general framework for efficient genome-wide association studies. *Biostatistics* 2009; **10**:297–309.
38 Haenisch S, Zimmermann U, Dazert E, Wruck CJ, Dazert P, Siegmund W, *et al.* Influence of polymorphisms of ABCB1 and ABCC2 on mRNA and protein expression in normal and cancerous kidney cortex. *Pharmacogenomics J* 2007; **7**:56–65.
39 Hartkoorn RC, Kwan WS, Shallcross V, Chaikan A, Liptrott N, Egan D, *et al.* HIV protease inhibitors are substrates for OATP1A2, OATP1B1 and OATP1B3 and lopinavir plasma concentrations are influenced by SLCO1B1 polymorphisms. *Pharmacogenetics and Genomics* 2010; **20**:112–120.
40 Niemi M. High plasma pravastatin concentrations are associated with single nucleotide polymorphisms and haplotypes of organic anion transporting polypeptide-C (OATP-C, SLCO1B1). *Pharmacogenetics* 2004; **14**:429–440.
41 Nishizato Y, Ieiri I, Suzuki H, Kimura M, Kawabata K, Hirota T, *et al.* Polymorphisms of OATP-C (SLC21A6) and OAT3 (SLC22A8) genes: consequences for pravastatin pharmacokinetics. *Clin Pharmacol Ther* 2003; **73**:554–565.
42 Michalski C, Cui Y, Nies AT, Nuessler AK, Neuhaus P, Zanger UM, *et al.* A naturally occurring mutation in the SLC21A6 gene causing impaired membrane localization of the hepatocyte uptake transporter. *J Biol Chem* 2002; **277**:43058–43063.
43 Janneh O, Jones E, Chandler B, Owen A, Khoo SH. Inhibition of P-glycoprotein and multidrug resistance-associated proteins modulates the intracellular concentration of lopinavir in cultured CD4T cells and primary human lymphocytes. *J Antimicrob Chemother* 2007; **60**:987–993.
44 Huisman MT, Smit JW, Crommentuyn KM, Zelcer N, Wiltshire HR, Beijnen JH, *et al.* Multidrug resistance protein 2 (MRP2) transports HIV protease inhibitors, and transport can be enhanced by other drugs. *AIDS* 2002; **16**:2295–2301.
45 Dussault I, Lin M, Hollister K, Wang EH, Synold TW, Forman BM. Peptide mimetic HIV protease inhibitors are ligands for the orphan receptor SXR. *J Biol Chem* 2001; **276**:33309–33312.
46 Haenisch S, May K, Wegner D, Caliebe A, Cascorbi I, Siegmund W. Influence of genetic polymorphisms on intestinal expression and rifampicin-type induction of ABCC2 and on bioavailability of talinolol. *Pharmacogenet Genomics* 2008; **18**:357–365.
47 Zhou Q, Sparreboom A, Tan EH, Cheung YB, Lee A, Poon D, *et al.* Pharmacogenetic profiling across the irinotecan pathway in Asian patients with cancer. *Br J Clin Pharmacol* 2005; **59**:415–424.
48 Naesens M, Kuypers DR, Verbeke K, Vanrenterghem Y. Multidrug resistance protein 2 genetic polymorphisms influence mycophenolic acid exposure in renal allograft recipients. *Transplantation* 2006; **82**:1074–1084.
49 Rau T, Erney B, Gores R, Eschenhagen T, Beck J, Langer T. High-dose methotrexate in pediatric acute lymphoblastic leukemia: impact of ABCC2 polymorphisms on plasma concentrations. *Clin Pharmacol Ther* 2006; **80**:468–476.
50 Anderson PL, Lamba J, Aquilante CL, Schuetz E, Fletcher CV. Pharmacogenetic characteristics of indinavir, zidovudine, and lamivudine therapy in HIV-infected adults: a pilot study. *J Acquir Immune Defic Syndr* 2006; **42**:441–449.
51 Kiser JJ, Carten ML, Aquilante CL, Anderson PL, Wolfe P, King TM, *et al.* The effect of lopinavir/ritonavir on the renal clearance of tenofovir in HIV-infected patients. *Clin Pharmacol Ther* 2008; **83**:265–272.
52 Crommentuyn KM, Kappelhoff BS, Mulder JW, Mairuhu AT, van Gorp EC, Meenhorst PL, *et al.* Population pharmacokinetics of lopinavir in combination with ritonavir in HIV-1-infected patients. *Br J Clin Pharmacol* 2005; **60**:378–389.
53 Hsu A, Isaacson J, Brun S, Bernstein B, Lam W, Bertz R, *et al.* Pharmacokinetic-pharmacodynamic analysis of lopinavir-ritonavir in combination with efavirenz and two nucleoside reverse transcriptase inhibitors in extensively pretreated human immunodeficiency virus-infected patients. *Antimicrob Agents Chemother* 2003; **47**:350–359.
54 Marzolini C, Tirona RG, Lee W, Gervasini G, Ho RH, Leake B, *et al.* Unexpected complexity in nuclear receptor activation by HIV protease inhibitors and induction of CYP enzymes and transporters. In: 11th Conference on Retroviruses and Opportunistic Infections, Feb 2004, San Francisco, Abstract 135 2009.
55 Mauss S, Schmutz G, Kuschak D. Unfavourable interaction of amprenavir and lopinavir in combination with ritonavir? *AIDS* 2002; **16**:296–297.
56 Arab-Alameddine M, Di IJ, Buclin T, Rotger M, Lubomirov R, Cavassini M, *et al.* Pharmacogenetics-based population pharmacokinetic analysis of efavirenz in HIV-1-infected individuals. *Clin Pharmacol Ther* 2009; **85**:485–494.

4.1.2.2. Original article

Association of Pharmacogenetic Markers with Premature Discontinuation of first-line anti-HIV Therapy: an Observational Cohort Study

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Journal of Infectious Diseases 2010; in press

Author contributions and the article are presented in section 4.1.1.3.

Comments on the article:

Given the high prevalence of treatment discontinuation in individuals under antiretroviral therapy [14], it is of prime importance to identify predictors susceptible to guide treatment combination or dosage.

In this aim, we retrospectively determined if genetic variations influencing first-line antiretroviral drug pharmacokinetics or pharmacodynamics, would be associated with drug tolerance at the population level, assessed through the phenotype of treatment discontinuation.

Individuals under tenofovir, abacavir, efavirenz, **lopinavir/ritonavir (LPV/r)** or atazanavir/ritonavir – containing regimen were included in the study.

For individuals under LPV/r-containing regimen, we assessed the 4 genetic variations associated in our previous work with changes in LPV/r clearance, as well as 8 genetic variants associated with dyslipidemia.

We failed to demonstrate any impact of these genetic variants on LPV/r treatment discontinuation rate. However, the small sample size contained in some groups and the retrospective aspect of the study might explain these results and emphasizes the need for prospective analyses.

4.2. Pharmacogenetics and Clearance of Hepatitis C Virus

4.2.1. Original article

Genetic Variation in *IL28B* is Associated with Chronic Hepatitis C and Treatment Failure: A Genome-Wide Association Study

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Gastroenterology 2010 April; 138(4):1338-1345, 1345.e1-7

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Study concept and design: A. Rauch, A. Telenti, P-Y. Bochud, F. Negro

Genotyping: P. Descombes

Statistical analysis: Z. Kutalik, T. Cai, J. S. Beckmann, S. Bergmann, A. Rauch

Resequencing and recombinant mapping: **J. di Iulio**, A. Telenti

Study supervision: A. Rauch, A. Telenti, P-Y. Bochud, F. Negro

Drafting of the manuscript: A. Rauch, A. Telenti, P-Y. Bochud, F. Negro, M. Bochud, D. Moradpour

Data and sample collection: all other authors

Critical revision of the manuscript for important intellectual content: all authors

Detailed personal contribution:

I contributed, with A. Telenti, in the establishment of a new approach to identify candidate causal variants responsible for the phenotype associated with the putatively non-functional hit SNP. This fine mapping strategy consisted in:

- *IL28B* promoter, exons, introns and 3'UTR resequencing (N=47)
- Haplotype inference and phylogenetic classification
- Data analysis and interpretation for candidate causal variants identification

Comments on the article:

Hepatitis C infection can result in either spontaneous/treatment-induced clearance or chronicity. As host genetics is believed to play a major role in the multifactorial interplay determining hepatitis C infection outcome, and although it has triggered much interest this last years, no key genetic variants have been identified in the candidate genes analyzed up to date.

We therefore performed a genome wide association study to identify host genetic markers associated with HCV infection outcome.

An intergenic SNP, rs8099917, reached genome-wide significance. As this SNP was part of a linkage disequilibrium block emcompassing interferon-λ3 (*IL28B*) locus, we investigated by resequencing and recombinant mapping strategy whether this SNP was tagging a functional SNP in *IL28B*. This analysis allowed the identification of distinct haplotypes and candidate SNPs associated with HCV infection outcome in *IL28B*.

This constituted the first lead to the causal allele and mechanism of action of *IL28B* for the natural control of HCV infection. These SNPs are prime candidates for functional assessment.

GASTROENTEROLOGY 2010;138:1338–1345

CLINICAL ADVANCES IN LIVER, PANCREAS, AND BILIARY TRACT

Genetic Variation in *IL28B* Is Associated With Chronic Hepatitis C and Treatment Failure: A Genome-Wide Association Study

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See editorial on page 1240.

BACKGROUND & AIMS: Hepatitis C virus (HCV) induces chronic infection in 50% to 80% of infected persons; approximately 50% of these do not respond to therapy. We performed a genome-wide association study to screen for host genetic determinants of HCV persistence and response to therapy. **METHODS:** The analysis included 1362 individuals: 1015 with chronic hepatitis C and 347 who spontaneously cleared the virus (448 were coinfected with human immunodeficiency virus [HIV]). Responses to pegylated interferon alfa and ribavirin were assessed in 465 individuals. Associations between more than 500,000 single nucleotide polymorphisms (SNPs) and outcomes were assessed by multivariate logistic regression. **RESULTS:** Chronic hepatitis C was associated with SNPs in the *IL28B* locus, which encodes the antiviral cytokine interferon lambda. The *rs8099917* minor allele was associated with progression to chronic HCV infection (odds ratio [OR], 2.31; 95% confidence interval [CI], 1.74–3.06; $P = 6.07 \times 10^{-9}$). The association was observed in HCV mono-infected (OR, 2.49; 95% CI, 1.64–3.79; $P = 1.96 \times 10^{-5}$) and HCV/HIV coinfected individuals (OR, 2.16; 95% CI, 1.47–3.18; $P = 8.24 \times 10^{-5}$). *rs8099917* was also associated with failure to respond to therapy (OR, 5.19; 95% CI, 2.90–9.30; $P = 3.11 \times 10^{-8}$), with the strongest effects in patients with HCV genotype 1 or 4. This risk allele was identified in 24% of individuals with spontaneous HCV clearance, 32% of chronically infected patients who responded to therapy, and 58% who did not respond ($P = 3.2 \times 10^{-10}$). Resequencing of *IL28B* identified distinct haplotypes that were associated with the clinical phenotype. **CONCLUSIONS: The association of the *IL28B* locus with natural and treatment-associated control of HCV indicates the importance of innate immunity and interferon lambda in the pathogenesis of HCV infection.**

Keywords: Hepatitis C; Genetics; Interferon; Interleukin-28.

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Hepatitis C virus (HCV) is a positive-stranded RNA virus that chronically infects 120 to 180 million people (ie, ~3% of the world population).[1] Infection with HCV induces a wide range of innate and adaptive immune responses that achieve permanent control of HCV in 20% to 50% of infected individuals.[2,3] Failure to clear

Abbreviations used in this paper: CI, confidence interval; HIV, human immunodeficiency virus; IFN, interferon; OR, odds ratio; SNP, single nucleotide polymorphism.

0016-5085/10/$36.00
doi:10.1053/j.gastro.2009.12.056

HCV leads to chronic hepatitis C. The morbidity and mortality associated with chronic hepatitis C are mainly attributable to its progression toward cirrhosis and hepatocellular carcinoma.[4] Standard therapy with pegylated interferon (IFN)-α and ribavirin fails to clear HCV in ~50% of chronically infected individuals.[5–8]

Host factors influence both the natural course of hepatitis C and response to therapy.[9–12] In 2 cohorts of pregnant women infected under similar conditions with anti-D immunoglobulin preparations contaminated with a single strain of HCV, half spontaneously cleared the infection and half progressed to chronic hepatitis C.[13,14] Among chronically infected patients, response to treatment differs, even among cases with similar HCV RNA levels and identical viral genotypes.[4–7] The response rates are associated with viral genotypes, ethnicity, and sex.[15–18]

Previous candidate gene studies reported the role of genetic polymorphisms of HLA,[9,12,19] killer immunoglobulin-like receptors,[20] chemokines, and interleukins as well as IFN-stimulated genes[21–25] on spontaneous HCV clearance. A recent candidate gene study showed that genetic variation in the *IL28B* gene, which encodes IFN-λ, is associated with spontaneous HCV clearance,[26] and 3 genome-wide association studies reported associations of SNPs in *IL28B* with response to antiviral therapy[27–29] in individuals infected with HCV genotype 1.

In this study, we systematically searched for common human genetic determinants of progression to chronic hepatitis C and of treatment failure using a genome-wide association study in 1362 HCV-infected individuals.

Patients and Methods

Patients were included from the Swiss Hepatitis C Cohort Study and the Swiss HIV Cohort Study, 2 multicenter studies performed at 8 major Swiss hospitals and their local affiliated centers,[30,31] and from the Medical Clinic for Hepatology and Gastroenterology, Medical University Charité Campus, Virchow-Klinikum Berlin, in Berlin, Germany. Written informed consent, including genetic testing, was mandatory for inclusion, and the study was approved by all local ethical committees. Due to the different genetic predictors of hepatitis C outcomes in racially diverse populations,[15,18] analyses were limited to the white population. Demographic characteristics including age, sex, HCV risk factors, HCV genotypes, alcohol consumption, markers for hepatitis B virus and human immunodeficiency virus (HIV) infection, HCV viral load, liver biopsy data, and HCV treatment were extracted from clinical databases.

Chronic HCV infection was defined as anti-HCV seropositivity (using enzyme-linked immunosorbent assay and confirmed by immunoblot or recombinant immunoblot assay) and detectable HCV RNA by quantitative or qualitative assays; spontaneous HCV clearance was defined as HCV seropositivity and undetectable HCV RNA in patients without previous antiviral treatment. To avoid the fluctuations of HCV RNA levels during the first year of infection (reviewed by Hoofnagle[32]), we determined HCV RNA levels at least 1 year after the first documented positive HCV serology. Patients who received at least 80% of the recommended dose of pegylated IFN-α/ribavirin were considered assessable for response to treatment. Sustained viral response was defined as an undetectable HCV RNA in serum more than >24 weeks after treatment termination; all other patients were considered nonresponders. Severe fibrosis was considered in patients with a METAVIR score ≥F3.

Genotyping of more than 500,000 common human polymorphisms was performed by the Genomics Platform of the National Center of Competence in Research "Frontiers in Genetics" at the University of Geneva in Geneva, Switzerland, by using Illumina Human1M-Duo, HumanHap550, or Human610W-Quad BeadChips (Illumina, San Diego, CA). Genotype calling was performed using the default settings of the BeadStudio software (Illumina). Calls with a genotyping score <0.2 were excluded from further analysis. Single nucleotide polymorphisms (SNPs) with a call rate <90% and individuals with a call rate <95% were excluded. To enable multiple platform analysis, genome-wide imputation was performed using MACH[33] based on measured SNPs with >90% call rate, minor allele frequency >1%, and Hardy–Weinberg P value $>10^{-7}$. SNPs with low imputation quality (r2-hat <0.3) were ignored. Whenever the measured genotype was available, it replaced the imputed value. Population stratification and relatedness were assessed using the ancestry principal components as previously described.[34–36] One of each genetically related/identical individual pair (relatedness >0.125) was excluded from further analysis.

To take into account the potential influence of HIV coinfection on spontaneous HCV clearance, HCV monoinfected and coinfected individuals were first analyzed separately; subsequently, we performed a genome-wide meta-analysis of the 2 cohorts. A meta-analysis of association signals obtained from each cohort was performed using inverse variance weighting. Association analysis was performed using a logistic regression model with exact maximum likelihood estimation. Covariates influencing the outcome in the univariate analysis ($P < 0.1$), along with the first 2 ancestry principal components, were included in the model. We used a mild P value cutoff as an inclusion criterion for covariates to avoid disregarding potentially important factors. To account for the fact that different genotyping platforms were used, we excluded any SNP with an allele frequency (among patients with chronic infection) that was significantly different (χ^2 test, $P < 10^{-4}$) between any 2 platforms. Genomic control was applied to the genome-wide P values yielding a λ of 1.04 (for the mono-infected cohort) or 1.02 (for the coinfected cohort). These values suggested very mild inflation and confirmed that possible

population stratification was sufficiently corrected by including the first 2 ancestry principal components in the models. Bonferroni correction was used to adjust for multiple testing; we used 5×10^{-8} as significance threshold.

Resequencing of the candidate locus and recombinant mapping[37] were performed for the purpose of mapping the candidate causal variant or genetic region using the primers indicated in Supplementary Table 1. We used PHASE version 2.1 software (University of Washington, Seattle, WA) for haplotype inference from population genotype data.

Results

Chronic Versus Spontaneously Cleared HCV Infection

The study included 1362 patients with HCV infection, among whom 347 had spontaneously cleared HCV infection and 1015 had progressed to persistent infection; 914 were HCV mono-infected and 448 were coinfected with HIV/HCV (Supplementary Table 2). Several SNPs near the *IL28* locus on chromosome 19 were associated with chronic HCV infection with genome-wide significance (Figure 1). The top hit *rs8099917* (odds ratio [OR], 2.31; 95% confidence interval [CI], 1.74–3.06; $P = 6.07 \times 10^{-9}$; Figure 1) is located in an ~80-kilobase region encoding 3 cytokines (ie, IL28B, IL28A, and IL29) as T (major allele) to G (minor allele) substitution 7554 base pairs upstream the start codon of *IL28B* (Supplementary Figure 1). The signal in the locus carried 7 SNPs with a P value $<10^{-5}$ (Supplementary Figure 1*A*). The recombination profile indicated that the signal encompassed the whole *IL28B* gene (Supplementary Figures 1 and 2). The effect was similar in HCV mono-infected (OR, 2.49; 95% CI, 1.64–3.79; $P = 1.96 \times 10^{-5}$) and HCV/HIV coinfected individuals (OR, 2.16; 95% CI, 1.47–3.18; $P = 8.24 \times 10^{-5}$). No SNP outside the *IL28B/A* locus reached genome-wide significance (Figure 1 and Supplementary Tables 3 and 4).

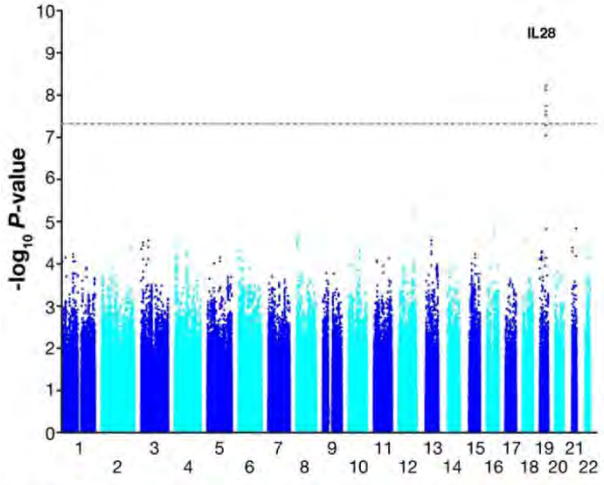

Figure 1. Manhattan plot for chronic versus spontaneously cleared HCV infection. The association values in $-\log_{10} P$ values are shown by chromosome. Genome-wide significance is indicated by the *horizontal line*. A genome-wide significant association signal is observed on chromosome 19. The signal maps to the *IL28* locus. *rs8099917* is the top hit.

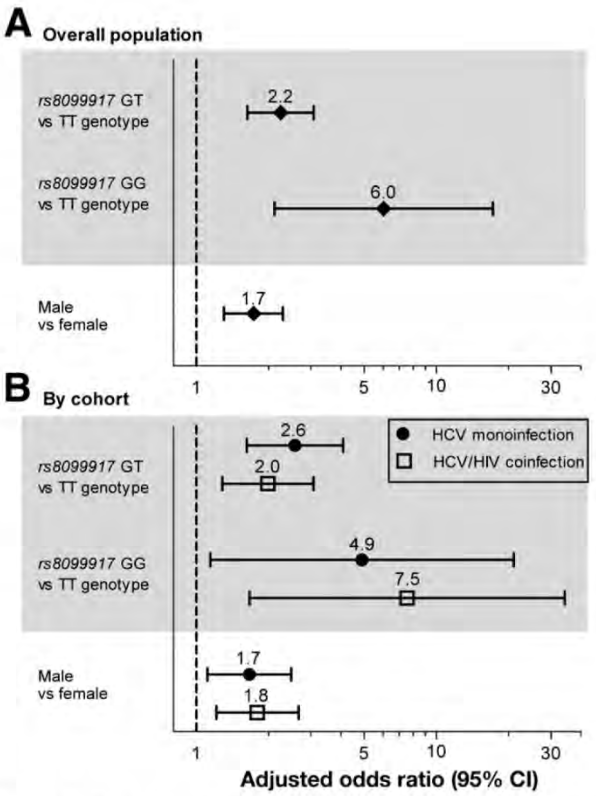

Figure 2. Predictors of chronic versus spontaneously cleared HCV infection. (*A*) Carriers of the rs8099917 G-risk genotypes had a higher risk of failing to spontaneously clear HCV infection and thus to progress to chronic infection. (*B*) The effect of *rs8099917* was similar in HCV mono-infected and HIV/HCV coinfected individuals. ORs were calculated by allele and were adjusted for sex and the first 2 ancestry principal components. Age was not included because the time point of acute HCV infection is unknown in most cases.

The frequencies of the *rs8099917* TT, GT, and GG genotypes were 0.58, 0.37, and 0.05 among patients with chronic infection versus 0.78, 0.21, and 0.01 among those with spontaneous clearance, respectively (Supplementary Table 5). In the analyses by genotype, both homozygous (GG: OR, 6.02; 95% CI, 2.10–17.21; $P = 8.10 \times 10^{-4}$) and heterozygous (GT: OR, 2.24; 95% CI, 1.63–3.07; $P = 6.63 \times 10^{-7}$) patients had a higher risk of chronicity compared with patients carrying the common genotype (TT) (Figure 2). Thus, the minor G allele increased the risk of chronicity and was defined as the risk allele. The effect of the genotypes was very similar for both the HCV mono-infected and HCV/HIV coinfected individuals (Figure 2).

As expected, male sex was associated with higher chronicity rates (OR, 1.67; 95% CI, 1.29–2.16; $P = 9.5 \times 10^{-5}$). The association between sex and HCV persistence was not modified relevantly by adjusting for *rs8099917* (OR, 1.80; 95% CI, 1.41–2.31; $P = 2.77 \times 10^{-6}$), and there was no significant interaction between sex and this SNP ($P > .05$).

Among those with chronic HCV infection, there was no significant association of *rs8099917* with HCV RNA levels (OR, 1.01; 95% CI, 0.81–1.25; $P = .94$).

Treatment Response

Next, we assessed whether the SNPs associated with chronicity also influenced response to pegylated IFN-α/ribavirin combination therapy. Among chronically infected patients, 465 were assessable for response to this treatment (all mono-infected). Factors associated with failure to treatment included HCV genotype 1 or 4 ($P < .001$), severe fibrosis ($P = .06$), male sex ($P = .05$), older age ($P < .01$), and higher pretreatment HCV RNA ($P = .04$). These variables were included as covariates in the logistic regression.

The frequencies of genotypes TT, GT, and GG for the previously discovered *rs8099917* were 0.42, 0.51, and 0.07 among patients with treatment failure versus 0.68, 0.29, and 0.03 among those with sustained viral response, respectively (Supplementary Table 5). In the analyses by genotypes, GG homozygous and GT heterozygous patients both had a higher risk of treatment failure compared with patients carrying the common genotype (TT) (Figure 3). Minor G allele carriers had a higher risk of treatment failure than the other patients (OR, 5.19; 95% CI, 2.90–9.30; $P = 3.11 \times 10^{-8}$), and the G allele was therefore defined as the risk allele.

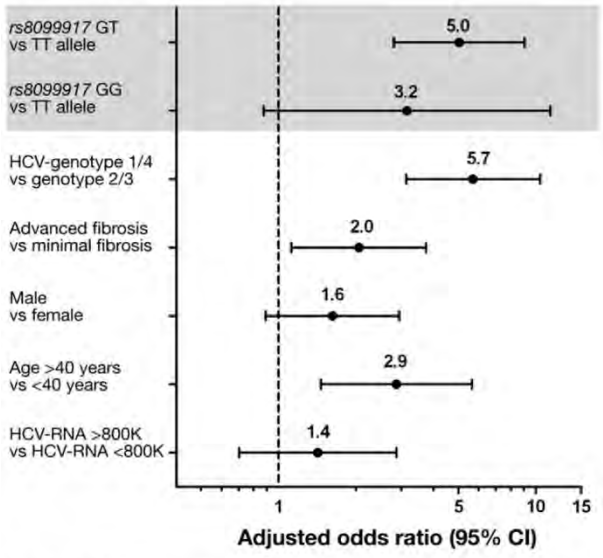

Figure 3. Predictors of failure to respond to pegylated IFN-α and ribavirin therapy. Carriers of the *rs8099917* G-risk genotypes had a higher risk of failing to respond to HCV treatment. ORs were calculated by allele and were adjusted for HCV genotypes, fibrosis stage, sex, age, baseline HCV viral load, and the first 2 ancestry principal components.

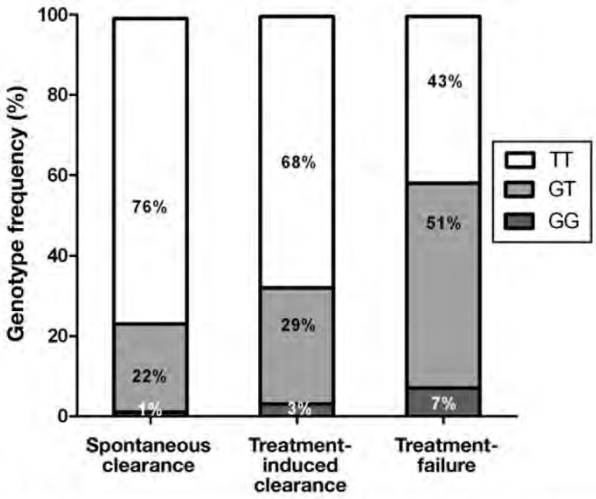

Figure 4. Distribution of genotypes in an infected population. Among 914 mono-infected patients, those with treatment failure have higher rates of carriage of the *rs8099917* G-risk allele than patients with treatment-induced clearance and patients with spontaneous clearance. There was a significant trend ($P = 4.83 \times 10^{-9}$) across the 3 groups.

Clinical Impact

Given that *rs8099917* was associated with both chronicity and treatment failure, we assessed data as a theoretical continuum, assuming the history of an infected population (Figure 4). Overall, the proportion of individuals carrying the risk allele increased progressively from a low frequency among patients with spontaneous clearance (24%) to an intermediate frequency among chronically infected patients with sustained viral response to treatment (32%) and to a highest frequency among chronically infected individuals who failed to respond to treatment (58%; $P = 3.2 \times 10^{-10}$).

A second aspect of importance in clinical care is viral genotype. Therefore, we assessed the joint contribution of host and pathogen genetic risk determinants. Patients were stratified in 4 groups, according to the viral genotypes (viral genotype 2 or 3 vs viral genotype 1 or 4) and host polymorphisms (host *rs8099917* G risk allele carriers vs noncarriers; Table 1). Treatment failure occurred in only 14% of patients with both low-risk parameters compared with 72% among those with both high-risk parameters (OR, 15.79; 95% CI, 8.37–29.76; $P = 1.48E\text{-}17$; Table 1). Among patients infected with genotypes 1 or 4, treatment failure occurred in 72% of risk allele carriers infected compared with only 39% of noncarriers (OR, 4.97; 95% CI, 2.56–9.66; $P = 2.13 \times 10^{-6}$). There was no significant association between genetic variation in *IL28B* and response to therapy among individuals infected with HCV genotype 2 or 3 (OR, 1.58; 95% CI, 0.77–3.25; $P = .18$).

Table 1. Joint Analysis of Viral and Host Genetic Determinants of Treatment Response

HCV genotype	*IL28B rs8099917* G allele	Treatment failure n	Treatment failure Frequency	Treatment success n	Treatment success Frequency	Failure (%)	OR (95% CI)[a]	*P* value
2/3	Absent	20	0.13	127	0.42	14		Referent
2/3	Present	17	0.11	66	0.22	20	1.62 (0.80–3.31)	1.81E-01
1/4	Absent	48	0.30	81	0.27	37	3.95 (2.19–7.12)	4.75E-06
1/4	Present	74	0.47	29	0.10	72	15.79 (8.37–29.76)	1.48E-17

[a]Adjusted for fibrosis stage, sex, age, baseline HCV viral load, and the first 2 ancestry principal components.

Recombinant Mapping

To map the candidate causal variant or genetic region tagged by *rs8099917*, we resequenced the *IL28B* locus. To maximize the likelihood of identifying the causal region, we performed recombinant mapping on DNA from individuals selected for having "concordant" or "discordant" genotype-phenotype constellations. Concordant referred to individuals homozygous for the common allele TT with clearance (n = 15) or homozygous for the *rs8099917* GG risk allele with chronic infection (n = 15). Discordant referred to individuals homozygous for the common allele TT with chronic infection (n = 15) or the rare individuals (n = 2) homozygous for the GG risk allele with HCV clearance.

Resequencing of the *IL28B* locus based on recombinant mapping identified 21 SNPs. Haplotype inference led to the identification of 2 main haplotype families (Figure 5). The first family carried the individuals with an HCV clearance phenotype. The second family of haplotypes carried most of the risk of chronicity. The sequence of the *IL28B* promoter and coding region in discordant individuals that progressed to chronic infection despite absence

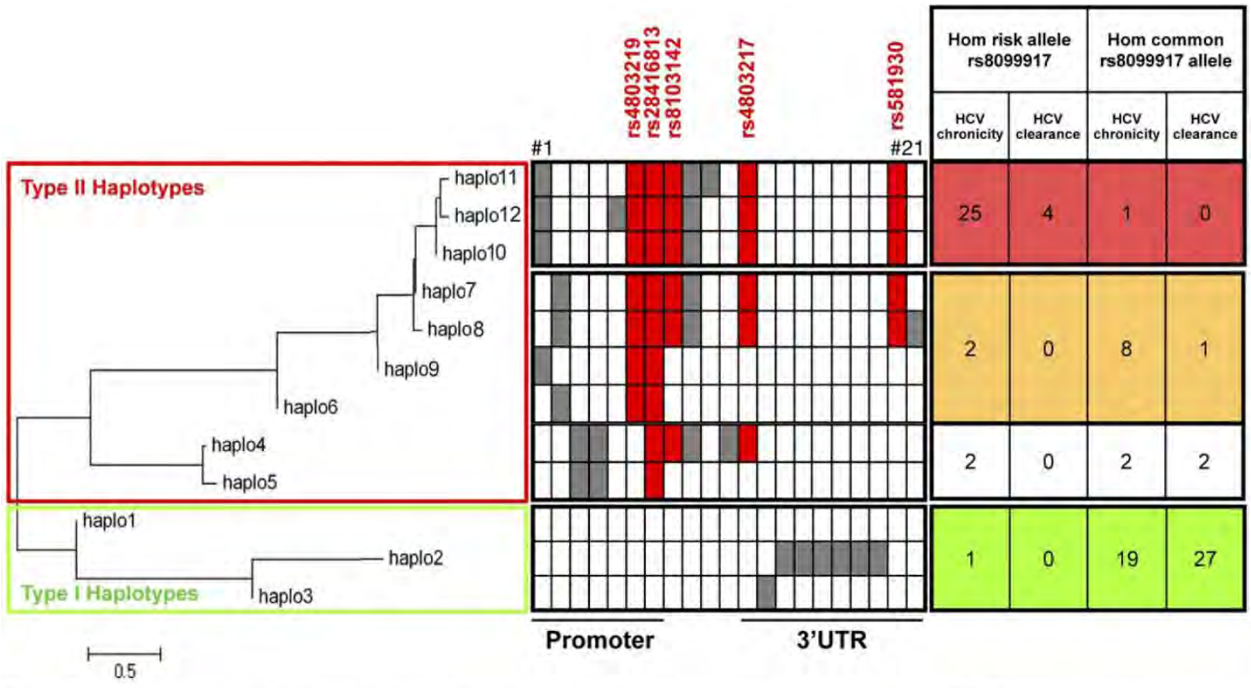

Figure 5. Resequencing, haplotype inference, and recombinant mapping of the candidate causal region in *IL28B*. Resequencing for the purpose of mapping the causal variant or region in *IL28B* was performed by using the primers indicated in Supplementary Table 5 to amplify 4279 base pairs. Sequencing was performed on DNA from 47 individuals representing the various constellations of marker genotype at *rs8099917* and the HCV clearance phenotype. This led to the identification of 21 SNPs (#1 to #21, presence of a polymorphism is indicated by a *shaded box*). Inference of the various haplotypes by using PHASE led to the proposal of a dendrogram with 2 main haplotypic families. The frequent haplotype 1 and derived haplotypes constitute the type I haplotype family, which is generally associated with HCV clearance. The frequent haplotype 10 and derived haplotypes constitute the type II haplotype family, which is generally associated with chronicity. Type II haplotypes are characterized by a defined structure that includes 2 promoter SNPs, a nonsynonymous K70R, and two 3′ untranslated region SNPs (marked *red*). The *numbers* in the *right panel* indicate the number of chromosomes with the respective haplotype – *rs8099917* combinations by clinical outcome.

of the risk allele of *rs8099917* was characteristic of that of individuals with the risk marker. Several specific SNPs were identified as candidates for being causal (Figure 5).

Discussion

This is the first genome-wide association study to report the influence of human genetic variation on the natural control of HCV infection. We found a similar effect of the same genetic marker (*rs8099917*, located near the *IL28B* gene) for both natural and treatment-induced control of HCV infection. The lowest carriage frequency of the risk allele (24%) was observed among persons with spontaneous clearance and increased to 32% among chronically infected patients who responded to treatment and to 58% among chronically infected patients who failed to respond. This observation is in line with recent studies that observed a strong association of genetic variation in *IL28B* with response to therapy[27–29] and with spontaneous HCV clearance.[26]

Our findings strongly point to a major role of the innate immunity in the control of HCV. The mapped region (19q13) encodes 3 cytokine genes (IL28A, IL28B, and IL29) that belong to the IFN-λ (also named type III IFN) family. IFN-λs interact with a transmembrane receptor to induce potent antiviral responses.[38–40] This antiviral activity is mediated through the activation of the JAK-STAT (IFN-αs, IFN-γs, and IFN-λs) and MAPK (IFN-αs and IFN-λs) pathways (reviewed by Li et al[39]). In vitro and in vivo models have shown the importance of IFN-λs in the immune response to several viral pathogens, including herpes simplex virus,[41,42] cytomegalovirus,[43] HIV,[44] and hepatitis B virus.[45] IFN-λ1 and IFN-λ2 blocked HCV replication in human hepatocytic cell lines.[46–48] IFN-λ has been proposed, and already tested, as a treatment of hepatitis C.[49,50] In a phase 1B trial of patients with chronic hepatitis C who are responders/relapsers, the drug had a robust activity against HCV and limited toxicity.[49,51] This low toxicity may be explained by a reduced tissue expression of the IFN-λ receptor compared with that of the IFN-α receptor.[49,50] Thus, IFN-λ is a promising molecule for the future treatment of hepatitis C, and a dose-ranging phase 2 trial is currently planned.

The genetic data from this study point to *IL28B* as a critical effector in the control of HCV. The mechanisms linking *rs8099917* with differential antiviral responses in HCV-infected patients need to be elucidated. The SNP is located 8 kilobases upstream of the start codon of *IL28B*. As part of a haplotype block that covers the full length of *IL28B*, it may interfere with transcription factors and influence gene expression or splicing. In 2 studies,[28,29] the presence of the *rs8099917* risk allele was associated with lower expression of IFN-λ.

Carriage of the *rs8099917* SNP was the strongest genetic predictor of both natural and treatment-induced control of HCV. This SNP was also the strongest predictor for response to HCV therapy in white[27,28] and Japanese[29] populations. Thomas et al reported a strong association of the *rs12979860* SNP with spontaneous HCV clearance.[26] *rs12979860* is not measured on the Illumina 550 chip and was not available in 149 individuals from our cohort who were genotyped with this chip. In the 1213 individuals with available data, carriage of *rs12979860* was highly associated with chronic HCV infection (OR, 1.95; 95% CI, 1.54–2.48; $P = 3.38 \times 10^{-8}$) and was in strong linkage disequilibrium with *rs8099917* ($D' = 0.98$; $r^2 = 0.5$).

SNPs associated with chronic infection may be in linkage disequilibrium with one or several coding SNP(s), or functional noncoding SNP(s), in *IL-28B* that modulate its function. For this purpose, we used recombinant mapping exploiting the various possible couplings of *rs8099917* and the clearance/chronicity phenotype. This led to the identification of 2 main haplotypes (and their derived haplotypes) in the study population. Each haplotypic family had a characteristic promoter and coding region that associates with the clinical phenotype. This constitutes a lead to the causal allele and mechanism of action of *IL28B* in clearance. Functional studies of gene expression and cytokine production among the different allele carriers will help in addressing this issue.

Our study shows that genetic variation in *IL28B* is genome-wide the strongest common human genetic determinant for the control of HCV infection. No SNP outside the *IL28B/A* locus reached genome-wide significance. This further underscores the particular role of innate immune responses for the control of HCV infection. The comparison with genome-wide associations in HIV infection is intriguing, because all significant determinants for the control of HIV were within the major histocompatibility complex on chromosome 6 in genes involved in adaptive immune responses.[52]

Our findings could have a substantial impact for prognosis and therapy. For example, individuals with HCV genotype 1 or 4 who carry the risk allele, particularly in homozygosis, will have a very low probability of natural or treatment-induced clearance. These individuals would be prime candidates for novel therapeutic strategies. However, emergence of HCV variants that are resistant to small molecules is almost inevitable if these drugs are not combined with other effective compounds.[53] Individuals with unfavorable host and viral genotypes treated with pegylated IFN-α/ribavirin and a small molecule would be treated with only one effective drug and therefore at high risk for selection of drug resistance mutations and treatment failure. Future studies need to address the question whether individuals with unfavorable host and viral genotypes would benefit from combining different antiviral molecules to maximize viral response and minimize the risk of drug resistance.

There was no significant association between genetic variation in *IL28B* and response to therapy among indi-

viduals infected with HCV genotype 2 or 3, indicating that the prognostic value of the risk allele for treatment response might be limited to individuals with difficult-to-treat HCV genotypes. Another point of clinical importance is the allele frequency of this variant in the population; in a survey of 5435 unrelated Swiss white subjects,[54] the carriage frequency of *rs8099917* was 17%. According to the Human Haplotype Map project (HapMap; www.hapmap.org), 15% to 19% of white people carry the rs8099917 minor allele. Carriage frequencies differ considerably (range, 2%–31%) in different ethnicities. These differences might therefore contribute to divergent clearance rates observed across populations.[5,6,15]

Taken together, the increasing evidence for the role of IFN-λ for both spontaneous and treatment-induced control of HCV infection opens new avenues for prognosis and treatment of HCV infection.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.12.056.

References

1. Shepard CW, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. Lancet Infect Dis 2005;5:558–567.
2. Thomas DL, Astemborski J, Rai RM, et al. The natural history of hepatitis C virus infection: host, viral, and environmental factors. JAMA 2000;284:450–456.
3. Rehermann B. Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. J Clin Invest 2009;119:1745–1754.
4. Lauer GM, Walker BD. Hepatitis C virus infection. N Engl J Med 2001;345:41–52.
5. Manns MP, McHutchison JG, Gordon SC, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001;358:958–965.
6. Fried MW, Shiffman ML, Reddy KR, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347:975–982.
7. Chung RT, Andersen J, Volberding P, et al. Peginterferon alfa-2a plus ribavirin versus interferon alfa-2a plus ribavirin for chronic hepatitis C in HIV-coinfected persons. N Engl J Med 2004;351:451–459.
8. Zeuzem S, Feinman SV, Rasenack J, et al. Peginterferon alfa-2a in patients with chronic hepatitis C. N Engl J Med 2000;343:1666–1672.
9. Asselah T, Bieche I, Paradis V, et al. Genetics, genomics, and proteomics: implications for the diagnosis and the treatment of chronic hepatitis C. Semin Liver Dis 2007;27:13–27.
10. Massard J, Ratziu V, Thabut D, et al. Natural history and predictors of disease severity in chronic hepatitis C. J Hepatol 2006;44:S19–S24.
11. Thio CL. Host genetic factors and antiviral immune responses to hepatitis C virus. Clin Liver Dis 2008;12:713–726, xi.
12. Yee LJ. Host genetic determinants in hepatitis C virus infection. Genes Immun 2004;5:237–245.
13. Kenny-Walsh E. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. Irish Hepatology Research Group. N Engl J Med 1999;340:1228–1233.
14. Muller R. The natural history of hepatitis C: clinical experiences. J Hepatol 1996;24:52–54.
15. Kau A, Vermehren J, Sarrazin C. Treatment predictors of a sustained virologic response in hepatitis B and C. J Hepatol 2008;49:634–651.
16. Rodriguez-Torres M, Jeffers LJ, Sheikh MY, et al. Peginterferon alfa-2a and ribavirin in Latino and non-Latino whites with hepatitis C. N Engl J Med 2009;360:257–267.
17. Liu CH, Liu CJ, Lin CL, et al. Pegylated interferon-alpha-2a plus ribavirin for treatment-naive Asian patients with hepatitis C virus genotype 1 infection: a multicenter, randomized controlled trial. Clin Infect Dis 2008;47:1260–1269.
18. Muir AJ, Bornstein JD, Killenberg PG. Peginterferon alfa-2b and ribavirin for the treatment of chronic hepatitis C in blacks and non-Hispanic whites. N Engl J Med 2004;350:2265–2271.
19. Thursz M, Yallop R, Goldin R, et al. Influence of MHC class II genotype on outcome of infection with hepatitis C virus. The HENCORE group. Hepatitis C European Network for Cooperative Research. Lancet 1999;354:2119–2124.
20. Khakoo SI, Thio CL, Martin MP, et al. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. Science 2004;305:872–874.
21. Knapp S, Hennig BJ, Frodsham AJ, et al. Interleukin-10 promoter polymorphisms and the outcome of hepatitis C virus infection. Immunogenetics 2003;55:362–369.
22. Knapp S, Yee LJ, Frodsham AJ, et al. Polymorphisms in interferon-induced genes and the outcome of hepatitis C virus infection: roles of MxA, OAS-1 and PKR. Genes Immun 2003;4:411–419.
23. Wietzke-Braun P, Maouzi AB, Manhardt LB, et al. Interferon regulatory factor-1 promoter polymorphism and the outcome of hepatitis C virus infection. Eur J Gastroenterol Hepatol 2006;18:991–997.
24. Yee LJ, Perez KA, Tang J, et al. Association of CTLA4 polymorphisms with sustained response to interferon and ribavirin therapy for chronic hepatitis C virus infection. J Infect Dis 2003;187:1264–1271.
25. Goulding C, McManus R, Murphy A, et al. The CCR5-delta32 mutation: impact on disease outcome in individuals with hepatitis C infection from a single source. Gut 2005;54:1157–1161.
26. Thomas DL, Thio CL, Martin MP, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 2009;461:798–801.
27. Ge D, Fellay J, Thompson AJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399–401.
28. Suppiah V, Moldovan M, Ahlenstiel G, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 2009;41:1100–1104.
29. Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105–1109.
30. Ledergerber B, Egger M, Opravil M, et al. Clinical progression and virological failure on highly active antiretroviral therapy in HIV-1 patients: a prospective cohort study. Swiss HIV Cohort Study. Lancet 1999;353:863–868.
31. Prasad L, Spicher VM, Zwahlen M, et al. Cohort profile: the Swiss Hepatitis C Cohort Study (SCCS). Int J Epidemiol 2007;36:731–737.
32. Hoofnagle JH. Course and outcome of hepatitis C. Hepatology 2002;36:S21–S29.
33. Li Y, Abecasis GR. Mach 1.0: rapid haplotype reconstruction and missing genotype inference. Am J Human Genet 2009:2290–2295.
34. Price AL, Patterson NJ, Plenge RM, et al. Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet 2006;38:904–909.

35. Novembre J, Stephens M. Interpreting principal component analyses of spatial population genetic variation. Nat Genet 2008;40: 646–649.
36. Lao O, Lu TT, Nothnagel M, et al. Correlation between genetic and geographic structure in Europe. Curr Biol 2008;18;1241–1248.
37. Degli-Esposti MA, Andreas A, Christiansen FT, et al. An approach to the localization of the susceptibility genes for generalized myasthenia gravis by mapping recombinant ancestral haplotypes. Immunogenetics 1992;35:355–364.
38. Sheppard P, Kindsvogel W, Xu W, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 2003;4:63–68.
39. Li M, Liu X, Zhou Y, et al. Interferon-lambdas: the modulators of antivirus, antitumor, and immune responses. J Leukoc Biol 2009;86:23–32.
40. Kotenko SV, Gallagher G, Baurin VV, et al. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol 2003;4:69–77.
41. Melchjorsen J, Siren J, Julkunen I, et al. Induction of cytokine expression by herpes simplex virus in human monocyte-derived macrophages and dendritic cells is dependent on virus replication and is counteracted by ICP27 targeting NF-kappaB and IRF-3. J Gen Virol 2006;87:1099–1108.
42. Ank N, West H, Bartholdy C, et al. Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. J Virol 2006;80:4501–4509.
43. Brand S, Beigel F, Olszak T, et al. IL-28A and IL-29 mediate antiproliferative and antiviral signals in intestinal epithelial cells and murine CMV infection increases colonic IL-28A expression. Am J Physiol Gastrointest Liver Physiol 2005;289:G960–G968.
44. Hou W, Wang X, Ye L, et al. Lambda interferon inhibits human immunodeficiency virus type 1 infection of macrophages. J Virol 2009;83:3834–3842.
45. Hong SH, Cho O, Kim K, et al. Effect of interferon-lambda on replication of hepatitis B virus in human hepatoma cells. Virus Res 2007;126:245–249.
46. Robek MD, Boyd BS, Chisari FV. Lambda interferon inhibits hepatitis B and C virus replication. J Virol 2005;79:3851–3854.
47. Zhu H, Butera M, Nelson DR, et al. Novel type I interferon IL-28A suppresses hepatitis viral RNA replication. Virol J 2005;2:80.
48. Marcello T, Grakoui A, Barba-Spaeth G, et al. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. Gastroenterology 2006;131:1887–1898.
49. Dodds MG, Hausman DF, Miller DM. Viral kinetic modeling during treatment with interferon lambda-1a in genotype 1 chronic hepatitis C patients. European Association for the Study of the Liver (EASL), 44th Annual Meeting, Copenhagen, Denmark. 1–4, 2009.
50. Lawitz E, Zaman A, Muir AJ. Interim results from a phase 1b dose-escalation study of 4 weeks of PEG-interferon lambda (PEG-rIL-29) treatment in subjects with hepatitis C virus (HCV) genotype 1 with prior virologic response and relapse to peginterferon alfa and ribavirin. Hepatology 2008;48:A–170.
51. Shiffman M, Lawitz E, Zaman A. PEG-IFN-lambda: antiviral activity and safety profile in a 4-week phase 1b study in relapsed genotype 1 hepatitis C infection. European Association for the Study of the Liver (EASL), 44th Annual Meeting, Copenhagen, Denmark. 1–4, 2009.
52. Fellay J, Shianna KV, Ge D, et al. A whole-genome association study of major determinants for host control of HIV-1. Science 2007;317:944–947.
53. Sarrazin C, Kieffer TL, Bartels D, et al. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. Gastroenterology 2007;132:1767–1777.
54. Firmann M, Mayor V, Vidal PM, et al. The CoLaus study: a population-based study to investigate the epidemiology and genetic determinants of cardiovascular risk factors and metabolic syndrome. BMC Cardiovasc Disord 2008;8:6.

Received October 8, 2009. Accepted December 29, 2009.

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Acknowledgments

The members of the Swiss Hepatitis C and HIV Cohort Studies are listed in the supplementary material.

A.R. and Z.K. contributed equally to this study, as did S.B., F.N., A.T., and P.-Y.B.

The authors thank Martin Rickenbach; Sandrine Estoppey for data management; Isabelle Durussel for genotyping; Anne–Laure Chanson, Arnold Probst, Ivana Rubino, and Thierry Roger for logistic assistance; Millan Ortiz for haplotype analysis; Thierry Calandra for support in all steps of the study and critical review of the manuscript; David Goldstein and Jacques Fellay for the contribution of genome-wide data; and Vincent Moser and Peter Vollenweider from the CoLaus study for providing the population frequency of *rs8099917*.

Conflicts of interest

The authors disclose no conflicts.

Funding

The Swiss Hepatitis C and HIV Cohort Studies are supported by the Swiss National Science Foundation (3347C0-108782/1, grant nos. 3347-069366, 3247-116862, and 3100A0-116323/1, and Swiss HIV Cohort Study grant 543), the Swiss Federal Office for Education and Sciences (03.0599), and the European Commission (LSHM-CT-2004-503359; VIRGIL Network of Excellence on Antiviral Drug Resistance). Genotyping in the Swiss Hepatitis C Cohort Study and statistical analyses were supported by the Leenaards Foundation and Debiopharm S.A. Genotyping in the Swiss HIV Cohort Study was supported by an unrestricted grant by Essex Chemie AG, the Novaris Research Foundation, and Infectigen, Switzerland. P.-Y.B. is supported by the Swiss National Science Foundation (32003B_127613/1), and the Leenaards Foundation.

4.2.2. Original Article

Estimating the Net Contribution of *IL28B* Variation to Spontaneous Hepatitis C Virus Clearance

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Study concept and design: A. Rauch, A. Telenti, **J. di Iulio**

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Study supervision: A. Rauch, A. Telenti

Drafting of the manuscript: **J. di Iulio**, A. Rauch,

Critical revision of the manuscript for important intellectual content: all authors

Detailed personal contribution:

I was the main person in charge of the project. I notably participated in the study concept and design, sample recruitment, data analyses and interpretation, drafting of the manuscript, as well as in the genetic characterization of individuals included in the study by performing:

1. *IL28B* candidate causal variant genotyping optimization

2. Multiple source cohort (N=389) genetic characterization, including :
    - Genotyping of the 4 candidate causal variants (rs4803219, rs28416813, rs8103142 & rs4803217)
    - Genotyping of the tag SNP rs12979860
    - Copy number variation assessment

3. Single source cohort (N=71) genetic characterization, including :
    - Genotyping of the 4 candidate causal variants (rs4803219, rs28416813, rs8103142 & rs4803217)
    - Genotyping of the tag SNP rs12979860
    - Genotyping of the tag SNP rs8099917

Comments on the article:

In our previous work, we identified an intergenic single nucleotide polymorphism (SNP) in the interferon-λ3 (*IL28B*) locus, rs8099917, significantly associated with HCV treatment and infection outcome. In the attempt of determining the causal allele, tagged by rs8099917, responsible for the natural and treatment induced control of HCV infection, we identified 4 SNPs in *IL28B* as candidates for being causal.

In this study, we optimized the method for genotyping these 4 candidate SNPs for further large scale studies. We then investigated the impact of these variants on HCV infection outcome in HIV/HCV co-infected individuals from the Swiss HIV Cohort Study. Finally, in order to determine the net contribution of *IL28B* variation, we analyzed these variants in pregnant women infected by the same HCV source, through exposition to contaminated immunoglobulin anti-D. This single source study population allowed for the control of co-factors such as HCV viral diversity, gender and co-infection (none were HBV or HIV co-infected).

Haplotypes carrying a SNP resulting in a non-synonymous amino acid change (K70R) could increase the predictive value of HCV infection outcome, in comparison to the tag SNP rs8099917, previously identified in our GWAS. However, these haplotypes were highly linked to rs12979860, a tagSNP identified by other groups [71, 72], and had nearly the same predictive value, making the assignment of the true causal variant difficult.

Estimating the Net Contribution of Interleukin-28B Variation to Spontaneous Hepatitis C Virus Clearance

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The identification of associations between interleukin-28B (*IL-28B*) variants and the spontaneous clearance of hepatitis C virus (HCV) raises the issues of causality and the net contribution of host genetics to the trait. To estimate more precisely the net effect of *IL-28B* genetic variation on HCV clearance, we optimized genotyping and compared the host contributions in multiple- and single-source cohorts to control for viral and demographic effects. The analysis included individuals with chronic or spontaneously cleared HCV infections from a multiple-source cohort (n = 389) and a single-source cohort (n = 71). We performed detailed genotyping in the coding region of *IL-28B* and searched for copy number variations to identify the genetic variant or haplotype carrying the strongest association with viral clearance. This analysis was used to compare the effects of *IL-28B* variation in the two cohorts. Haplotypes characterized by carriage of the major alleles at *IL-28B* single-nucleotide polymorphisms (SNPs) were highly overrepresented in individuals with spontaneous clearance versus those with chronic HCV infections (66.1% versus 38.6%, $P = 6 \times 10^{-9}$). The odds ratios for clearance were 2.1 [95% confidence interval (CI) = 1.6-3.0] and 3.9 (95% CI = 1.5-10.2) in the multiple- and single-source cohorts, respectively. Protective haplotypes were in perfect linkage ($r^2 = 1.0$) with a nonsynonymous coding variant (rs8103142). Copy number variants were not detected. *Conclusion:* We identified *IL-28B* haplotypes highly predictive of spontaneous HCV clearance. The high linkage disequilibrium between *IL-28B* SNPs indicates that association studies need to be complemented by functional experiments to identify single causal variants. The point estimate for the genetic effect was higher in the single-source cohort, which was used to effectively control for viral diversity, sex, and coinfections and, therefore, offered a precise estimate of the net host genetic contribution. (HEPATOLOGY 2011;000:000-000.)

Hepatitis C virus (HCV) infections resolve spontaneously in 30% to 50% of cases.[1] Sex, ethnicity, jaundice, and coinfections with human immunodeficiency virus (HIV) and hepatitis B virus affect spontaneous HCV clearance.[2] Recently, genetic variation in the interleukin-28B gene (*IL-28B*) was shown to strongly predict viral clearance,[3-5] with similar effects found in HCV-monoinfected and HIV/HCV-coinfected individuals.[3,4] The two strongest genetic predictors for spontaneous HCV clearance, the rs12979860 C allele and the rs8099917 T allele, are located 3 and 8 kb upstream of the *IL-28B* gene, respectively.[3] However, at this stage, it is uncertain whether these polymorphisms play a causal role or are merely tagging one or more unknown causal variants. To identify the functional variant tagged by the

Abbreviations:: CI, confidence interval; CNV, copy number variation; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IL, interleukin; PCR, polymerase chain reaction; SNP, single-nucleotide polymorphism.

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Received December 17, 2010; accepted February 16, 2011.

associated single-nucleotide polymorphism (SNP), we performed comprehensive genetic mapping of the region to identify the most plausible causal SNP for spontaneous HCV clearance.

HCV-specific immune responses influence clinical outcomes and strongly depend on the viral genotype.[2,6] Ideally, the identification of the optimal host genetic marker for spontaneous HCV clearance would control for this viral variable. However, because the genotype of the infecting virus is unknown in most infected individuals once they have cleared the virus, an assessment of the relative contribution of host genetics versus viral genetics to HCV clearance is rarely possible. Single-source outbreaks provide a unique opportunity for studying the net host genetic effects independently of the viral genotype and diversity. Therefore, we assessed the influence of *IL-28B* variants on spontaneous HCV clearance in a cohort of women infected by an identical HCV genotype 1b strain through contaminated anti-D immunoglobulins, and we compared the genetic effects in this single-source source and a multiple-source cohort.

The aim of this study was to improve the prediction of spontaneous HCV clearance through (1) the optimization of *IL-28B* genotyping and (2) the estimation of the net host genetic effects with a single-source outbreak.

Patients and Methods

Study Population. The multiple-source cohort included 389 randomly selected HIV/HCV-coinfected individuals (200 with spontaneous HCV clearance and 189 with chronic hepatitis C). The study participants were recruited through the Swiss HIV Cohort Study. The cohort included HIV-infected individuals coinfected with diverse HCV genotypes and was heterogeneous with respect to sex, viral coinfections, and age (Supporting Table 1).

Individuals from the single-source outbreak were part of a cohort of 704 women infected with HCV genotype 1b through contaminated anti-D immunoglobulins as described elsewhere.[7] Infections occurred between May 1977 and November 1978 in Ireland. From this cohort, we included those currently attending routine clinical follow-up; 27 experienced spontaneous HCV clearance, and 44 had chronic hepatitis C. All study participants gave informed consent, which included genetic testing.

Spontaneous HCV clearance was defined as HCV seropositivity (determined with an enzyme-linked immunosorbent assay and confirmed by immunoblotting) and negative HCV RNA findings by quantitative or qualitative assays more than 12 months after HCV seropositivity. *Chronic hepatitis C* was defined as HCV seropositivity and detectable HCV RNA more than 12 months after HCV seropositivity. Demographic characteristics are shown in Supporting Table 1.

Genotyping and Copy Number Variation (CNV) Determination. Because our previous genome-wide association study clearly indicated that the association signal maps to *IL-28B*, we further explored this gene in more detail. On the basis of our previous work on

The Swiss HIV Cohort Study is supported by the Swiss National Science Foundation (grants 3345CO-100935 and 324700-112655 and Swiss HIV Cohort Study grant 543) and by the Swiss HIV Cohort Study Research Foundation. Genotyping in the Swiss HIV Cohort Study is supported by an unrestricted grant from the Novartis Research Foundation. Paul Klenerman and Karen Fitzmaurice are supported by the Wellcome Trust, the James Martin 21st Century School (Oxford, United Kingdom), and the National Institute for Health Research Biomedical Research Centre (Oxford, United Kingdom). The supporters had no role in the study design, collection, analysis, or interpretation of data.

Julia di Iulio, Amalio Telenti, and Andri Rauch designed the study, established phenotypes, contributed to statistical analyses, and wrote the article. Julia di Iulio, Margalida Rotger, and Angela Ciuffi performed the genotyping, which was supervised by Amalio Telenti. Jacques Fellay and Paul Klenerman contributed to the study design and writing. All other authors contributed to the data and sample collection at the different study centers, and all authors provided a critical review of the article.

The members of the Swiss HIV Cohort Study are M. Battegay, E. Bernasconi, J. Böni, H. C. Bucher, P. Bürgisser, A. Calmy, S. Cattacin, M. Cavassini, R. Dubs, M. Egger, L. Elzi, M. Fischer, M. Flepp, A. Fontana, P. Francioli (president of the Swiss HIV Cohort Study, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland), H. Furrer (chairman of the clinical and laboratory committee), C. Fux, M. Gorgievski, H. Günthard (chairman of the scientific board), H. Hirsch, B. Hirschel, I. Hösli, C. Kahlert, L. Kaiser, U. Karrer, C. Kind, T. Klimkait, B. Ledergerber, G. Martinetti, B. Martinez, N. Müller, D. Nadal, M. Opravil, F. Paccaud, G. Pantaleo, A. Rauch, S. Regenass, M. Rickenbach (head of the data center), C. Rudin (chairman of the mother and child substudy), P. Schmid, D. Schultze, J. Schüpbach, R. Speck, P. Taffé, A. Telenti, A. Trkola, P. Vernazza, R. Weber, and S. Yerly.

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View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.24263

Potential conflict of interest: Andri Rauch and Jacques Fellay are co-applicants for patents (currently being evaluated) on the original finding of the interleukin-28B association with spontaneous and treatment-induced hepatitis C virus clearance.

Additional Supporting Information may be found in the online version of this article.

the resequencing of the *IL-28B* locus,[3] the genotyping of four candidate causal SNPs (rs8403219, rs28416813, rs8103142, and rs4803217) was performed by TaqMan allelic discrimination (ABI-Prism 7000 SDS software, Applied Biosystems) with custom Assays-on-Demand products from Applied Biosystems; this was preceded by a pre-amplification step. Primers and probes are shown in Supporting Table 2. In addition, the tag SNP rs12979860 was assessed with a custom TaqMan assay designed by Ge et al.,[8] and the tag SNP rs8099917 was genotyped with an Assays-on-Demand product provided by Applied Biosystems (C__11710096_10). Haplotype inference was performed with Phase 2.1 software (University of Washington, Seattle, WA).

An analysis of CNVs (deletions, insertions, and inversions) was initiated through an inspection of the structural variation at the *IL-28B* locus and 1 Mb (1 million nucleotides) upstream and downstream of the gene in chromosome 19 (as captured in the 1000 Genomes Project; go to http://www.1000genomes.org for the March 2010 data release).[9] The analysis included calls from both pilot 1 [179 samples sequenced with low-pass shotgun (2-6×) coverage] and pilot 2 [6 individuals with high (>30×) coverage] of the 1000 Genomes Project. CNV calls from the 1000 Genomes Project are reported as validated, unvalidated, and invalidated on the basis of a number of algorithms, experimental data, and replication across various samples and populations.

The presence of CNVs was then experimentally assessed by quantitative real-time polymerase chain reaction (PCR); the primers and probes are shown in Supporting Table 2. The CNV assessment with real-time PCR included a clone containing one copy of *IL-28B* and the housekeeping gene hydroxymethylbilane synthase as a control (Supporting Table 2). Because the quantitative PCR approach allowed the detection of CNVs only in a limited region of the gene (set by the location of the chosen primers), we further assessed potential CNVs with primer combinations that would amplify a PCR product only in the presence of CNVs (Supporting Fig. 1). The targeted PCR across potential CNVs was internally controlled by the identification of the expected amplification fragment or larger/shorter fragments.

Statistics. Genetic associations were assessed by logistic regression. The analyses were adjusted for covariates significantly associated with spontaneous HCV clearance in univariate analyses. Haplotype analyses were performed with the haplologit command in the Stata software package (version 10.0). This analysis included haplotypes exceeding a frequency of 2/N (the default threshold). The estimates of haplotype effects were adjusted for significant covariates in logistic regression models.

Results

Genotyping Candidate Causal Variants. Our previous genome-wide association study identified rs8099917 as the strongest predictor of spontaneous HCV clearance.[3] This variant is located within a genomic block encompassing the *IL-28B* gene and is, therefore, susceptible to reflecting the signal resulting from a functional variant in *IL-28B*. To identify the potentially causal variant tagged by rs8099917, *IL-28B* had been previously resequenced in 41 HIV/HCV-coinfected individuals with the four extreme genotype/phenotype combinations: chronic or spontaneously cleared hepatitis C and homozygosity for the protective or risk alleles.[3] Candidate causal SNP selection was based on location, frequency among the main haplotype families, and linkage disequilibrium.[3] This approach led to the prioritization of four SNPs as prime candidates for causality (Fig. 1A). These four SNPs were genotyped along with the two tagging SNPs that were most strongly associated with spontaneous HCV clearance in previous studies (rs8099917 and rs129879860[3,4]). Twelve distinct haplotypes could then be derived from the six genotyped SNPs, as shown in Fig. 1A. The haplotypes were further categorized according to the classification used in our previous study into two main families (Fig. 1B). Type I haplotypes were characterized by the presence of the common allele for all candidate SNPs (Fig. 1B).

Genotyping Assay Optimization. Because of the high homology between the *IL-28B* and *IL-28A* sequences (96% amino acid identity[10]), we first performed a pre-amplification step in order to selectively genotype *IL-28B* polymorphisms in the TaqMan assays. The four candidate SNPs were then genotyped from the pre-amplified product. However, we observed discrepancies in up to 19.5% of cases for rs8103142 between the TaqMan results and the previous resequencing results.[3] Discrepancies were present for various pre-amplification primer combinations (marked with dashed lines in Fig. 2), with more frequent homozygosity found with the TaqMan assay; this suggested the preferential amplification of only one allele in some individuals. Further optimization of the pre-amplification step identified primer combinations that correctly identified all genotypes (marked with solid lines in Fig. 2). Primer combinations that included a

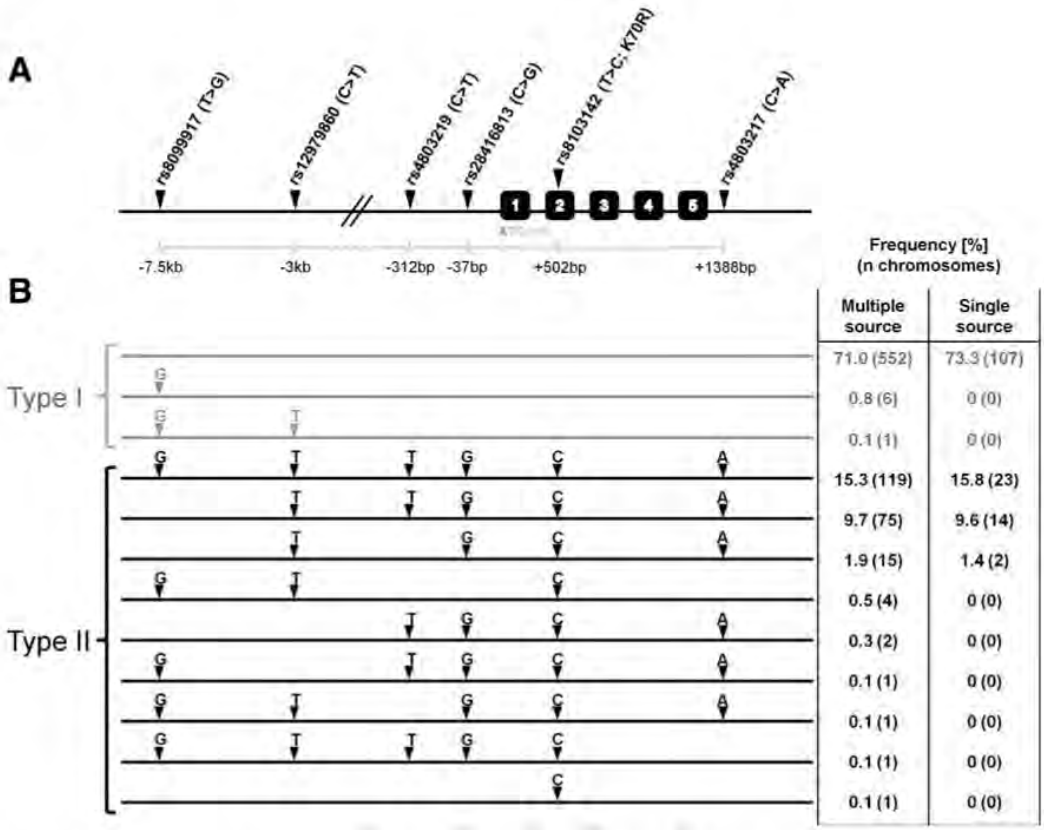


Fig. 1. *IL-28B* genotyping and haplotype inference. (A) Two tagging SNPs (rs8099917 and rs129879860) and four candidate causal SNPs in *IL-28B* were genotyped in all study participants. Boxes 1 to 5 denote exons. (B) Haplotype inference identified 12 distinct haplotypes divided into two main families (types I and II). Arrowheads indicate the presence of the minor allele.

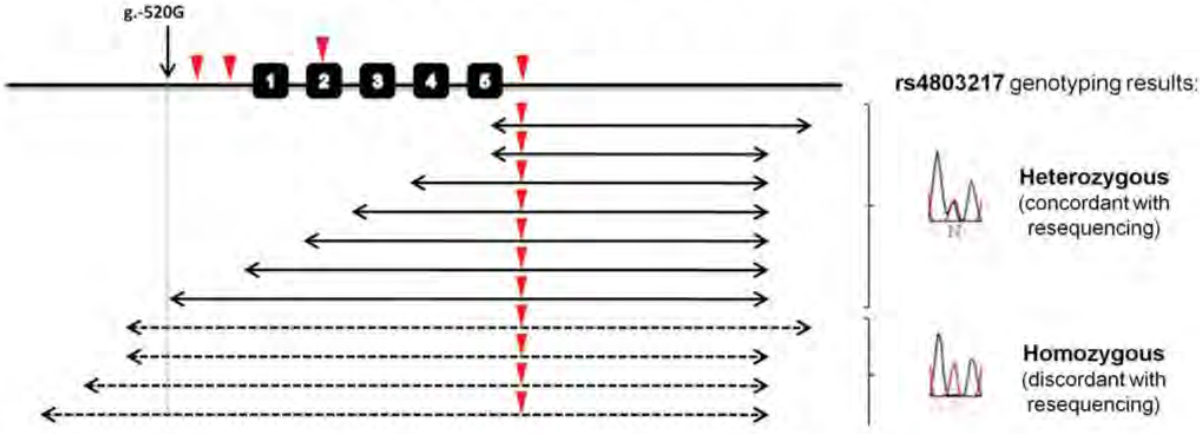


Fig. 2. Primer pair optimization for the pre-amplification step. Because discordant results were obtained by genotyping and resequencing, several primer combinations were tested for the pre-amplification step preceding the genotyping. The arrows show the different PCR products amplified for the pre-amplification step. Arrows with dashed lines indicate discordant results for a given SNP (heterozygous by resequencing and homozygous by the TaqMan assay), whereas arrows with solid lines indicate concordant results for a given SNP (heterozygous by both resequencing and TaqMan assay). A primer combination containing a forward primer located upstream of position g.-520G yielded discordant results in up to 19.5% of the individuals (for rs8103142). The red triangles show the location of the four genotyped SNPs.

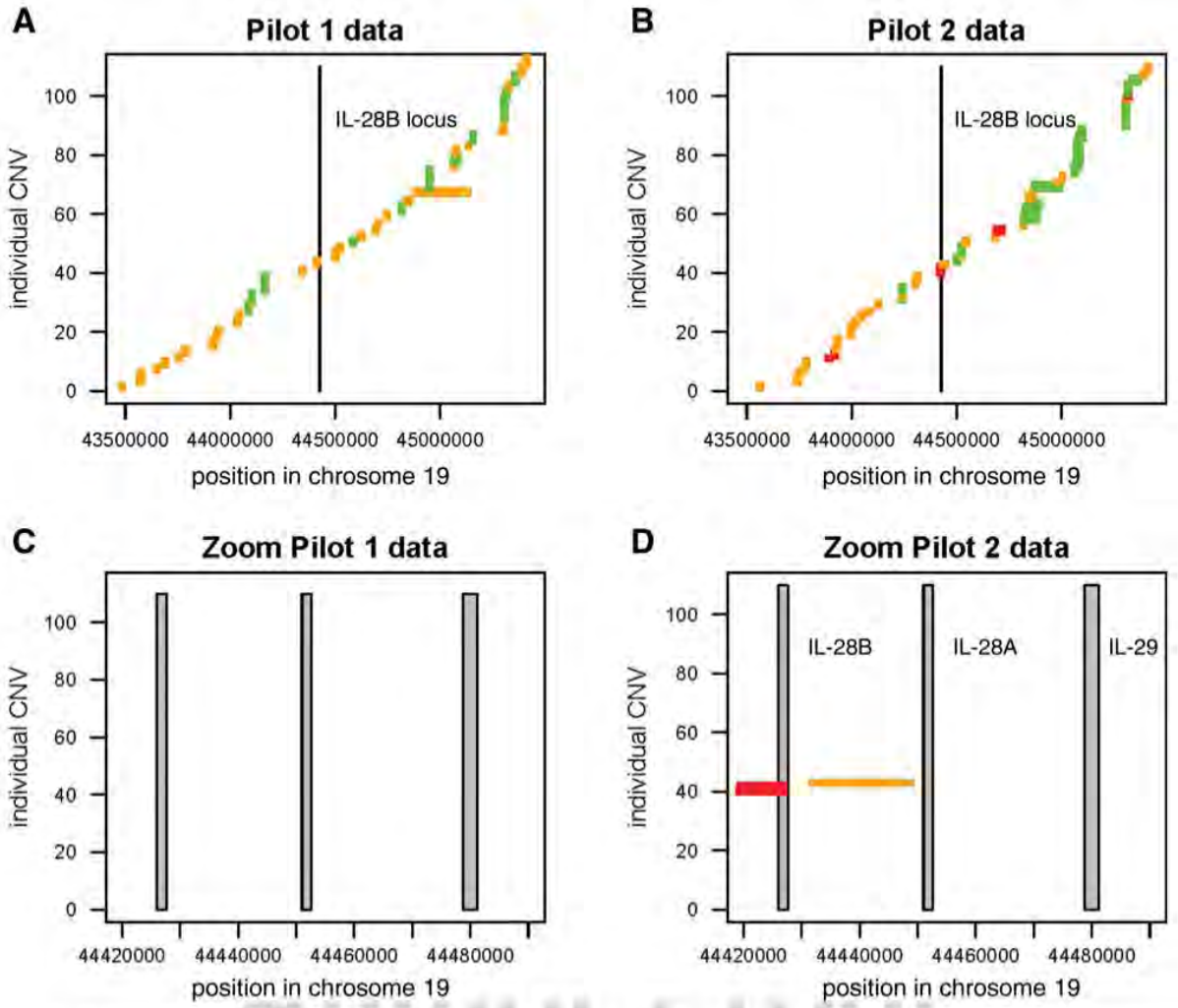


Fig. 3. CNVs at the *IL-28B* locus. (A,B) CNV data from the 1000 Genomes Project (pilots 1 and 2) for 1 Mb upstream and downstream of the *IL-28B* locus (black, vertical lines) in chromosome 19. The *y* axis and the horizontal, color lines represent individual observations of possible CNVs (orange, not yet confirmed/unvalidated; red, refuted/invalidated; green, confirmed/validated). (C,D) Close-ups of the locus. The gray, vertical lines indicate the chromosomal locations of *IL-28B*, *IL-28A*, and *IL-29*.

forward primer downstream of position g.-520G gave concordant results. Primers and probes that were used in the optimized genotyping assay are listed in Supporting Table 2.

CNVs. To further characterize the genetic region, we assessed whether CNVs (deletions, insertions, and inversions) exist in the *IL-28B* region. An analysis of pilot 1 data from the 1000 Genomes Project identified 113 possible instances of CNVs in the window of 2 Mb around the *IL-28B* locus (Fig. 3A). These correspond to 107 deletions and 6 insertions; 43 of these have been validated, and validation is pending for 70. An analysis of the pilot 2 data identified 110 possible instances of CNVs; these correspond to 106 deletions, 3 duplications, and 1 inversion, and 48 have been validated. Validation is pending for 53, and 9 have been invalidated (Fig. 3B). However, none of the reported CNVs directly involve *IL-28B*, with the exception of an inversion (yet to be validated) reported in one individual in the *IL-28B–IL-28A* intergenic region (Fig. 3C,D).

We could not identify any CNV with quantitative real-time PCR using primers amplifying a region of 121 bp that spanned exon 5 and the 3′-untranslated region (data not shown). The customized PCR described in Supporting Fig. 1A revealed potential gene duplications; however, these were entirely explained by artifactual formation of gene copies due to PCR-induced recombination of repetitive regions in the *IL-28B* region (Supporting Fig. 1B).[11]

Table 1. Association Between the *IL-28B* Genotype and HCV Clearance (Multiple-Source Cohort)

	SNP	Minor Allele Frequency		Odds Ratio for Spontaneous Clearance (Noncarrier Versus Carrier)	P Value*
		HCV Clearance	HCV Chronicity		
Tagging SNPs	rs8099917	0.12	0.23	2.5 (1.6-3.9)	9×10^{-5}
	rs12979860	0.20	0.36	3.0 (1.9-4.5)	5×10^{-7}
Candidate causal SNPs	rs4803219	0.19	0.32	2.6 (1.7-3.8)	1×10^{-5}
	rs28416813	0.20	0.35	3.0 (1.9-4.5)	5×10^{-7}
	rs8103142	0.21	0.36	3.0 (2.0-4.6)	2×10^{-7}
	rs4803217	0.20	0.35	3.0 (2.0-4.6)	3×10^{-7}

*P values were adjusted for sex and coinfection with hepatitis B.

Predictive Value of Candidate SNPs for Spontaneous HCV Clearance in the Multiple-Source Cohort. Using the optimized pre-amplification step, we genotyped the four candidate causal variants in the entire cohort. All SNPs were highly associated with spontaneous HCV clearance and had a stronger effect in comparison with the tagging SNP rs8099917 identified in our previous genome-wide association study[3] (Table 1). Using nested regression models, we observed that rs8099917 was not significantly associated after an adjustment for candidate SNPs ($P > 0.1$ for all comparisons), whereas the candidate causal variants remained significantly associated with spontaneous HCV clearance after we accounted for rs8099917 ($P < 0.001$ for all comparisons). The linkage disequilibrium between rs8099917 and the candidate causal variants was moderate ($r^2 \sim 0.4$; Fig. 4), whereas the other tag SNP, rs12979860,[8] was highly linked to the candidate variants ($r^2 \geq 0.85$, Fig. 4).

Predictive Value of Candidate SNPs for Spontaneous HCV Clearance in the Single-Source Cohort. We next investigated the net host genetic contribution to spontaneous HCV clearance in the single-source cohort. Again, all genotyped SNPs were significantly associated with spontaneous HCV clearance. The candidate causal SNPs were more strongly associated than the tagging SNP rs8099917 and were in perfect linkage disequilibrium with rs129879860, except for rs4803219 ($r^2 = 0.93$; Table 2).

IL-28B *Haplotypes and Spontaneous HCV Clearance.* We next examined the association of *IL-28B* haplotypes with spontaneous HCV clearance. In both cohorts, haplotypes characterized by carriage of the major alleles at *IL-28B* candidate SNPs had the strongest association with clearance (Table 3). These type I haplotypes (Fig. 1B) were clearly enriched in individuals with spontaneous HCV clearance, and there was a greater association in the single-source cohort (Fig. 5). Homozygosity for type I haplotypes was highly linked to the rs12979860 CC genotype ($r^2 = 0.97$).

Discussion

Genetic variation in *IL-28B* is the strongest known genetic predictor of natural and treatment-induced clearance of HCV infection. Although there is overwhelming evidence for the association of SNPs with HCV infection outcomes, the causal genetic variant has not been identified yet. Using a comprehensive and optimized genetic analysis of *IL-28B*, we evaluated SNPs in the coding, promoter, and 3′-untranslated regions of the gene that could represent causal variants. Haplotypes characterized by carriage of the major alleles at the *IL-28B* candidate SNPs (type I haplotypes) were highly overrepresented in individuals with

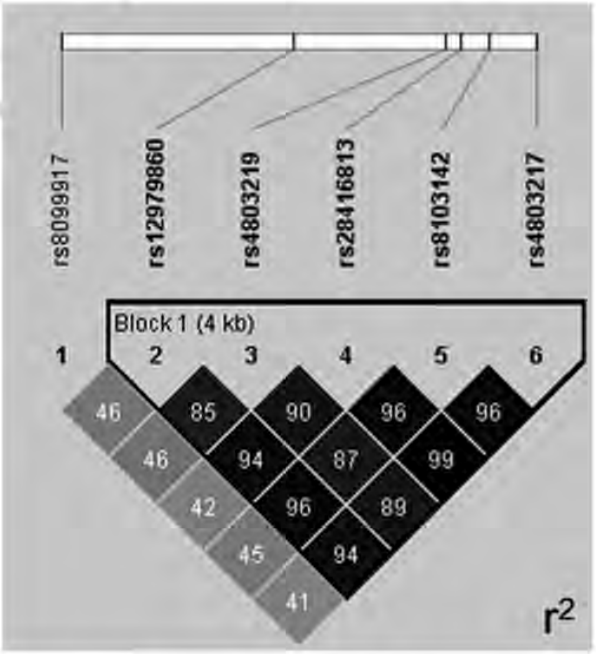


Fig. 4. Linkage disequilibrium plot for the multiple-source cohort. The linkage disequilibrium between the four candidate causal SNPs (rs4803219, rs28416813, rs8103142, and rs4803217) and the two previously identified tagging SNPs is shown.

Table 2. Association Between the *IL-28B* Genotype and HCV Clearance (Single-Source Cohort)

	SNP	Minor Allele Frequency		Odds Ratio for Spontaneous Clearance (Noncarrier Versus Carrier)	*P* Value*
		HCV Clearance	HCV Chronicity		
Tagging SNPs	rs8099917	0.08	0.20	3.3 (1.0-11.2)	0.05
	rs12979860†	0.12	0.34	4.1 (1.4-11.8)	0.008
Candidate causal SNPs	rs4803219	0.11	0.33	4.6 (1.6-13.6)	0.005
	rs28416813†	0.13	0.34	4.1 (1.4-11.8)	0.008
	rs8103142†	0.13	0.34	4.1 (1.4-11.8)	0.008
	rs4803217†	0.13	0.34	4.1 (1.4-11.8)	0.008

*P values were adjusted for sex and coinfection with hepatitis B.
†In perfect linkage disequilibrium in this cohort.

spontaneous HCV clearance versus those with chronic HCV infections. Homozygosity for the rs12979860 C allele (CC genotype) tagged ($r^2 = 0.97$) homozygosity for the protective type I haplotypes. Protective type I haplotypes were in perfect linkage ($r^2 = 1.0$) with a nonsynonymous coding variant (rs8103142) that has also been highly associated with treatment response.[8,12] Although rs8099917 was only moderately linked to the SNPs in the *IL-28B* gene, there was a high linkage of rs12979860 with the four candidate causal SNPs in both cohorts. The candidate causal SNPs and haplotypes were, therefore, better tagged by rs12979860 than rs8099917. Furthermore, rs12979860 and the candidate causal SNPs remained significantly associated with HCV clearance after we accounted for rs8099917. In contrast, rs8099917 was not significantly associated after an adjustment for candidate causal SNPs. Therefore, we conclude that candidate causal SNPs and rs12979860 are better predictors of spontaneous HCV clearance than rs8099917. The candidate SNPs are in very high linkage disequilibrium (Fig. 4). This indicates that association studies are not sufficient to identify the causal SNPs, at least in Caucasians. Functional studies are needed to define which variants ultimately influence the control of HCV infection.

Even if protective *IL-28B* genotypes were clearly enriched in individuals with spontaneous HCV clearance in both cohorts, the point estimate of the odds ratio was approximately 2-fold higher in the single-source cohort. Women homozygous for the protective type I haplotypes were approximately 4 times more likely to spontaneously clear their HCV infection in comparison with women carrying at least one type II haplotype. Because of the inherent control of clinical, demographic, and viral factors in the single-source cohort, this point estimate represents an optimal assessment of the net host genetic contribution. This finding is in line with a very recent report by Tillmann and colleagues,[13] who found a strong association between the rs12979860 CC genotype and spontaneous HCV clearance in a German single-source cohort. In the multiple-source cohort, the effects of *IL-28B* haplotypes on spontaneous HCV clearance were similar in men [odds ratio = 2.3, 95% confidence interval (CI) = 1.5-4.2] and women (odds ratio = 2.3, 95% CI = 1.5-3.6). Furthermore, *IL-28B* has very similar effects on spontaneous HCV clearance in HIV/HCV-coinfected and HCV-monoinfected individuals.[3-5] Although the inherent heterogeneity between the two cohorts makes it difficult to compare predictors of spontaneous HCV clearance, it is unlikely that the larger effect in the single-source cohort can be explained entirely by the different distributions of sex and coinfection with HIV. The influence of viral genotypes on spontaneous HCV clearance rates is poorly understood. This is mainly due to the paucity of acute hepatitis C cohorts in which the viral genotype can be

Table 3. Association Between the *IL-28B* Haplotypes and HCV Clearance

IL-28B SNP in the Promoter/Coding Region and 3′-Untranslated Region*				Multiple-Source Cohort		Single-Source Cohort	
rs4803219 (C>T)	rs28416813 (C>G)	rs8103142 (T>C)	rs4803217 (C>A)	Odds Ratio (95% CI)†	*P* Value	Odds Ratio (95% CI)†	*P* Value
0	0	0	0	2.1 (1.6-3.0)	2×10^{-6}	3.9 (1.5-10.2)	0.005
0	0	1	0	1.4 (0.2-8.6)	0.7	−‡	−‡
0	1	1	1	0.3 (0.1-0.9)	0.04	−‡	−‡
1	1	1	1	0.5 (0.3-0.7)	2×10^{-5}	0.3 (0.09-0.7)	0.006

*0 = major allele; 1 = minor allele.
†Odds ratios were adjusted for *IL-28B* haplotypes, sex, and coinfection with hepatitis B.
‡The numbers were too small for odds ratio calculations.

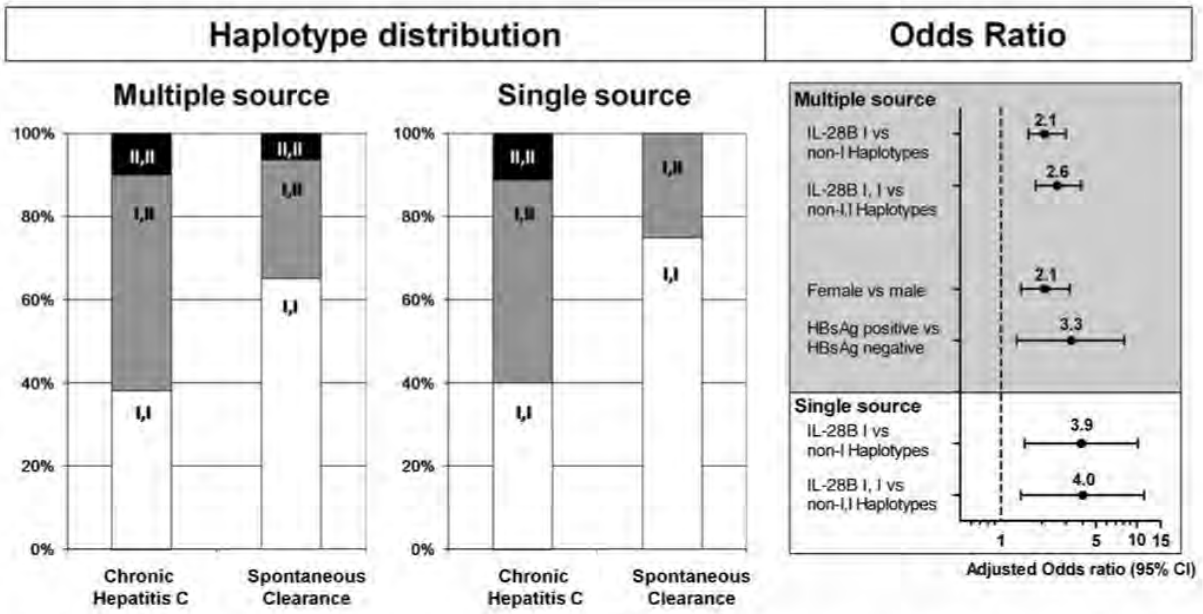


Fig. 5. *IL-28B* haplotypes and spontaneous HCV clearance in the multiple- and single-source cohorts. The odds ratios were adjusted for *IL-28B* haplotypes, sex, and coinfection with hepatitis B. Abbreviation: HBsAg, hepatitis B surface antigen.

assessed in individuals who spontaneously clear their infections. The limited overlap of T cell responses between HCV genotypes[6] suggests that the viral genotype influences the host immune response to HCV. It is, therefore, not surprising that the effect of *IL-28B* variants was larger in the single-source cohort, which was used to control for viral effects. Although the difference in the odds ratios between the single- and multiple-source cohorts was not statistically significant with overlapping CIs, it is interesting to note that the highest odds ratios for spontaneous HCV clearance were observed in the Irish and German single-source cohorts. This underscores the advantages of single-source cohorts for genetic association studies of infectious diseases because these cohorts allow the net host genetic determinants to be assessed independently of pathogen-associated variability.

Our study also identified substantial technical complexity in the genetic analysis of the *IL-28B* locus. First, the high homology between *IL-28A* and *IL-28B* requires pre-amplification of *IL-28B* before the genotyping of SNPs by TaqMan in order to avoid unspecific amplification of *IL-28A*, which can result in erroneous genotypes. Second, the pre-amplification step needs optimized primer combinations; upstream of position g.-520G, the forward primer does not reliably amplify both alleles, and this can result in erroneous genotype distributions for SNPs in the promoter, coding, and 3′-untranslated regions of *IL-28B*. Tagging SNPs can be genotyped reliably without a pre-amplification step because these are not located in a region with high homology with *IL-28A*. Therefore, our findings do not alter the conclusions of previous reports on the impact of the tagging SNPs rs12979860 and rs8099917 on the control of HCV infection. Third, repetitive homology regions can artifactually simulate CNVs. The study also proceeded to an analysis of CNVs, which were a consideration because of the close proximity of the *IL-28B* and *IL-28A* paralogs. The analysis of CNVs captured in the 1000 Genomes Project identified several validated CNVs near *IL-28B*; however, none of these encompassed the *IL-28B* gene. Furthermore, neither real-time nor targeted PCR identified CNVs. It is, therefore, unlikely that CNVs play a role in the strong influence of *IL-28B* variation on HCV infection outcomes. Completion of the 1000 Genomes Project, which will yield many more samples sequenced with high coverage, may lead to a better understanding of the genetic structure around the *IL-28B* locus.

Our study has confirmed and extended previous observations. We have confirmed the high linkage of rs12979860 to candidate causal *IL-28B* SNPs observed in the Individualized Dosing Efficacy Versus Flat Dosing to Assess Optimal Pegylated Interferon Therapy cohort,[8] and we have additionally described the influence of these variants on spontaneous HCV clearance. Furthermore, we have demonstrated that the

rs12979860 CC genotype tags homozygosity for protective type 1 haplotypes that are characterized by carriage of major alleles of *IL-28B* candidate causal SNPs. We have also described the substantial complexity of the genetic analysis of the *IL-28B* locus, and we have presented a new approach to identifying samples with particular *IL-28B* genotypes for functional studies. Our study has also confirmed the strong effect of *IL-28B* variation in the German single-source cohort,[13] has extended the genetic assessment, and has provided comparative estimates of multiple- and single-source cohorts and thereby effectively defined the net host contribution to the phenotype. Finally, previous studies that assessed the potential role of CNVs in HCV infection outcomes[8] relied on the information provided by HapMap (see http://hapmap.ncbi.nlm.nih.gov). Because this approach provides relatively poor discrimination for detecting CNVs, we performed an extensive screening of the region with the 1000 Genomes Project data and real-time and targeted PCR. These studies and the current work jointly represent a definitive assessment of genetic variation at the *IL-28B* locus and indicate the limits of genetic approaches to the understanding of the mechanism of action of *IL-28B*.

In conclusion, SNPs located in the promoter, coding, and 3′-untranslated regions of *IL-28B* are highly associated with spontaneous HCV clearance. These SNPs are prime candidates for functional assessment. The method described here can be useful to identify samples with particular *IL-28B* genotypes for functional studies. We also have confirmed that for large-scale studies, rs12979860 genotyping is a reliable method for predicting the presence or absence of the putative causal haplotypes in *IL-28B* associated with spontaneous HCV clearance. The striking association of *IL-28B* genotypes with spontaneous HCV clearance in a single-source cohort underscores the role of host genetic variation in the control of HCV infection, which is independent of viral effects.

References

1. Thomas DL, Astemborski J, Rai RM, Anania FA, Schaeffer M, Galai N, et al. The natural history of hepatitis C virus infection: host, viral, and environmental factors. JAMA 2000;284:450-456.
2. Rauch A, Gaudieri S, Thio C, Bochud PY. Host genetic determinants of spontaneous hepatitis C clearance. Pharmacogenomics 2009;10:1819-1837.
3. Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, Mueller T, et al. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology 2010;138:1338-1345.
4. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 2009;461:798-801.
5. Grebely J, Petoumenos K, Hellard M, Matthews GV, Suppiah V, Applegate T, et al. Potential role for interleukin-28B genotype in treatment decision-making in recent hepatitis C virus infection. HEPATOLOGY 2010;52:1216-1224.
6. Rauch A, James I, Pfafferott K, Nolan D, Klenerman P, Cheng W, et al. Divergent adaptation of hepatitis C virus genotypes 1 and 3 to human leukocyte antigen-restricted immune pressure. HEPATOLOGY 2009;50:1017-1029.
7. Kenny-Walsh E. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. N Engl J Med 1999;340:1228-1233.
8. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399-401.
9. Durbin RM, Abecasis GR, Altshuler DL, Auton A, Brooks LD, Gibbs RA, et al. A map of human genome variation from population-scale sequencing. Nature 2010;467:1061-1073.
10. Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, et al. IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 2003;4:63-68.
11. Kanagawa T. Bias and artifacts in multitemplate polymerase chain reactions (PCR). J Biosci Bioeng 2003;96:317-323.
12. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 2009;41:1100-1104.
13. Tillmann HL, Thompson AJ, Patel K, Wiese M, Tenckhoff H, Nischalke HD, et al. A polymorphism near IL28B is associated with spontaneous clearance of acute hepatitis C virus and jaundice. Gastroenterology 2010;139:1586-1592.

5. DISCUSSION

5.1. Genetics

In this work, we have explored the influence of host genetics on drug disposition and disease susceptibility by single candidate gene, large scale candidate gene and genome wide association study approaches. All three approaches have pros and cons that should be carefully weighted before choosing the most adapted technique.

5.1.1. Single Candidate Gene Analysis

Single candidate gene analysis approach arose from the belief that phenotypic traits were controlled by a single gene [4]. Although this proves true for Mendelian diseases and some pharmacogenetic traits (typically showing a bimodal distribution), most diseases and phenotypic traits are believed to result from the interplay of several genes, moving the paradigm “one gene, one disease” to “complex disease”, where single variants are able to explain only 1% to 5% of the phenotypic variation. Although single candidate gene approaches may be reductionistic and result in non-replication of most studies [4, 73], it can be a cost effective and efficient tool in the settings of extensive *a priori* knowledge of the involved proteins.

In this work, we used this approach to investigate the impact of *CYP2A6* genetic variants on EFV exposure. *CYP2A6* is not expected to have a clinically relevant impact on EFV metabolism in the general population as it is involved in a minor EFV metabolic pathway representing only 7% of EFV metabolism. However, we could point out the influence of *CYP2A6* genotype on EFV exposure among individuals that had limited CYP2B6 function, emphasizing that single candidate gene approach might be helpful in the setting of extensive *a priori* knowledge of the involved proteins.

5.1.2. Large Scale Candidate Gene Analysis

Large scale candidate gene analysis approach aims at providing some understanding of the interactions among several candidate genes and how these contribute to a particular trait [4]. This strategy involves a larger screen of potential associations, which is in line with the new paradigm consisting in linking a trait to a pathway or a biological system, rather than linking a phenotype to a single gene [74]. However, like single candidate gene analysis, it does not allow the identification of unexpected associations, as there is an *a priori* hypothesis of the genes possibly involved in the phenotypic trait. In addition, a pre-defined SNP array might not allow a complete coverage of all variants present in a gene. This is notably the case for copy number variants, polymorphisms present in genes that have highly homologous counter pairs (such as pseudogenes) [75], and rare/private mutations. Finally, as previously mentioned, the SNPs identified in these studies are not necessarily the causal variants responsible for the detected effect, but might rather tag them.

In this work, this approach was used to investigate the impact of genetic variants, potentially susceptible to influence drug pharmacokinetics, on LPV clearance. Two of the hit SNPs identified were located in *SLCO1B1*. While the first was known to be functional, the second was not. By resequencing and a fine mapping strategy, the study identified a SNP leading to a non synonymous amino acid change likely to be responsible for the detected effect and which had not formerly been associated with LPV clearance. This method also allowed the identification of two other SNPs in other genetic loci, overall emphasizing that large scale candidate gene approach is helpful in identifying candidate genes for further functional analysis or more comprehensive genetic mapping.

5.1.3. Genome Wide Association Studies (GWAS)

Genome wide association studies aim at discovering biological pathways involved in a given phenotype. This strategy implies a whole genome scan for potential associations and in recent years it has been the state of the art approach to identify candidate genes involved in "complex diseases". Contrary to single candidate gene and large scale candidate gene analyses, there is no *a priori* hypothesis of the involved genes in the phenotypic trait. However, genome-wide SNP arrays do not allow a complete coverage of all variants present in a gene, which is exacerbated by the fact that solely variants with a minor allelic frequency (MAF) of at least 0.05 are assessed in GWAS. Usually the lower the MAF is, the higher becomes the level of detectable contribution of the variant to a phenotype. This highlights the fact that GWAS are aimed at identifying biological pathways and genes involved in a given phenotype rather than variants with the highest relative risk [4]. Finally, the SNP arrays should be customized according to the study population. This is notably the case for African individuals, who tend to be more difficult to tag because of higher recombination rate and therefore a larger number of SNP is needed to allow a full characterization [4].

In this work, this approach was used to investigate the impact of genetic variants on HCV treatment and infection outcome. Several SNPs located in the *IL28* locus were identified. As the hit SNP was not likely to be functional, the study proceeded to resequencing and fine mapping. This strategy served to identify four candidate functional SNPs. In addition, at the time of analysis, the encoded protein, interferon λ3, had not been yet neither investigated nor associated with HCV infection outcome, emphasizing that GWAS approach might be helpful in identifying candidate genes for further functional analysis or more comprehensive genetic mapping.

5.2. Pharmacogenetics of Antiretroviral Agents

Pharmacogenetics involves the assessment of the influence of genetics on either pharmacokinetic or pharmacodynamic drug outcome. Although drug pharmacodynamics is a critical outcome, we focused in this work in genes involved in drug pharmacokinetics for three reasons:

1. Drug pharmacokinetics is primarily determined by ADME related genes. As the ADME pathway is common for all drugs, it narrows the window of genes susceptible to be implicated.
2. Drug pharmacodynamic outcome is dependant of the drug pharmacokinetic properties. Indeed, functional genetic variation in ADME genes can account for much of the variation in drug toxicity and response phenotypes [26].
3. In the case of antiretroviral treatment, the drug pharmacodynamic outcome is not only related to host genetics but also to viral genetics. Given the lack of error proofing of the viral polymerase, there is a facilitated path to viral mutation that affect drug efficacy.

5.2.1. Efavirenz

We have improved the understanding of efavirenz pharmacogenetics. This was achieved by investigating genetic variation in accessory metabolic pathways, and by resequencing (including the identification of a new null *CYP2A6* allele). The composite analysis of genetic variation in main and accessory metabolic pathways identified individuals that, in the setting of multiple non-functional alleles, experienced extremely high drug levels.

The very complete understanding of the genetic determinants of efavirenz pharmacokinetics allowed (i) the *in vivo* dissection of efavirenz metabolism, and (ii) the development of a population pharmacokinetics model with genetic covariates.

These new findings might help a better understanding of efavirenz associated neuropsychotoxicity. The correlation between treatment naïve individual's genotype and the time to discontinuation/dose reduction of efavirenz treatment showed that individuals with impaired function in both main and accessory metabolic pathways interrupted their medication more often (71.2%) than the remaining individuals (28.1%) during the first year of treatment, mainly because of toxicity.

Finally, we could demonstrate that TDM-guided dose adjustment could be predicted with nearly the same precision by genetic assessment. This tends to the final objective of making efavirenz genetic based dose adjustment possible. The advantage here is the possibility to determine the appropriate dose before treatment start, and therefore before experiencing treatment failure or toxicity.

5.2.2. Lopinavir

We improved the understanding of LPV/r pharmacogenetics. This was achieved by investigation of the role of genetic variation in ADME related genes. The composite analysis of genetic variation in three replicated loci (*SLCO1B1*, *ABCC2*, *CYP3A* locus) identified individuals that, in the setting of homozygocity for *SLCO1B1*4* (which has been associated with differences in substrate-specific transport kinetics) experienced extremely high LPV/r clearance, whereas in the setting of multiple alleles associated with decrease of function in either *SLCO1B1*, *ABCC2* or *CYP3A* locus, individuals experienced significantly lower LPV/r clearance.

The better understanding of the genetic determinants of LPV/r pharmacokinetics allowed the development of a population pharmacokinetics model with genetic covariates and highlights the potential use of the array for other antiretroviral drugs.

Despite the high pharmacokinetic relevance of these variants, their clinical impact could not be demonstrated through the retrospective assessment of the correlation between treatment naïve individual's genotype and LPV/r discontinuation rate. In this study, homozygocity for *SLCO1B1*4* was considered as a protective trait against treatment discontinuation given the lower risk of treatment toxicity originated from increased clearance. However, at the opposite, this could also be seen as a risk factor for treatment discontinuation, as individuals might experience higher risk of treatment failure caused by sub-therapeutic drug levels. Overall emphasizing that, although we failed to detect any clinical relevance of these variants, these results should be interpreted with caution and would need further prospective assessment.

5.3. Pharmacogenetics and Clearance of Hepatitis C Virus

We improved the understanding of shared genetic determinants of treatment-induced and spontaneous hepatitis C clearance. This could be achieved through several steps, including whole genome scan to detect variants associated with HCV infection outcome and then resequencing and fine mapping of the candidate gene *IL28B*, to identify candidate functional variants. The net contribution of *IL28B* was finally assessed in a single source cohort of pregnant women infected through the same HCV inoculum, by controlling for determinant cofactors such as viral diversity, gender and co-infection. In this cohort, individuals carrying one or more of the candidate functional SNPs were up to 5 times less likely to spontaneously clear the virus than individuals with the reference allele at the same positions.

Together with other groups' work [72, 76], this helped in the understanding of HCV pathogenesis, shifting the focus of HCV research towards innate immunity and opening the perspectives for future work, notably development of novel therapies such as exogenous interferon-λ3. Noteworthy, type III interferon receptor expression is tissue dependent - at the opposite of type I interferon receptor ubiquitous expression – potentially reducing the high prevalence toxicity experienced under pegylated interferon α therapy.

6. CONCLUSION

6.1. General Conclusion

In this work, three genetic approaches (single candidate gene analysis, large scale pathway analysis and genome-wide association study) were applied to investigate interindividual variability in either drug disposition/response or disease susceptibility. Each of those approaches could demonstrate the importance of the genetic background on these phenotypes.

Through the single candidate gene approach we could demonstrate, by comprehensively analyzing *CYP2A6*, that EFV metabolism was critically dependent of the accessory metabolic pathway in the setting of impaired CYP2B6 function. Therefore, individuals with impaired main and accessory EFV metabolic pathways might benefit from a genotype-guided dose adjustment. This would reduce not only the treatment discontinuation rate but also the treatment cost. This might be of relevance in population where *CYP2B6* and *CYP2A6* loss-of-function alleles have a high prevalence, such as in Africans.

Through large scale ADME related gene analysis and fine mapping strategy we could demonstrate the role of OATP1B1 in LPV/r transport by identifying two functional variants influencing LPV/r clearance. At the time of analysis, this was the first lead to a pharmacogenetic determinant for LPV/r dispositioin. These results emphasize the discovery potential of large scale candidate gene analyses and the benefit of such arrays for drugs with ADME pathways that are poorly characterized.

Through genome-wide association study and fine mapping strategy, we could demonstrate that hepatitis C treatment-induced or spontaneous clearance was strongly dependent of interferon-λ3, which was not known to be involved in HCV infection at the time of analyses, and neither had been considered as a candidate. Those results emphasize the pathway discovery potential of genome-wide association studies.

Overall, this work illustrates the constantly expanding possibilities offered in pharmacogenetics/genomics research since the completion of the Human Genome Project, the International HapMap Project, the 1000 Genomes Project, and the availability of new high throughput genotyping and sequencing technologies. These are the bases for future initiatives in personalized medicine and individualized drug therapy.

7. REFERENCES

7.1. Articles and Books

1. *Finishing the euchromatic sequence of the human genome.* Nature, 2004. **431**(7011): p. 931-45.
2. Lander, E.S., et al., *Initial sequencing and analysis of the human genome.* Nature, 2001. **409**(6822): p. 860-921.
3. *The International HapMap Project.* Nature, 2003. **426**(6968): p. 789-796.
4. Nebert, D.W., G. Zhang, and E.S. Vesell, *From human genetics and genomics to pharmacogenetics and pharmacogenomics: past lessons, future directions.* Drug Metab Rev, 2008. **40**(2): p. 187-224.
5. Slatkin, M., *Linkage disequilibrium--understanding the evolutionary past and mapping the medical future.* Nat Rev Genet, 2008. **9**(6): p. 477-85.
6. *A haplotype map of the human genome.* Nature, 2005. **437**(7063): p. 1299-1320.
7. Frazer, K.A., et al., *A second generation human haplotype map of over 3.1 million SNPs.* Nature, 2007. **449**(7164): p. 851-61.
8. Carlson, C.S., et al., *Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium.* Am J Hum Genet, 2004. **74**(1): p. 106-20.
9. Hazuda, D., M. Iwamoto, and L. Wenning, *Emerging pharmacology: inhibitors of human immunodeficiency virus integration.* Annu Rev Pharmacol Toxicol, 2009. **49**: p. 377-94.
10. De Clercq, E., *Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV.* Int J Antimicrob Agents, 2009. **33**(4): p. 307-20.
11. Long, M.C., J.R. King, and E.P. Acosta, *Pharmacologic aspects of new antiretroviral drugs.* Curr HIV/AIDS Rep, 2009. **6**(1): p. 43-50.
12. Temesgen, Z., D. Warnke, and M.J. Kasten, *Current status of antiretroviral therapy.* Expert.Opin.Pharmacother., 2006. **7**(12): p. 1541-1554.
13. Quinn, T.C., *HIV epidemiology and the effects of antiviral therapy on long-term consequences.* AIDS, 2008. **22 Suppl 3**: p. S7-12.
14. Chen, R.Y., et al., *Duration of highly active antiretroviral therapy regimens.* Clin Infect Dis, 2003. **37**(5): p. 714-22.
15. Rotger, M., C. Csajka, and A. Telenti, *Genetic, Ethnic, and GenderDifferences in the Pharmacokineticsof Antiretroviral Agents.* Curr.HIV./AIDS Rep., 2006. **3**(3): p. 118-125.
16. Telenti, A. and U.M. Zanger, *Pharmacogenetics of anti-HIV drugs.* Annu Rev Pharmacol Toxicol, 2008. **48**: p. 227-56.
17. Lubomirov, R., C. Csajka, and A. Telenti, *An ADME pathway approach for pharmacogenetic studies of anti-HIV therapy.* Pharmacogenomics., 2007.
18. Evans, W.E. and H.L. McLeod, *Pharmacogenomics--drug disposition, drug targets, and side effects.* N Engl J Med, 2003. **348**(6): p. 538-49.
19. Crettol, S., N. Petrovic, and M. Murray, *Pharmacogenetics of phase I and phase II drug metabolism.* Curr Pharm Des, 2010. **16**(2): p. 204-19.
20. Ingelman-Sundberg, M., *Pharmacogenetics of cytochrome P450 and its applications in drug therapy: the past, present and future.* Trends Pharmacol Sci, 2004. **25**(4): p. 193-200.
21. Ingelman-Sundberg, M. and C. Rodriguez-Antona, *Pharmacogenetics of drug-metabolizing enzymes: implications for a safer and more effective drug therapy.* Philos Trans R Soc Lond B Biol Sci, 2005. **360**(1460): p. 1563-70.

22. Zanger, U.M., et al., *Functional pharmacogenetics/genomics of human cytochromes P450 involved in drug biotransformation.* Anal Bioanal Chem, 2008. **392**(6): p. 1093-108.
23. Lewis, D.F., *57 varieties: the human cytochromes P450.* Pharmacogenomics, 2004. **5**(3): p. 305-18.
24. Dobrinas, M. and C.-B. Eap. *Editorial : Cytochrome P4503A pharmacogenetics*. HIV PGX 2:2 2007 22.04.2009]; Available from: http://www.hiv-pharmacogenomics.org/
25. Ingelman-Sundberg, M., et al., *Influence of cytochrome P450 polymorphisms on drug therapies: pharmacogenetic, pharmacoepigenetic and clinical aspects.* Pharmacol Ther, 2007. **116**(3): p. 496-526.
26. Grossman, I., *ADME pharmacogenetics: current practices and future outlook.* Expert Opin Drug Metab Toxicol, 2009. **5**(5): p. 449-62.
27. Sissung, T.M., et al., *Pharmacogenetics of membrane transporters: an update on current approaches.* Mol Biotechnol, 2010. **44**(2): p. 152-67.
28. Kerb, R., *Implications of genetic polymorphisms in drug transporters for pharmacotherapy.* Cancer Lett, 2006. **234**(1): p. 4-33.
29. Giacomini, K.M.a.S., Y., *Membrane transporters and drug response*, in *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, L.L. Brunton, Lazo, J. S. and Parker, K. L., Editor. 2005, McGraw-Hill: New York, USA. p. 41-70.
30. Kis, O., et al., *The complexities of antiretroviral drug-drug interactions: role of ABC and SLC transporters.* Trends Pharmacol Sci, 2010. **31**(1): p. 22-35.
31. Huang, Y., et al., *Genetic variations and gene expression of transporters in drug disposition and response.* Expert Opin Drug Metab Toxicol, 2008. **4**(3): p. 237-54.
32. Link, E., et al., *SLCO1B1 variants and statin-induced myopathy--a genomewide study.* N Engl J Med, 2008. **359**(8): p. 789-99.
33. *Bristol-Myers Squibb Company. SUSTIVA® (efavirenz). Prescribing information; Available at http://www.bms.com/products/Pages/prescribing.aspx (last accessed 21 April 2009)*
34. Desta, Z., et al., *Impact of CYP2B6 polymorphism on hepatic efavirenz metabolism in vitro.* Pharmacogenomics, 2007. **8**(6): p. 547-58.
35. Mutlib, A.E., et al., *Identification and characterization of efavirenz metabolites by liquid chromatography/mass spectrometry and high field NMR: species differences in the metabolism of efavirenz.* Drug Metab Dispos., 1999. **27**(11): p. 1319-1333.
36. Ward, B.A., et al., *The cytochrome P450 2B6 (CYP2B6) is the main catalyst of efavirenz primary and secondary metabolism: implication for HIV/AIDS therapy and utility of efavirenz as a substrate marker of CYP2B6 catalytic activity.* J.Pharmacol.Exp.Ther., 2003. **306**(1): p. 287-300.
37. Belanger, A.S., et al., *Glucuronidation of the antiretroviral drug efavirenz by UGT2B7 and an in vitro investigation of drug-drug interaction with zidovudine.* Drug Metab Dispos, 2009. **37**(9): p. 1793-6.
38. Staszewski, S., et al., *Efavirenz plus zidovudine and lamivudine, efavirenz plus indinavir, and indinavir plus zidovudine and lamivudine in the treatment of HIV-1 infection in adults. Study 006 Team.* N.Engl.J.Med., 1999. **341**(25): p. 1865-1873.
39. Csajka, C., et al., *Population pharmacokinetics and effects of efavirenz in patients with human immunodeficiency virus infection.* Clin.Pharmacol.Ther., 2003. **73**(1): p. 20-30.
40. Marzolini, C., et al., *Efavirenz plasma levels can predict treatment failure and central nervous system side effects in HIV-1-infected patients.* AIDS (London), 2001. **15**(1): p. 71-75.

41. Pereira, S.A., et al., *Intra-individual variability in efavirenz plasma concentrations supports therapeutic drug monitoring based on quarterly sampling in the first year of therapy.* Ther Drug Monit, 2008. **30**(1): p. 60-6.

42. Ofotokun, I., S.K. Chuck, and J.E. Hitti, *Antiretroviral pharmacokinetic profile: a review of sex differences.* Gend Med, 2007. **4**(2): p. 106-19.

43. Burger, D.M., et al., *The effect of the CYP2C19*2 heterozygote genotype on the pharmacokinetics of nelfinavir.* British Journal of Clinical Pharmacology (Oxford), 2006. **62**(2): p. 250-252.

44. Rotger, M., et al., *Influence of CYP2B6 polymorphism on plasma and intracellular concentrations and toxicity of efavirenz and nevirapine in HIV-infected patients.* Pharmacogenet.Genomics, 2005. **15**(1): p. 1-5.

45. Rotger, M., et al., *Predictive value of known and novel alleles of CYP2B6 for efavirenz plasma concentrations in HIV-infected individuals.* Clin.Pharmacol.Ther., 2007. **81**(4): p. 557-566.

46. Tsuchiya, K., et al., *Homozygous CYP2B6 *6 (Q172H and K262R) correlates with high plasma efavirenz concentrations in HIV-1 patients treated with standard efavirenz-containing regimens.* Biochem.Biophys.Res.Commun., 2004. **319**(4): p. 1322-1326.

47. Haas, D.W., et al., *Pharmacogenetics of efavirenz and central nervous system side effects: an Adult AIDS Clinical Trials Group study.* AIDS (London), 2004. **18**(18): p. 2391-2400.

48. *Abbott Laboratories. Kaletra® (lopinavir/ritonavir). Prescribing information; Available at http://www.rxabbott.com/pdf/kaletratabpi.pdf (last accessed 09 september 2010)*

49. *Food and Drug Administration. Kaletra® (lopinavir/ritonavir). FDA approval label; Available at http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction =Search.Label_ApprovalHistory#apphist (last accessed 09 september 2010)*

50. Kumar, G.N., et al., *In vitro metabolism of the HIV-1 protease inhibitor ABT-378: species comparison and metabolite identification.* Drug Metab Dispos, 1999. **27**(1): p. 86-91.

51. Kumar, G.N., et al., *Potent inhibition of the cytochrome P-450 3A-mediated human liver microsomal metabolism of a novel HIV protease inhibitor by ritonavir: A positive drug-drug interaction.* Drug Metab Dispos, 1999. **27**(8): p. 902-8.

52. Letendre, S.L., et al., *Lopinavir with Ritonavir Reduces the HIV RNA Level in Cerebrospinal Fluid.* Clin Infect Dis, 2007. **45**(11).

53. Chandwani, A. and J. Shuter, *Lopinavir/ritonavir in the treatment of HIV-1 infection: a review.* Ther Clin Risk Manag, 2008. **4**(5): p. 1023-33.

54. Gonzalez de Requena, D., et al., *Correlation between lopinavir plasma levels and lipid abnormalities in patients taking lopinavir/ritonavir.* AIDS Patient Care STDS, 2003. **17**(9): p. 443-5.

55. Gutierrez, F., et al., *Lopinavir plasma concentrations and changes in lipid levels during salvage therapy with lopinavir/ritonavir-containing regimens.* J Acquir Immune Defic Syndr, 2003. **33**(5): p. 594-600.

56. Molto, J., et al., *Variability in non-nucleoside reverse transcriptase and protease inhibitors concentrations among HIV-infected adults in routine clinical practice.* British Journal of Clinical Pharmacology (Oxford), 2007.

57. van der Leur, M.R., et al., *A retrospective TDM database analysis of interpatient variability in the pharmacokinetics of lopinavir in HIV-infected adults.* Ther Drug Monit, 2006. **28**(5): p. 650-3.

58. Janneh, O., et al., *Inhibition of P-glycoprotein and multidrug resistance-associated proteins modulates the intracellular concentration of lopinavir in cultured CD4 T cells and primary human lymphocytes.* J Antimicrob Chemother, 2007. **60**(5): p. 987-93.
59. Agarwal, S., D. Pal, and A.K. Mitra, *Both P-gp and MRP2 mediate transport of Lopinavir, a protease inhibitor.* Int J Pharm, 2007. **339**(1-2): p. 139-47.
60. van Waterschoot, R.A., et al., *Effects of cytochrome P450 3A (CYP3A) and the drug transporters P-glycoprotein (MDR1/ABCB1) and MRP2 (ABCC2) on the pharmacokinetics of lopinavir.* Br J Pharmacol, 2010. **160**(5): p. 1224-33.
61. Hartkoorn, R.C., et al., *HIV protease inhibitors are substrates for OATP1A2, OATP1B1 and OATP1B3 and lopinavir plasma concentrations are influenced by SLCO1B1 polymorphisms.* Pharmacogenet Genomics, 2010. **20**(2): p. 112-20.
62. Kohlrausch, F.B., et al., *The impact of SLCO1B1 polymorphisms on the plasma concentration of lopinavir and ritonavir in HIV-infected men.* Br J Clin Pharmacol, 2010. **69**(1): p. 95-8.
63. Williams, G.C. and P.J. Sinko, *Oral absorption of the HIV protease inhibitors: a current update.* Adv Drug Deliv Rev, 1999. **39**(1-3): p. 211-238.
64. *World Health Organization. Hepatitis C; Available at http://www.who.int/csr/disease/ hepatitis/Hepc.pdf (last accessed 09 september 2010)*
65. Rauch, A., et al., *Host genetic determinants of spontaneous hepatitis C clearance.* Pharmacogenomics, 2009. **10**(11): p. 1819-37.
66. McHutchison, J.G., *Understanding hepatitis C.* Am J Manag Care, 2004. **10**(2 Suppl): p. S21-9.
67. Rehermann, B., *Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence.* J Clin Invest, 2009. **119**(7): p. 1745-54.
68. Hoofnagle, J.H., *Course and outcome of hepatitis C.* Hepatology, 2002. **36**(5 Suppl 1): p. S21-9.
69. Gale, M., Jr. and E.M. Foy, *Evasion of intracellular host defence by hepatitis C virus.* Nature, 2005. **436**(7053): p. 939-45.
70. Kenny-Walsh, E., *Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. Irish Hepatology Research Group.* N Engl J Med, 1999. **340**(16): p. 1228-33.
71. Ge, D., et al., *Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance.* Nature, 2009. **461**(7262): p. 399-401.
72. Thomas, D.L., et al., *Genetic variation in IL28B and spontaneous clearance of hepatitis C virus.* Nature, 2009. **461**(7265): p. 798-801.
73. Tabor, H.K., N.J. Risch, and R.M. Myers, *Candidate-gene approaches for studying complex genetic traits: practical considerations.* Nat Rev Genet, 2002. **3**(5): p. 391-7.
74. Bader, J.S., *Systems approaches for pharmacogenetics and pharmacogenomics.* Pharmacogenomics, 2008. **9**(3): p. 257-62.
75. Rieder, M.J., et al., *The environmental genome project: reference polymorphisms for drug metabolism genes and genome-wide association studies.* Drug Metab Rev, 2008. **40**(2): p. 241-61.
76. Tillmann, H.L., et al., *A polymorphism near IL28B is associated with spontaneous clearance, of acute hepatitis C virus and jaundice.* Gastroenterology, 2010.

7.2. Websites

101. http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml/

102. http://www.hapmap.org/

103. http://www.1000genomes.org/page.php

104. http://www.fda.gov/ForConsumers/byAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118915.htm

105. http://www.hiv-pharmacogenomics.org/

106. www.cypalleles.ki.se

107. http://pharmacogenetics.ucsf.edu/cgi-bin/Study.py

8. SUPPLEMENTARY FILES

8.1. Pharmacogenetics of Antiretroviral Agents

8.1.1. Efavirenz

8.1.1.1. Original article

In Vivo Analysis of Efavirenz Metabolism in Individuals with Impaired CYP2A6 Function

Table S1: Demographic and genetic characteristics of the study population

Characteristic	Value	% study population
Sex (No.)		
Men	124	73
Women	45	27
Age (y)		
Median (Range)	47 (30-73)	-
Body weight (kg)		
Median (Range)	77.5 (44-101)	-
Height (cm)		
Median (Range)	179 (153-193)	-
Ethnicity (No.)		
Caucasians	142	83
Africans	16	10
Hispanics	6	4
Asians	5	3
PIs (No.)		
Ritonavir	20	13
Saquinavir	4	3
Lopinavir	15	9
Atazanavir	18	11
NRTIs (No.)		
Lamivudine	116	72
Stavudine	15	9
Didanosine	29	18
Tenofovir	29	18
Emtricitabine	4	2
Zidovudine	81	50
Entry inhibitors (No.)		
Enfuvirtide	4	2
Efavirenz (No.)		
200 mg	1	<1
300 mg	1	<1
400 mg	3	2
600 mg	162	96
700 mg	1	<1
800 mg	1	<1
***CYP2B6* genotype (No.)**		
Hom Ref	77	44
Het LOF	53	33
Hom LOF	23	14
Het GOF	16	9

NRTIs, nucleoside reverse transcriptase inhibitors. PIs, Protease inhibitors. Hom, homozygous. Het, heterozygous. Ref, reference allele. LOF, decrease/loss-of-function allele. GOF, gain-of-function allele.

Table S2: Primers and probes for *CYP2A6* genotyping

Allele	fSNP	Meth.	Primers	Probe	Size	T°	Ref
CYP2A6							
**1H, *1J*	g.-745A>G	Seq.	F: 5'- CCCTCGAATGTGATCTTCTC –3'; R: 5'- CAGCGGGTTCTCCCAGAAAG –3'	-	582 bp	60°C	This study
**9, *13, *15*	g.-48T>G	Taq.	Assay on demand from Applied Biosystems: C__30634332_10		-	60°C	AB
**2*	g.1799T>A	Taq.	Assay on demand from Applied Biosystems: C__27861808_60		-	60°C	AB
**17*	g.5065G>A	Taq.	Assay on demand from Applied Biosystems: C__34816076_20		-	60°C	AB
**7*, **10*, **19*	g.6558T>C	Seq.	F: 5'-TGCAAGTGTACCTGGCAGGAAA-3'; R: 5'- CGCATCTTCCCCCCATTCTTATA –3'	-	654 bp	62°C	[1]
**5*	g.6582G>T						
**12*	GC	Seq.	F : 5'- GGGGGTGAAGGATCCCAGTACT-3'; R : 5'- GTCCCCTGCTCACCGCCA-3'	-	1474 bp	65°C	[1]
**4/ *1X2*	CNV	qPCR	Assay on demand from Applied Biosystems: HS0001002_cn		-	60°C	AB
β-Globin							
**1*	CNV	qPCR	F : 5'-GGCAACCCTAAGGTGAAGGC-3'; R : 5'-GGTGAGCCAGGCCATCACTA-3'	VIC-5'-CATGGCAAGAAAGTGCTCGGTGCCT-3' TAMRA	67 bp	60°C	[2]

SNP, single nucleotide polymorphism. fSNP, functional SNP. Meth., Methods. Seq., Sequencing. Taq., TaqMan allelic discrimination. qPCR, quantitative PCR. AB, Applied Biosystems. GC, gene conversion. CNV, copy number variations. Position numbering of the functional SNPs refers to genomic (g.) DNA (bp1=A of ATG). F, forward. R, reverse

1. Haberl M, Anwald B, Klein K, Weil R, Fuss C, Gepdiremen A*, et al*. Three haplotypes associated with CYP2A6 phenotypes in Caucasians. *Pharmacogenet Genomics* 2005; **15**(9)**:** 609-624.

2. Gonzalez E, Kulkarni H, Bolivar H, Mangano A, Sanchez R, Catano G*, et al*. The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. *Science* 2005; **307**(5714)**:** 1434-1440

Table S3: Primers used for *CYP2A6* resequencing

Region	Primers	Size	T°	Ref
Promoter	F: 5'- CCCTCGAATGTGATCTTCTC –3'; R: 5'-CAGCGGGTTCTCCCAGAAAG –3'	582 bp	60°C	This study
Promoter + Exon 1	F: 5'-GGCTGTGTCCCAAGCTAGGCA-3'; R: 5'-GACTCTGGTCCACACTGGTCAAC-3'	428 bp	62°C	[1]
Exon 2	F : 5'-TCCCTGACTGTGAGAACCTGGGT-3'; R : 5'-GATGGGGAGGGAAGACCAGACT-3'	407 bp	62°C	[1]
GC In 2	F : 5'-GGGGGTGAAGGATCCCAGTACT-3'; R : 5'- GTCCCCTGCTCACCGCCA-3'	1474 bp	65°C	[1]
Exon 3	F: 5'-CTCTGACTGAGTTTGCAGCTCTG-3'; R:5'-AACGCGCGCGGGTTCCTCGT-3'	379 bp	62°C	[1]
Exon 4	F: 5'-GCGCTGGGAATTTGGCTCAACAA-3'; R: 5'-GGGGACACTGTCTGGAGGGC-3'	375 bp	62°C	[1]
Exon 5	F: 5'-GCCCCACTGAAATACCTAAACAAC-3'; R: 5'-CTGCCTGCCCCACTCCCAGA-3'	389 bp	62°C	[1]
Exon 6	F: 5'-CCCTCTTTCCACCTTTGGTCTGA-3'; R: 5'-ATCAGTGCAGACATTTTCAATATTTTAATAT-3'	450 bp	62°C	[1]
Exon 7	F: 5'-TGATGTCTGTTCTGTTATGAATGCTCTACT-3'; R: 5'-GACAGGGTCTAGAAAGCTTCTAATGT-3'	423 bp	62°C	[1]
Exon 8	F: 5'-GTCCCCCAAACTCCTGCCTAGA-3'; R: 5'-TACACCGCAGAGAGGGGAGGA-3'	451 bp	62°C	[1]
Exon 9	F: 5'-GGTTCACCATTGTTACATCTCTTATAGAAAGAAAT-3'; R: 5'-TCCTGCCCCCAGTCTTAGCTG-3'	473 bp	62°C	[1]
GC 3' UTR region	F: 5'-TGCAAGTGTACCTGGCAGGAAA-3'; R: 5'-CGCATCTTCCCCCCATTCTTATA-3'	654 bp	62°C	[1]

GC, Gene conversion. In, intron 2. UTR, untranslated region. F, forward. R, reverse

1. Haberl M, Anwald B, Klein K, Weil R, Fuss C, Gepdiremen A*, et al*. Three haplotypes associated with CYP2A6 phenotypes in Caucasians. *Pharmacogenet Genomics* 2005; **15**(9)**:** 609-624

Table S4: Primers used to amplify and resequence *CYP2A6*34*

Region amplified	PCR-Primers	Size	T°
Promoter *CYP2A7->* intron 5 *CYP2A6*	F: 5'–CCTCCGCAACAGAAGACCTC –3'; R: 5'–TTGAATGGGCCTGTGTCATC –3'	4010 bp	65°C
Exon 1 *CYP2A7->* intron 5 *CYP2A6*	F: 5'–TCCCAAGCTAGGTGGCATTC–3'; R: 5'–TTGAATGGGCCTGTGTCATC–3'	3692 bp	65°C
Exon 2 *CYP2A7->* intron 5 *CYP2A6*	F : 5'–GTGAAGGATCCCAGTACTTG–3'; R : 5'–TTGAATGGGCCTGTGTCATC–3'	3176 bp	65°C
Exon 3 *CYP2A7->* intron 5 *CYP2A6*	F : 5'–TCTCTGCGCATCTCTATCTG–3'; R : 5'–TTGAATGGGCCTGTGTCATC–3'	2260 bp	65°C
Exon 4 *CYP2A7->* intron 5 *CYP2A6*	F: 5'–CGCACGGTGAGTAAGGTTCC–3'; R:5'–TTGAATGGGCCTGTGTCATC–3'	1819 bp	65°C

Region sequenced	Sequencing-Primers	Size	T°
Exon 1	F: 5'–TCCCAAGCTAGGTGGCATTC–3'; R: 5'–CCCAGCACCGAGATGTCAAG –3'	552 bp	50°C
Exon 2	F: 5'–GTGAAGGATCCCAGTACTTG–3'; R: 5'–ATGGAGAGGCCACAGTGAAG –3'	407 bp	50°C
Exon 3	F: 5'–TCTCTGCGCATCTCTATCTG–3'; R: 5'–TCAGAGGTCTGAGGAGAATC–3'	639 bp	50°C
Exon 4	F: 5'–CGCACGGTGAGTAAGGTTCC–3'; R: 5'–GGCTTTTGTTCAGGTGCTCAG –3'	1111 bp	50°C
Recombinant region	F: 5'–AGTTGCACCAGAAGCCTGTC–3'; R: 5'–GGCTTTTGTTCAGGTGCTCAG –3'	569 bp	50°C

Exons 5 to 9 were re-sequenced with the primers shown in Supplementary Table S3.

Table S5: Association of *CYP2A6* genotype and EFV plasma exposure

	CYP2A6 alleles (n)				Median log_{10} EFV AUC (μg*h/ml)	Range log_{10} EFV AUC (μg*h/ml)	n
	Ref	DOF<50%	DOF≥50%	LOF			
***CYP2B6* het GOF**	2	-	-	-	1.445	1.389-1.654	8
	1	1	-	-	1.590	1.456-1.611	3
	1	-	1	-	1.362	1.248-1.598	3
	1	-	-	1	1.668	-	1
	-	1	1	-	1.596	-	1
***CYP2B6* reference**	2	-	-	-	1.590	1.188-1.919	51
	1	1	-	-	1.680	1.567-2.129	10
	1	-	1	-	1.569	1.306-1.892	9
	1	-	-	1	1.552	1.526-1.578	2
	-	1	1	-	1.790	1.621-1.957	3
	-	-	2	-	1.673	-	1
	-	-	1	1	1.769	-	1
***CYP2B6* het LOF**	2	-	-	-	1.716	1.458-2.164	30
	1	1	-	-	1.629	1.480-1.975	8
	1	-	1	-	1.727	1.590-1.911	8
	1	-	-	1	1.666	1.632-1.783	3
	-	1	1	-	1.839	1.806-2.011	3
	-	-	1	1	1.734	-	1
***CYP2B6* hom LOF**	2	-	-	-	2.177	1.687-2.639	12
	1	1	-	-	2.264	1.850-2.317	5
	1	-	1	-	2.518	2.286-2.635	3
	-	1	1	-	2.482	-	1
	-	-	2	-	2.464	-	1
	-	-	-	2	2.989	-	1

Hom, homozygous. Het, heterozygous. Ref, reference. DOF, decrease-of-function. LOF, loss-of-function.

Table S6: Genetic profiles selected for metabolite analysis

	***CYP2A6* alleles (n)**				**Individuals**
	Reference	**DOF<50%**	**DOF≥50%**	**LOF**	**(n)**
***CYP2B6* reference alleles**	2	-	-	-	11
	1	1	-	-	8
	1	-	1	-	3
	-	1	1	-	3
	-	-	2	-	1
	-	-	1	1	1
***CYP2B6* hom LOF**	2	-	-	-	12
	1	1	-	-	5
	1	-	1	-	2
	-	-	2	-	1
	-	-	-	2	1

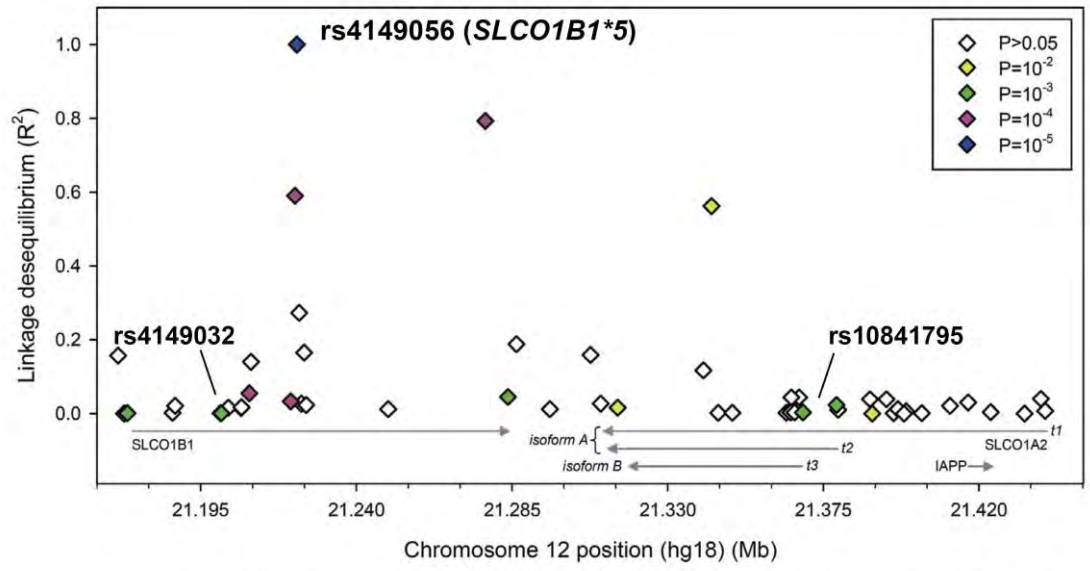
Hom, homozygous. Het, heterozygous. DOF, decrease-of-function. LOF, loss-of-function.

8.1.2. Lopinavir

8.1.2.1. Original article

ADME Pharmacogenetics – Investigation of the Pharmacokinetics of the Antiretroviral Agent Lopinavir

Figure S1: Analysis of the *SLCO1B1-1A2* cluster. The upper panel presents the linkage disequilibrium (LD) pattern (R^2) for the study-wide significant SNP rs4149056. SNPs are colored according to the association P value in Step 1. The dbSNP rs number of the two independent signals are shown. The lower panel depicts the various LD blocks in the regions. The analysis indicates the presence of association signals independent of the study-side hit.



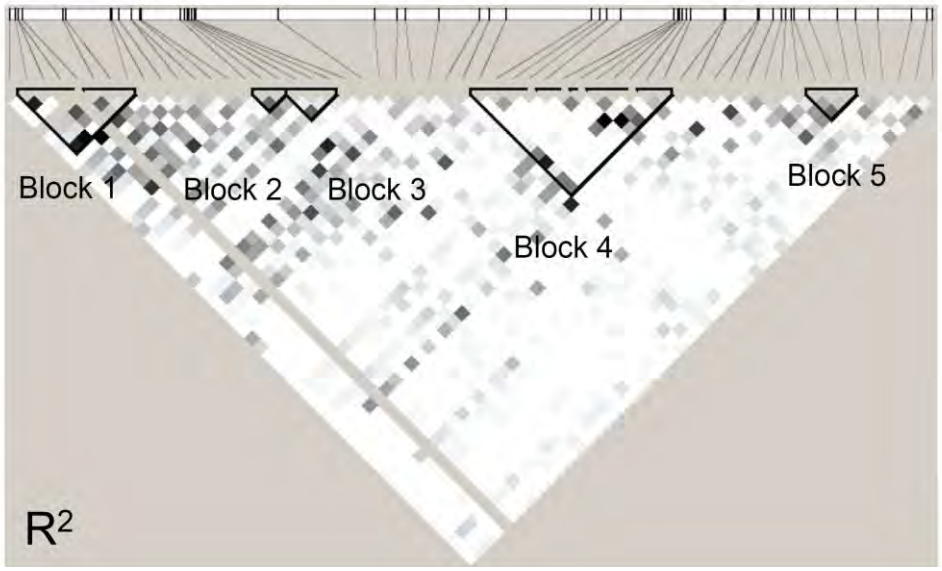


Figure S2: *SLCO1B1* resequencing. 49 polymorphisms were identified by resequencing *SLCO1B1* gene in 24 individuals of the discovery population. There were 3 polymorphisms in the promoter region (*blue*), 7 in the exons, three of which were synonymous (*black*) and 4 non-synonymous (*purple*), 28 polymorphisms in the exon-intron boundaries (*gray*) and 11 in the 3'UTR region (*green*). The position refers to the genomic DNA (g.). Seven of the SNPs have not been previously identified (*new*). The dbSNP rs ID for the known SNPs are described in the **Supplementary Table S7**

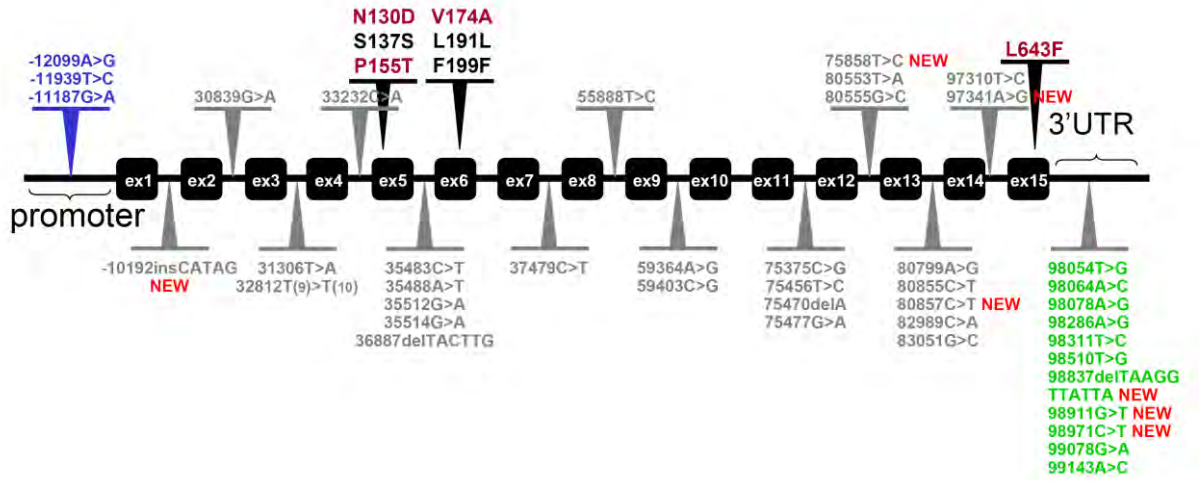
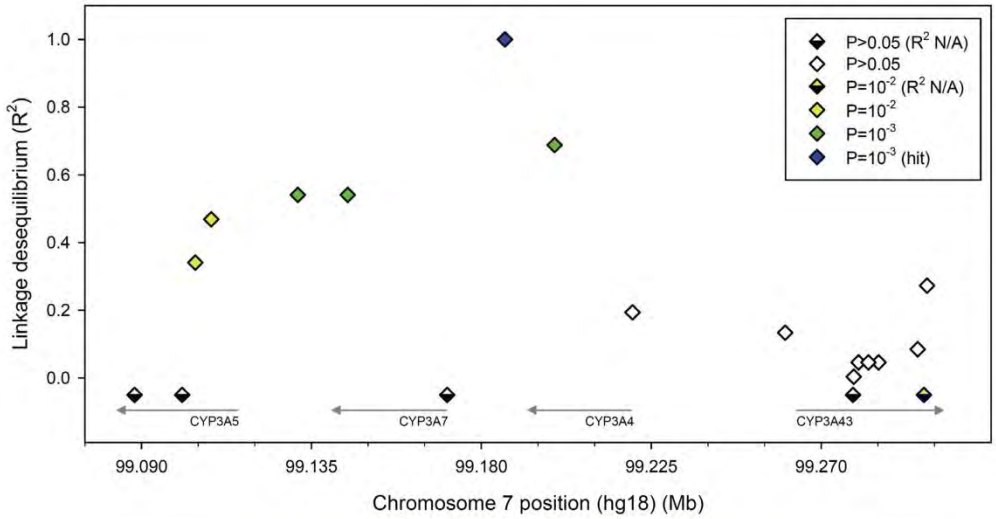


Figure S3: Analysis of the *CYP3A* cluster. The upper panel presents the linkage disequilibrium (LD) pattern (R^2) for the highest significant SNP rs6945984 in the *CYP3A locus*. SNPs are colored according to the association P value in Step 1. The lower panel depicts the two LD blocks in the regions. The analysis do not indicates the presence of association signals independent of the rs6945984.



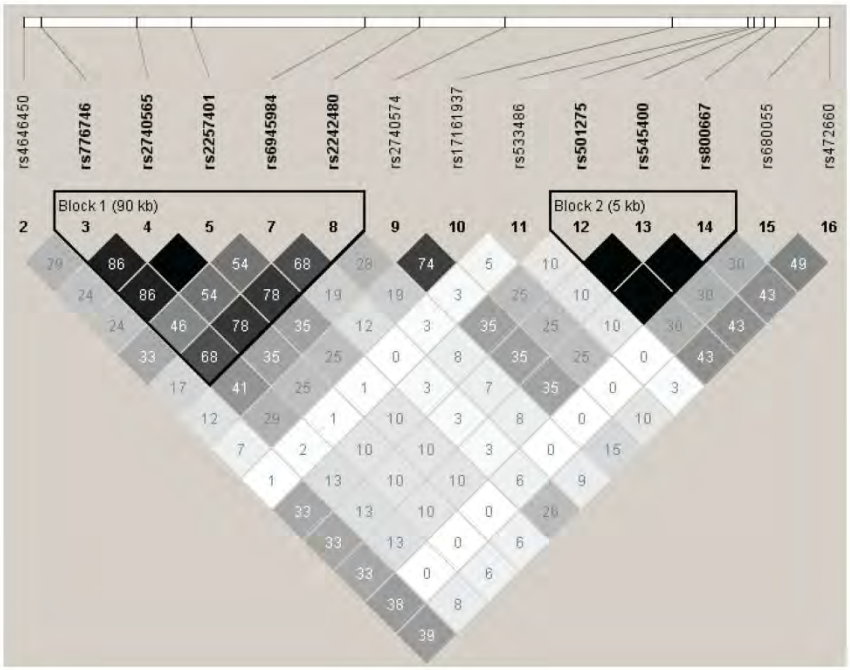


Figure S4: Ritonavir concentrations. Ritonavir observed concentrations (circles) in 96 patients with population average prediction (solid line) and 90 % prediction interval (dashed lines).

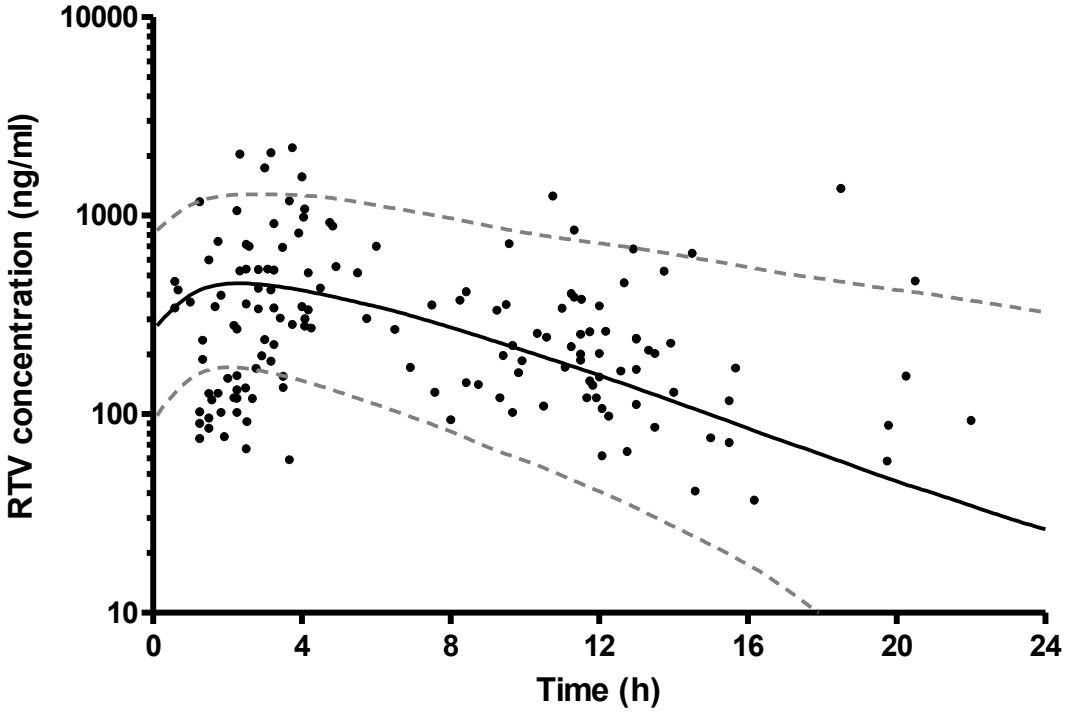


Figure S5: Influence of genetic variants on ritonavir and lopinavir average predicted clearance in the sub-set of 96 individual from the replication population (n=148).

A. Average predicted ritonavir (RTV) clearance among the individuals with different genotypes of *ABCC2* rs717620. The percentage and the direction of differences respect the homozygote carriers have been shown.

B. Average predicted lopinavir (LPV) clearance by the model including the RTV plasma levels among the individuals behave the different genetic score groups. The score group «-1,0,1» represents *SLCO1B1*4* Het variant «-1» carriers, for Hom Ref allele «0» carriers in any of the four SNPs included, and Het variant carriers in any of the three SNPs (rs4149056, rs717620, and rs6945984), finally the score group «2,3» represents the carriers of 2 or 3 variant alleles in in any of *SLCO1B1* rs4149056 (*SLCO1B1*5*), *ABCC2* rs717620 or rs6945984 in the *CYP3A* cluster variants. The percentage and the direction of differences respect the homozygote carriers have been shown.

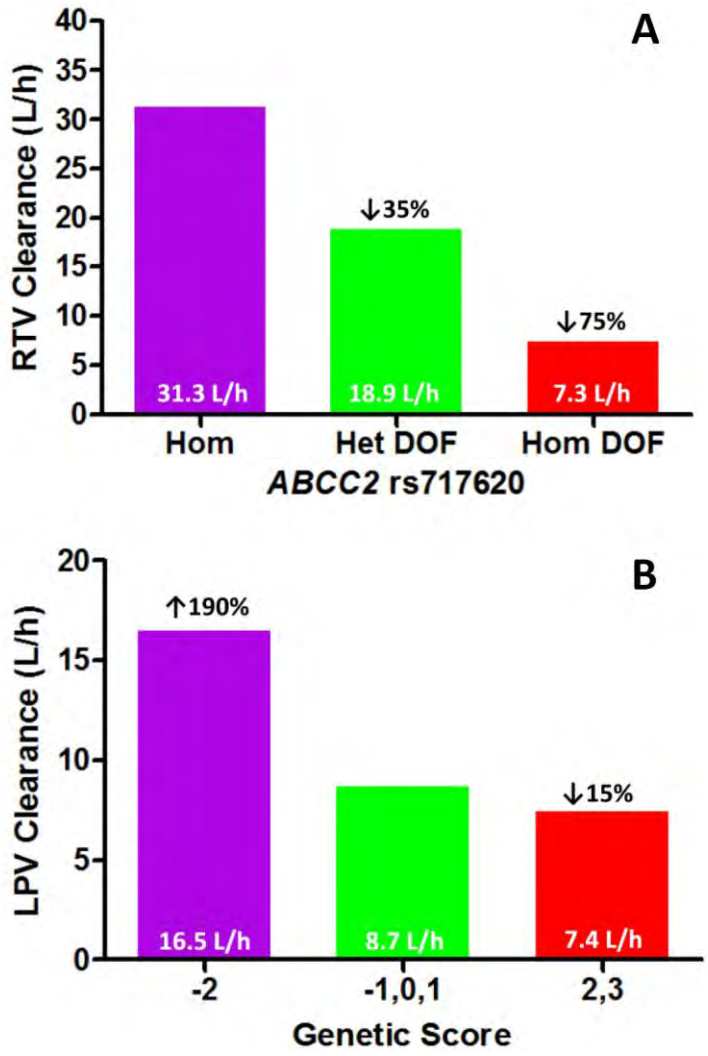


Figure S6. Lopinavir population plasma concentration prediction. Prediction for the 148 individuals in the replication study in relation to the three genetic score groups (see **Figure 4**). Population predictions of the corresponding genetic score groups were represented by black lines, and the 90% prediction interval is shown by gray dotted lines. The circles represent the individual plasma concentrations. A) Individual homozygous for rs11045819 (*SLCO1B1*4*) only; B) Reference group includes individuals with one or less variant alleles of *SLCO1B1*4* and in total less than two variant alleles in *SLCO1B1* (**5*, rs4149056), *ABCC2* (rs717620) and *CYP3A cluster* (rs6945984); C) individuals carrying two or more alleles associated with diminished function in *SLCO1B1* (**5*, rs4149056), *ABCC2* (rs717620) and *CYP3A cluster* (rs6945984).

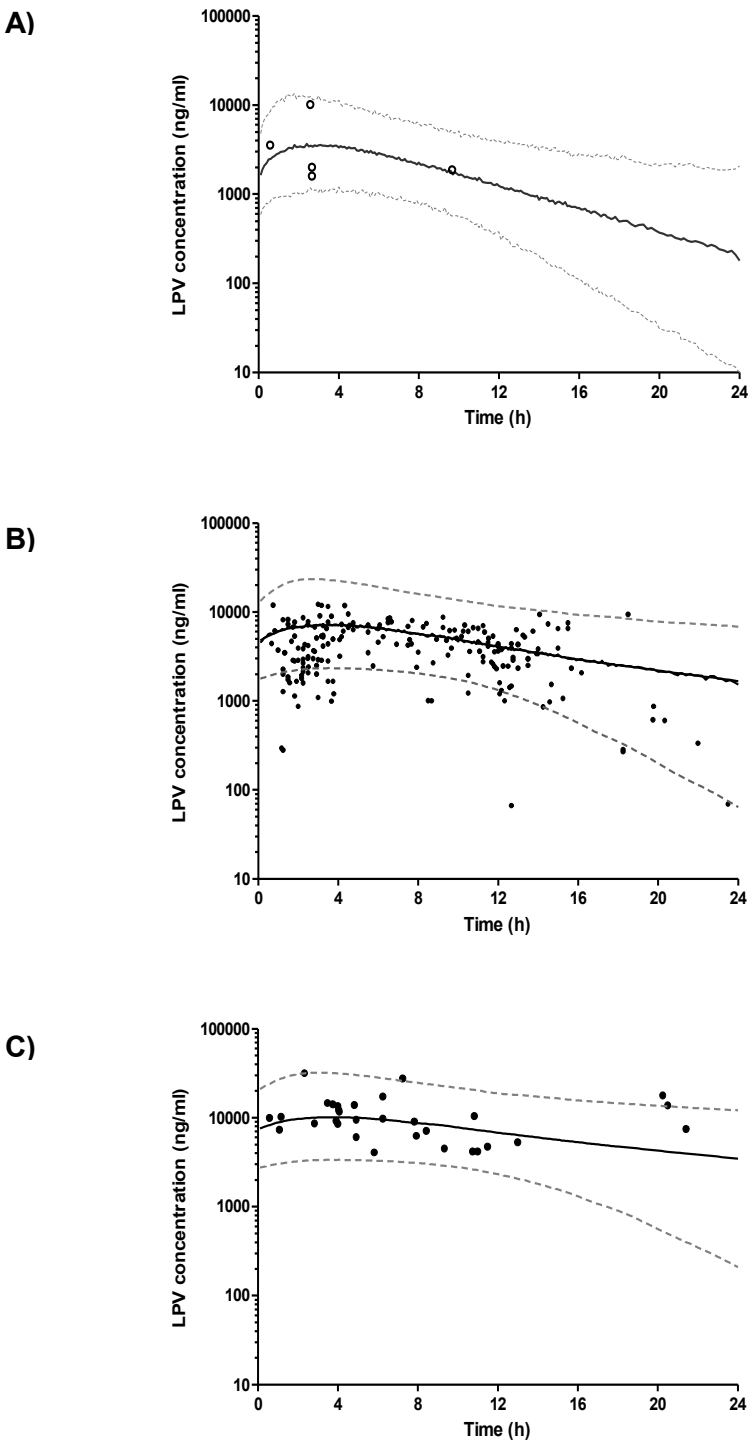


Table S1,Table S2 (lists of the genes and variants included in the ADME array) and **Table S6** (list of the P-value obtained for the variants included in the array) are not presented here because of their size, but are available on Pharmacogenetics and Genomics website ().

Table S3: commercially available TaqMan® SNP Genotyping Assays used for genotyping the nine variants in the replication study.

Gene symbol	dbSNP_rs#	Alleles change	AB Assay ID
SLCO1B1	rs4149056	T>C(*5)	C__30633906_10
	rs4149032	C>T	C___1901709_10
	rs11045819	C>A (*4)	NA *
	rs11045891	A>C	C__31106921_10
	rs17328763	T>C	C__33090767_10
ABCC2	rs717620	C>T	C___2814642_10
CYP3A_locus	rs6945984	T>C	C__29879759_10
SLCO1A2	rs10841795	A>G	C__25605906_20
SLCO2B1	rs1077858	A>G	C__27163766_10

* The rs11045819 was genotyped by direct sequencing using the same primers for *SLCO1B1* exon 5 amplification described in the Supplementary Table S4.

Table S4*:* Primers used for *SLCO1B1* resequencing.

Region	Primers	Size	T°	Ref
Promoter part 1	F: 5'- GTGCCAGGCATTATGTAAGG –3'; R: 5'-GTAAGAATCTACTGGCGAGTTG –3'	797 bp	65°C	This study
Promoter part 2	F: 5'- TTAACAGGCATAATCTTTGGTCTC –3'; R: 5'- TCTTTGCTTGGTACATAAGTATGG –3'	817 bp	63°C	This study
Exon 1	F: 5'- CAGGTGGTATCTCCAGTCTC -3'; R: 5'- GAGTCTAGTGTGATCAGTCAATG -3'	850 bp	65°C	This study
Exon 2	F : 5'- TGACCTAGCAGAGTGGTAAC -3'; R : 5'- TTCCTAAATATGTCGTGATCAATCC -3'	580 bp	63°C	This study
Exon 3	F: 5'- CTGGAAATGCTGCCTTTGAG -3'; R:5'- GCAGTTATGACAACCACAAATG -3'	690 bp	64°C	This study
Exon 4	F: 5'- TTCATTCCAGTATAATCCAGTCAAC -3'; R: 5'- AATCTTATAGGCAAAGACGTACAG -3'	697 bp	63°C	This study
Exon 5	F: 5'- TATCTTTCTTGCTGGACACTTC -3'; R: 5'- GGCAGGTTTATCATCCAGTTC -3'	564 bp	65°C	This study
Exons 6-7	F: 5'- GGACTAATACACCATATTGTCAAAG -3'; R: 5'- ATTAAGCAAAGGACTATTGAAAGAG -3'	718 bp	61°C	F: Morimoto R: This study
Exon 8	F: 5'- GGCAATGACAACAATATCATGAC -3'; R: 5'- ACCAGGGCATAGTACAGTTC -3'	847 bp	64°C	This study
Exon 9	F: 5'- GGTATTGCAGGCTATTCTCAC -3'; R: 5'- TTGTAAGTAACTGAGTCTTGATTTC -3'	679 bp	61°C	This study
Exon 10	F: 5'- TGTATGTAACAAACATGCACATTC -3'; R: 5'- CCCACTACTAATTGTAAGTACCAC -3'	619 bp	63°C	This study
Exon 11	F: 5'- CCCTCTTTCTCTGCTTTCAC-3'; R: 5'- TCACAATAACAGATAATACTGGAAAC-3'	407 bp	61°C	This study
Exon 12	F: 5'- GTTCTAGGCACTAATTTCTTTGTTTC-3'; R: 5'- ATTATTAATTACAGCCTTGAGAGTTC-3'	626 bp	61°C	This study
Exon 13	F: 5'- ATAAAGTCTGTTCTAACCACTTCC-3'; R: 5'- GAATTCTCCTTTAGGTCCATCAC-3'	592 bp	63°C	This study
Exon 14	F: 5'- GTCAATGACATTACTACATGATTTGG-3'; R: 5'- AATGAGATACGAGATTGCTTGATAC-3'	474 bp	63°C	This study
Exon 15	F: 5'- CAGAGGCAACTAGAGTATAGTTTC-3'; R: 5'- AGTTCATAGGCTTATTTATACTTCCAC-3'	572 bp	64°C	This study
3'UTR part 1	F: 5'- AAACAGCATTGCATTGATTCAG-3'; R: 5'- GACTAAGAGACGTGGGAAAG-3'	836 bp	64°C	This study
3'UTR part 2	F: 5'-TAGCCACTCTCATACTTATCTTAGC -3'; R: 5'-GCATCAAACAAGTCTATTGACATC -3'	860 bp	65°C	This study

UTR, untranslated region. F, forward. R, reverse

Ref: Morimoto et al. Drug Metab Pharmacokinet 2004; 19(6): 453-455 [PMID: 15681900].

Table S5: Concomitant drugs in the discovery and replication populations.

Concomitant drugs	Discovery			Replication	
	Total (n=638)	Cases (n=121)	Controls (n=92)	Total (n=148)	RTV (n=96)*
	n (%)	n (%)	n (%)	n (%)	n (%)
Anti-HIV drugs					
PIs					
Atazanavir	97 (15.20)	22 (18.18)	10 (10.87)	13 (8.78)	9 (9.38)
Saquinavir	49 (7.68)	8 (6.61)	10 (10.87)	5 (3.38)	4 (4.17)
Amprenavir	48 (7.52)	7 (5.79)	8 (8.70)	3 (2.03)	2 (2.08)
Nelfinavir	11 (1.72)	3 (2.48)	0 (0.00)	0 (0.00)	0 (0.00)
Indinavir	6 (0.94)	3 (2.48)	1 (1.09)	0 (0.00)	0 (0.00)
Tipranavir	11 (1.72)	0 (0.00)	4 (4.35)	0 (0.00)	0 (0.00)
Duranavir	5 (0.78)	1 (0.83)	0 (0.00)	0 (0.00)	0 (0.00)
NNRTIs					
Efavirenz	119 (18.65)	19 (15.70)	22 (23.91)	22 (14.86)	15 (15.63)
Nevirapina	44 (6.90)	7 (5.79)	6 (6.52)	10 (6.76)	5 (5.21)
Etravirine	1 (0.16)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
NRTIs					
Lamivudine	377 (59.09)	71 (60.68)	50 (55.56)	76 (51.35)	43 (44.79)
Abacavir	134 (21.00)	20 (17.09)	30 (33.33)	0 (0.00)	0 (0.00)
Stavudine	42 (6.58)	7 (5.98)	7 (7.78)	6 (4.05)	1 (1.04)
Zidovudine	229 (35.89)	35 (29.91)	32 (35.56)	48 (32.43)	25 (26.04)
Tenofovir	239 (37.46)	55 (47.01)	29 (32.22)	76 (51.35)	59 (61.46)
Emtricitabine	29 (4.55)	6 (5.13)	2 (2.22)	36 (24.32)	32 (33.33
Zalcitabine	1 (0.16)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Didanosine	120 (18.81)	24 (20.51)	17 (18.89)	22 (14.86)	12 (12.50)
EIs					
Enfuvirtide	28 (4.39)	9 (7.69)	6 (6.67)	0 (0.00)	0 (0.00)
Maraviroc	1 (0.16)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
IIs					
Raltegravir	1 (0.16)	0 (0.00)	0 (0.00)	3 (2.03)	1 (1.04)
CYP interacting drugs**					
CYP inhibitors	22 (3.45)	6 (4.96)	5 (5.43)	3 (2.03)	5 (5.21)
CYP inducers	4 (0.63)	0 (0.00)	1 (1.09)	0 (0.00)	0 (0.00)
CYP inhibitors + inducers	6 (0.94)	1 (0.83)	1 (1.09)	2 (1.35)	2 (2.08)

CYP, cytocrome P450 enzymes; *EIs*, entry inhibitors; *IIs*, Integrase inhibitors; *NNRTIs*, nonnucleoside reverse transcriptase inhibitors; *NRTIs*, nucleoside reverse transcriptase inhibitors; *PIs*, protease inhibitors.

* Individuals with lopinavir and ritonavir plasma levels available.

** Non-antiretroviral CYP interacting drugs.

Table S7: Polymorphisms identified by *SLCO1B1* resequencing.

Location	SNP position*	Amino acid Change	dbSNP rs #
promoter	g.-12099A>G		rs4149013
	g.-11939T>C		rs17328763
	g.-11187G>A		rs4149015
intron 1	g.-10192insCATAG		NEW
intron 2	g.30839G>A		rs7295464
intron 3	g.31306T>A		rs2291073
	g.32812T(9)>T(10)		rs4149094
intron 4	g.33232C>A		rs4149036
exon 5	c.388A>G	N130D	rs2306283
	c.411G>A	S137S	rs11045818
	c.463C>A	P155T	rs11045819
intron 5	g.35483C>T		rs11045820
	g.35488A>T		rs4149044
	g.35512G>A		rs4149045
	g.35514G>A		rs4149046
	g.36887delTACTTG		rs61760243
exon 6	c.521T>C	V174A	rs4149056
	c.571T>C	L191L	rs41149057
	c.597C>T	F199F	rs2291075
intron 7	g.37479C>T		rs2291076
intron 8	g.55888T>C		rs7957274
intron 9	g.59364A>G		rs4149066
	g.59403C>G		rs4149067
intron 11	g.75375C>G		rs4149070
	g.75456T>C		rs4149071
	g.75470delA		rs4149100
	g.75477G>A		rs4149072
intron 12	g.75858T>C		NEW
	g.75876T>A		NEW
	g.80553T>A		rs11045873
	g.80555G>C		rs11045874
intron 13	g.80799A>G		NEW
	g.80855C>T		rs4149077
	g.80857C>T		NEW
	g.82989C>A		rs12814646
	g.83051G>C		rs4149080
intron 14	g.97310T>C		rs12815795
	g.97341A>G		NEW
exon 15	c.1929A>C	L643F	rs34671512
3'UTR	g.98054T>G		rs4149087
	g.98064A>C		rs11045891
	g.98078 A>G		rs4149088
	g.98286A>G		rs11045892
	g.98311T>C		rs11045893
	g.98510T>G		rs12372157
	g.98837delTAAGGTTATTA		NEW
	g.98911G>T		NEW
	g.98971C>T		NEW
	g.99078G>A		rs12370842
	g.99143A>C		rs11045896

* Position numbering refers to genomic DNA (indicated as g.) according to NT_009714.17 or to coding DNA (indicated as c.) according to NM_006446.3; base pair +1 = A of ATG start codon of *SLCO1B1* gene.

8.2. Pharmacogenetics and Clearance of Hepatitis C Virus

8.2.1. Original article

Genetic Variation in *IL28B* is Associated with Chronic Hepatitis C and Treatment Failure: A Genome-Wide Association Study

Figure S1: *IL28* locus recombination pattern

A. Association of SNPs with chronic (versus spontaneously resolved) HCV infection in the *IL28B/A* and *IL-29* locus. The lowest P values are located within a low recombination rate region that encompasses the *IL28B* gene. The area is separated from the *IL28A* gene by a high recombination peak. This suggests that the association targets a haplotype block containing IL28B. Thus, *IL28B* is more likely to be associated with spontaneous clearance than *IL28A*. Measured SNPs, as opposed to inferred SNPs, are indicated by a X.

B. The association with treatment failure shows the same pattern as for chronic HCV infection and further supports an association with *IL28B*.

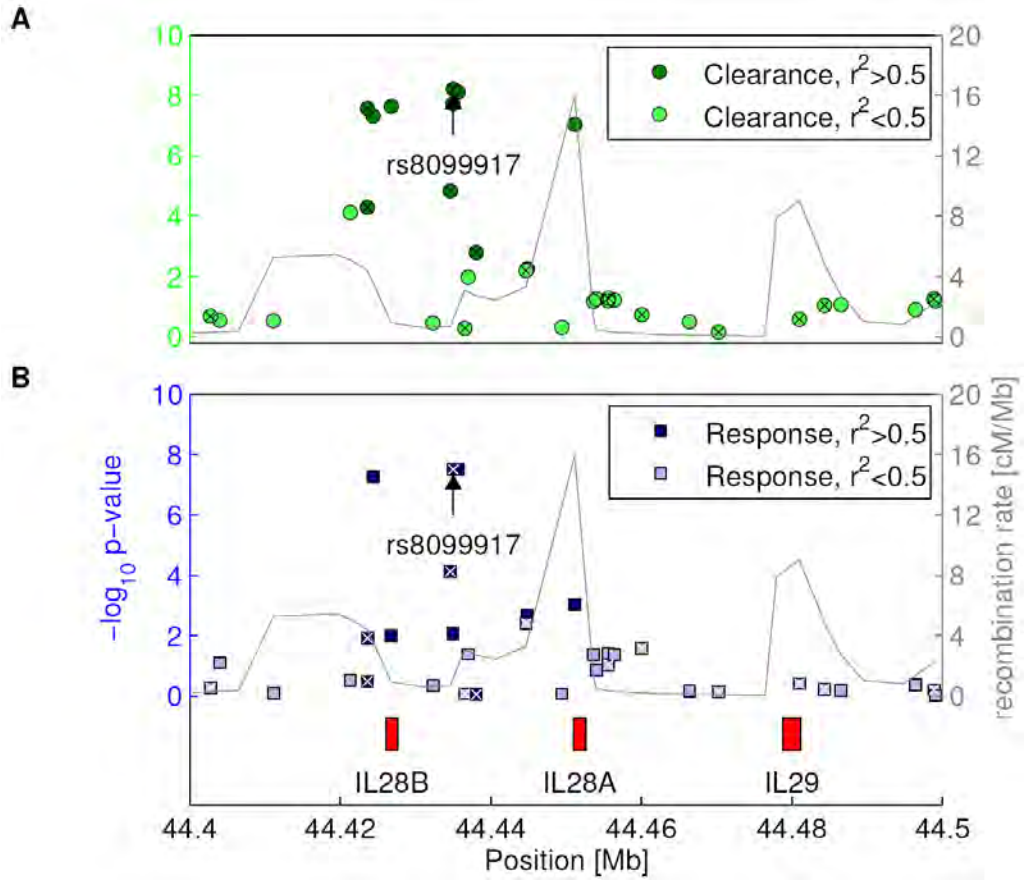
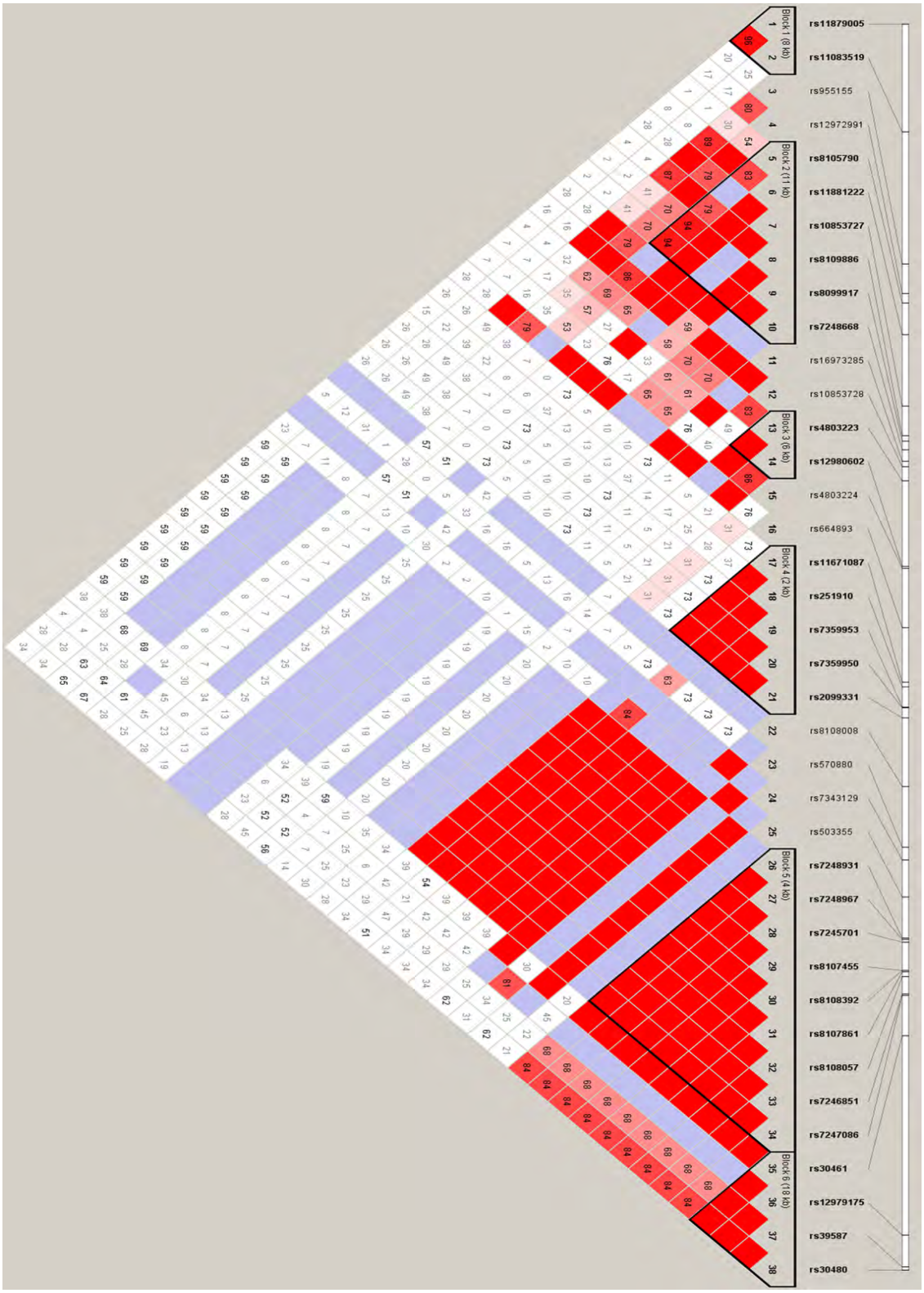


Figure S2: Pair-wise LD (r^2) pattern of the *IL28* region



Supplementary Table 1. Primers Used to Amplify and Resequence *IL28B*

Region amplified	Polymerase chain reaction primers	Size (*base pairs*)	Temperature (°C)
Promoter *IL28B*	Forward, 5′-GGTGGCCTGAGTTTCAGTTC-3′; reverse, 5′-CCCGGTCATGTCTGTGTC-3′	1500	62
Exons and introns *IL28B*	Forward, 5′-GTGGGCAGCCTCTGCATTC-3′; reverse, 5′-CAAATACATAAATAGCGACTGGGTGAC-3′	1476	62
3′UTR *IL28B*	Forward, 5′-CTTCCGCCAGTCATGCAAC-3′; reverse, 5′- AGCAGGCACCTTGAAATGTC-3′	1450	65
Region sequenced	**Sequencing primers**	**Size (*base pairs*)**	**Temperature (°C)**
Promoter part 1	Forward, 5′-GGTGGCCTGAGTTTCAGTTC-3′; reverse, 5′-TGCCCAGAGGCCAATATTTC-3′	512	50
Promoter part 2	Forward, 5′-CCTTCGTCACACCTCAATTC-3′; reverse, 5′-GGAAGGTATGTTCCCAAGAG-3′	581	50
Promoter part 3	Forward, 5′-GAGCAGGTGGAATCCTCTTG-3′; reverse, 5′-CCCGGTCATGTCTGTGTC-3′	529	50
Exons-introns part 1	Forward, 5′-GTGGGCAGCCTCTGCATTC-3′; reverse, 5′-AGCAGAAGCGACTCTTCC-3′	540	50
Exons-introns part 2	Forward, 5′-GGCTAACCTGTGCCTTTG-3′; reverse, 5′-GGAGCTGGGAGAGGATATG-3′	505	50
Exons-introns part 3	Forward, 5′-CTGACGCTGAAGGTTCTG–3′; reverse, 5′-CAAATACATAAATAGCGACTGGGTGAC-3′	577	50
3′UTR part 1	Forward, 5′-CTTCCGCCAGTCATGCAAC-3′; reverse, 5′-TCAAGTGATCCTCCCAACTC-3′	582	50
3′UTR part 2	Forward, 5′-CCTGGATGTGATTGCTCAAG-3′; reverse, 5′-GGTGGAGAATGACACTCTG-3′	565	50
3′UTR part 3	Forward, 5′-TGAGCTGCTGGAACAAAG-3′; reverse, 5′-AGCAGGCACCTTGAAATGTC-3′	443	50

UTR, untranslated region.

Supplementary Table 2. Demographics

	End point spontaneous hepatitis C clearance						End point response to therapy	
	Mono-infected		Coinfected		Total		Mono-infected	
	Chronic infection	Spontaneous clearance	Chronic infection	Spontaneous clearance	Chronic infection	Spontaneous clearance	Nonresponders	Sustained responders
n	779	135	236	212	1015	347	168	297
Median age (y) (interquartile range)	44.66 (14.17)	43.61 (16.81)	33.00 (10.00)	34.00 (7.00)	41.86 (14.71)	36.00 (12.00)	46.80 (12.02)	42.66 (14.71)
Male sex	489 (0.63)	61 (0.45)	158 (0.67)	110 (0.52)	647 (0.64)	171 (0.49)	114 (0.68)	181 (0.61)
Hepatitis B surface antigen positive[a]	8 (0.02)	3 (0.03)	8 (0.04)	20 (0.10)	16 (0.03)	23 (0.08)	2 (0.02)	3 (0.02)
HCV genotypes								
1	373 (0.48)	NA	98 (0.42)	NA	471 (0.46)	NA	101 (0.60)	87 (0.29)
2	80 (0.10)		7 (0.03)		87 (0.09)		8 (0.05)	52 (0.18)
3	223 (0.29)		71 (0.30)		294 (0.29)		28 (0.17)	134 (0.45)
4	67 (0.09)		24 (0.10)		91 (0.09)		18 (0.11)	16 (0.05)
Other/unknown	36 (0.05)		36 (0.15)		72 (0.07)		13 (0.08)	8 (0.03)
Log HCV RNA (median, IQR)[b]	5.87 (0.98)	0 (0)	6.09 (1.16)	0 (0)	5.90 (1.03)	0 (0)	5.94 (0.85)	5.83 (1.15)
Heavy drinker[c]							30 (0.21)	32 (0.12)
Liver biopsy[d]							122 (0.73)	202 (0.68)
Severe fibrosis							54 (0.44)	54 (0.27)
Severe inflammation							22 (0.18)	45 (0.22)
Steatosis							2 (0.50)	20 (0.40)

NOTE. All values are expressed as n (proportion).
NA, not applicable.
[a]Information on hepatitis B surface antigen before the measurement of HCV RNA was missing in 466 individuals.
[b]HCV RNA at set point (for clearance end point) and before treatment (for treatment end point).
[c]Heavy drinker was defined as use of more than 40 g alcohol per day for more than 5 years.
[d]Biopsy data before treatment were missing in 128 patients. Severe fibrosis and inflammation were defined by a METAVIR score ≥3 and steatosis by the presence of steatosis in >5% of hepatocytes.

Supplementary Table 3. Top 20 Hits ($P < 5 \times 10^{-5}$) by Chromosome for the Association With Chronic Versus Spontaneously Resolved HCV Infection

rs no.	Chromosome	Position (*base pairs*)	Gene	Distance (*kilobase*)	Minor allele frequency	r2-hat	Alleles		*P* value	Effect size	SE
rs3806575	2	217071179	*RPL37A*	1	0.05	0.67	G	C	4.29E-05	−1.00	0.24
rs3765098	3	1390195	*CNTN6*	0	0.48	1.00	T	C	4.51E-05	−0.41	0.10
rs4685148	3	14403792	*SLC6A6*	15	0.09	0.95	T	C	3.11E-05	−0.77	0.19
rs661777	3	52440226	*SEMA3G*	2	0.06	0.86	G	A	2.84E-05	1.14	0.27
rs7621695	3	196961660	*MUC4*	0	0.36	0.34	G	A	2.52E-05	−0.77	0.18
rs4689126	4	7518216	*SORCS2*	0	0.27	0.90	T	C	3.20E-05	−0.52	0.12
rs9349739	6	9705195	*OFCC1*	300	0.49	0.99	T	C	5.01E-05	0.42	0.10
rs880122	6	165988331	*PDE10A*	0	0.03	0.48	C	A	4.25E-05	−1.49	0.36
rs4278174	8	5910879	*MCPH1*	341	0.15	0.92	T	A	2.68E-05	−0.58	0.14
rs6984231	8	10264638	*MSRA*	0	0.46	0.93	G	A	1.72E-05	0.43	0.10
rs6584313	10	83542130	*NRG3*	83	0.22	0.97	G	A	4.78E-05	0.51	0.12
rs4463881	12	90832436	*BTG1*	229	0.29	0.95	T	C	3.61E-05	0.45	0.11
rs11612078	12	112692736	*RBM19*	52	0.13	0.90	G	A	6.19E-06	−0.68	0.15
rs9538873	13	60165634	*TDRD3*	120	0.19	0.94	T	G	3.49E-05	0.62	0.15
rs765771	16	63647863	*CDH11*	0	0.40	0.96	T	C	1.47E-05	0.47	0.11
rs8091335	18	31282121	*C18orf37*	20	0.49	0.97	C	A	2.85E-05	0.42	0.10
rs8099917	**19**	**44435005**	***IL28B***	**8**	**0.21**	**1.00**	**T**	**G**	**6.07E-09**	**0.84**	**0.14**
rs1297152	21	14615347	*STCH*	50	0.41	1.00	T	C	4.20E-05	−0.42	0.10
rs7280694	21	42418487	*UMODL1*	0	0.35	0.98	T	C	1.47E-05	0.46	0.11
rs5753723	22	30332294	*SFI1*	0	0.20	1.00	T	C	4.58E-05	0.48	0.12

NOTE. Only one SNP is reported for each locus. The top hit *rs8099917* appears in bold.
r2-hat, imputation accuracy.

Supplementary Table 4. Association of SNPs in the *IL28/IL29* Locus With Chronic Versus Spontaneously Resolved HCV Infection

Rs number	Chromosome	Position [bp]	Imputed	Gene	Distance [kb]	MAF	r2-hat	Alleles		P-value	Effect size	SE
rs11879005	19	44402743	0	SYCN	16	0.42	1	T	C	2.49E-01	−0.12	0.1
rs12975799	19	44403977	1	SYCN	17	0.42	0.92	G	A	3.29E-01	0.1	0.11
rs11083519	19	44411103	1	IL28B	15	0.42	0.88	T	A	3.39E-01	−0.1	0.11
rs955155	19	44421319	1	IL28B	5	0.25	0.92	G	A	7.39E-05	0.49	0.12
rs12972991	19	44423587	0	IL28B	3	0.25	1	C	A	7.23E-05	−0.47	0.12
rs12980275	19	44423623	0	IL28B	2	0.32	1	G	A	3.03E-08	−0.63	0.11
rs8105790	19	44424341	1	IL28B	2	0.24	0.81	T	C	5.24E-08	0.81	0.15
rs11881222	19	44426763	1	IL28B	0	0.32	0.98	G	A	2.48E-08	−0.64	0.12
rs10853727	19	44432303	1	IL28B	5	0.10	0.93	T	C	3.42E-01	0.16	0.17
rs8109886	19	44434602	0	IL28B	7	0.45	1	C	A	1.51E-05	0.44	0.1
rs8113007	19	44434943	1	IL28B	7	0.31	0.98	T	A	1.82E-08	−0.66	0.12
rs8099917	**19**	**44435005**	**0**	**IL28B**	**8**	**0.21**	**1**	**T**	**G**	**6.07E-09**	**0.84**	**0.14**
rs7248668	19	44435661	1	IL28B	8	0.21	0.97	G	A	7.59E-09	0.84	0.15
rs16973285	19	44436536	0	IL28B	9	0.10	1	T	C	5.22E-01	−0.11	0.16
rs10853728	19	44436986	1	IL28B	10	0.30	0.41	G	C	8.22E-03	−0.44	0.16
rs4803223	19	44438059	0	IL28B	11	0.16	0.98	G	A	1.68E-03	−0.46	0.15
rs12980602	19	44444660	0	IL28A	6	0.22	1	T	C	6.24E-03	0.34	0.12
rs4803224	19	44444854	1	IL28A	6	0.27	0.75	G	C	5.12E-03	0.36	0.13
rs664893	19	44449412	1	IL28A	2	0.10	0.79	G	A	4.80E-01	−0.13	0.19
rs576832	19	44451122	1	IL28A	0	0.32	0.7	G	C	9.04E-08	0.71	0.13
rs11671087	19	44453630	1	IL28A	1	0.21	0.99	T	C	6.04E-02	0.23	0.12
rs251910	19	44453989	1	IL28A	1	0.27	0.94	T	C	4.69E-02	−0.23	0.12
rs7359953	19	44455553	1	IL28A	3	0.21	1	G	A	5.87E-02	0.23	0.12
rs7359950	19	44455605	0	IL28A	3	0.21	1	T	C	4.82E-02	−0.24	0.12
rs2099331	19	44456390	1	IL28A	4	0.21	1	T	G	5.50E-02	0.24	0.12
rs11665818	19	44460056	0	IL28A	7	0.19	1	G	A	1.78E-01	0.17	0.13
rs570880	19	44466370	1	IL29	12	0.10	0.86	T	A	2.80E-01	−0.19	0.18
rs503355	19	44470224	0	IL29	9	0.07	1	T	C	6.58E-01	−0.09	0.21
rs30461	19	44480955	0	IL29	0	0.11	1	G	A	2.27E-01	−0.19	0.16
rs194014	19	44484300	0	IL29	3	0.09	1	G	A	8.03E-02	0.3	0.17
rs251903	19	44486490	1	LRFN1	3	0.09	0.95	G	C	7.51E-02	0.3	0.17
rs12979175	19	44496394	1	LRFN1	0	0.07	0.96	G	A	1.17E-01	0.3	0.19
rs39587	19	44498837	0	LRFN1	1	0.09	0.99	G	A	4.90E-02	−0.34	0.17
rs30480	19	44499053	1	LRFN1	1	0.09	0.96	G	A	5.73E-02	0.32	0.17

NOTE. The list contains both imputed and measured SNPs. The top hit *rs8099917* appears in bold.

Supplementary Table 5. Association of the *rs8099917* SNP With Spontaneous and Treatment-Induced Control of HCV Infection and Association of *rs8099917* With Chronic Versus Spontaneously Resolved HCV Infection

	Chronic infection, n (proportion)		Spontaneous clearance, n (proportion)		Inheritance	Multivariate[a] OR (95% CI)	*P* value
Mono-infected							
IL28B-7554 genotype							
TT	441	0.57	103	0.76	Recessive	3.69 (0.86–15.79)	7.79E-02
GT	297	0.38	30	0.22	Dominant	2.75 (1.75–4.32)	1.07E-05
GG	41	0.05	2	0.01	Additive[b]	2.49 (1.64–3.79)	1.96E-05
Coinfected							
IL28B-7554 genotype							
TT	145	0.61	166	0.78	Recessive	6.36 (1.40–28.84)	1.65E-02
GT	78	0.33	44	0.21	Dominant	2.22 (1.45–3.48)	2.35E-04
GG	13	0.06	2	0.01	Additive[b]	2.16 (1.47–3.18)	8.25E-05
Combined[c]							
IL28B-7554 genotype							
TT	586	0.58	269	0.78	Recessive	4.79 (1.68–13.67)	3.37E-03
GT	375	0.37	74	0.21	Dominant	2.46 (1.80–3.35)	1.23E-08
GG	54	0.05	4	0.01	Additive[b]	2.31 (1.74–3.06)	6.07E-09

Association of *rs8099917* with treatment failure

	Treatment failure, n (proportion)		Treatment success, n (proportion)		Percentage of failure	Inheritance	Multivariate[d] OR (95% CI)	*P* value
Mono-infected								
IL28B-7554 genotype								
TT	71	0.42	201	0.68	26	Rec	1.80 (0.51–6.37)	3.62E-01
GT	85	0.51	86	0.29	50	Dom.[b]	5.19 (2.90–9.30)	3.11E-08
GG	12	0.07	10	0.03	55	Add	3.45 (2.12–5.61)	6.30E-07

NOTE. Because patients with clearance are frequently undetected in the population, percentages of chronic infection versus spontaneous clearance were not calculated. For the end point of spontaneous HCV clearance, we could not adjust for age because the time point of acute HCV infection is unknown in most cases.

[a]Logistic regression estimate adjusted for population stratification and sex.

[b]Most likely model.

[c]Meta-analysis with inverse variance weighting.

[d]Logistic regression estimate adjusted for population stratification, sex, age, viral genotype, HCV RNA level, and liver fibrosis stage.

Appendix 1. Cohort Members

The members of the Swiss HCV Cohort Study are F. Negro (Chairman, University Hospitals, Geneva, Switzerland), A. Hadengue, L. Kaiser, L. Rubbia-Brandt, D. Moradpour (Lausanne, Chairman of Scientific Committee), P. Burgisser, P. Francioli, S. Estoppey-Younes, F. Schoni-Affolter, M. Rickenbach (Head of Data Center), G. Martinetti, A. Cerny, V. Masserey Spicher, M. Gorgievski, J. F. Dufour, H. Hirsch, H. Heim, B. Helbling, B. Müllhaupt, S. Regenass, R. Malinverni, C. Meyenberger, J. Borovicka, G. Dollenmaier, and G. Cathomas.

The members of the Swiss HIV Cohort Study are M. Battegay, E. Bernasconi, J. Böni, H. C. Bucher, P. Bürgisser, A. Calmy, S. Cattacin, M. Cavassini, R. Dubs, M. Egger, L. Elzi, M. Fischer, M. Flepp, A. Fontana, P. Francioli (President of the Swiss HIV Cohort Study), H. Furrer (Chairman of the Clinical and Laboratory Committee), C. A. Fux, M. Gorgievski, H. F. Günthard (Chairman of the Scientific Board), H. H. Hirsch, B. Hirschel, I. Hösli, C. Kahlert, L. Kaiser, U. Karrer, C. Kind, T. Klimkait, B. Ledergerber, G. Martinetti, N. Müller, D. Nadal, F. Paccaud, G. Pantaleo, A. Rauch, S. Regenass, M. Rickenbach (Head of Data Center), C. Rudin (Chairman of the Mother & Child Substudy), P. Schmid, D. Schultze, F. Schöni-Affolter, J. Schüpbach, R. Speck, B. M. de Tejada, P. Taffé, A. Telenti, A. Trkola, P. Vernazza, R. Weber, and S. Yerly.

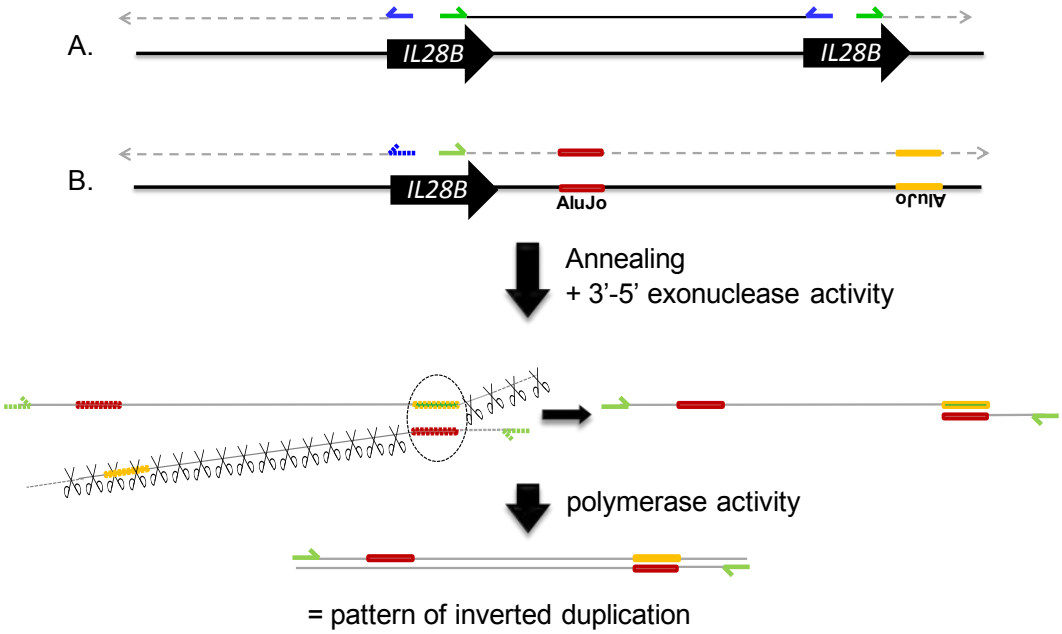
8.2.2. Original article

Estimating the Net Contribution of *IL28B* Variation to Spontaneous Hepatitis C Virus Clearance

Supplementary Figure S1: *IL28B* copy number investigation and artifact.

A. An approach consisting of a forward primer (in green) located downstream a reverse primer (in blue) was performed in order to identify potential gene duplication.

B. The presence of repetitive and complementary region (AluJo) in *IL28B* 3'UTR resulted in the artificial formation of chimeras by template switching.



Supplementary Table S1: Demographics

	SHCS		Single Source Cohort[#]	
Characteristics	**Spontaneous clearance**	**Chronic infection**	**Spontaneous clearance**	**Chronic infection**
N	200	189	27	44
Age (median, IQR)	39 (35-43)	39 (34-42)	29 (25-31)	28 (26-33)
Female sex, n (%)	95 (47)	60 (31)*	27 (100)	44 (100)
HBs positive, n(%)	20 (10)	7 (4)**	0 (0)	0 (0)
HCV genotypes				
1	unknown	75 (40)	27 (100)	44 (100)
2		5 (3)		
3		56 (30)		
4		21 (11)		
Other / unknown		72 (17)		
Log HCV RNA (median, IQR) [$]	0 (0)	6.0 (5.7-6.8)		

NA: Not applicable. *P=0.02, **P=0.002

[$] HCV RNA at set point

[#] All HIV negative

Supplementary Table S2: Primers and Probes for *IL28B* genotyping.

Rs number	position	Primers	Probe	Size	Ref
IL28B					
Preamplification step		F: 5'-GAGCAGGTGGAATCCTCTTG-3'; R: 5'- AGCAGGCACCTTGAAATGTC–3'	-	3308 bp	This study
rs8099917	g.-7558A>G	Assay on demand from Applied Biosystems: C__11710096_10		unknown	AB
rs12979860	g.-3180G>A	F: 5'-GCGCGGAGTGCAATTCAAC-3'; R: 5'- GCCTGTCGTGTACTGAACCA–3'	VIC 5„-TGGTTCGCGCCTTC-3„- MGBNFQ 6FAM 5„-CTGGTTCACGCCTTC-3„- MGBNFQ	66 bp	[1]
rs4803219	g.-312G>A	F: 5'- AGGCTGTGTTTTCACTTTTCCTACA-3'; R: 5'- GTAATTCCTGCCTGAGCTCCAT–3'	VIC 5„-TCTGTCAGGGATAAAA-3„- MGBNFQ 6FAM 5„-TTCTGTCAGAGATAAAA-3„- MGBNFQ	88 bp	AB (custom assay)
rs28416813	g.-37G>C	F: 5'- CAGCCCCTGCCCTCAG-3'; R: 5'- TGTCACAGAGAGAAAGGGAGCT–3'	VIC 5„-TGGGCAGCCTCTGCAT-3„- MGBNFQ 6FAM 5„-TGGGCAGCCTCTCCAT-3„- MGBNFQ	60 bp	AB (custom assay)
rs8103142	g.502A>G	F: 5'- CTAACCTGTGCCTTTGCTGTCTA-3'; R: 5'- GCCTCAGGTCCCAGGTC–3'	VIC 5„-AGCGGCACTTGCAG-3„- MGBNFQ 6FAM 5„-AGCGGCACCTGCAG-3„- MGBNFQ	94 bp	AB (custom assay)
rs4803217	g.1388G>T	F: 5'- GCCAGTCATGCAACCTGAGATTTTA-3'; R: 5'- AAATACATAAATAGCGACTGGGTGACA–3'	VIC 5„-TAGCCACTTGGCTTAAT-3„- MGBNFQ 6FAM 5„-TTAGCCACTTGTCTTAAT-3„- MGBNFQ	82 bp	AB (custom assay)
CNV	exon5-3'UTR	F: 5'- CCTGAATTGTGTTGCCAGC-3'; R: 5'- CATAAATAGCGACTGGGTGAC–3'	6FAM-5'- ACCCTTCCGCCAGTCATGC-3' MGBNFQ	121bp	This study
HMBS (encoding PBGD)					
**1*	CNV	F : 5'-AAGGGATTCACTCAGGCTCTTTC-3'; R : 5'-GGCATGTTCAAGCTCCTTGG-3'	VIC-5'-CCGGCAGATTGGAGAGAAAAGCCTGT-3'-MGBNFQ	75 bp	[2]

SNP, single nucleotide polymorphism. AB, Applied Biosystems. CNV, copy number variations. Position numbering of the SNPs refers to genomic (g.) DNA (bp1=A of ATG) in the sense of the gene (opposite the sense of the genome, as *IL28B* is encoded in the negative strand). F, forward. R, reverse (according to the gene and not the genome)

1. Ge, D., et al., Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature*, 2009. **461**(7262): p. 399-401.

2. Konig, R., et al., Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. *Cell*, 2008. **135**(1): p. 49-60.

9. APPENDIX

9.1. Original letter (Pharmacotherapy. 2008; 28(9): 215e-216e)

ALTERNATIVE VIEWPOINTS

Efavirenz Dose Adjustment in HIV Patients with Impaired CYP2B6 Function

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Efavirenz dosage adjustment in several patients with high EFV levels and presenting CNS toxicity have been successfully achieved in Switerland over the past seven years but many others have not beneficiated from dosage reduction owing to the lack of prospective studies evaluating the safety and clinical benefit of reduced dosage regimens.

Key Words: Pharmacokinetics, Pharmacogenomics, Antiretrovirals.
(Pharmacotherapy 2008;28(9):215e–216e)

The recent Case Report by Torno et al[1] comes at the right time to stimulate debate on dosage individualization in ART treatment, based on genotypic and phenotypic considerations. Efavirenz, a non-nucleoside reverse transcriptase inhibitor, is commonly used at fixed dosage regimen of 600 mg daily despite its very large interindividual variability[2, 3] and although dose ranging phase II studies have shown good virological efficacy at 400 mg or 200 mg daily.[4] Among various factors, recent evidence shows that polymorphisms of *CYP2B6*, but also of *CYP2A6* and *3A4/A5*, account for more than half of this pharmacokinetic variability, thus suggesting that unusual efavirenz concentrations profile are strongly related to patients' genetic constitution.[5, 6]

As part of the Swiss HIV Cohort Study, we are prospectively following patients under ART, a number of whom are offered concentration levels determinations. In the past 16 months, 85 patients under efavirenz benefited from efavirenz blood measurements. A dosage reduction from 600 mg to 400 mg (n = 6, 7.0 %) or to 200 mg (n=3, 3.5 %) daily had been applied to those patients to either reverse SNC toxicity (n = 8) or as a preventive measure (n = 1) in presence of multiple co-morbidities. No virological breakthrough was observed for up to 7 years of follow up. Dosage adjustment based on therapeutic drug monitoring (TDM) is slowly becoming standard of care for efavirenz-containing regimens as for other antiretroviral agents in Switzerland. However, no dose reduction was made for another 21 individuals (25% of patients) with efavirenz plasma concentrations at the higher tail of the therapeutic target. This evidence points out that dosage individualization in ART treatment, based on genotypic and/or phenotypic considerations is still not widely performed; this might probably be due to a lack of prospective controlled trials evaluating the safety and clinical benefit of reduced dosage regimens. Whether the

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systematic genetic testing of *CYP2B6* might either replace or complement TDM to guide efavirenz-based therapy should also be prospected,[1, 7] considering the contribution of accessory pathways on efavirenz elimination and the modulation of cytochrome activity by induction or inhibition.

References

1. **Torno MS, Witt MD, Saitoh A, et al**. Successful use of reduced-dose efavirenz in a patient with human immunodeficiency virus infection: Case report and review of the literature. Pharmacotherapy 2008;28(6):782–787
2. **Csajka C, Marzolini C, Fattinger K, et al**. Population pharmacokinetics and effects of efavirenz in patients with human immunodeficiency virus infection. Clin Pharmacol Ther 2003;73:20–30.
3. **Kappelhoff B, Huitema A, Yalvaç Z, et al**. Population pharmacokinetics of efavirenz in an unselected cohort of HIV-1 infected individuals. Clin Pharmacokinet 2005; 44(8):849–861.
4. **Hicks C, Hass D, Seekins D. et al**. A Phase 1, double-blind, placebo-controlled, dose ranging study to assess the antiretroviral activity and safety of DMP 266 (efavirenz, SUSTIVA®) in combination with open-Label zidovudine (ZDV) with Lamivudine (3TC). In 5th Conference on Retroviruses and Opportunistic Infections 1998. Abstract 698
5. **Rotger M, Tegude H, Colombo S et al**. Predictive value of known and novel alleles of *CYP2B6* for efavirenz plasma concentrations in HIV-infected individuals. Clin Pharmacol Ther 2007;81:557–566.
6. **Di Iulio J, Rotger M, Lubomirov R et al**. Genetic variation in accessory metabolic pathways is associated with extreme efavirenz exposure in individuals with impaired *CYP2B6* function. In 15th Conference on Retroviruses and Opportunistic Infections, Boston 2008. Abstract L-142.
7. **Rotger M, Telenti A**. Optimizing efavirenz treatment: *CYP2B6* genotyping or therapeutic drug monitoring? Eur J Clin Pharmacol 2008;64:335–336.

Authors' Reply

We appreciate and concur with the excellent points made by Dr. Csajka and his colleagues. Prospective, controlled trials are needed to evaluate the value of genetic testing in conjunction with therapeutic drug monitoring to guide individualized dosing of efavirenz-based therapy. We applaud the efforts of Dr. Csajka and his colleauges and the Swiss HIV Cohort study in addressing this need. We eagerly await the final results of their study.

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9.2. **Original article** [Antimicrobial Agents and Chemotherapy. 2010 Nov; 54(11):4619-25]

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2010, p. 4619–4625 Vol. 54, No. 11
0066-4804/10/$12.00 doi:10.1128/AAC.00712-10

Pharmacokinetics and Pharmacogenomics of Once-Daily Raltegravir and Atazanavir in Healthy Volunteers[▽]

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Received 25 May 2010/Returned for modification 17 July 2010/Accepted 28 August 2010

Atazanavir inhibits UDP-glucuronyl-transferase-1A1 (UGT1A1), which metabolizes raltegravir, but the magnitude of steady-state inhibition and role of the *UGT1A1* genotype are unknown. Sufficient inhibition could lead to reduced-dose and -cost raltegravir regimens. Nineteen healthy volunteers, age 24 to 51 years, took raltegravir 400 mg twice daily (arm A) and 400 mg plus atazanavir 400 mg once daily (arm B), separated by ≥3 days, in a crossover design. After 1 week on each regimen, raltegravir and raltegravir-glucuronide plasma and urine concentrations were measured by liquid chromatography-tandem mass spectrometry in multiple samples obtained over 12 h (arm A) or 24 h (arm B) and analyzed by noncompartmental methods. *UGT1A1* promoter variants were detected with a commercially available kit and published primers. The primary outcome was the ratio of plasma raltegravir C_{tau}, or concentration at the end of the dosing interval, for arm B (24 h) versus arm A (12 h). The arm B-to-arm A geometric mean ratios (95% confidence interval, *P* value) for plasma raltegravir C_{tau}, area under the concentration-time curve from 0 to 12 h ($AUC_{0–12}$), and raltegravir-glucuronide/raltegravir $AUC_{0–12}$ were 0.38 (0.22 to 0.65, 0.001), 1.32 (0.62 to 2.81, 0.45), and 0.47 (0.38 to 0.59, <0.001), respectively. Nine volunteers were heterozygous and one was homozygous for a *UGT1A1* reduction-of-function allele, but these were not associated with metabolite formation. Although atazanavir significantly reduced the formation of the glucuronide metabolite, its steady-state boosting of plasma raltegravir did not render the C_{tau} with a once-daily raltegravir dose of 400 mg similar to the C_{tau} with the standard twice-daily dose. *UGT1A1* promoter variants did not significantly influence this interaction.

In 2007, 4 million HIV-infected patients were taking antiretroviral medications in countries with low to middle income levels, according to the World Health Organization (WHO) (24). According to the 2009 update (23) of the WHO treatment guidelines (22), recommended first-line therapies should include two nucleoside reverse transcriptase inhibitors (NRTI) and either efavirenz or nevirapine. However, efavirenz is classified by the United States Food and Drug Administration (FDA) as a "D" drug during pregnancy, due to teratogenic potential demonstrated in monkeys and in retrospective human data, which means it is not recommended for women of child-bearing age who cannot practice reliable contraception. Nevirapine has a "black box" warning advising of increased risk of severe or fatal hepatotoxicity if used in women with $CD4^+$ cell counts of >250 cells/ml. Therefore, there is room for additional first line and even salvage agents that are potentially affordable, safe, well-tolerated, and independent of current, widely used first- or second-line therapies. Raltegravir could be one of these new drugs, but its high cost, which is prohibitive for most of the world's HIV-infected patients (16), warrants investigation into strategies, such as boosting, to reduce the dose.

Raltegravir was approved by the FDA in October 2007. It is the first-in-class and, currently, sole FDA-approved inhibitor of viral integrase, an HIV-1-specific enzyme that is required for viral replication (9). Inhibition of integrase prevents the insertion of linear HIV-1 DNA into the host cell genome, a critical step in the life cycle of the virus. Raltegravir is primarily metabolized by UDP-glucuronosyl transferase 1A1 (UGT1A1) and not by the P450 cytochromes.

Atazanavir-mediated inhibition of UGT1A1, which also conjugates bilirubin, results in mild hyperbilirubinemia in most patients treated with atazanavir. When given as a single dose in addition to a steady-state regimen of atazanavir 400 mg daily in healthy volunteers, the maximum concentration (C_{max}), 12-h postdose concentration (C_{12}), and area under the time-concentration curve (AUC) of raltegravir were 53%, 95%, and 72% higher, respectively, than when raltegravir was given alone (11). With ritonavir-boosted atazanavir, these metrics were 10 to 20% lower, which is not surprising given the ability of ritonavir to induce drug-metabolizing enzymes, including glucuronyl transferase, in addition to its inhibitory effect on cytochrome P450 2D6 (CYP2D6), CYP3A4, or P-glycoprotein (6).

The combination of raltegravir and atazanavir has potential as an initial or salvage regimen. Both drugs are potent inhibitors of HIV-1 replication, have minimal impact on the lipid profile, are generally well tolerated, and do not require refrigeration. We hypothesized that at steady state, boosting of raltegravir plasma concentrations by atazanavir would be sufficient

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▽ Published ahead of print on 7 September 2010.

to permit a reduced daily dose of raltegravir by eliminating one of the two doses in the standard regimen of 400 mg twice daily. Such a dosing regimen could lower the cost of raltegravir and increase the feasibility of combination with atazanavir, which is routinely dosed once daily. We also hypothesized that the *UGT1A1* genotype may influence the magnitude of a raltegravir-atazanavir interaction.

(Data from the investigation were presented in part at the 5th International AIDS Society Conference on HIV Pathogenesis, Treatment and Prevention, Cape Town, South Africa, July 2009.)

MATERIALS AND METHODS

Study design and population. We conducted an open-label, crossover pharmacokinetic study in healthy adult volunteers over the age of 18 years. The University of Southern California (USC) Institutional Review Board approved the study, and all volunteers gave written informed consent to participate.

Volunteers were eligible if they were free from any chronic medical condition that would potentially alter the pharmacokinetics of either raltegravir or atazanavir or any psychiatric condition that might affect adherence to the study protocol. No concomitant medications except occasional ibuprofen or acetaminophen were allowed. Female participants using hormonal contraception were asked to refrain from use beginning 2 weeks prior to the study and to practice alternate forms of contraception. Avoidance of these disallowed medications was verified by participant self-report. Participants self-identified their racial category as Caucasian, Asian, or African American and their ethnicity as Hispanic or not.

Study procedures. Prior to receiving any study medication, all participants passed baseline screening with normal results for physical examination, complete blood cell count, serum electrolytes, liver enzymes (alanine and aspartate aminotransferase), serum creatine kinase, serum bilirubin, and 12-lead electrocardiogram (ECG), which was reviewed by a licensed cardiologist. HIV infection was excluded by establishing <50 HIV RNA copies/ml of blood (NucliSens; bioMerieux, United States) in order to avoid the scenario of administration of suboptimal antiretroviral therapy to a newly infected patient yet to seroconvert. Participants were then randomized to start one of two unblinded treatment arms according to a prespecified random number list with a block size of 2 to ensure equal allocation. In arm A, participants took one raltegravir 400-mg tablet orally twice daily; in arm B they took one raltegravir 400-mg tablet plus two atazanavir 200-mg capsules, both orally once daily.

Study medications for both arms A and B were obtained by open-market purchase, stored at recommended temperatures in the USC Investigational Drug Service (IDS) pharmacy, and dispensed by IDS pharmacists in bottles fitted with MEMS electronic caps which recorded the time and day each time the bottle was opened. Study staff instructed the participants on the nature, purpose, and use of the MEMS caps and asked them to only open the bottles when the medications were to be taken and to take the medications immediately, i.e., to not save them for later. Each participant's electronic dosing record was then uploaded into the MEMS software to obtain an accurate dosing record for pharmacokinetic modeling. As a backup, participants were also provided with a written medication log to record dates and times of all doses. Although participants were instructed to take all study medications in a semifasted state at least 1 h prior to or 2 h after eating, the medication log also contained space to record any food intake within this window.

After 7 days of dosing in arm A or B, participants were admitted to the USC General Clinical Research Center (GCRC) in the early morning, with no food consumption since the evening prior. A predose blood sample for pharmacokinetic and *UGT1A1* genotypic analysis was obtained; the participant voided his/her bladder, and the urine was discarded. After a witnessed dose of study medications, further blood samples were obtained at 1, 2, 4, 8, and 12 h after the dose. Standardized meals were provided, beginning 1 h after study medication intake. All urine was collected and combined into 2-h aliquots. After the last 12-h sample, participants were discharged from the GCRC. For arm B, participants were given a container to collect the remaining 12-h production of urine as a single aliquot stored in the refrigerator at home. The next morning, 24 h after the last dose of study medications, they returned for a final blood sample, bringing the collected urine. Verification of complete urine collection was by participant self-report and analyzing the volume returned for outliers, defined as ml/h/kg of body weight of urine produced above or below 1.5 times the interquartile range. All participants were to complete both arms A and B, with a minimum 3-day period between arms.

Sample analysis. After collection, samples were stored on ice, and within 30 min they were centrifuged at 1,500 × *g* (2,500 rpm) for 10 min. Plasma and urine aliquots were frozen at −70°C until batch analysis. Raltegravir and atazanavir concentrations in plasma and urine were determined by high-performance liquid chromatography coupled to tandem mass spectrometry (LC–MS-MS) after protein precipitation with acetonitrile using an adaptation of our previously reported methods (3, 5). We used the same LC–MS-MS methodology with modification for the assay of raltegravir-glucuronide. Briefly, aliquots (100 μl) of plasma or diluted urine (20-fold with ultrapure water) were mixed with a 100-μl volume of internal standard solution (2 μg/ml raltegravir-glucuronide-d3 [Toronto Research Chemicals, Inc., North York, Canada] in methanol/water [50:50]). The resulting sample was subjected to protein precipitation with acetonitrile (200 μl) and vortexed. The mixture was centrifuged at 4°C for 10 min at 20,000 × *g* (14,000 rpm). The supernatant (200 μl) was transferred into glass high-performance liquid chromatography (HPLC) vials and was diluted with 400 μl methanol–ammonium formate 20 mM (1:1). Chromatographic separations were done on an Atlantis dC_{18} column (2.1 by 50 mm, 3 μm; Waters, Milford, MA). The mobile phase used for chromatography was 2 mM ammonium acetate in ultrapure water (buffer A) and acetonitrile (solvent B), both containing 0.1% formic acid. The mobile phase was delivered using a stepwise gradient elution program: 2% acetonitrile (solvent B) at 0 min, 80% B at 6 min at a flow rate of 0.3 ml/min. The second part of the run included 1 min of rinsing with 80% B followed by a reequilibration step to the initial solvent up to 12 min. This chromatographic program allows an excellent separation of raltegravir-glucuronide and raltegravir, which were eluted at 4.28 min and 5.46 min, respectively. Raltegravir-glucuronide quantification was performed by selected reaction monitoring (SRM) in the negative mode, using the *m*/*z* transitions 619.1→316.0 and 622.1→319.0 for raltegravir-glucuronide and raltegravir-glucuronide-d3, respectively. The range of the calibration curve for raltegravir-glucuronide was established up to 10,000 ng/ml, with a lower limit of quantification of 10 ng/ml. The laboratory participates in an international external quality assurance program for antiretroviral drug analysis (Stichting Kwaliteitsbewaking Klinische Geneesmiddelanalyse en Toxicologie [KKGT; Association for Quality Assessment in TDM and Clinical Toxicology], The Hague, Netherlands).

For *UGT1A1* genotyping, DNA was extracted from blood using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Due to the small study population size, we focused on the most common reduction-of-function allele, *UGT1A1*28*, which is a TA insertion in the TATA box of the promoter region: $A[TA]_7TAA$. The reference allele, **1*, is $A[TA]_6TAA$. *UGT1A1*28* occurs with an allelic frequency of 0.39 in Caucasians, 0.43 in Africans, and 0.16 in Asians (1). Genotyping of *UGT1A1*28* was performed by direct sequencing using forward (5′-AAGTGAACTCCCTGCTACCTT-3′) and reverse (5′-CCACTGGGATCAAC-AGTATCT-3′) primers as described by Monaghan et al. (17) This method also permitted the detection of *UGT1A1*36* ($A[TA]_5TAA$), *UGT1A1*37* ($A[TA]_8TAA$), and *UGT1A1*81* (g.-64G>C), which have increased, decreased, and similar function to **1*, respectively.

Sample size. Our primary endpoint was the geometric mean ratio (GMR) of the concentrations for each dosing strategy at the end of the dosing interval (C_{tau}), i.e., the raltegravir 24-hour concentration (C_{24}) when dosed once daily versus the 12-hour concentration (C_{12}) when dosed twice daily. The relationship between raltegravir plasma concentrations and outcomes has not been established. Since the ratio of steady-state C_{12} with 100 mg twice daily to 400 mg twice daily is 0.35 (10) and the virologic efficacy of these two doses was similar in one study (15), we chose a sample size of 20 to ensure that if the mean primary endpoint GMR was 0.8, the two-sided 95% confidence interval (CI) about the mean GMR would be greater than or equal to 0.4, with an alpha of 0.05 and a power of 0.8, assuming a GMR standard deviation of 0.65.

Data analysis. Summary statistics, comparative statistics, and noncompartmental raltegravir pharmacokinetic analyses were performed using R 2.9.2 (Vienna, Austria). The area under the concentration-time curve (AUC) was calculated using the linear trapezoidal algorithm. Apparent total plasma clearance of oral drug ($CL_{t/F}$) was calculated using the formula $CL_{t/F}$ = dose/AUC, where the AUC is that in plasma during the dosing interval at steady state. Renal clearance (CL_R) was calculated as CL_R = RAL(urine)/AUC, where RAL(urine) is the amount of raltegravir excreted in the urine during the dosing interval and the AUC is that in plasma during the same time interval. Terminal elimination, β, was calculated by least-square fitting of the concentrations at the last sample time points for each arm. The apparent fraction of raltegravir eliminated unchanged in the urine after oral dosing (Fe/*F*) was calculated as the ratio of Ae/(dose/*F*), where Ae is the amount excreted in urine. Note the dependence of both total plasma clearance and fractional excretion of unchanged raltegravir on oral bioavailability, *F*, such that both will be underestimated relative to intravenous

TABLE 1. Population demographics

Patient characteristic	Value
Age [median (range)]	28.6 years (24.3–51.1)
Weight [median (range)]	68.2 kg (49.3–86.0)
Race/ethnicity [no. (%)]	
Caucasian, non-Hispanic	9 (45)
Caucasian, Hispanic	3 (15)
Asian	5 (25)
African American	3 (15)
Female [no. (%)]	13 (65)

administration if F is less than 1. According to the package insert for raltegravir, absolute F has not been established.

Raltegravir and raltegravir-glucuronide concentrations in plasma and urine and AUCs were $\log_{10}$ transformed to satisfy assumptions of normality and first compared as ratios of sequence (i.e., arm A first or arm B). The GMRs for raltegravir C_{max} ($P = 0.97$), C_{12} ($P = 0.42$), or 12-h AUC ($P = 0.36$) were similar whether participants started with arm A or B, indicating that the crossover design did not bias the results, for example, due to carryover effects from the first study arm to the second arm; hence, sequence effect was omitted from further ratios of arm B to arm A, which were tested using the single-sample Student's t test for the probability of difference from 0 or $\log_{10}$ (1).

A *UGT1A1* diplotype score was generated for each participant by summing the function of both alleles, with −1 for reduced function (**28*, **37*), 0 for function similar to that of the reference allele (**1*, **81*), and +1 for increased function (**36*). The association between the *UGT1A1* score as an ordered categorical variable and the log-transformed raltegravir-glucuronide/raltegravir ratio at each sample time point was tested by linear mixed-effect modeling, with participants as the random effect and score and atazanavir use as the fixed effects. The association between score and the log-transformed ratio of AUCs for raltegravir-glucuronide and raltegravir was tested by multivariate linear regression, adjusting for atazanavir use. Fisher's exact test was used to test association between score and race/ethnicity.

RESULTS

Population characteristics. Twenty-one adults were screened for participation. One was excluded due to an abnormal electrocardiogram. A second volunteer completed arm A but dropped out of the study before starting arm B due to a relapse of her chronic gastroesophageal reflux that developed during her washout period and was not judged to be study related. Therefore, of the 20 enrolled, 19 participants completed both study arms. Demographic characteristics of the study population are shown in Table 1.

Adherence to the protocol was excellent. From MEMS logs and tablet or capsule counts, 524 (99.8%) of 525 prescribed doses were taken, with 478 (91.0%) at the prescribed time plus-or-minus 25% of the dosing interval. The MEMS logs were in good agreement with the written medication logs. As indicated on the written logs, 464 (88.3%) of the doses were taken in a semifasted state (not within 1 h before to 2 h after food), according to instructions. Study medications were well tolerated, with occasional mild nausea reported. Two participants developed rashes 1 and 2 days after completing the study (arm B in both cases) which were self-limited, did not interfere with daily activities, and judged as possibly study drug related. Both participants had tolerated arm A without incident. Repetition of the screening laboratories at the end of all study activities did not detect abnormalities in any participant.

Assay performance. In plasma and urine, the raltegravir and raltegravir-glucuronide mean bias and percent coefficient of variation (CV%) were each <5%. Likewise, plasma atazanavir had a mean bias and CV% of <5%.

Plasma pharmacokinetics. Plasma concentration-time profiles for raltegravir, raltegravir-glucuronide, and atazanavir are shown in Fig. 1. Pharmacokinetic characteristics of raltegravir and its metabolite in both arms A and B are shown in Table 2. With regard to the primary endpoint, the GMR of C_{tau} for arm B to arm A was 0.38, with a 95% confidence interval of 0.22 to

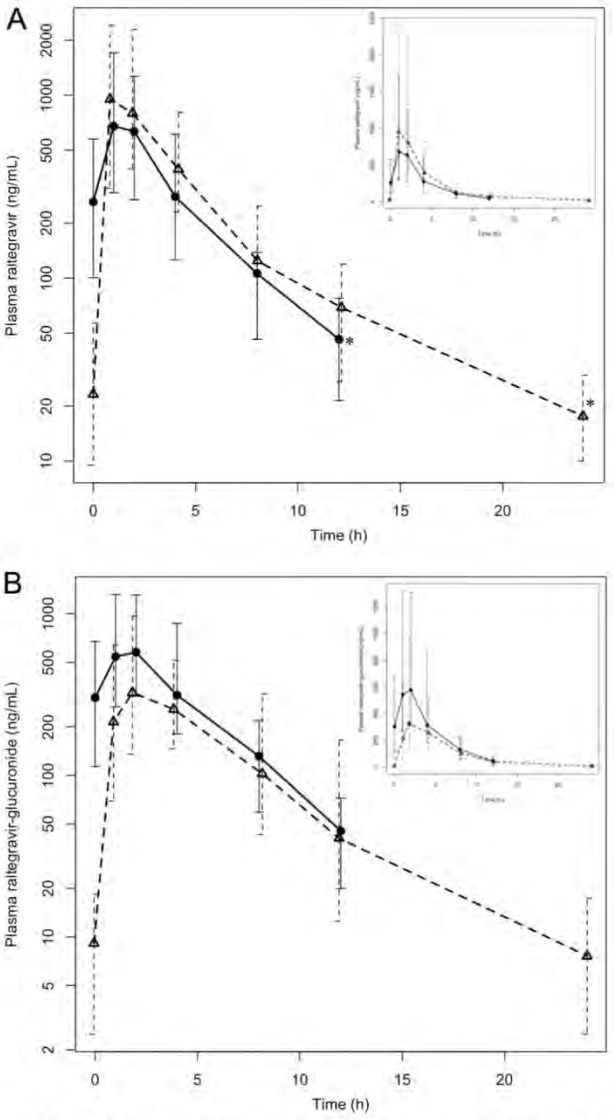

FIG. 1. Observed concentration-time profiles for raltegravir with and without atazanavir (A) and raltegravir-glucuronide with and without atazanavir (B). Solid and dashed dark lines are the geometric mean concentrations at each time point for arm A (raltegravir 400 mg twice daily) and arm B (raltegravir 400 mg plus atazanavir 400 mg once daily), respectively. Insets are in linear scales. *, comparison of these two concentrations (C_{tau}) formed the primary endpoint. Whisker bars indicate the geometric interquartile range.

TABLE 2. Comparison of raltegravir and raltegravir-glucuronide pharmacokinetics with and without coadministered atazanavir

Type of sample, drug, and pharmacokinetic parameter[a]	GM (range) of results for arm[b]		GMR for arm B/arm A (95% CI, *P* value)
	A	B	
Plasma			
Raltegravir			
C_{tau} (ng/ml)	46 (<10–257)	18 (4–100)	0.38 (0.22–0.65, 0.001)
C_{min} (ng/ml)	40 (<10–257)	13 (4–89)	0.32 (0.21–0.51, 0.001)
C_{max} (ng/ml)	1,016 (112–9,997)	1,402 (219–8,696)	1.37 (0.62–3.02, 0.40)
C_{12} (ng/ml)	46 (<10–257)	69 (11–1,812)	1.49 (0.59–3.75, 0.38)
AUC (ng · h/ml)			
0–12 h	3,532 (465–22,245)	4,657 (610–35,236)	1.32 (0.62–2.81, 0.45)
12–24 h	ND[c]	875 (177–16,517)	ND
0–tau	3,532 (465–22,245)	6,085 (840–41,303)	1.72 (0.79–3.75, 0.15)
a.m./p.m. trough	5.64 (0.29–71.00)	1.32 (0.30–34.50)	0.23 (0.08–0.69, 0.01)
β	0.26 (0.09–0.48)	0.11 (0.05–0.30)	0.45 (0.31–0.66, 0.001)
Raltegravir-glucuronide			
C_{tau} (ng/ml)	45 (<10–512)	8 (3–92)	0.16 (0.09–0.30, 0.001)
C_{min} (ng/ml)	39 (<10–512)	5 (3–85)	0.14 (0.08–0.22, 0.001)
C_{max} (ng/ml)	799 (157–3,264)	458 (50–2,507)	0.57 (0.29–1.12, 0.10)
C_{12} (ng/ml)	45 (<10–511)	41 (3–1,271)	0.90 (0.31–2.61, 0.84)
AUC (ng · h/ml)			
0–12 h	3,267 (562–12,466)	2,036 (181–13,230)	0.62 (0.33–1.20, 0.14)
12–24 h	ND	342 (0–5,361)	ND
Raltegravir-glucuronide AUC_{0-12}/raltegravir AUC_{0-12}	0.95 (0.39–2.14)	0.43 (0.23–0.96)	0.47 (0.38–0.59, 0.001)
β	0.30 (0.16–0.46)	0.14 (0.04–0.30)	0.60 (0.43–0.84, 0.006)
Urine			
Raltegravir			
Amt (mg)			
0–12 h	11.66 (1.80–102.03)	10.81 (1.96–48.68)	0.93 (0.43–2.00, 0.84)
12–24 h	ND	0.41 (0.046–4.08)	ND
Raltegravir-glucuronide			
Amt (mg)			
0–12 h	82.50 (18.57–239.65)	84.51 (18.16–325.83)	1.02 (0.53–1.99, 0.94)
12–24 h	ND	7.03 (2.02–59.74)	ND
Raltegravir-glucuronide/raltegravir [amt (mg) 0–12 h]	7.08 (1.94–13.32)	7.81 (3.68–24.73)	1.10 (0.90–1.36, 0.33)
Raltegravir clearance			
$CL_{r/F}$ (ml/min)	1,887 (300–14,352)	1,096 (161–7,940)	0.58 (0.27–1.26, 0.16)
CL_R (ml/min)	55 (25–264)	39 (11–84)	0.70 (0.49–1.01, 0.06)
Fe/*F* (%)	2.9 (0.5–25.5)	3.5 (0.7–27.5)	1.21 (0.53–2.77, 0.63)
Raltegravir-glucuronide CL_R (ml/min)	421 (151–1,559)	692 (221–1,790)	1.64 (1.11–2.44, 0.02)

[a] C_{tau}, concentration at the end of the dosing interval, i.e., 12 h for arm A and 24 h for arm B; C_{min}, minimum concentration (regardless of time, not always at time tau, the end of the dosing interval); C_{max}, maximum concentration; C_{12}, 12–h concentration; AUC, area under the concentration-time curve; β, terminal slope, such that ln(2)/β is half-life; $CL_{r/F}$, apparent total plasma clearance of oral drug; CL_R, renal clearance of plasma drug; Fe/*F*, apparent fraction of oral drug excreted unchanged.

[b] Arm A, raltegravir 400 mg twice daily; arm B, raltegravir 400 mg + atazanavir 400 mg once daily.

[c] ND, not determined.

0.65, which did not meet the study definition of sufficient raltegravir boosting by atazanavir—a threshold of ≥0.4 for the lower boundary of the 95% confidence interval. The GM plasma raltegravir C_{max}, C_{12}, and 12-hour AUC were 37%, 49%, and 32% higher, respectively, in arm B, but none of these were statistically significant. When dosed with atazanavir, the GM total apparent plasma clearance of raltegravir was 42% lower than when dosed alone, but this difference was not significant due to nearly 50-fold intrasubject variability in both study arms. The terminal raltegravir concentration-time slope, β [ln(2)/β = half life], was significantly lower in arm B, indicating that atazanavir slowed the elimination of raltegravir, but the atazanavir AUC was not significantly associated with β in arm B ($P = 0.86$).

Of note, in arm A the geometric mean (GM) predose (C_0) morning concentration is over 5-fold higher than the evening C_{12}, suggesting a circadian or diurnal variation in raltegravir absorption when dosed twice daily. For this reason, we did not try to extrapolate the 12-hour raltegravir AUC in arm A to 24 h by doubling it.

Just as the raltegravir-glucuronide C_{tau} and C_{min} were lower in arm B than in arm A (Table 2) for raltegravir, the C_{max}, C_{12}, and 12-h AUC were similar between the two arms, and β was reduced in arm B. However, the 12-h AUC for raltegravir-

TABLE 3. Participant's *UGT1A1* genotype, functionality score, race, ethnicity, and plasma raltegravir-glucuronide/raltegravir geometric mean ratio by study arm

Participant	Race	Hispanic	Genotype[a]	Score[b]	Plasma raltegravir-glucuronide/raltegravir GMR in arm[c]	
					A	B
1	Caucasian	No	*1/*1	0	1.00	0.45
2	Caucasian	Yes	*1/*1	0	0.97	0.68
3	Caucasian	No	*1/*28	−1	0.81	0.53
4	Caucasian	No	*1/*28	−1	1.99	1.04
5	Asian	No	*1/*28	−1	1.09	0.60
6	Caucasian	No	*1/*1	0	0.98	0.25
7	Asian	No	*1/*1	0	2.16	0.54
8	Caucasian	Yes	*1/*1	0		
9	Caucasian	No	*28/*28	−2	0.47	0.27
10	Caucasian	No	*1/*28	−1	1.50	0.62
11	Asian	No	*1/*1	0	1.25	0.51
12	Caucasian	No	*1/*1	0	1.76	0.42
13	Asian	No	*1/*81	0	0.51	0.33
14	Asian	No	*1/*81	0	0.70	0.52
15	African American	No	*1/*28	−1	1.04	0.40
16	Caucasian	Yes	*1/*X	−1	1.02	0.52
17	African American	No	*1/*28	−1	0.77	0.43
18	Caucasian	No	*1/*28	−1	1.02	0.45
19	African American	No	*1/*28	−1	1.00	0.69
20	Caucasian	No	*1/*1	0	0.93	0.31
Avg					1.02†	0.47†

[a] **1*, reference sequence $A(TA)_6TAA$; **28*, $A(TA)_7TAA$; **81*, g.-64G>C; **X* $A(TA)_9TAA$.

[b] Score, *UGT1A1* score as described in Materials and Methods.

[c] Arm A, raltegravir alone; arm B, raltegravir with atazanavir. Participant 8 did not complete the pharmacokinetic study but was genotyped. †, $P < 0.0001$.

glucuronide relative to that of raltegravir was 53% lower when coadministered with atazanavir than without atazanavir ($P < 0.001$).

Urinary data. All participants reported full collection of urine from 12 to 24 h during the arm B pharmacokinetic study. The GM (range) volume collected during this interval was 1.0 (0.5 to 2.0) ml/kg of bodyweight/h, with an interquartile range of 0.8 to 1.4, indicating no outliers. As shown in Table 2, urinary clearance of unchanged raltegravir, consistent with extensive metabolism, was minimal relative to total clearance, with an apparent fractional excretion of about 3%, which was similar between the two study arms. Urinary clearance of raltegravir-glucuronide was higher when dosed with atazanavir, but the significance of this is unclear.

Glucuronidation and UGT1A1 genotypes. *UGT1A1* genotypes are shownin Table 3. All 20 enrolled participants were genotyped, but as mentioned, participant 8 did not complete the pharmacokinetic study. Eight (40%) of the 20 were homozygous for the reference allele (**1*), with 6 TA repeats in the TATA box. Nine (45%) were heterozygous for promoter variants associated with reduced UGT1A1 function. All but one of these were carrying *UGT1A1*28*. The remaining reduced-function allele, which we have termed **X*, had 9 TA repeats in the TATA box (8) and occurs with such low frequency that it has not received an official designation. One participant (5%) was homozygous for *UGT1A1*28*, which is associated with Gilbert's syndrome. All patients had normal bilirubin at screening and at completion of the study. There were no increased-function alleles in this small population. By Fisher's exact test, there was no association between genotype and race ($P = 0.16$) or ethnicity ($P = 0.19$).

Although the only participant who was homozygous for 2 reduced-function alleles had the lowest raltegravir-glucuronide/raltegravir ratio regardless of atazanavir dosing, overall by linear mixed-effect modeling, participants with 1 or 2 reduced-function alleles had on average a nearly identical ratio compared to those with the reference *UGT1A1*1* (1.08-fold versus the reference; 95% CI 0.80 to 1.44, $P = 0.90$) when adjusted for the effect of atazanavir exposure (0.46-fold versus no atazanavir; 95% CI 0.39 to 0.54, $P < 0.001$). By linear regression, again adjusting for atazanavir, the ratio of the raltegravir-glucuronide AUC to the raltegravir AUC was no different in those with reduced-function alleles than in those with the reference allele (1.19-fold change; 95% CI 0.62 to 2.30, $P = 0.59$).

Atazanavir plasma levels. The atazanavir GM (range) C_{max} was 3,439 (290 to 6,299) ng/ml, C_{24} was 83 (13 to 495) ng/ml, and AUC was 18.2 (2.8 to 47.2) μg · h/ml. Given that participants took atazanavir on an empty stomach, these observations agree closely with those reported in the Reyataz package insert (http://www.bms.com/products/Pages/prescribing.aspx) for healthy volunteers at steady state with 400 mg once daily, when corrected for fasting conditions that lower C_{max} and AUC by 64% and 58%, respectively, to values of 3,412 ng/ml and 17.2 μg · h/ml.

DISCUSSION

In this healthy adult volunteer population, despite reduced formation of plasma raltegravir-glucuronide, there were only small (and nonsignificant) increases in raltegravir plasma concentrations when raltegravir was dosed once daily with atazanavir. We found that the degree of raltegravir boosting in plasma by atazanavir was insufficient to ensure that raltegravir 400 mg once daily would result in concentrations at the end of the dosing interval that would be at least similar to those with current standard dosing. Overall, at steady state, the boosting effect of atazanavir (without ritonavir) on raltegravir was less than in a previously reported single-dose study (11).

Does the lack of boosting by atazanavir mean that once-daily raltegravir dosing is out of the question? We do not believe that our data force this conclusion, for three reasons. The first reason is that a minimally effective plasma concentration relative to *in vitro* viral susceptibility has not been established for raltegravir. In early dose-ranging studies, 100 mg twice daily in treatment-naïve patients had the same 48-week virologic and immunologic efficacy as doses up to 600 mg twice daily (15). In triple-class experienced patients, the 24-week virologic efficacy was the same in the 200-mg twice-daily group as in the 600-mg twice-daily group, although the CD4 cell count gains in the 200-mg group were less than in the other groups (7).

The second reason to consider once-daily raltegravir dosing as a still-viable strategy is the persistent binding of the drug to its intracellular target, the preintegration complex. Although there are as yet few published data on the intracellular pharmacokinetics of raltegravir, it is known *in vitro* to disassociate from the preintegration complex with a half-time that is longer than the half-time of the complex itself, effectively making the binding irreversible (9). This may be analogous to zidovudine: recognition that the active moiety had a prolonged intracellu-

lar half-life enabled a decrease in dosing frequency from five times to twice daily (20). We did not measure intracellular concentrations in this study.

The third reason that we cannot exclude the possibility of 400-mg once-daily dosing is simply that this was a healthy-volunteer study that cannot provide any efficacy data. Without a clearly defined pharmacokinetic-pharmacodynamic target, extrapolation to an effect in HIV-infected patients is impossible.

Although 400-mg once-daily raltegravir (50% of the currently recommended dose for both naïve and experienced patients) is attractive from cost and adherence perspectives, and despite the previous arguments, data from this study nonetheless highlight concerns with this dosing strategy. First, as is obvious from the results in Fig. 1 and the wide concentration ranges shown in Table 2, absorption of this drug is clearly erratic, even in the relatively controlled circumstances of this clinical study, and this raises the worrisome possibility that some patients with once-daily dosing may have particularly low concentrations, which may compromise efficacy if and when a concentration-response relationship can be described.

Second, our overall observed plasma concentrations were lower than those reported by the manufacturer in phase I studies of the same dose in young, healthy, adult participants. The AUC from 0 to 12 h ($AUC_{0–12}$) for arm A was only 31% (10) or 46% (11) of the reported $AUC_{0–12}$, depending on the study. However, our $AUC_{0–12}$ is very similar to that found in other pharmacokinetic studies that included a group receiving raltegravir 400 mg twice daily (2, 12, 13, 19). Despite the lack of food-drug interaction reported in the package insert (http://www.isentress.com), food has subsequently been shown to have a major effect on the absorption profile (18), and this may have caused the diminished concentrations relative to the more stringent fasting conditions in the manufacturer's studies, since participants in our study were allowed to eat 1 h after a dose. These lower concentrations cause one to question what concentrations were achieved in the large phase III efficacy studies, with less regulation of food intake, since none have actually reported raltegravir concentrations in participants with or without virologic suppression.

Our data suggested a circadian rhythm to raltegravir pharmacokinetics, evidenced by the arm A morning raltegravir trough concentration that was 5-fold higher than in the evening. We are the first to report this, but careful inspection of the previous pharmacokinetic study (see their Fig. 1B, inset) with atazanavir from the manufacturer shows a 3-fold higher morning trough compared to the evening trough (11). In our study, when dosed once daily, this trough-to-trough variation was not noted. Because of the similar rate of raltegravir decline in both arms, as shown in Fig. 1, this circadian rhythm is likely due to differential absorption patterns of morning versus evening doses. However, without overnight data, we are unable to verify this hypothesis. The significance is that one should use caution if extrapolating 12-h to 24-h raltegravir exposures for a regimen with two daily doses.

Especially when one considers the recent, disappointing results of the SWITCHMRK study, in which treatment-experienced, virologically suppressed patients who switched their boosted lopinavir to raltegravir were more likely to lose virologic control than those who continued lopinavir (4), it would seem that if a once-daily 400-mg raltegravir strategy were to have any application, efficacy from the ongoing 800-mg once-daily study (http://www.clinicaltrials.gov/ct2/show/NCT00745823) must first be demonstrated and the relationship of plasma and/or intracellular raltegravir concentrations to efficacy should be established.

UGT1A1 genetic variants, while common even in this small population, did not significantly influence the degree of raltegravir glucuronidation or raltegravir plasma exposure. This is in contrast to the modest effect on plasma exposure demonstrated in a previous study (21). It is likely that the high degree of variability in raltegravir plasma concentrations in our study obscured any pharmacogenomic effects on either overall raltegravir exposure or the degree of glucuronidation. Furthermore, only one of the 19 patients with genomic and pharmacokinetic data had a homozygous reduced-function diplotype (**28*/**28*). Lastly, it is possible that there were polymorphisms in other regions of the gene that we did not sequence but that are associated with changes in function (e.g., **6* and **60*) (1). However, these are present at a much lower frequency in the racial/ethnic groups in this study (1, 14) and, therefore, would be unlikely to contribute significantly to the substantial observed variation in raltegravir pharmacokinetics.

In summary, although atazanavir reduced the formation of the glucuronide metabolite, its steady-state boosting did not render a once-daily raltegravir dose of 400 mg pharmacokinetically similar to the standard twice-daily dose in terms of the concentration at the end of the dosing interval. Conversely, the observed concentrations of atazanavir when dosed with raltegravir were similar to those in the package insert, when adjusted for fed state, indicating that raltegravir did not influence the pharmacokinetics of atazanavir. As the relationship between raltegravir plasma exposure and efficacy is unclear, there may still be a rationale to test a simpler and cheaper single 400-mg daily dose of raltegravir, but because of highly variable interindividual plasma concentrations, it should be tested in a pilot study that includes measurement of extra- and intracellular drug concentrations. Atazanavir in such a trial would appear to add no pharmacological benefit. Results from the ongoing study of 800 mg once daily and better characterization of the relationship between plasma/intracellular concentrations and efficacy are crucial prerequisites of any study of reduced-dose raltegravir.

ACKNOWLEDGMENTS

We thank Andrew Hill for his advice and the study volunteers for their time and effort.

This work was supported by National Institute of Allergy and Infectious Diseases grant no. K23 AI076106 (M.N.), National Institute of Biomedical Imaging and Bioengineering grant no. R01 EB005803 (R.J. and M.N.), Swiss National Science Foundation (SNF) grants no. 324700-112655 and 32430-124943 (A.F. and J.D.), SNF REQUIP grant no. 326000-121314/1 (L.D.), and a grant from Médecins Sans Frontières.

REFERENCES

1. **Beutler, E., T. Gelbart, and A. Demina.** 1998. Racial variability in the UDP-glucuronosyltransferase 1 (UGT1A1) promoter: a balanced polymorphism for regulation of bilirubin metabolism? Proc. Natl. Acad. Sci. U. S. A. **95:**8170–8174.
2. **Blanco, J., M. Martinez-Rebollar, M. Calvo, I. Perez, M. Larrousse, M. Martin, R. Lopez, M. Brunet, J. Gatell, and J. Mallolas.** 2009. Pharmacokinetic interaction between raltegravir and tipranavir in HIV-1 infected patients, abstr. P-23. 10th Int. Workshop Clin. Pharmacol. HIV Ther.
3. **Colombo, S., A. Beguin, A. Telenti, J. Biollaz, T. Buclin, B. Rochat, and L. Decosterd.** 2005. Intracellular measurements of anti-HIV drugs indinavir,

amprenavir, saquinavir, ritonavir, nelfinavir, lopinavir, atazanavir, efavirenz and nevirapine in peripheral blood mononuclear cells by liquid chromatography coupled to tandem mass spectrometry. J. Chromatogr. B. **819:**259–276.

4. **Eron, J., J. Andrade, R. Zajdenverg, C. Workman, D. Cooper, B. Young, X. Xu, B. Nguyen, R. Leavitt, and P. Sklar.** 2009. Switching from stable lopinavir/ritonavir-based to raltegravir-based combination ART resulted in a superior lipid profile at week 12 but did not demonstrate non-inferior virologic efficacy at week 24, abstr. 70aLB. 16th Conf. Retrovir. Opportunistic Infect.
5. **Fayet, A., A. Béguin, B. Zanolari, S. Cruchon, N. Guignard, A. Telenti, M. Cavassini, H. F. Günthard, T. Buclin, J. Biollaz, B. Rochat, and L. A. Decosterd.** 2009. A LC-tandem MS assay for the simultaneous measurement of new antiretroviral agents: raltegravir, maraviroc, darunavir, and etravirine. J. Chromatogr. B. **877:**1057–1069.
6. **Foisy, M. M., E. M. Yakiwchuk, and C. A. Hughes.** 2008. Induction effects of ritonavir: implications for drug interactions. Ann. Pharmacother. **42:**1048–1059.
7. **Grinsztejn, B., B. Nguyen, C. Katlama, J. M. Gatell, A. Lazzarin, D. Vittecoq, C. J. Gonzalez, J. Chen, C. M. Harvey, and R. D. Isaacs.** 2007. Safety and efficacy of the HIV-1 integrase inhibitor raltegravir (MK-0518) in treatment-experienced patients with multidrug-resistant virus: a phase II randomised controlled trial. Lancet **369:**1261–1269.
8. **Guillemette, C., R. C. Millikan, B. Newman, and D. E. Housman.** 2000. Genetic polymorphisms in uridine diphospho-glucuronosyltransferase 1A1 and association with breast cancer among African Americans. Cancer Res. **60:**950–956.
9. **Hazuda, D., M. Iwamoto, and L. Wenning.** 2009. Emerging pharmacology: inhibitors of human immunodeficiency virus integration. Annu. Rev. Pharmacol. Toxicol. **49:**377–394.
10. **Iwamoto, M., L. Wenning, A. Petry, M. Laethem, M. De Smet, J. Kost, S. Merschman, K. Strohmaier, S. Ramael, K. Lasseter, J. Stone, K. Gottesdiener, and J. Wagner.** 2008. Safety, tolerability, and pharmacokinetics of raltegravir after single and multiple doses in healthy subjects. Clin. Pharmacol. Ther. **83:**293–299.
11. **Iwamoto, M., L. A. Wenning, G. C. Mistry, A. S. Petry, S. Y. Liou, K. Ghosh, S. Breidinger, N. Azrolan, M. J. Gutierrez, W. E. Bridson, J. A. Stone, K. M. Gottesdiener, and J. A. Wagner.** 2008. Atazanavir modestly increases plasma levels of raltegravir in healthy subjects. Clin. Infect. Dis. **47:**137–140.
12. **Jackson, A., G. Moyle, V. Watson, D. Back, S. Khoo, C. Higgs, K. Armenis, A. Pozniak, B. Gazzard, and M. Boffito.** 2009. Variability in steady-state raltegravir pharmacokinetics, impact of ezatimibe? abstr. P-25. 10th Int. Workshop Clin. Pharmacol. HIV Ther.
13. **Jones, A., J. Talameh, K. Patterson, N. Rezk, H. Prince, and A. Kashuba.** 2009. First-dose and steady-state pharmacokinetics of raltegravir in the genital tract of HIV-uninfected women, abstr. O-86. 10th Int. Workshop Clin. Pharmacol. HIV Ther.
14. **Kaniwa, N., K. Kurose, H. Jinno, T. Tanaka-Kagawa, Y. Saito, M. Saeki, J. Sawada, M. Tohkin, and R. Hasegawa.** 2005. Racial variability in haplotype frequencies of UGT1A1 and glucuronidation activity of a novel single nucleotide polymorphism 686C> T (P229L) found in an African-American. Drug Metab. Dispos. **33:**458–465.
15. **Markowitz, M., B. Nguyen, E. Gotuzzo, F. Mendo, W. Ratanasuwan, C. Kovacs, G. Prada, J. O. Morales-Ramirez, C. S. Crumpacker, R. D. Isaacs, L. R. Gilde, H. Wan, M. D. Miller, L. A. Wenning, and H. Teppler.** 2007. Rapid and durable antiretroviral effect of the HIV-1 integrase inhibitor raltegravir as part of combination therapy in treatment-naive patients with HIV-1 infection: results of a 48-week controlled study. J. Acquir. Immune Defic. Syndr. **46:**125–133.
16. **Medecins Sans Frontieres.** 2009. Untangling the Web of antiretroviral price reductions. Campaign for Access to Essential Medicines, Medecins Sans Frontieres, Geneva, Switzerland.
17. **Monaghan, G., M. Ryan, R. Seddon, R. Hume, and B. Burchell.** 1996. Genetic variation in bilirubin UPD-glucuronosyltransferase gene promoter and Gilbert's syndrome. Lancet **347:**578–581.
18. **Nachman, S., E. Acosta, A. Wiznia, H. Teppler, M. Long, B. Homony, B. Graham, C. Worrell, P. Samson, E. Handelsman, and P1066 Protocol Team.** 2008. Raltegravir pharmacokinetics and safety in adolescents: preliminary results from IMPAACT P1066, abstr. H-4059a. Abstr. 48th Intersci. Conf. Antimicrob. Agents Chemother. (ICAAC)-Infect. Dis. Soc. Am. (IDSA) 46th Annu. Meet. American Society for Microbiology and Infectious Diseases Society of America, Washington, DC.
19. **Rhame, F., M. Long, and E. Acosta.** 2008 Pharmacokinetics (PK) of coadministered raltegravir (RAL) and lopinavir-ritonavir (KAL) in healthy adults, abstr. TUPE0075. XVII Int. AIDS Conf., Mexico City, Mexico.
20. **Stretcher, B. N., A. J. Pesce, J. A. Murray, P. E. Hurtubise, W. H. Vine, and P. T. Frame.** 1991. Concentrations of phosphorylated zidovudine (ZDV) in patient leukocytes do not correlate with ZDV dose or plasma concentrations. Ther. Drug Monit. **13:**325–331.
21. **Wenning, L., A. Petry, J. Kost, B. Jin, S. Breidinger, I. DeLepeleire, E. Carlini, S. Young, T. Rushmore, F. Wagner, N. Lunde, F. Bieberdorf, H. Greenberg, J. Stone, J. Wagner, and M. Iwamoto.** 2009. Pharmacokinetics of raltegravir in individuals with UGT1A1 polymorphisms. Clin. Pharmacol. Ther. **85:**623–627.
22. **World Health Organization.** 1 January 2006. Antiretroviral therapy for HIV infection in adults and adolescents. World Health Organization, Geneva, Switzerland.
23. **World Health Organization.** 30 November 2009. Rapid advice: antiretroviral therapy for HIV infection in adults and adolescents. World Health Organization, Geneva, Switzerland.
24. **World Health Organization.** 30 September 2009. Towards universal access: scaling up priority HIV/AIDS interventions in the health sector. September 2009 progress report. World Health Organization, Geneva, Switzerland.

9.3. Original article [Hepatology. 2011 Feb;53(2):396-405]

Effect of Immune Pressure on Hepatitis C Virus Evolution: Insights From a Single-Source Outbreak

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The host's immune response to hepatitis C virus (HCV) can result in the selection of characteristic mutations (adaptations) that enable the virus to escape this response. The ability of the virus to mutate at these sites is dependent on the incoming virus, the fitness cost incurred by the mutation, and the benefit to the virus in escaping the response. Studies examining viral adaptation in chronic HCV infection have shown that these characteristic immune escape mutations can be observed at the population level as human leukocyte antigen (HLA)–specific viral polymorphisms. We examined 63 individuals with chronic HCV infection who were infected from a single HCV genotype 1b source. Our aim was to determine the extent to which the host's immune pressure affects HCV diversity and the ways in which the sequence of the incoming virus, including preexisting escape mutations, can influence subsequent mutations in recipients and infection outcomes. *Conclusion:* HCV sequences from these individuals revealed 29 significant associations between specific HLA types within the new hosts and variations within their viruses, which likely represent new viral adaptations. These associations did not overlap with previously reported adaptations for genotypes 1a and 3a and possibly reflected a combination of constraint due to the incoming virus and genetic distance between the strains. However, these sites accounted for only a portion of the sites in which viral diversity was observed in the new hosts. Furthermore, preexisting viral adaptations in the incoming (source) virus likely influenced the outcomes in the new hosts. (HEPATOLOGY 2010;000:000-000)

After infection with hepatitis C virus (HCV), outcomes are variable: spontaneous resolution of the infection is observed in approximately 30% of individuals, but for others, chronic infection develops. Factors such as age, gender, and host genetic variants have been associated with different infection

Abbreviations: HCV, hepatitis C virus, HLA, human leukocyte antigen, IL-28B, interleukin-28B, MHC, major histocompatibility complex, NS, nonstructural, OR, odds ratio, SNP, single-nucleotide polymorphism.

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Received April 25, 2010; accepted November 5, 2010.

This work was supported by the National Health and Medical Research Council of Australia (program grant 384702), the Wellcome Trust, the National Institute for Health Research through the Biomedical Research Centre Programme (Oxford, United Kingdom), the James Martin 21st Century School (Oxford, United Kingdom), and the National Institutes of Health through a U19 agreement (National Institute of Allergy and Infectious Diseases grant 1U19AI082630-01). The contribution to this project by Shahzma Merani was conducted while she was receiving a University International Stipend award and a Scholarship for International Research Fees award from the University of Western Australia as well as funding from the Centre for Clinical Immunology and Biomedical Statistics of Murdoch University.

The hepatitis C virus sequences described in this article have been submitted to GenBank with the following accession numbers: HM106522 to HM106981.

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outcomes[1,2] (reviewed by Rauch et al.[3]). Study cohorts that capture all individuals exposed to the virus, such as HCV single-source outbreak cohorts[4,5] and cohorts of individuals who have a high risk of HCV exposure,[6] have been particularly important in delineating relevant viral and host factors associated with the outcome of HCV infection. Such studies corroborate other studies indicating that a host's T cell response to HCV, including genes involved in regulating this response, is an important correlate of infection outcome.[7-11]

T cell immune responses are stimulated by the presentation of processed viral peptides (epitopes) by human leukocyte antigen (HLA) molecules to $CD4^+$ and $CD8^+$ T cells. This host-virus interaction is dependent on the sequence of the viral epitope and surrounding regions, which play a role in peptide processing and presentation to T cells. Viral adaptations can reduce the binding affinity of the peptide to the HLA molecule and result in poor peptide cleavage or poor T cell recognition; these factors can subvert host immune control (reviewed by Bowden and Walker[12]). The importance of immune control in HCV infection has been illustrated in studies showing that mutations in $CD8^+$ T cell epitopes contribute to viral persistence in both chimpanzees and humans.[13,14] Accordingly, the extent to which the virus can adapt to the host's immune response is likely to be an important factor in determining infection outcome. These adaptations are dependent on the sequence of the incoming virus and the balance between the fitness cost incurred by these mutations[15] and their benefit to the virus due to immune escape.

It is unclear how much genetic diversity observed in HCV is the result of host immune pressures. Recent studies have suggested that viral adaptation can be observed at both the individual level[16,17] and the population level.[18,19] For example, genetic studies examining HCV sequences in the context of the HLA repertoire of a host population have shown associations between specific polymorphisms across the viral genome and HLA types within individuals in a host population.[18,19] These HLA-associated viral polymorphisms are thought to represent viral adaptations and tag regions of the viral genome that are under *in vivo* T cell pressure. However, HCV evolution is shaped by evolutionary forces that include genetic drift and both positive and purifying selection pressures.[20,21] It is likely that all these factors exert their influence simultaneously on the virus and affect the ability of the virus to adapt to new selection pressures and/or revert in a new host.

A previous study of an Irish HCV single-source cohort showed evidence of immune selection in known T cell targets.[22] In this study, we compared HCV sequences from 63 individuals with genotype 1b infection from this single-source outbreak[5] to identify sites likely representing new T cell targets in the HCV genome and to determine the extent to which host immune pressures on the virus affected sequence diversity in the cohort. Knowledge of the incoming viral sequence also allowed us to determine whether preexisting viral adaptations could predict beneficial or detrimental host HLA alleles within the cohort with respect to infection outcomes.

Patients and Methods

Study Population. The study population was part of a cohort of women who had been infected with HCV between May 1977 and November 1978 in Ireland through the administration of anti-D immunoglobulin that had been contaminated with an HCV genotype 1b virus originating from a single individual.[5] From this original cohort, we studied 63 individuals with chronic HCV infection; a subset (n = 15) was selected on the basis of the carriage of HLA-A*03, an allele that was previously shown to be protective in this cohort.[8] A comparison of the HLA alleles found in this cohort and those in another Irish population is in the Supporting Information.

Serum samples from the subjects were collected between 1996 and 2002 and were stored at −80°C. Written, informed consent was obtained from participants, and local institutional review board approval was obtained by all centers contributing to the study.

Viral RNA Extraction. Viral RNA was extracted from serum samples with the QIAamp Viral RNA mini kit (Qiagen) or the Cobas Amplicor HCV specimen preparation kit (version 2.0, Roche) according to each manufacturer's instructions.

HLA Genotyping. Two-digit resolution HLA class I (HLA-A, HLA-B, and HLA-C) typing was performed at St. James Hospital (Dublin, Ireland).[8]

Interleukin-28B (IL-28B) Genotyping. Genotyping of the single-nucleotide polymorphism (SNP) rs12979860 upstream of the *IL-28B* gene was performed for 34 subjects as previously described.[23]

Bulk Viral Sequencing. HCV sequencing was performed as previously described.[18,19] Briefly, three reverse-transcription polymerase chain reactions were performed to cover the core to nonstructural 5B (NS5B) region. The first-round products were used as templates in nested second-round polymerase chain reactions containing generic or genotype-specific primers. Amplicons were bulk-sequenced with the BigDye

Terminator version 3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's recommendations, and electropherograms were edited with Assign (Conexio Genomics). Mixtures were identified in which the secondary peak was greater than 20% of the main peak.

HCV sequences in this study have been submitted to GenBank (accession numbers HM106522 to HM106981). Supporting Information Table 1 lists the mean sequence coverage by protein.

An analysis of the viral sequences for testing the single-source nature of this outbreak can be found in the Supporting Information.

Ultradeep Sequencing. To identify minor quasispecies below the detection threshold of bulk sequencing methods, ultradeep sequencing was carried out with the 454 Life Sciences platform (Roche Applied Science) for two individuals (HLA-A*03$^+$/HLA-B*08$^-$ and HLA-A*03$^-$/HLA-B*08$^-$). With the previously described amplification method, polymerase chain reaction templates were obtained that covered NS3 (positions 3494-4530) and NS5A to NS5B (positions 7335-8356). Amplicons were quantified and pooled for each individual. Library preparation and sequencing were performed according to the manufacturer's protocol. Data were collected and analyzed with Roche and public license software programs. All sequence reads were aligned to the source sequence (AF313916) with GS Reference Mapper software (Roche). The threshold for mixtures was set at 1% with 100-fold or greater coverage.

HLA-Associated Viral Polymorphisms. Associations between HLA alleles and amino acid distributions at each residue of the HCV proteins were assessed with Fisher's exact test for classification as consensus or nonconsensus amino acid. A false discovery rate analysis was carried out, and q values were obtained as reported previously.[19] Only sequences with $\geq$50% sequence coverage for each respective protein were used. Analyses were carried out with Spotfire S+ 8.1 (TIBCO, Somerville, MA). Associations with a P value $\leq$0.01 for Fisher's exact test of consensus versus nonconsensus are reported. An assessment of possible confounding by founder effects via viral cluster stratification and the Mantel-Haenszel procedure, as described by Rauch et al.,[19] indicated that no correction for significant associations was necessary, and this was consistent with the sequences originating from a single source. In addition, because P values associated with relatively small frequencies can be affected by small numbers of misclassified cases, we restricted our analysis to associations for which there were five or more nonconsensus amino acids and five or more carriers of the HLA allele.

Sliding-Window Analysis. In order to identify viral escape that might not be captured with a single amino acid approach, an analysis was conducted as described previously, except that *adaptation* was defined as nonconsensus at any residue within sliding windows of nine amino acids, which represented typical peptide sizes for HLA class I molecules. Significant sites of associations were identified as strings of significant values, whereas the window slid over any residues containing strong associations or combinations of associations. We restricted the analysis to cases that had all amino acids in the window. Associations with $P \leq 0.01$ were reported.

Covariation. Residue covariation was assessed with Fisher's exact test for classification as consensus or nonconsensus amino acid. Covariation based on a sequence with $\geq$90% coverage was reported; covarying sites had $P \leq 0.001$ for amino acid versus amino acid comparison and $P \leq 0.0001$ for amino acid versus nucleotide comparison. Because of the exploratory nature of this part of the analysis, no adjustment was made for multiple comparisons.

Peptide Prediction for HLA-Associated Viral Polymorphism Sites. Flanking sequences of the identified HLA-associated viral polymorphisms and sites of common divergence from the source sequence were entered into the epitope prediction software SYFPEITHI[24] to identify putative epitopes based on a cutoff score of 20 with the highest scoring peptide reported. HLA-associated viral polymorphism sites were compared against published genotype 1 epitopes found in the Immune Epitope Database (http://www.immuneepitope.org).

Viral Sequence Diversity. Sequence diversity from the source sequence (AF313916) was determined with the Highlighter program (available at http://www.lanl.gov) for NS3 and NS5B to identify sites of synonymous and nonsynonymous substitutions for sequences with greater than 50% sequence coverage. Genetic diversity was determined with the Kimura two-parameter model, and differences in the rate of nonsynonymous and synonymous changes (ds/dn) were obtained with the modified Nei and Gojobori method with MEGA version 3.1.[25]

IL-28B–*Associated Viral Polymorphisms.* We assessed associations between the presence or absence of the minor allele rs12979860 and consensus or nonconsensus amino acids at each residue of the HCV proteins via Fisher's exact test. Because of the smaller number of subjects with typing available for this part of the analysis, no assessment of false

Table 1. HLA Class I-Associated Viral Polymorphisms

Protein	Residue	Source Amino Acid	Cohort Amino Acid†	Variant Amino Acid in the Cohort	HLA	OR	*P* Value	*q* Value	Sliding Window*	Predicted Epitope	Published Epitope‡
E1	299	E	E	V/R/Q/L/K	C*16	24.00	0.003	0.298	291, 292, 298, 299		
	373	I	I	V	C*16	18.00	0.006	0.357	365		
E2	393	A	A	G	C*06	11.00	0.008	0.739			
	397	R	S	H/R/L/F/S/N	A*01	0.05	0.010	0.739			
	400	A/T	T	A/V	C*07	0.08	0.005	0.730			
	403	F	F	L	A*02	30.00	0.004	0.730			SLLAPGAKQNV
	405	S	S	T/A	C*16	0.04	0.006	0.730			
	577	D	D	N/B	A*01	0.12	0.010	0.739			
	625	T	T	S	B*08	11.00	0.006	0.730			
P7	790	F	F	I/I	B*07	11.00	0.001	0.037	786-794		
NS2	834	H	H	Q	B*08	15.00	0.001	0.045	836-838	SPHYKVFL	
	951	D	D	N	A*02	12.00	0.003	0.108	950-955		
					C*05	11.00	0.004	0.148			
NS3	1040	L	L	F	B*07	12.00	0.007	0.162	1036-1038		
	1087	T	T	A	A*03	17.00	0.001	0.030	1083-1091	TVYHGAGTK	
	1088	K	K	R	A*03	57.00	2.86×10^{-6}	0.000			
	1130	L	L	P/M	***B*14***	17.00	0.009	0.245			
					C*08	36.00	0.001	0.035	1126-1132		
	1282	V	V	I	A*02	25.00	0.005	0.128	1278	NIRTGVRTI	
					B*14	35.00	0.001	0.035	1282, 1283		
					C*08	26.00	0.002	0.060	1282, 1283		
	1370	I	I	T/V/D	B*07	0.09	0.002	0.066			
NS4B	1958	K	K	R	C*08	10.00	0.009	0.059	1960		
NS5A	2143	E	E	D	C*16	17.00	0.008	0.553			
NS5B	2518	K	K	R	A*03	11.00	0.001	0.032	2520, 2521		SLTPPHSAK
					C*08	9.70	0.005	0.203			
	2609	S	S	P	***B*35***	29.00	9.30×10^{-5}	0.000	2605-2613		
					C*04	19.00	3.82×10^{-4}	0.019	2608-2613		
	2821	R	R	K	B*18	21.00	0.005	0.203	2821-2825		

For amino acids, numbering begins from the start of the polyprotein. Bold and italicized HLA alleles are likely to form part of an MHC haplotype. Underlined amino acids are sites of interest. E2 contains HLA-associated sites that fall within the hypervariable region (393, 397, 400, 403, and 405).

*Median point.

†Consensus amino acid.

‡Amino acid difference in two source sequences (AF313916 and DQ061375-DQ061378, which are separated by a forward slash).

discovery rates was made, and $P \leq 0.01$ was used to indicate significance.

Results

HLA-Associated Viral Polymorphisms: Putative Viral Adaptations in the New Hosts Reflecting Sites of Immune Pressure. We determined whether there were associations between the expression of particular HLA alleles in subjects in this cohort and specific polymorphisms in their viral sequences (putative viral adaptations) reflecting areas under *in vivo* T cell immune pressure. We identified 29 HLA-associated viral polymorphisms with $P \leq 0.01$ for 23 sites along the HCV genome (Table 1 and Supporting Information Fig. 3). In some instances, HLA alleles from different loci were associated with the same site, and we have previously shown that these associations can be explained in part by the linkage disequilibrium observed within the major histocompatibility complex (MHC).[18] Among those associations shown in Table 1, three HLA-B/C combinations are associated with common MHC haplotypes. The *q* values for associations within some of the proteins are high with respect to others (particularly E2) and possibly reflect smaller sample sizes in these proteins (Supporting Information Table 1).

Two HLA-associated viral polymorphisms fell within previously published epitopes (HLA-A*02 epitope in E2 404 SLLAPGAKQNV and HLA-A*03 epitope in NS5B 2518 SLTPPHSAK; Table 1). Furthermore, three HLA-associated viral polymorphisms fell within predicted epitopes as determined by the peptide binding prediction program SYFPEITHI[24] (Table 1). The limited number of matches between known epitopes and putative viral adaptation sites may be the result of the small number of published HCV epitopes in the literature and its focus on common HLA types. Several of the putative viral adaptations are associated with HLA-C alleles for which there are either no or few known HLA-restricted epitopes or characterized binding properties.

None of the associations shown in Table 1 overlap with the findings of our previous studies examining HLA-associated viral polymorphisms for genotype 1.[18,19] However, the previous study had a much larger number of genotype 1a sequences in the data set than 1b sequences; because the sequences in this single-source cohort were all genotype 1b, it was likely that we would observe differential escape profiles similar to what we had seen between genotypes 1a and 3a but to a lesser extent between genotype 1 subtypes (1a and 1b). Furthermore, in contrast to the subjects in the previous cross-sectional studies, the subjects in this study were infected from a single-source strain.

Window Analysis Identifies Additional Areas Under T Cell Pressure. Areas under HLA-specific immune pressure that can accommodate more than one site of variation may not be detected by our initial single amino acid approach. Accordingly, a sliding-window analysis (with a size reflective of a typical HLA class I epitope) was also performed to examine areas under HLA-specific immune pressure in which more than one site might be relevant for escape. As expected, several of the HLA-associated viral polymorphisms identified with a single-site analysis were identified with the window analysis (Table 1). However, the single-site associations found in highly variable regions in E2 were not identified in the window analysis, probably because of the higher level of variation found in this region in comparison with other proteins that may occur in some cases when the variation is not related to adaptation (as tested here) and may hinder the ability to find specific HLA associations with any change(s) within a window. There were three examples [E2 and HLA-C*06 with a median position of 537, odds radio (OR) = 28; NS2 and HLA-B*08 within windows of 875-878, OR = 0.026-0.039; and NS5A and HLA-B*08 with a median position of 2132, OR = 26] for which the window analysis identified HLA-associated substitutions that were not found to be significant in the single-site analysis. These cases suggested that multiple sites within a target region may be under immune pressure (Supporting Information Fig. 4). This observation is consistent with our own study and other studies showing different escape profiles within epitopes, including the immunodominant HLA-B*08 epitope (1395-1403) in NS3[17] and the protective HLA-B*27 epitope (2841-2849) in NS5B.[11]

Overall, the number of associations found with either the single-site analysis or the sliding-window analysis represented only a portion of the 184 variable sites across the viral genome that fit the inclusion criteria described in the methods (18 of 163 if the highly variable region in E2 is excluded because this area is likely to also be under other strong selective pressures).

Source and Causes of Viral Adaptation. We then examined the pattern of synonymous and nonsynonymous changes in these sequences to determine if purifying selection was acting across the HCV genome and potentially restricting the ability of the virus to adapt to new selection pressures or revert to unadapted forms. Figure 1 shows the pattern of these changes in each individual with respect to the source within the NS3 and NS5B proteins. It is apparent that there are a greater number of synonymous changes with respect to nonsynonymous changes in this region (indicating purifying or negative selection). Similar results were observed for other proteins (data not shown).

Covarying Sites in the Genome Likely to Reflect Networks Within the HCV Genome. As previously suggested, purifying selection may reflect the existence of covarying sites in the HCV genome.[26] Here we identified sites of covariance by assessing amino acid sites in a pairwise manner per protein and genome-wide for sequences with greater than 90% sequence coverage. Only results with $P < 0.001$ were reported because adjustments for multiple comparisons were not made in this analysis. Thirteen of 25 paired sites of significant covariance were within the same protein, whereas 12 of 25 fell in different proteins. For the majority of pairs of covariant sites, one or both sites fell at a reported HLA-associated viral polymorphism site, within a known epitope, or at a common site of reversion from the source. Four of the 25 paired sites fell at an HLA-associated site in Table 1. In particular, two HLA-A*03–associated sites at positions 1087 and 1088 in NS3 fell within a confirmed HLA-A*03 epitope in which variation at both sites is required to restore replicative efficacy (K.F., unpublished data, 2010); this reflects the potential compensatory nature of these covariations.

Fig. 2 shows a linear trend for many covarying sites suggesting that many fell in close proximity to one another but not necessarily in the same protein. Interestingly, clusters of covarying sites appeared to connect sites across the genome and particularly other proteins with NS5A. One group contained sites in only one protein (NS3 sites 1644F/Y, 1647A/T, and 1656A/T), whereas another group contained sites in three proteins (NS2 908R/K, NS3 1173S/L, and NS5A 2279R/K). These links may further restrict the ability of the virus to adapt or revert quickly and suggest critical interactions between the HCV proteins. We extended this analysis to assess covariation at amino acid and synonymous sites to identify potential constraints on codon

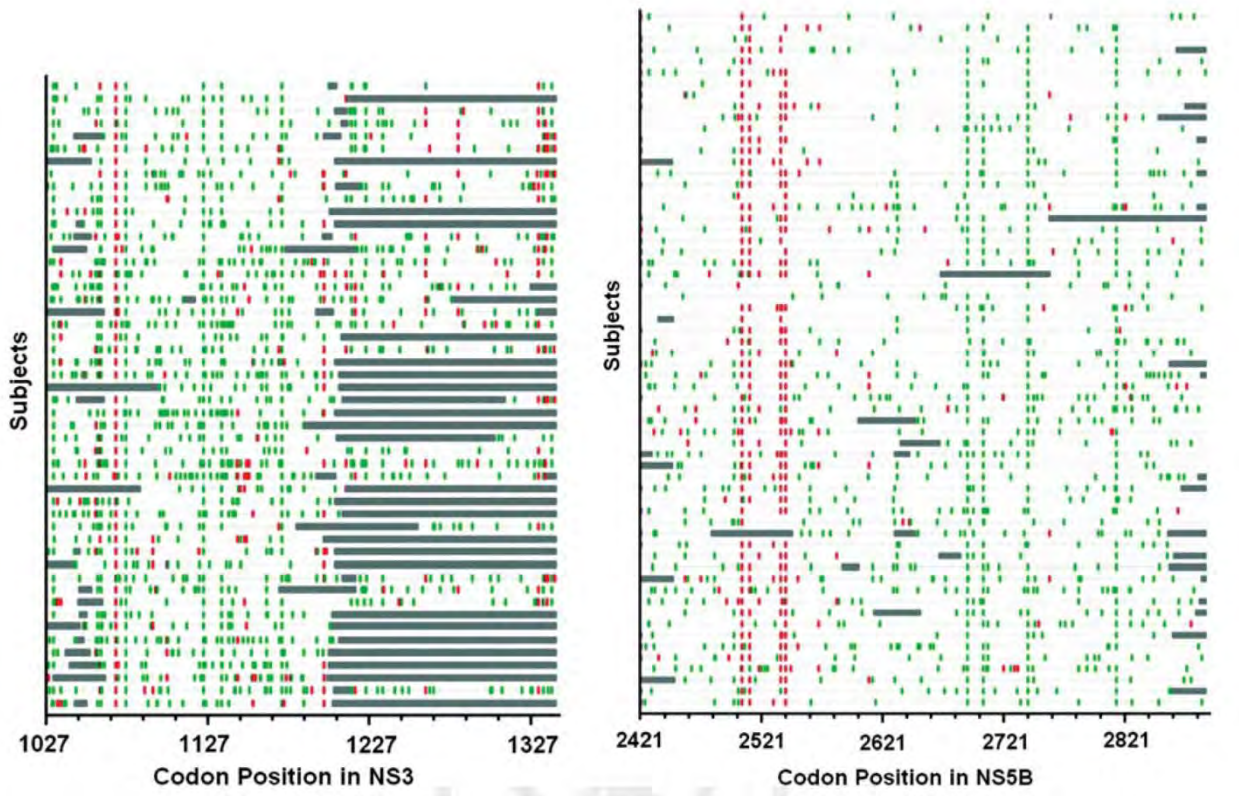

Fig. 1. Highlighter plot of synonymous and nonsynonymous substitutions in NS3 and NS5B with respect to the source sequence (AF313916). The plot was created with Highlighter (available at http://www.lanl.gov). Red lines denote nonsynonymous substitutions, green lines indicate synonymous substitutions, and gray regions show unsequenced sections.

usage (and subsequent amino acid changes) and identified four amino acid sites associated with synonymous changes in other proteins.

Relevance of Viral Adaptations in the New Hosts and Preexisting Ones in the Source in Infection Outcomes. Although the host immune pressure is one of several forces shaping HCV diversity, it is likely that only a small number of selected viral adaptations in the sequence may affect infection outcomes. In this cohort, HLA-A*03 was shown to be protective,[8] and we selected chronic HCV–infected individuals with HLA-A*03 for this study to identify viral adaptations in these individuals that may have affected their infection outcomes. Three viral polymorphisms were associated with HLA-A*03 in this study (Table 1). Two of the associations were in NS3 at positions 1087 and 1088 within a predicted epitope for HLA-A*03. As mentioned previously, this epitope was subsequently shown to be a true *in vivo* target of the immune response (NS3 1080 TVYHGAGTK; K.F., unpublished data, 2010; Fig. 3A) and reflected a drop in the SYFPEITHI-predicted binding score from 34 for the wild type to 21 for the putative escape peptide. Another HLA-A*03–associated viral polymorphism at position 2518 in NS5B was within the previously characterized genotype 1a epitope SLTPPHSAK (Fig. 3B). Half of the HLA-A*03 individuals had a polymorphism at these sites in both regions. These results suggest that these two viral epitopes are important immune targets and that escape within the targets may influence the outcome.

Further analysis of the quasispecies at the NS3 1087 and 1088 sites in HLA-A*03^{+} and HLA-A*03^{-} subjects was performed with ultradeep sequencing. Table 2 reveals the lack of a source sequence at amino acid position NS3 1088 in the HLA-A*03 subject with complete amino acid replacement but 100% retention of the source sequence in the HLA-A*03^{-} subject. The two subjects had the same amino acid at position 1087 (unadapted), but codon usage was different between the two.

Previous studies have found other HLA alleles to be associated with chronic infection that are specific to this cohort, such as alleles HLA-A*01, HLA-B*08, and HLA-C*07[8] (these alleles most likely correspond to a single MHC haplotype). It has been suggested that the

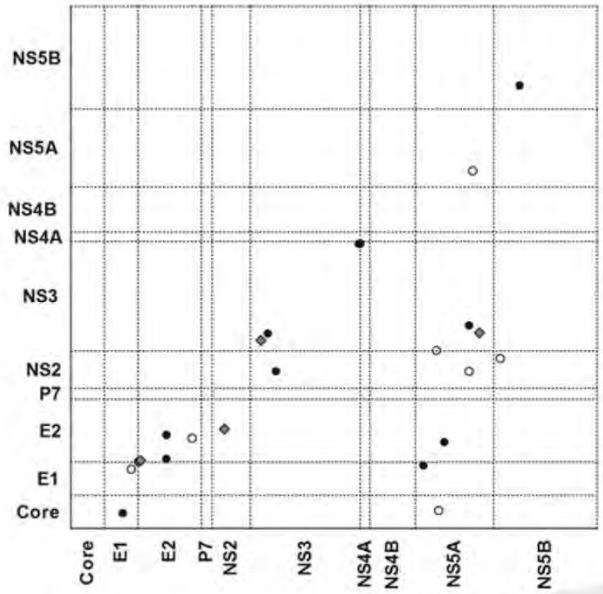

Fig. 2. Covarying sites ($P < 0.001$) in the HCV genome represented as coordinates. Open diamonds indicate that one or both sites fall within an epitope or at an association site, and dark diamonds indicate that the sites do not fall within either. Many covariant sites fall in close proximity to one another in the genome (illustrated by the linear trend); however, there are groupings that suggest strong covariation between residues within NS5A and residues within other proteins. Sequence coverage was not found to be a function of covariant site identification.

Table 2. Ultradeep Sequencing Reveals a Lack of a Source Sequence at Putative Viral Adaptation Sites (NS3 1087 and 1088) in a Subject With HLA-A3 but 100% Maintenance of the Source Sequence in an HLA-A3$^-$ Subject

	Position						HLA*	
Sequence	1087			1088			A*03$^+$	A*03$^-$
Source		T			K		0	346
	A	C	A	A	A	G		
Variant 1		T			K		0	685
	A	C	T	A	A	G		
Variant 2		T			R		249	0
	A	C	A	A	G	G		
Variant 3		T			R		1111	0
	A	C	G	A	G	G		

The HLA-A3$^+$ subject carried only species that differed at the amino acid level from the source. Although the two subjects had the same amino acid at position 1087, codon usage was different. The region had more than 1000-fold coverage.

*Number of sequence reads with a corresponding variant or source combination.

association between infection outcomes and specific HLA alleles may be due to preexisting viral adaptations in the incoming virus that may facilitate the evasion of host immune responses with the corresponding HLA types.[27] Here we tested this hypothesis by examining the source sequence for escape mutations within known epitopes as well as putative viral adaptations

A

	NS3	1080	1081	1082	1083	1084	1085	1086	1087	1088	Count
	Source (AF313916)	T	V	Y	H	G	A	G	T	K	
A*03+		.	.	.	.	.	.	.	A	R	5
		.	.	.	.	.	.	.	A/.	R	1
		.	.	.	.	.	.	.	.	R	2
		-	-	.	.	.	.	.	.	R	1
		.	.	.	.	.	.	.	S/.	.	1
		.	.	.	.	.	.	.	.	.	2
A*03-		.	.	.	.	.	.	.	.	.	27
		.	.	.	.	.	.	.	A	R	1
		-	-	-	-	.	.	.	.	.	1
		-	-	.	.	.	.	.	A	.	1
		.	A	.	.	.	.	.	.	.	1
		-	-	.	.	.	.	.	.	.	1

B

	NS5B	2510	2511	2512	2513	2514	2515	2516	2517	2518	2519	2520	Count
	Source (AF313916)	K	L	T	P	P	H	S	A	K	S	K	
A*03+		.	.	.	.	.	.	.	.	R	.	.	6
		M	.	.	.	.	.	.	.	R	.	.	1
		R	.	.	.	.	.	.	.	R	.	.	1
		M	.	.	.	.	.	.	.	.	.	.	4
		.	.	.	.	.	.	.	.	.	.	.	1
		R	.	.	.	.	.	.	.	.	.	.	1
		K/R	.	.	.	.	.	.	.	.	.	.	1
A*03-		M	.	.	.	.	.	.	.	.	.	.	31
		.	.	.	.	.	.	.	.	.	.	.	8
		.	.	.	.	.	.	.	.	R	.	.	2
		M	.	.	.	.	.	.	.	R/.	.	.	2
		.	.	.	.	.	H/R	.	.	.	.	.	1
		K/M	.	.	.	.	.	.	.	.	.	.	1
		R/M	.	.	.	.	.	.	.	.	.	.	1

Fig. 3. HLA-A*03–associated viral polymorphisms at (A) positions 1087 and 1088 in NS3 and (B) position 2518 in NS5B. Sequences in regions of interest (from Table 1) are displayed for HLA-A*03$^+$ and HLA-A*03$^-$ subjects. The sequence identity with the source sequence is identified by a dot. Amino acid mixtures at a site are separated by a forward slash. The number of individuals with a particular sequence is shown in the count column. The lysine (K) to arginine (R) substitution at 2518 (8 of 15 HLA-A*03$^+$ subjects versus 4 of 47 HLA-A*03$^-$ subjects) resulted in a change in the SYFPEITHI-predicted binding score from 27 to 21. Only one HLA-A*03 individual with chronic infection did not have a polymorphism at the 1087 or 1088 site in NS3 or at the 2518 site in NS5B.

Table 3. Ultradeep Sequencing Reveals a Lack of a Source Sequence at Position 1397 in the Immunodominant HLA-B*08 Epitope in NS3 (HSKKKCDEL) in Two HLA-B*08$^-$ Subjects

	Position									HLA*	
Sequence	1397			1398			1399			A*03$^+$/B*08$^-$	A*03$^-$/B*08$^-$
Source		R			K			K		0	0
	A	G	G	A	A	A	A	A	A		
Variant 1		K			K			K		526	358
	A	A	G	A	A	G	A	A	A		

*Number of sequence reads with a corresponding variant or source combination.

identified in our previous genetic study of chronic HCV infection.[18,19]

Initially, we examined the immunodominant epitope for HLA-B*08 in NS3 (1395 HSKKKCDEL) and the protective HLA-B*27 epitope in NS5B (2841 ARMILMTHF). The region in the source containing the HLA-B*27 epitope in NS5B had the unmutated form. However, the HLA-B*08 epitope in NS3 in the source sequence had a preexisting viral adaptation in the epitope (arginine at position 3), which subsequently reverted in 8 of 11 subjects without HLA-B*08 and was retained in 5 of 8 subjects who expressed HLA-B*08. Although the numbers in the two groups were not significantly different ($P = 0.18$), they supported other studies showing reversion from an arginine to lysine at position 3 in this epitope when there was no immune pressure; this is suggestive of a fitness cost.[15] This HLA-B*08 epitope was previously studied in this cohort with similar results.[15,22] The fitness cost of this substitution was further supported by the results from the ultradeep sequencing of two HLA-B*08$^-$ subjects in this region, who showed complete reversion from the source escape mutation at position 3 of the epitope (Table 3).

Viral adaptation in the source sequence at a site in the HLA-B*08 immunodominant epitope likely to incur a fitness cost suggests that the source may have been an HLA-B*08$^+$ individual. We suggest that this could potentially reduce the ability of hosts with HLA-B*08 to control the virus via the reduction of good immune targets, and this reflects the association of this allele with poor outcomes in this cohort. Additional association sites with HLA-B*08$^+$ individuals found in this study may represent alternative targets for HLA-B*08 along the HCV genome. Furthermore, Table 1 and Fig. 1 list HLA-associated viral polymorphisms that have an OR less than 1 and represent the maintenance of the consensus sequence (which for most sites in Table 1 is the same as the source) for the specific HLA type; this possibly reflects that the source sequence is pre-adapted at these sites. Interestingly, this occurs for alleles within the MHC haplotypes HLA-A*01, HLA-B*08, and HLA-C*07, which are associated with poor outcomes.

Other Selective Pressures Likely to Affect HCV Evolution. In order to determine how other host immune pressures may affect HCV evolution, we assessed possible associations between HCV polymorphisms in this cohort and an SNP that tags the *IL-28B* gene encoding interferon-λ3 and recently has been associated with infection outcome.[2] We found one significant association between homozygosity for the major allele of rs12979860 (associated with good outcome) and variation at position 849 in NS2 ($P = 0.006$). We also tested for additional effects of the *IL-28B* SNP on the HLA-associated polymorphisms. After adjustments for HLA, among the positions identified in Table 1, *IL-28B* was associated with a polymorphism ($P = 0.036$) only at position 2609 of NS5B, which harbors the strong HLA-B*35/HLA-C*04 association. The significance of the HLA-B*35 association with nonconsensus after adjustments for the *IL-28B* SNP is $P = 0.00004$, whereas for HLA-B*35 alone, the P value is 0.0001. There was no significant interaction between the effects of HLA-B*35 and *IL-28B* ($P > 0.9$), and this suggests that they act independently. Further studies examining the association between variations that tag *IL-28B* and HCV evolution are warranted and should be performed on larger cohorts including subjects with different treatment and infection outcomes.

Discussion

Here we illustrate that the incoming viral sequence, host immune pressure, and covariation play important roles in shaping HCV viral diversity. Specifically, we identified 29 significant HLA-associated viral polymorphisms ($P \leq 0.01$; 23 sites) within the cohort that likely reflect viral adaptations. Some of these sites fall within published and/or predicted T cell epitopes. The use of a sliding-window analysis accounting for more

than a single escape variant within a T cell target identified a small number of additional potential regions under T cell pressure, and this supported other studies showing that escape can require the accumulation of escape mutations[28] or that viral escape sites are often mutually exclusive because of the fitness cost.[15,18]

The number of significant HLA-associated viral polymorphism sites identified in this study is only a small proportion of the sites (23/184) across the HCV genome showing variation in the cohort; this is possibly due to the relatively small sample size or suggests that the host immune pressure has a targeted influence on HCV diversity. This would be expected because the immune system sees the viral polyprotein as a set of peptides, and only a small number of these peptides are likely to be presented to the immune system. Furthermore, the lack of significant overlap with previously reported adaptations for genotypes 1a and 3a likely reflects the constraint of the incoming virus and differential viral adaptation pathways on genotype 1b versus other circulating genotypes due to the genetic distance between these strains. It should be noted that although we did not show HLA class II–associated viral polymorphisms, it is likely that, in addition to what we observed for HLA class I alleles, some of the variations correspond to the expression of specific HLA class II alleles.

To appreciate the extent to which both positive and purifying selections influence HCV diversity, we examined the number of synonymous and nonsynonymous changes across the genome for this single-source cohort. An abundance of synonymous changes indicated purifying selection that would to some extent limit the plasticity of HCV. Covariations that become fixed across the HCV genome may also restrict the ability of HCV to adapt to the host's immune response and revert when it enters a new non–HLA-matched host. We examined the genome for covarying sites and showed that although covariation did occur locally within proteins, there were also a number of sites that were linked to sites more distant in the genome. Furthermore, several of these sites were putative viral adaptation sites.

Access to the source viral sequence from this single-source cohort allowed the identification of preexisting escape mutations across the genome. A known escape mutation at position 3 of the immunodominant HLA-B*08 NS3 epitope was found in the source sequence. This mutation was for the most part retained in HLA-B*08 subjects but had reverted in most HLA-B*08$^-$ subjects. Furthermore, deep sequencing revealed no traces of the escape mutant in two B*08$^-$ individuals, and this supports the fitness cost that may be incurred by the escape mutation. Importantly, existing adaptation in the incoming virus may affect infection outcomes in individuals expressing the appropriate HLA type. The pre-adaptation of the source sequence to HLA-B*08 may account for the observed lack of protection of HLA-B*08 in this cohort.

The single-source cohort studied here has provided us an opportunity to obtain a better understanding of viral diversity and the ways in which different forces can shape viral diversity at the population level.

Acknowledgment: The authors thank the patients and clinical staff who participated in this study and their colleagues at the Centre for Clinical Immunology and Biomedical Statistics of Murdoch University.

References

1. Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. Lancet 1997;349:825-832.
2. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 2009;461:798-801.
3. Rauch A, Kutalik Z, Descombes P, Cai T, Iulio J, Mueller T, et al. Genetic variation in IL28β is associated with chronic hepatitis C and treatment failure: a genome wide association study. Gastroenterology 2010;138:1338-1345.
4. Wiese M, Grungreiff K, Guthoff W, Lafrenz M, Oesen U, Porst H. Outcome in a hepatitis C (genotype 1b) single source outbreak in Germany—a 25-year multicenter study. J Hepatol 2005;43:590-598.
5. Kenny-Walsh E. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. N Engl J Med 1999;340: 1228-1233.
6. Dore GJ, MacDonald M, Law MG, Kaldor JM. Epidemiology of hepatitis C virus infection in Australia. Aust Fam Physician 2003;32:796-798.
7. Missale G, Bertoni R, Lamonaca V, Valli A, Massari M, Mori C, et al. Different clinical behaviors of acute hepatitis C virus infection are associated with different vigor of the anti-viral cell-mediated immune response. J Clin Invest 1996;98:706-714.
8. McKiernan SM, Hagan R, Curry M, McDonald GSA, Kelly A, Nolan N, et al. Distinct MHC class I and II alleles are associated with hepatitis C viral clearance, originating from a single source. HEPATOLOGY 2004;40:108-114.
9. Fanning LJ, Levis J, Kenny-Walsh E, Wynne F, Whelton M, Shanahan F. Viral clearance in hepatitis C (1b) infection: relationship with human leukocyte antigen class II in a homogeneous population. HEPATOLOGY 2000;31:1334-1337.
10. Barrett S, Ryan E, Crowe J. Association of the HLA-DRB1*01 allele with spontaneous viral clearance in an Irish cohort infected with hepatitis C virus via contaminated anti-D immunoglobulin. J Hepatol 1999;30:979-983.
11. Neumann-Haefelin C, McKiernan S, Ward S, Viazov S, Spangenberg HC, Killinger T, et al. Dominant influence of an HLA-B27 restricted CD8+ T cell response in mediating HCV clearance and evolution. HEPATOLOGY 2006;43:563-572.
12. Bowden DG, Walker CM. Mutational escape from CD8 cell immunity: HCV evolution, from chimpanzees to man. J Exp Med 2005;201: 1709-1714.
13. Erickson AL, Kimura Y, Igarashi S, Eichelberger J, Houghton M, Sidney J, et al. The outcome of hepatitis C virus infection is predicted by escape mutations in epitopes targeted by cytotoxic T lymphocytes. Immunity 2001;15:883-895.

14. Cox AL, Mosbruger T, Mao Q, Liu Z, Want XH, Yang HC, et al. Cellular immune selection with hepatitis C virus persistence in humans. J Exp Med 2005;201:1741-1742.
15. Salloum S, Oniangue-Ndza C, Neumann-Haefelin C, Hudson L, Giugliano S, Siepen MAD, et al. Escape from HLA-B*08-restricted CD8 T cells by hepatitis C virus is associated with fitness costs. J Virol 2008;82:11803-11812.
16. Cox AL, Mosbruger T, Lauer GM, Pardoll D, Thomas DL, Ray SC. Comprehensive analyses of CD8+ T cell responses during longitudinal study of acute human hepatitis C. HEPATOLOGY 2005;42:104-112.
17. Timm J, Lauer GM, Kavanagh DG, Sheridan I, Kim AY, Lucas M, et al. CD8 epitope escape and reversion in acute HCV infection. J Exp Med 2004;200:1593-1604.
18. Gaudieri S, Rauch A, Park LP, Freitas E, Herrmann S, Jeffrey G, et al. Evidence of viral adaptation to HLA class I-restricted immune pressure in chronic hepatitis C virus infection. J Virol 2006;80:11094-11104.
19. Rauch A, James I, Pfafferott K, Nolan D, Klenerman P, Cheng W, et al. Divergent adaptation of hepatitis C virus genotypes 1 and 3 to human leukocyte antigen-restricted immune pressure. HEPATOLOGY 2009;50:1017-1029.
20. Manzin A, Solforosi L, Debiaggi M, Zara F, Tanzi E, Romano L, et al. Dominant role of host selective pressure in driving hepatitis C virus evolution in perinatal infection. J Virol 2000;74:4327-4334.
21. Kuntzen T, Timm J, Berical A, Lewis-Ximenez LL, Jones A, Nolan B, et al. Viral sequence evolution in acute hepatitis C virus infection. J Virol 2007;81:11658-11668.
22. Ray SC, Fanning L, Wang XH, Netski DM, Kenny-Walsh E, Thomas DL. Divergent and convergent evolution after a common-source outbreak of hepatitis C virus. J Exp Med 2005;201:1753-1759.
23. Urban TJ, Thomas AJ, Bradrick B, Fellay J, Schuppan D, Cronin KD, et al. IL28 genotyping is associated with differential expression of intrahepatic interferon-stimulated genes in chronic hepatitis C patients. HEPATOLOGY 2010; 52.
24. Rammensee H, Bachmann J, Emmerich NN, Bachor OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 1999;50:213-219.
25. Kumar S, Nei M, Dudley J, Tamura K. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 2008;9:299-306.
26. Campo DS, Dimitrova Z, Mitchell RJ, Lara J, Khudyakov Y. Coordinated evolution of the hepatitis C virus. Proc Natl Acad Sci USA 2008;105:9685-9690.
27. Giugliano S, Ruhl M, Neumann-Haefelin C, Wiese M, Thimme R, Roggendorf M, et al. Differences in the source sequence of two HCV genotype 1b outbreaks within immunodominant CD8 epitopes are associated with differential outcome. Paper presented at: 16th International Symposium on Hepatitis C and Related Viruses; October 2009; Nice, France.
28. Dazert E, Neumann-Haefelin C, Bressanelli S, Fitzmaurice K, Kort J, Timm J, et al. Loss of viral fitness and cross-recognition by CD8+T cells limit HCV escape from a protective HLA-B27-resticted immune response. J Clin Invest 2009;119:276-286.