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Analyse statistique de l'impact des mutations génotypiques du VIH-1 sur la réponse virologique au traitement antirétroviral

Statistical analysis of the impact of HIV-1 genotypic mutations on virological response to antiretroviral therapy

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Résumé

Les mutations de résistance génotypiques constituent un problème majeur pour l'optimisation du traitement antirétroviral chez les patients infectés par le VIH-1 naïfs au traitement ou prétraités. Cependant, l'analyse de l'impact des mutations sur la réponse au traitement est compliquée par i) le nombre élevé de mutations, ii) la colinéarité possible entre ces mutations, iii) le faible nombre de patients inclus dans les études et iv) la définition du critère de jugement. Les objectifs de cette thèse sont 1) de donner une vue d'ensemble et de discuter, en collaboration avec le réseau européen NEAT (European AIDS treatment network), les critères de jugement utilisés dans les essais cliniques récents et ceux utilisés lors de l'analyse des mutations de résistance, 2) d'évaluer l'impact des mutations génotypiques sur la réponse au traitement chez les patients naïfs dans le cadre d'une grande collaboration Européenne (EuroCoord-CHAIN) et 3) de comparer des méthodes adaptées pour les données à haute-dimension dans le but de construire un score génotypique pour la prédiction de la réponse virologique chez les patients prétraités. Les critères de jugement composites sont les plus utilisés dans les essais cliniques récents mais un critère purement virologique devrait être utilisé pour l'analyse de l'impact des mutations génotypiques. Les mutations de résistance transmises impactent sur la réponse à la première ligne de traitement si le traitement antirétroviral n'est pas adapté au génotype du virus du patient. L'analyse en composantes principales et l'analyse partial least square avaient une bonne capacité à prédire la réponse virologique mais étaient guère meilleures que le score génotypique. Nous allons continuer à travailler sur la comparaison des ces méthodes utilisant des critères de jugement différents dans le cadre de notre collaboration avec le Forum for collaborative HIV research.

Summary

Genotypic resistance mutations are a major concern for antiretroviral treatment optimisation in HIV-1 infected treatment naïve and treatment experienced patients. However, the analysis of the impact of genotypic mutations on treatment outcome is hampered by methodological issues such as the i) high number of possible mutations, ii) the potential collinearity between mutations, iii) the low number of patients included in those studies and iv) the definition of a virological endpoint. The objective of this thesis are 1) to give an overview and to discuss endpoints used in recent clinical trials in collaboration with European AIDS treatment network (NEAT) and those used in the context of drug resistance analysis, 2) to investigate the impact of genotypic resistance mutations on treatment outcome in treatment naïve patients in a huge European collaboration EuroCoord-CHAIN and 3) to compare methods adapted for high-dimensional data in order to construct a genotypic score to predict treatment outcome in treatment experienced patients. We saw that most of the endpoints used in recent clinical trials are composite endpoints but pure virological outcomes should be used for the evaluation of drug resistance mutations. Transmitted drug resistance mutations impact on virological outcome of initial antiretroviral therapy if the treatment of the patient is not adapted to the viral genotype the patient is harbouring. Principal component analysis and partial least square showed a good performance but had only a slightly better predictive capacity for a virologal outcome compared to the genotypic score. We continue working on the comparison of these and other methods using different endpoints in the context of a collaboration with the Forum for collaborative HIV research.

Mots clés : VIH-1, mutations génotypiques, résistances au traitement antirétroviral, définition du critère de jugement, analyse statistique
Key words: HIV-1, genotypic mutations, antiretroviral drug resistance, endpoint definition, statistical analysis

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Résumé substantiel

Introduction

Depuis 1996 la thérapie antirétrovirale combinée (combination antiretroviral therapy, cART) combinant au moins trois médicaments antirétroviraux est le traitement de référence pour les patients infectés par le virus d'immunodéficience humaine (VIH). A ce jour, 24 molécules sont disponibles et sont divisées en 6 classes : les inhibiteurs nucléosidiques de la transcriptase inverse (INRT), les inhibiteurs non nucléosidiques de la transcriptase inverse (INNRT), les inhibiteurs de la protéase, les inhibiteurs de fusion, les inhibiteurs de l'intégrase et les inhibiteurs de chémokine co-recepteur 5 (CCR5).

La thérapie antirétrovirale n'est pas capable d'éradiquer l'infection par le VIH. Le but principal de la thérapie antirétroviral est d'empêcher une progression de l'infection. Pour cela, la thérapie antirétrovirale a comme objectif de baisser l'ARN VIH (charge virale) au dessous de 50 copies/ml (limite de détection pour la plupart des techniques de mesure disponibles dans le commerce) et de maintenir la charge virale au dessous de cette borne aussi longtemps que possible [1-3]. Ceci permet d'une part une meilleure restauration immunitaire et d'autre part empêche la sélection des virus potentiellement résistants au traitement. Les CD4 infectés quiescents et d'autres types cellulaires à durée de vie longue constituent un réservoir qui est la cause de la persistance à vie du virus [4-6]. Ce réservoir contient aussi des mutants archivés pendant les phases de réplication virale [7-12].

Mutations génotypiques et résistance antirétrovirale

Les échecs thérapeutiques peuvent être liés à différentes causes (défaut d'adhérence, concentration plasmatique insuffisante…). La conséquence est une suppression incomplète de la réplication du virus. Cette dernière facilite le développement des résistances contre les antirétroviraux : c'est le résultat d'une sélection de mutations génotypiques qui permet au virus de se répliquer en présence du traitement.

Les mutations pertinentes peuvent apparaître dans les gènes de la transcriptase inverse, de la protéase, de la gp41 ou de l'intégrase. Les mécanismes de résistance sont différents selon les classes des antirétroviraux et peuvent aussi être différents selon l'antirétroviral dans une même classe [13]. Le plus souvent, seule une combinaison de certaines mutations provoque une résistance : cela dépend des positions des mutations et leur impact sur la structure de la protéine (encodé par le gène). De plus, les mutations rencontrées n'ont pas toutes la même importance. Par exemple, les mutations génotypiques de la protéase virale peuvent être distinguées en des mutations primaires sélectionnées en premier lieu lors d'un

échappement au traitement et des mutations secondaires qui s'accumulent à la suite d'une mutation primaire. Une mutation primaire se développe sous la pression d'un médicament et se trouve le plus souvent dans le centre actif de la protéase [14, 15]. Une mutation secondaire seule est rarement la cause d'une résistance mais elle peut aggraver la résistance lorsqu'elle est combinée avec une mutation primaire [16].

Epidémiologie des mutations de résistance antirétrovirale

La résistance acquise pendant le traitement antirétroviral est répandue chez des patients traités avec une charge virale détectable. La probabilité d'avoir une résistance contre au moins une molécule antirétrovirale (selon l'algorithme d'interprétation de l'ANRS) était de 88% chez des patients traités avec une charge virale >1000 copies/mL utilisant des données de 24 centres en France et un centre de Suisse [17]. Dans une étude réalisée en Royaume-Uni 80% des patients traité avec un test génotypique avaient au moins une mutations majeure de la liste IAS (une liste de mutations de référence internationale) [18].

La prévalence de résistance aux antirétroviraux transmise est très variable selon les pays, le groupe à risque d'infection par le VIH et le moment de la réalisation d'un test génotypique après l'infection. En Europe, la prévalence de la résistance transmise (avoir au moins une mutation de résistance) chez les patients naïfs de traitements antirétroviraux est estimée entre 10 à 15% [19-25]. Des études en Amérique du Nord décrivent des prévalences jusqu'à 25% [26, 27]. L'impact potentiel de la résistance transmise reste controversé. La proportion des patients avec succès virologique (suppression de la charge virale) [26, 28] ainsi que le temps jusqu'au succès virologique [19, 29, 30] et la réponse immunologique [19, 26, 29, 30] ne sont pas significativement différents entre les patients avec et sans mutations transmises dans la plupart des études. Toutefois, il y a une tendance à une meilleure réponse virologique chez les patients sans résistance transmise [19, 30] et le temps jusqu'à la suppression virologique était plus court chez les patients infectés par des souches virales susceptibles [31, 32]. Cependant, dans toutes ces études, la proportion de patients avec une résistance transmise était restreinte, limitant la puissance statistique de ces études. L'impact des mutations de résistance sur la réponse clinique au long terme reste incertain en particulier dans des populations spécifiques (enfants, personnes âgées). Seule une étude avec une taille d'échantillon suffisamment grande pourrait répondre à ce type de question.

Impact de la résistance antirétrovirale sur la prise en charge thérapeutique

Méthode de détermination de la résistance antirétrovirale

Deux types de test sont utilisés pour déterminer la résistance antirétrovirale utilisée en pratique clinique : les tests génotypiques et les tests phénotypiques. Les premiers consistent en un séquençage des gènes du VIH concernés (e.g. protéase) pour détecter des mutations conduisant à une résistance antirétrovirale. Les tests phénotypiques mesurent la réplication du virus en culture cellulaire en présence ou absence d'une molécule antirétrovirale donnée. Les tests génotypiques ne permettent que l'analyse de la population virale majoritaire soit la population qui représente au moins 20% à 30% de la population virale totale circulante dans le plasma. Les populations minoritaires ne sont pas détectées en routine. Il y a des techniques permettant de les analyser mais pour l'instant l'analyse des populations minoritaires est réservée aux protocoles de recherche. Les tests phénotypiques sont plus chers, nécessitent plus de temps et sont pas utilisés en pratique clinique.

Aujourd'hui, il est fortement recommandé de réaliser un test génotypique avant la mise en place d'un traitement ou avant le début d'un nouveau traitement pour que les patients puissent être traités de façon adéquate [1, 3, 13]. Le traitement est ainsi adapté aux souches virales circulantes afin d'assurer une efficacité optimale.

Interprétation des données de tests génotypiques

Il existe plusieurs algorithmes habituellement utilisés pour interpréter le génotype, notamment ceux de l'ANRS [33], de HIVdb Stanford [34] et de Rega [34, 35]. Ils sont utilisés pour classer le virus comme 'susceptible', 'possiblement résistant' ou 'résistant'. Un score génotypique construit à partir des informations *in vitro* et une combinaison des informations donnée par les mutations génotypiques avant la mise sous traitement et la charge virale après l'initiation d'un nouveau traitement antirétroviral [36, 37] est calculé pour établir la classification. Il existe aussi des systèmes d'interprétation utilisant des méthodes bioinformatiques telles que le SVM (Support Vector Machine) [38] ou une combinaison de plusieurs méthodes bioinformatiques [39] construites en utilisant les informations génotypiques en combinaison avec des données phénotypiques ou des données cliniques.

L'activité d'une combinaison de plusieurs molécules peut aussi être déterminée en utilisant les algorithmes et est généralement exprimée en termes de GSS (genotypic sensitivity score) représentant le nombre de molécules actives dans la stratégie thérapeutique.

Méthodes de détermination de la susceptibilité du virus à un traitement donné

Données

Pour pouvoir associer les mutations détectées à une molécule donnée, les patients inclus dans une étude pour déterminer par exemple un score génotypique ou pour valider des algorithmes existants ont une molécule en commun. Par exemple, tous les patients commençant une nouvelle molécule (par exemple l'inhibiteur de protéase darunavir) en combinaison avec différentes autre molécules sont inclus. Les mutations de la protéase déterminées avant la mise en place du darunavir sont corrélées avec la réponse au traitement pour déterminer les mutations de la protéase associées à un échec au traitement sous darunavir. Ces données peuvent être issues d'essais cliniques ou des cohortes de patients infectés par le VIH.

Les données utilisées pour étudier l'impact des mutations de résistance sur la réponse virologique sont constituées des résultats de test génotypiques déterminés avant la mise en place d'un traitement ou avant le début d'un nouveau traitement. Les mutations sont codées comme variables binaires. Si la mutation sur une position donnée, par exemple I47A, est présente, la variable prend la valeur 1 et 0 sinon. Les variables à expliquer sont soit des résultats d'un test phénotypique soit la réponse virologique mesurée par la charge virale.

Les résultats d'un test phénotypique représentent la concentration médicamenteuse nécessaire pour inhiber la réplication du virus du patient (la valeur donnée est soit la concentration nécessaire pour inhiber 50% ou 90% de la réplication). Il s'agit d'une variable quantitative. La réponse virologique peut être considérée soit comme variable quantitative (e.g. la différence de la charge virale entre deux dates) ou comme variable binaire (e.g. le succès est codé 1 si la charge virale descend au dessous d'un certain seuil).

Difficultés méthodologiques

L'analyse de données est complexe d'une part à cause du nombre élevé de mutations possibles par rapport au nombre de patients habituellement inclus dans les études cliniques et d'autre part à cause de la colinéarité possible entre des mutations (par exemple la présence d'une mutation seulement en présence d'une ou plusieurs autres mutations pour compenser une capacité réplicative diminuée). Le nombre élevé de variables/mutations en comparaison avec le faible nombre de patients peut mener à un sur-ajustement conduisant à une mauvaise validité externe du modèle statistique (capacité à prédire la réponse virologique chez un

patient en dehors de l'étude). De plus, toutes les mutations ne contribuent pas de la même façon et avec le même poids à la résistance.

Score génotypique

Une méthode simple pour gérer les difficultés citées précédemment est de résumer l'information des mutations génotypiques dans un score génotypique. Ce score est normalement lié à une molécule donnée. Un score génotypique est la somme des mutations de résistance observées chez un patient donné. Par la suite, nous allons décrire la construction d'un score génotypique simple, i.e. sans pondération des mutations pour le calcul du score. La liste des mutations servant à déterminer le score génotypique peut être obtenue par des stratégies différentes. Normalement deux étapes principales sont nécessaires. D'abord les mutations issues d'une liste constituée par un groupe d'experts internationaux (e.g. IAS-USA) sont évaluées en analyse univariable si elles ont une prévalence entre 10% et 90%. Une mutation est sélectionnée si le degré de significativité statistique est <0,25 [36, 37]. Avec les mutations ainsi sélectionnées un premier score est déterminé pour chaque patient dans la base de données. Par exemple, la première sélection contient 6 mutations V32I, I47A, I50V, V77I, I84V et L90M. Le score est égal à S = V32I + I47A + I50V + V77I + I84V + L90M. Une mutation prend la valeur 1 s'il est présent est 0 sinon (le score peut varier de 0 à 6). Dans cet exemple, un patient avec les mutations I47A, I50V et L90M aura un score de 3. Dans un deuxième temps, les mutations à inclure dans la sélection finale sont déterminés par des méthodes de sélection pas à pas. Chaque mutation de la première sélection est éliminée une par une pour calculer de nouveau des scores génotypiques pour chaque patient. Ainsi pour chaque sélection de 5 mutations, l'association entre le score (variable ordinale) et la réponse virologique est analysée en utilisant des tests statistiques. La seléction finale est la liste conduisant à l'association la plus forte avec la réponse virologique [37]. Dans cette approche les mutations considérées pour le score ont toutes le même poids. Un score prenant en compte le fait qu'il y avait des mutations associées avec une meilleure réponse virologique a déjà été considéré [40, 41]. Il a été proposé que les mutations associées à l'échec virologique contribuent au score par +1 et les mutations associées avec succès virologique contribuent au score par -1 [40, 41].

Le score génotypique a aussi l'inconvénient de ne pas pouvoir prendre en compte l'interaction possible entre les mutations. Par exemple, il est possible que l'effet d'une mutation soit plus fort ou plus faible en présence d'une autre mutation.

Une fois le score génotypique établi, un seuil est déterminé pour prédire la réponse virologique (par exemple avoir un score >5 signifie résistant, 4 signifie possiblement résistant et <4 signifie pas d'évidence de résistance). Sa valeur prédictive doit aussi être testée en ajustant sur les facteurs de confusion probables comme le taux de CD4 initial, etc..

Méthodes alternatives

Il existe des méthodes alternatives permettant de résumer l'information des mutations, d'allouer des poids différents ou de prendre en compte les interactions entre les mutations.
L'analyse en composantes principales (ACP) et la régression partial least square (PLS) ont été proposées pour la réduction du nombre des variables prédictives corrélées [42-44]. L'objectif de l'analyse en composante principale est de trouver des variables dites latentes utilisant une transformation linéaire des variables prédictives. Les variables latentes sont aussi appelées des composantes principales et elles peuvent par exemple être utilisées dans un modèle de régression comme variable prédictive. L'analyse en composantes principales a déjà été utilisée pour déterminer des groupes de mutations corrélées [45] et pour prédire le phénotype [46]. La régression PLS cherche également des variables dites latentes, les composantes PLS. La différence principale entre la régression PLS et l'analyse en composantes principales est que la régression PLS utilise aussi la variable à expliquer (e.g. la réponse virologique) pour déterminer les composantes. Les composantes peuvent également être utilisées comme variables explicatives dans un modèle de régression. Les mutations ainsi résumées dans une composante principale ou une composante PLS sont représentées par des poids différents dans le calcul de celles-ci.

D'autres méthodes pour prédire la réponse au traitement ou le phénotype à partir des informations génotypiques ont été testées ou comparées, notamment les réseaux neuronaux [47, 48], la méthode Lasso [46], les arbres de décision [49], les random forests [50]. Une méthode globale (superlearner) a été proposée, combinant plusieurs des méthodes statistiques citées [51]. Rabinowitz *et al.* ont montré que des machines à vecteur de support et la méthode Lasso (least absolute shrinkage and selection operator) avaient la meilleure performance en utilisant le génotype pour prédire le phenotype *in vitro* [46] par rapport à la ridge regression, aux réseaux neuronaux, l'analyse en composantes principales, les arbres de décision et la sélection pas à pas. L'avantage de la méthode Lasso est que les résultats sont faciles à interpréter car les coefficients estimés sont directement liés aux variables prédictives (et pas au vecteur de support).

Définition de la réponse virologique

La définition de la réponse virologique utilisée d'une part pour déterminer les mutations liées à la réponse virologique mais aussi pour construire un système d'interprétation à l'aide des méthodes bioinformatiques est très variable. Les définitions utilisées sont par exemple, 1) la différence entre la charge virale à 3 mois (ou 6 mois) et la charge virale à la mise sous traitement, 2) avoir une charge virale en dessous de la limite de détection, 3) avoir une décroissance de la charge virale d'au moins 1 $\log_{10}$ jusqu'à 3 mois ou un critère composite basé sur une chute d'au moins 1 $\log_{10}$ ou une charge virale au dessous du seuil de détectabilité. La charge virale est censurée due au seuil de détectabilité. Le plus souvent une imputation de la valeur seuil ou la moitié de la valeur seuil est utilisée pour calculer par exemple la différence entre la charge virale à 3 mois et la valeur de la charge virale à la mise sous traitement. Il a été démontré que ces méthodes d'imputation simple conduisent à des estimations biaisées [52-54].

Plan et objectifs

L'analyse de l'impact des mutations génotypiques sur la réponse au traitement chez les patients prétraités et les patients naïfs est importante car le choix d'un traitement optimal est crucial. Cependant, l'analyse des données génotypiques est compliquée à cause de problèmes méthodologiques. Premièrement, la définition d'un critère de jugement n'est pas simple. Deuxièmement, la définition de la résistance et en particulier la création des algorithmes génotypiques pour des nouvelles molécules est très souvent compliquée par le nombre élevé des mutations et le faible nombre des patients inclus dans ces études.

Le premier objectif était de décrire la définition des critères de jugement utilisé dans les essais cliniques du VIH et de discuter leurs limites méthodologiques. Le chapitre 4.1 décrit des problèmes méthodologiques en particulier pour des critères de jugement composites. Ce travail a été publié dans Clinical Trials 2010. Le travail est le résultat des discussions avec le groupe de travail des statisticiens du réseau européen NEAT (European AIDS treatment network) pendant la conception de l'essai NEAT001/ANRS143 (NCT01066962 [55]). Le chapitre 4.2 donne un vue d'ensemble sur les critères de jugement utilisés dans les études évaluant l'impact des mutations transmises sur la réponse virologique et les critères utilisés dans des études déterminant un score génotypique.

Le deuxième objectif était d'analyser l'impact des mutations de résistance transmises sur la réponse au traitement pendant la première année de traitement antirétroviral. Pour ce travail nous sommes particulièrement intéressés aux effets des mutations transmises chez les

patients traités avec une combinaison de traitement prédite comme non affectée par les mutations présentes. Un manuscrit (chapitre 5) de ce travail est en cours de finalisation et une soumission au Lancet est prévue. Le projet a été réalisé comme projet pilote entre les réseaux européens EuroCoord (méta-collaboration de cohortes européennes de patients infectés par le VIH) et CHAIN (réseau d'experts européen de résistance du VIH au traitement).

Le troisième objectif était d'évaluer des méthodes alternatives à la construction d'un score génotypique pour l'analyse des mutations génotypiques chez les patients prétraités. En particulier, nous nous sommes intéressés à l'utilisation de méthodes permettant de résumer l'information génotypique et à la question si l'information résumée peut-être utilisée pour prédire la réponse virologique au traitement. Le chapitre 6.1 résume l'application d'ACP et PLS en comparaison avec la construction d'un score génotypique et a été publié dans BMC Medical Research Methodology 2008. De plus, l'adaptation de la méthode Lasso pour la prise en compte de la censure à gauche des marqueurs virologiques est un projet en cours et présenté dans le chapitre 6.2. Cette méthode va être appliquée en collaboration avec le Forum for collaborative HIV research (plateforme internationale pour faciliter la recherche sur le VIH).

Production scientifique liée à la thèse

Articles

Wittkop L, Smith C, Fox Z, Sabin C, Richert L, Aboulker JP, Philipps A, Chêne G, Babiker A, Thiébaut R on behalf of NEAT WP4. Methodological issues in the use of composite endpoints in clinical trials: examples from the HIV field. *Clin Trials* 2010;7(1):19-35.

Wittkop L, Commenges D, Pellegrin I, Breilh D, Neau D, Lacoste D, Pellegrin JL, Chêne G, Dabis F, Thiébaut R. Alternative methods to analyse the impact of HIV mutations on virological response to antiviral therapy. *BMC Med Res Methodol* 2008,8:68.

The EuroCoord-CHAIN joint project writing committee:
Wittkop L, Günthard HF, de Wolf F, Dunn D, Cozzi-Lepri A, de Luca A, Kücherer C, Obel N, von Wyl V, Masquelier M, Stephan C, Torti C, Antinori A, García F, Judd A, Porter K, Thiébaut R, Castro (née Green) H, van Sighem AI, Colin C, Kjaer J, Lundgren JD, Paredes R, Pozniak A, Clotet B, Phillips A, Pillay D*, Chêne G* and the EuroCoord-CHAIN study group (* contributed equally to this work) Impact of transmitted drug resistance on virological and immunological response to initial combination Antiretroviral Therapy (en cours de finalisation)

Communications dans un congrès avec comité de lecture

Communications orales

L Wittkop on behalf of the EuroCoord-CHAIN project team
Impact of transmitted drug resistance (TDR) on virological and immunological response to initial combination Antiretroviral Therapy (cART) – EuroCoord-CHAIN joint project. AIDS 2010, July 18-23, 2010, Vienna, Austria (**Late breaker**)

Communications affichées

L Wittkop on behalf of the EuroCoord-CHAIN project team
Impact of transmitted drug resistance (TDR) on virological response to initial combination Antiretroviral Therapy (cART). Abstracts of the XVIII International HIV & Hepatitis Drug Resistance Workshop & curative strategies, June 08-12, 2009, Dubrovnik, Croatia. *Antivir Ther* 2010, 14 Suppl 2: A124 (**Best poster prize**)

L Wittkop on behalf of the EuroCoord-CHAIN project team
Impact of transmitted drug resistance on virological response to initial combination Antiretroviral Therapy (cART)-regimen. International workshop on HIV observational databases**,** March 25-27, 2010, Sitges, Spain

L Wittkop, I Pellegrin, D Commenges, D Breilh, D Neau, D Lacoste, J-L Pellegrin, G Chêne, F Dabis, R Thiébaut for the ANRS Co3 Aquitaine Cohort. Comparison of principal component analysis and partial least square with the construction of a genotypic score to analyse the impact of HIV protease mutations on virological response upon HAART. International workshop on HIV observational databases, March 27-30 2008, Malaga, Spain.

Table of contents

Preamble

The human immunodeficiency virus (HIV) is a lentivirus belonging to the family of retroviruses [56]. Main target cells of HIV are cells of the human immune system such as CD4 positive T lymphocytes (CD4 cells), macrophages and dendritic cells. Over time HIV infection leads to the deterioration of the immune system which is indicated by a progressive decrease of the CD4 cell count. HIV ultimately leads to the acquired immunodeficiency syndrome (AIDS) and death.

First cases of AIDS were described since 1981 and HIV was first isolated from a patient with lymphadenopathy by Francoise Barré Sinoussi and Luc Montagnier in 1983 [57]. Since then more than 25 million people died from HIV infection and in December 2008 33.4 million people were living with HIV, most of them in resource limited settings [58]. The current standard regimen for initial treatment of HIV infected patients is the combination antiretroviral therapy (cART) introduced in 1996.

The general treatment goal is to suppress HIV RNA load (viral load) to less than 50 copies/mL in naïve and in treatment experienced patients [1-3, 13]. The choice of an initial treatment is important as it can have long standing consequences for future therapy. The selection of the drugs depends on factors like viral load, number of CD4 cells, toxicity and compliance etc. Furthermore, genotypic testing is now recommended for treatment experienced patients [1-3, 13] but also for treatment naïve patients as prevalence of transmitted drug resistance is between 10% and 15% in Europe [19, 21, 23, 24, 28, 59-61] and up to 25% in North America [26, 27].

Chapter 1 introduces some basic virological and biological information for better understanding of antiretroviral treatment targets and resistance testing. Additionnally, the epidemiological background of drug resistance in HIV-1 infected patients is described.

Chapter 2 describes methodological issues involved in the analysis of virological response according to genotypic data. It presents an overview on the methodological literature applied to analyse genotypic data for the sake of predicting treatment outcome. Additionally, methods we used to interprete genotypic data in treatment naïve patients and methods we used for analysing genotypic data in treatment experienced patients are outlined.

1 Antiretroviral treatment and drug resistance

1.1 Biology of the human immunodeficiency virus

The description of the structure and the life cycle of HIV in the following chapters are far from being complete but aim at giving a short overview of the molecular biology of the virus for a better understanding of antiretroviral treatment targets and emergence of antiretroviral drug resistance.

1.1.1 Structure

The HIV is a spherical particle with a diameter of approximately 100 nm. The outer surface "envelope" is formed by a lipid bilayer (originating from the host cell membrane) in which the viral glycoprotein gp41 is embedded. The viral glycoprotein gp120 is attached to gp41 (see Figure 1).

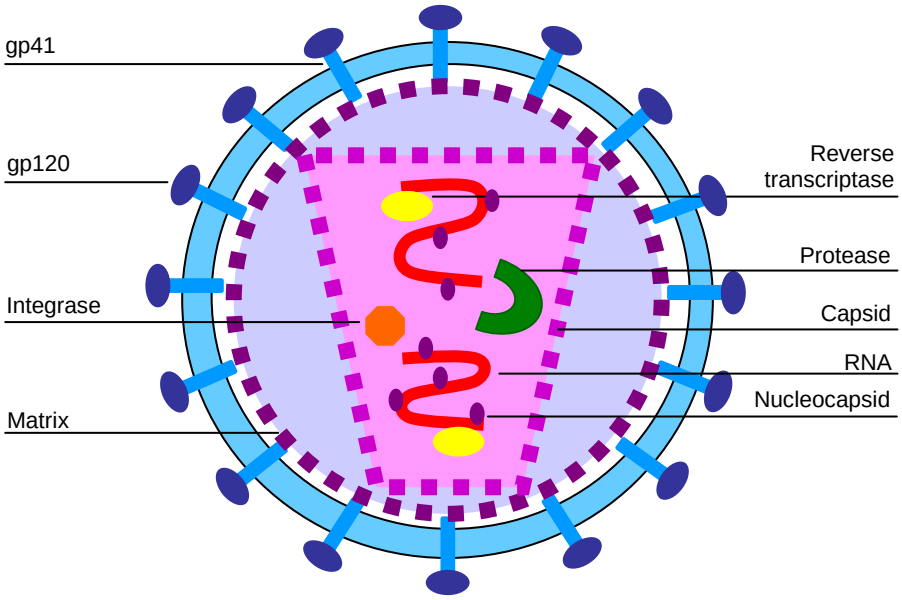

Figure 1: Simplified structure of the human immunodeficiency virus (adapted from [62, 63]). At the inner side of the envelope there is a layer of the matrix protein p17. The capsid is formed by the capsid protein p24 and found inside of a mature HIV particle. The capsid contains two copies of the viral RNA (ribonucleic acid) encoding for all necessary proteins of the virus (e.g. viral enzymes reverse transcriptase and protease). Nucleocapsid proteins (p7 and p6) are bound to the viral RNA. The functional viral proteins, i.e. reverse transcriptase, protease and integrase are also located inside the viral capsid.

1.1.2 Genome

The genome of HIV is composed of two copies of positive single-stranded ribonucleic acid (RNA) with a length of approximately 9500 base pairs. The genome harbours nine open reading frames encoding three structural genes called group antigen (*gag*), polymerase (*pol*)

and envelope (*env*), two regulatory genes (*tat* and *rev*) and four accessory genes (*nef*, *vif*, *vpu* and *vpr*) [62-64] (see Figure 2).

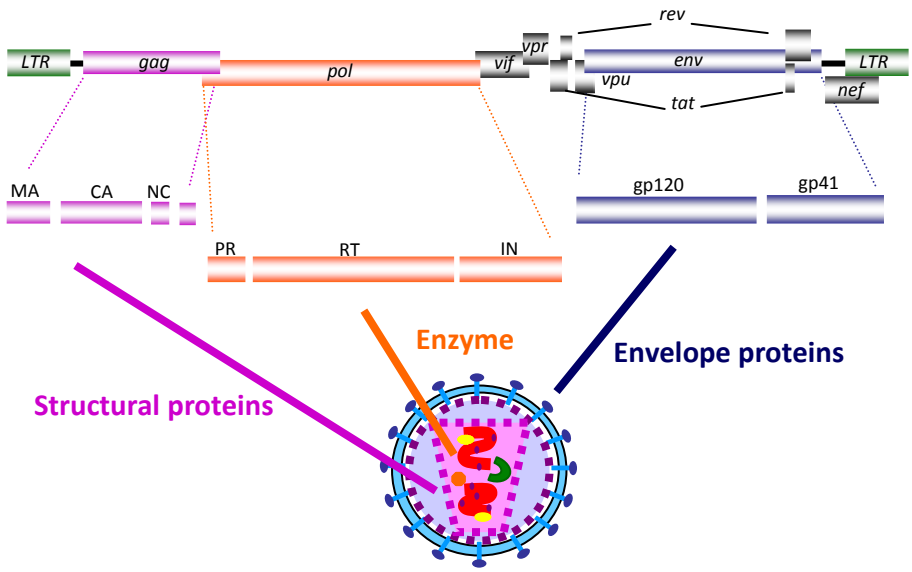

Figure 2: Simplified genomic organisation of human immunodeficiency virus 1 (adapted from [65]). Depicted are the nine open reading frames *gag*, *pol*, *env*, *vif*, *vpr*, *vpu*, *rev*, *tat* and *nef* that are flanked by two long terminal repeats (LTRs). *gag* (group antigen) encodes the structural proteins of the virus, i.e. the matrix protein (MA), the capsid protein (CA) and the nucleocapsid proteins (NC). *pol* (polymerase) encodes three viral enzymes, i.e. protease (PR), reverse transcriptase (RT) and integrase (IN). *env* (envelope) encodes for two envelope proteins, i.e. the glycoproteins gp120 and gp41.

The *gag* gene encodes the matrix protein (MA) p17, the capsidprotein (CA) p24 and nucleo-capsid protein (NC) p7 and protein p6. The *pol* gene encodes the viral enzymes, i.e. protease, reverse transcriptase and integrase, and the *env* gene encodes the glycoproteins (gp) 120 and gp41 envelope. The products of the regulatory and accessory genes are involved in different processes during the life cycle of the virus, e.g. nuclear export or regulation of transcription [62-64]. Both ends of the HIV genome are flanked by so-called long terminal repeats (LTRs), which play a regulatory role during the transcription of the viral genetic material into viral proteins by interacting with host cell enzymes of the transcriptional machinery [66].

1.1.3 HIV life cycle

The HIV replication cycle can be summarised in several steps (see Figure 3) [62-64]. First, HIV binds with its envelope protein gp120 to at least two specific receptors, to the CD4 receptor and to the chemokine co-receptors CCR5 or CXCR4 (Figure 3A). The binding to the co-receptor CCR5 triggers the protein gp41 mediated fusion of the HIV envelope with the host cell membrane (Figure 3B). After the envelope has been fused the HIV capsid containing the HIV genome and viral proteins are released into the cytoplasm. The capsid and

nucleocapsid are then dissolved, a feature called “Uncoating”, so that the genetic material and viral proteins are released into the cytoplasm (Figure 3C).

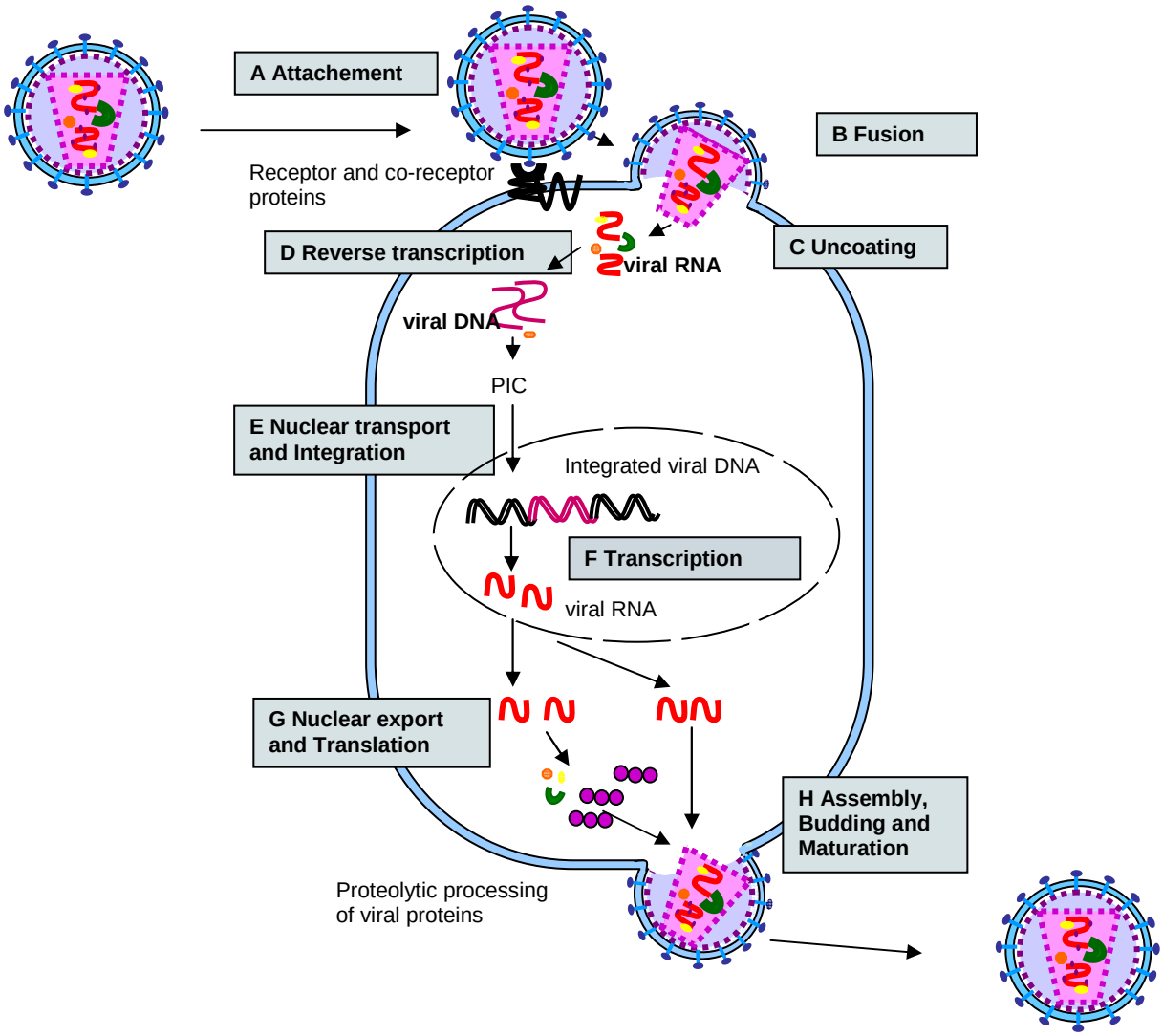

Figure 3: Overview of the HIV life cycle (adapted from [67, 68]). PIC: preintegration complex.

The viral genetic material – the RNA – must be converted into DNA (desoxyribonucleic acid) to be replicated in the host cell. This process is mediated by a viral enzyme called reverse transcriptase (Figure 3D). In the next step the viral DNA is transported to the nucleus as part of a so-called pre-integration complex (PIC) consisting of the viral DNA, the integrase and cellular proteins. The viral DNA is then inserted into the host DNA by the viral enzyme integrase. Once integrated in the host genome, the cellular RNA polymerase transcribes the viral genetic material into viral messenger RNA (mRNA, Figure 3F). This mRNA is exported to the cytoplasm where again host proteins translate the viral mRNA into the viral subunit proteins e.g. GAG, POL (Figure 3G).

The virus subunits are assembled and viral RNA packed before new particles are released from the cell surface. The outer envelope contains parts of the host cell membrane in which the viral envelope proteins gp120 and gp41 are embedded. The viral subunits must be separated to form a mature virus, i.e. GAG protein must be separated from POL protein and further GAG protein must be divided into the matrix, capsid and nucleocapsid proteins. This separation, or cleavage, is accomplished by the viral enzyme called protease and is necessary for the production of mature virus particles (Figure 3H). The typical conical form of the HIV capsid is only found in mature capsid and therefore the protease needs to cleave the GAG protein in its different sub-proteins (amongst others in the Matrixprotein, Capsidprotein and nucleocapsid protein). This step takes place after budding (not distinguished in Figure 3).

1.1.4 Genetic diversity

Even in absence of antiretroviral treatment, several mechanisms lead to the huge diversity and variability of HIV. First, the reverse transcriptase of HIV presents a high error rate because it lacks a proof reading mechanism [69]. Errors during the transcription process entail that the DNA copy of the virus is not an exact mirror of the RNA. This mechanism leads to permanent evolution of the HIV genome and also plays an important role in the emergence of HIV drug resistance mutations, which will be described in more details in section 1.3. Second, if a patient is infected with two different HIV strains then one RNA strain of each virus can be packed in one viral particle. A virus containing RNA strains from two different HIV strains can then infect new cells and lead to retroviral recombination during the reverse transcription step. Retroviral combination occurs because the reverse transcriptase can switch between the two strains and form one DNA strain containing genetic material of the two different strains [70-72]. A large variety of HIV subtypes and so-called circulating recombinant forms (CRFs) have been described.

1.1.4.1 HIV types, groups and subtypes

There exist two major types of HIV: HIV-1 and HIV-2. HIV-1 is the predominant HIV virus worldwide. HIV-2 is mainly found in West Africa and in countries with immigrants from this region [73].

HIV-1 can be divided into at least three groups: group M (major), group O (outlier), and group N (nonmajor, nonoutlier). Group M is the predominant circulating HIV-1 group and over 90% of the HIV infections are due to infections with a group M virus [74]. Group O is mainly found in central Africa and was first identified in Cameroon as was Group N, which is

very rare [75, 76]. Another new virus that is distinct from virus belonging to group M, O and N was found in Cameroon in 2009 and was classified in group P [77].

HIV-1 group M can be further subdivided into subtypes denoted with letters and sub-subtypes (or clades) denoted with numbers and so called circulating recombinant forms (CRFs). To date the following subtypes have been described A1, A2, A3, A4, B, C, F1, F2, G, H, J and K [74].

CRFs are the result of genetic recombination of different HIV strains. Recombination occurs if a patient is co-infected with different strains or more likely if a patient becomes super-infected with a second strain. At least three patients without direct epidemiological linkage must be infected to define a strain as a CRF. The name reflects the sequence of discovery and the subtype composition, e.g. CRF02_AG was the second CRF found and is a recombination between subtypes A and G. To date 48 CRFs have been discovered [78].

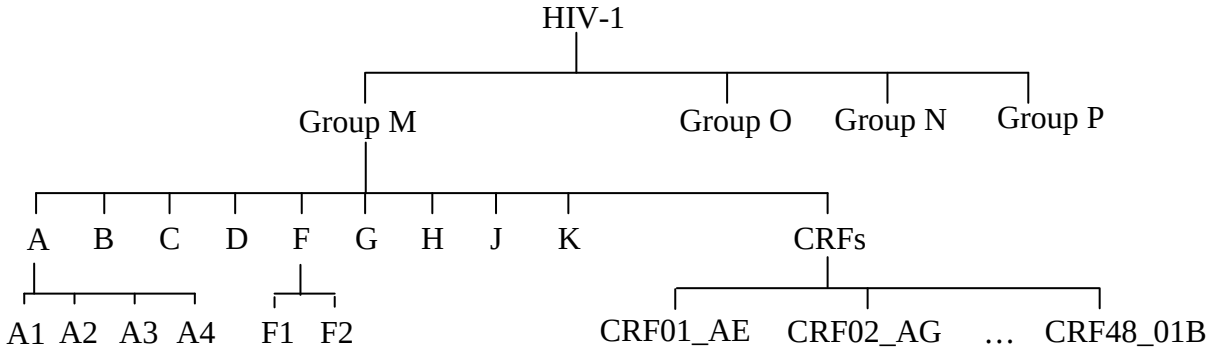

Figure 4: Overview of HIV-1 groups and subtypes

Of note, the nomenclature and classification of HIV subtypes is an evolving process, e.g. the former "subtype E" does no longer exists as it was found to be a circulating recombinant form containing components from subtype A [79, 80].

1.1.4.2 Distribution of HIV-1 subtypes worldwide

Subtype B accounts for 12% of the worldwide HIV infection but is the predominant virus in Western Europe, North and Middle America and Australia (see Figure 5). The most prevalent subtype is subtype C, which accounts for 50% of the worldwide HIV infection and is mainly found in India, China, and South and East Africa [81].

The most common CRFs are CRF01_AE and CRF02_AG with a prevalence of around 5% worldwide [81].

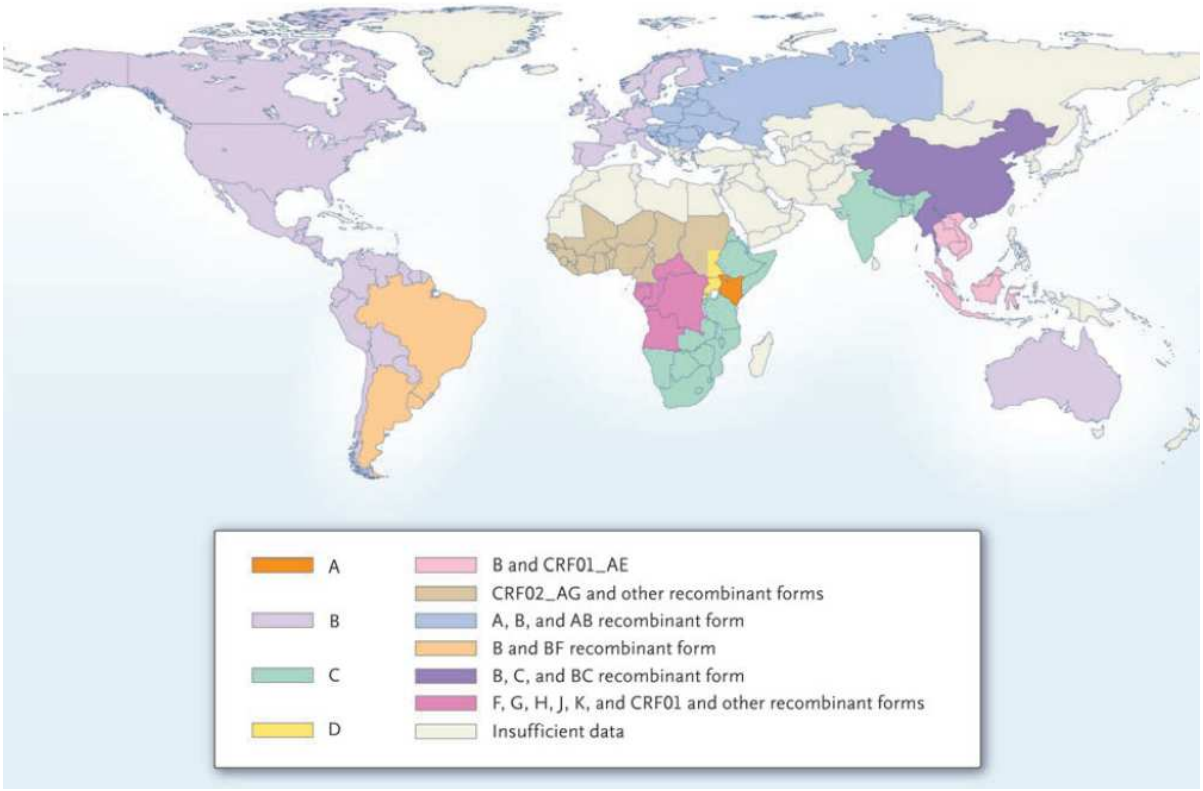

Figure 5: Global distribution of HIV subtypes and Circulating recombinant forms (CRFs) (from [74])**.**

1.2 Antiretroviral treatment

Antiretroviral treatment has remarkably decreased disease progression and mortality [82-86]. The following chapter gives an overview of currently available drugs, drug targets, the standard of care therapy and general aims of antiretroviral therapy.

1.2.1 Antiretroviral drug targets – Different classes of drugs available

Antiretroviral drugs target different steps during the HIV life cycle. Drugs targeting the same step during the life cycle are summarised into a drug class. Antiretroviral drugs targets are the host cell co-receptor CCR5, the envelope protein gp41, the reverse transcriptase, the integrase and the protease (see Figure 6). CCR5 antagonists block the co-receptor CCR5 and thus prevent the HIV virus from entering the cell because gp41 mediated fusion cannot take place (Figure 6A).

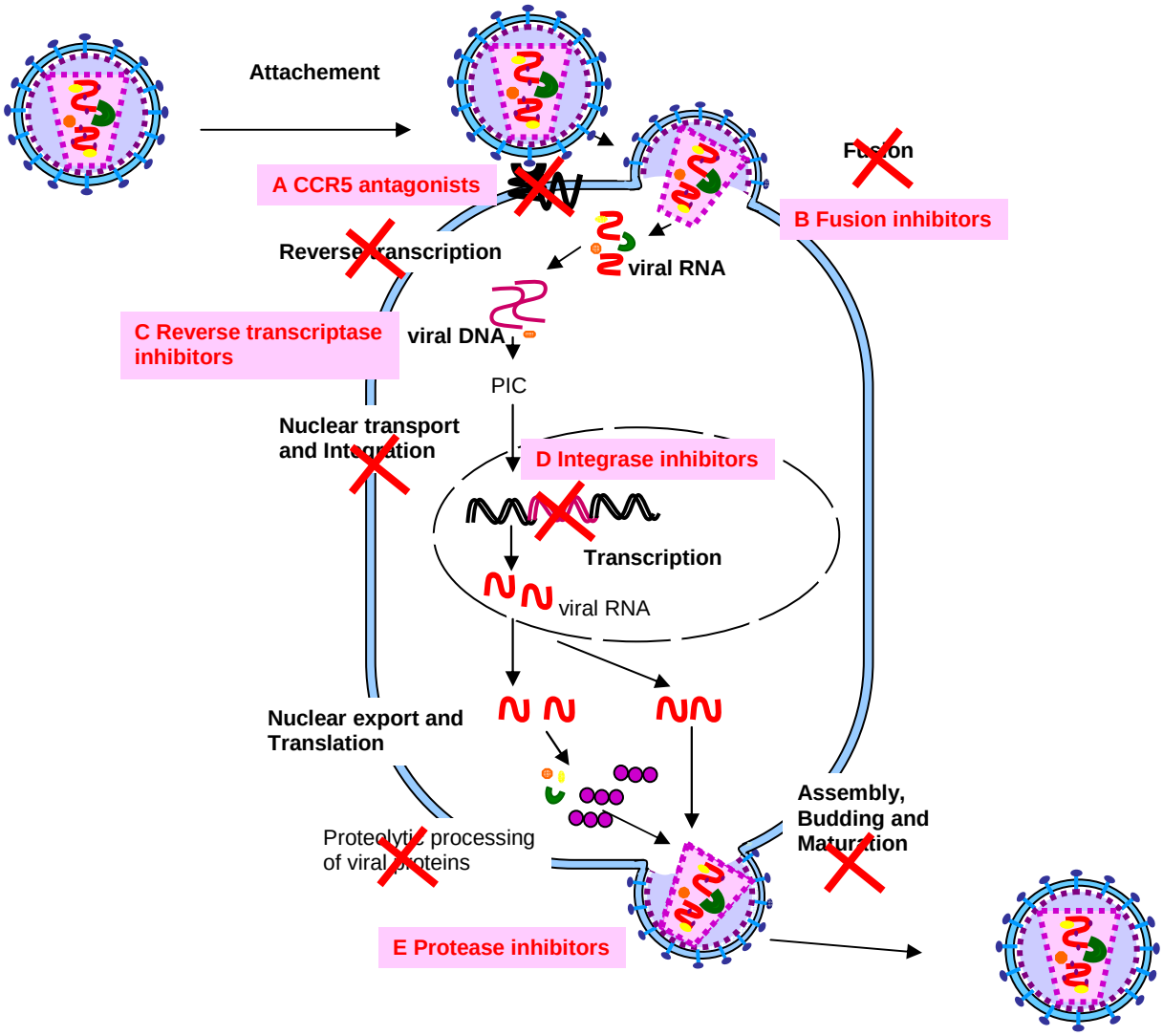

Figure 6: Antiretroviral targets in the virus life cycle (adapted from [67, 68]).

Fusion inhibitors disrupt the interaction of gp41 with the host cell membrane and thus inhibit the fusion of the virus with the cell membrane and ultimately inhibit the entry of the virus into the host cell (Figure 6B).

Reverse transcriptase inhibitors can be divided into two classes, NRTIs and NNRTIs. Both act as terminator of the DNA chain elongation via different mechanisms during the reverse transcription process (Figure 6C). HIV-integrase catalyses amongst others the insertion of the viral DNA into the host genome ('strand transfer'). The integrase strand transfer inhibitors block this process (Figure 6D). The HIV protease is an essential enzyme for virus maturation and thus inhibition of the protease leads to the production of immature, non-infectious HIV (Figure 6E).

1.2.2 Currently approved drugs

Zidovudine was the first antiretroviral drug approved by the United States Food and Drug Administration (FDA) in 1987. Today 26 drugs from six drug classes are approved by the FDA or the European Medicines Agency (EMEA) (see Table 1) and available for antiretroviral treatment [87, 88]. Further, fixed dose combinations of 2 or 3 NRTIs exist since 1997 and 2000, respectively. The first fixed dual drug class combination containing 2 NRTIs plus 1 NNRTI was approved in 2006 by the FDA and 2007 by the EMEA.

The protease inhibitor ritonavir is not recommended to be used as a single protease inhibitor anymore. However as ritonavir inhibits a particular liver enzyme cytochrome P450-3A4 that metabolizes protease inhibitors, a low dose of ritonavir can be used to enhance other protease inhibitors. A protease inhibitor plus ritonavir is also referred to as a boosted PI regimen.

Table 1: Overview of antiretroviral drugs used in the treatment for HIV infection.

Approval (FDA or EMEA)	Generic Name
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	
1987	zidovudine (ZDV, AZT)
1991	didanosine (ddI)
1992	zalcitabine (ddC) (no longer marketed)
1994	stavudine (d4T)
1995	lamivudine (3TC)
1997	lamivudine + zidovudine
1998	abacavir (ABC)
2003	emtricitabine (FTC)
2000	abacavir + zidovudine + lamivudine
2000	enteric coated didanosine (ddI EC)
2001	tenofovir (TDF)
2004	abacavir + lamivudine
2004	tenofovir + emtricitabine
Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)	
1996	nevirapine (NVP)
1997	delavirdine (DLV)
1998	efavirenz (EFV)
2008	etravirine
Protease Inhibitors (PIs)	
1995	saquinavir mesylate (SQV)
1996	indinavir (IDV)
1996	ritonavir (RTV)
1997	saquinavir (no longer marketed)
1997	nelfinavir mesylate (NFV)
1999	amprenavir (APV)
2000	lopinavir + ritonavir (LPV/RTV)
2003	Fosamprenavir Calcium (FOS-APV)

Table 1 (continued)

Approval (FDA or EMEA)	Generic Name
2003	atazanavir sulfate (ATV)
2005	tipranavir (TPV)
2006	darunavir
Fusion Inhibitors	
2003	enfuvirtide (T-20)
Entry Inhibitors - CCR5 co-receptor antagonist	
2007	maraviroc
HIV integrase strand transfer inhibitors	
2007	raltegravir
Multi Drug Class combination (2NRTI + 1NNRTI)	
2006	efavirenz + emtricitabine + tenofovir

FDA: U.S. Food and drug administration; EMEA: European Medicine Agency.

1.2.3 Combination antiretroviral therapy

Since 1996 combination antiretroviral therapy (cART) is recommended as standard of care regimen. CART consists of at least three antiretroviral drugs from two different treatment classes.

The choice of an initial treatment is very important as a life long treatment must be considered [1, 3] and the selection can have consequences for future therapy. The initial cART should be chosen appropriately based on resistance testing, previous disease history, social and demographic status, virological and immunological markers etc. to give the patient the highest chance for a successful treatment.

The most common treatment combinations for the initiation of cART recommended by recent treatment guidelines [1-3] are

1) 2 NRTIs + 1 NNRTI
2) 2 NRTIs + 1 ritonavir boosted PI.

The most recent recommendations for antiretroviral treatment of Adult HIV infection of the International AIDS Society-USA panel recommend also the combination 2 NRTIs + 1 INSTI for initial cART [3]. In Europe, this combination is to date only recommended as an alternative initial regimen because of the limited data on long-term tolerance and the more rapid selection of resistant variants in the case of virological failure compared to initial combinations containing ritonavir boosted PIs [1, 2, 89].

Treatment combinations for treatment experienced patients can be more sophisticated and contain for example three class treatments such as 2 NRTIs + 1 NNRTI + 1 Fusion inhibitor or regimen composed of PIs and NNRTIs.

1.2.4 Goals of antiretroviral therapy and monitoring treatment efficacy

HIV can not be eradicated and thus the main goal of antiretroviral therapy is to prevent disease progression, the development of AIDS and to prolong the patient's life. There are two main clinical markers used as surrogate markers to judge the status of the patient and the efficacy of treatment: 1) the viral load - the number of free virus per mL of patient's plasma - and 2) CD4 cell count - the number of CD4 cells per µL of patient's blood. After initiation of cART, viral load should be suppressed below 50 copies/mL (detection limit of current standard of care tests) after 6 months of therapy in treatment naïve and treatment experienced patients [1-3]. Further, antiretroviral therapy aims also at restoring the immune system and maintaining the CD4 cell count above 500 cells/µL [1].

Other treatment goals are for example a good tolerability of the treatment, a good quality of life and the reduction of mother to child transmission.

Treatment success should be monitored by regularly measuring the viral load and CD4 cell count. Assessment of antiretroviral toxicity depends on specific drugs and underlying co-morbidities (i.e. renal insufficiency) and intervals are two to four weeks after initiation and six to 12 months after stabilisation of the disease.

1.2.5 Viral load evolution and persistence of HIV after start of antiretroviral therapy

After treatment start the HIV viral load declines to below the detection limit after 3 – 6 months. First, there is a rapid decline of viral load followed by a slower decline that ultimately leads to viral load levels below the detection limit. The first decline can be attributed to the clearance of short living CD4 cells ($t_{1/2}$ = 1.2 days) and the second phase probably due to long-lived productively infected cells ($t_{1/2}$ = 27.2 days), activation of latently infected cells and release into the blood of virions from sanctuary sites (Figure 7, [90, 91]).

In patients with a viral load below the detection limit for several years, low level viremia can still be detected by more sensitive assays [93]. Blips are transient episodes of detectable viremia (by standard assays) and can occur even after long term suppression of viral load (Figure 7) [94].

Resting memory CD4 cells serve as a cellular viral reservoir and no virus is produced by these cells unless they are activated [4, 95, 96]. The half life of HIV infected memory CD4 cells was estimated to be 44 months and eradication would need over 60 years of suppressive cART [97-99].Other cell types, e.g. monocytes or dentritic cells and anatomical sites, e.g. the central nervous system, may also constitute a reservoir for HIV (for a review see [4]).

The viral reservoir serves as an archive for all forms of virus, i.e. wild-type and resistant mutants [7-12] and see section 1.5.3).

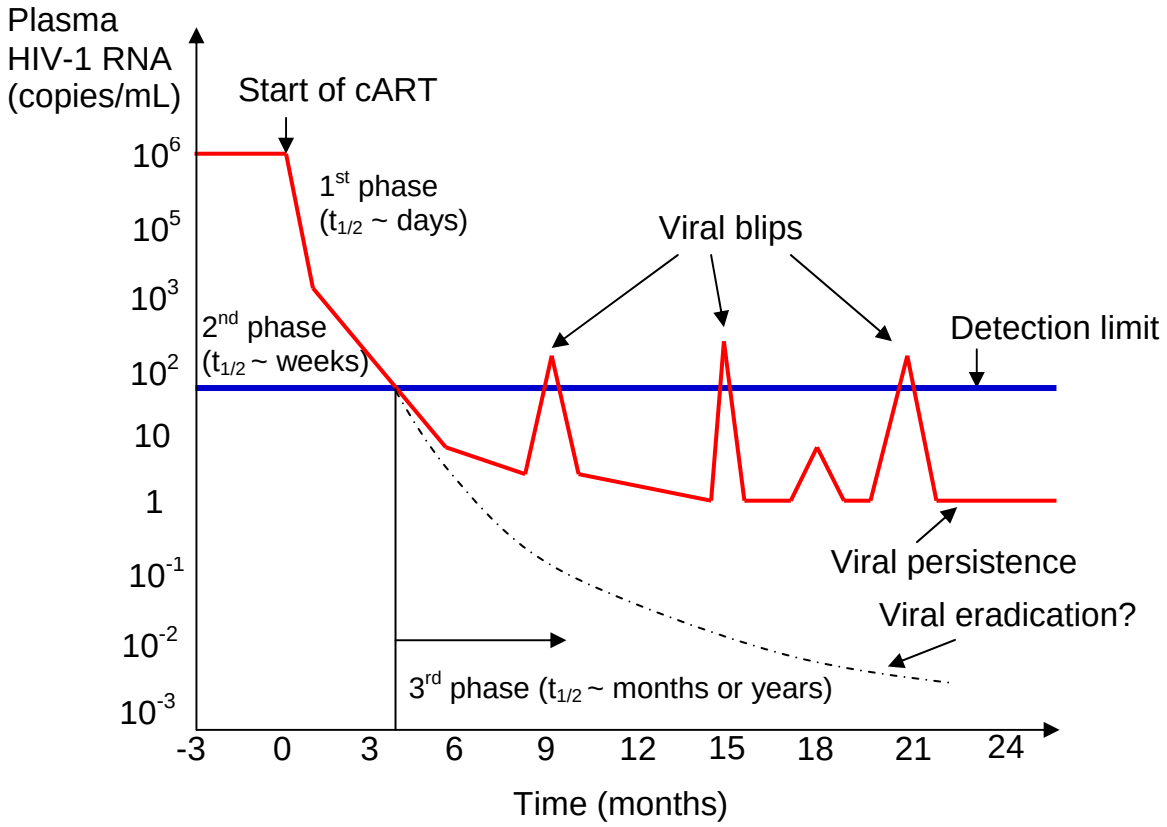

Figure 7: Evolution of plasma viral load after the start of cART (adapted from [92]). cART: combination antiretroviral therapy, $t_{1/2}$: half life of infected cells.

1.3 Antiretroviral drug resistance

Antiretroviral drug resistance is characterised by the need of higher concentrations of antiretroviral drugs to achieve viral suppression. Finally, antiretroviral drug resistance leads to the loss of the ability of the treatment to inhibit the viral replication. Mutations (changes in the genome in comparison with a wild type strain of the virus) in the viral genome, especially in regions targeted by antiretroviral drugs, e.g. reverse transcriptase, are the cause of antiretroviral drug resistance.

We can distinguish between primary/transmitted drug resistance and secondary/acquired drug resistance. Transmitted drug resistance is detected in treatment naïve patients and is due to the infection of the patient with a virus already carrying drug resistance mutations. Acquired drug resistance is detected in antiretroviral experienced patients. Antiretroviral drug resistance can

be specific for a given drug or can also affect drugs of the same drug class, so-called "cross-resistance".

Drug resistance is an inevitable consequence of antiretroviral therapy. Drug resistance is influenced by many factors such as the ability of the regimen to suppress replication, the adherence to and tolerability of antiretroviral treatment, pharmacokinetics, the availability and continuity of drug supply and access to care. Virological factors also play a role for the development of drug resistance, for example the so-called "genetic barrier" of a drug to develop resistance (number of mutations required to reduce the antiretroviral activity) and the relative fitness (replicative capacity under drug exposure) of resistant variants. The genetic barrier is different for each drug, and the higher the genetic barrier of a given drug the more rare the development of drug resistance.

1.3.1 Pathogenesis of drug resistance

The reverse transcriptase of HIV is error-prone and this leads to a high mutation rate with, in average, one error per progeny virion [100]. Given the high replication rate of approximately 10 million new viral particles [101] and the high error rate of the HIV reverse transcriptase, any single mutant could be generated per day [101]. Thus, HIV infection in a single individual is characterised by a heterogenous viral population called "quasipecies" [102], this means that the circulating viruses are not represented by a unique virus genotype but by heterogenous genotypes that are genetically related.

Most mutations are dead ends for the virus as they result in a considerable loss of replicative capacity. Some mutations have no effect or even a beneficial effect on the replicative capacity of the virus. In the absence of therapy the wild type virus is the virus with the better ability to replicate and is thus the most abundant one.

Viruses that represent more than 20% are the so-called major virus population whereas viruses that represent < 20% of the whole virus population constitute the minor virus population.

Viral mutants with a fitness advantage under therapy can rapidly overgrow the wild type virions in the presence of this therapy (see Figure 8). Under drug selection pressure, complete replacement of wild type virus by drug resistant virus can occur within 14-28 days [103]. Persistent viral replication under antiretroviral therapy can lead to the accumulation of more mutations resulting in an increased resistance or an improved fitness.

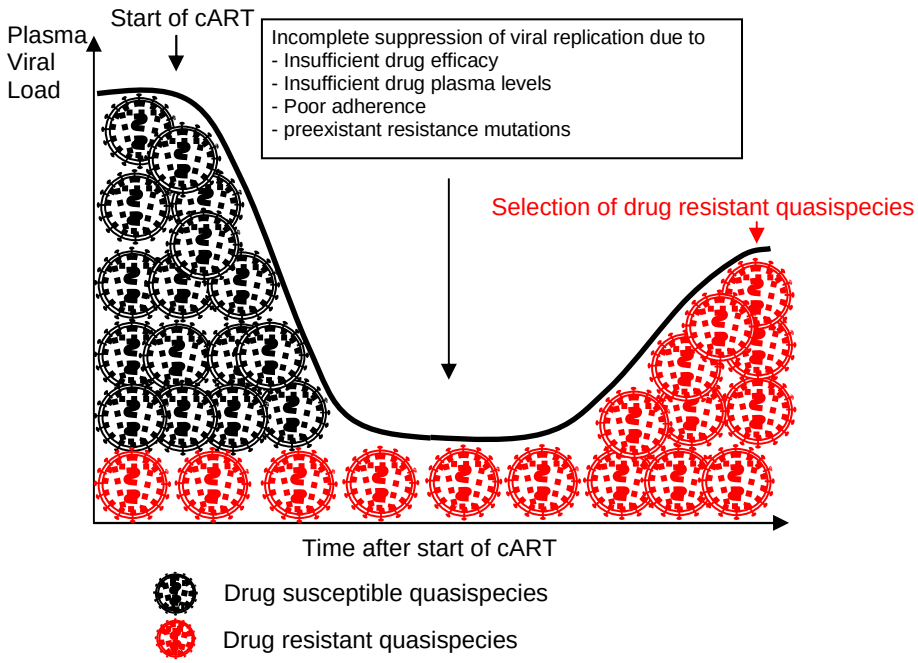

Figure 8: Simplified schematic illustration of selective drug pressure. After treatment initiation the drug susceptible quasispecies (in black) are declining. Incomplete suppression of viral replication leads to the selection of a drug resistant quasispecies (in red) able to replicate in the presence of antiretroviral drug. cART: combination antiretroviral therapy.

1.4 Resistance testing

Genotypic and phenotypic resistance tests are available to determine the sensitivity of HIV for a specific drug. For optimal assay performance, a minimum vial load of 500-1,000 copies/mL is required for genotypic and phenotypic testing.

1.4.1 Phenotypic testing

Phenotypic resistance tests quantify drug sensitivity directly. They are based on viral replication in cell culture in the presence of different drug concentrations. The most common summary measure of a phenotypic test is the 50% inhibitory concentration (IC50) that is the drug concentration needed to reduce viral replicative activity by 50%. The IC50 of the test strain is compared to the IC50 of a reference strain and reported as fold change. The fold change reflects thus the difference in drug concentration needed to obtain a 50% suppression of viral activity. Phenotypic testing however takes a long time and is expensive. In clinical routine genotypic resistance tests are used to determine resistance mutations in the viral genome.

1.4.2 Genotypic testing

After amplification by a technique called polymerase chain reaction (PCR), the nucleotide sequences of regions encoding the molecular targets of antiretroviral therapy from the

patient's viral population are determined by population sequencing. This implies that their detection requires an abundance in mutants of at least 20 to 30% in the total viral population.

The nucleotide sequence of the patient's viral population is compared to a reference wild-type sequence. This alignment allows the determination of differences in the genome. Mutations in a patient's virus population, when they are not silent (i.e. a silent mutation is a change in the nucleotide sequence without impacting on the amino acid sequence), lead to amino acid changes on the protein encoded by the portion of the genome that was sequenced, e.g. protease or reverse transcriptase (see Figure 9).

The amino acid of the reference strain is reported first, followed by the codon and the amino acid of the tested virus strain. The example from Figure 9, in which the aminoacid Methionin (M) at position 46 is replaced by Leucin (L), would be reported as protease mutation M46L.

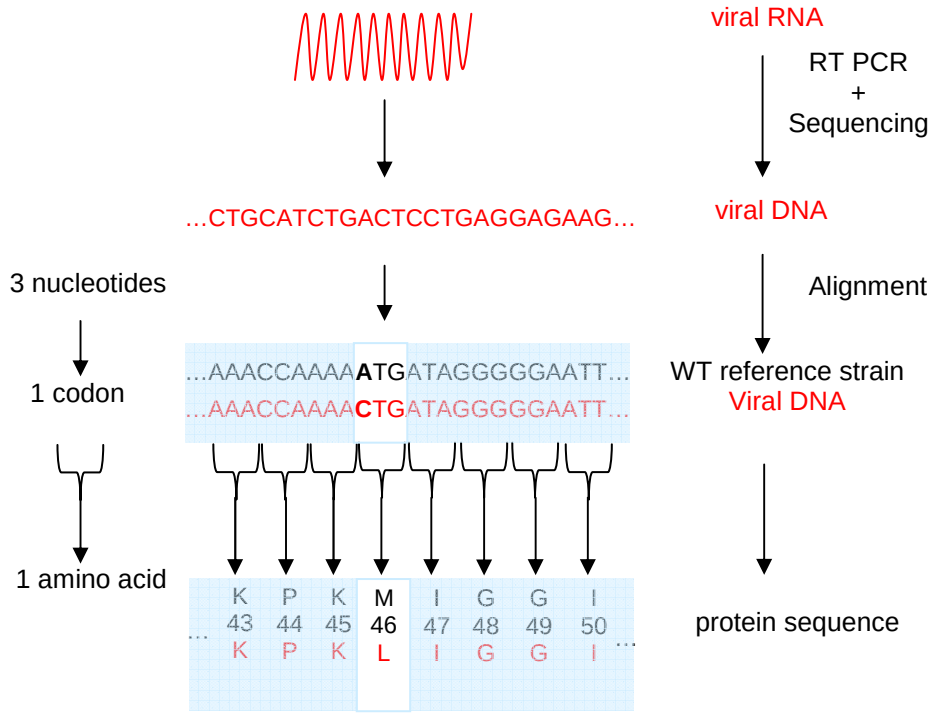

Figure 9: Overview of genotypic testing. The viral RNA of the patient's major population is amplified using reverse transcriptase polymerase chain reaction (RT PCR) and the nucleotide sequence of the DNA is then obtained. The viral DNA is then aligned (compared) to a wild type (WT) reference strain (positions 43 to 50 of the protease are used for the example). Finally, the encoded protein sequence is determined. In the above example, the amino acid Leucine (L) is found at position 46 of the protease on the viral DNA instead of a Methionin (M) at the same codon on the WT reference strain.

1.4.3 Minority resistance testing

New techniques such as ultradeep 454 sequencing, allele-specific polymerase chain reaction or single genome sequencing allow the detection of minority resistant variants going down to

a detection sensitivity of ≤1% [104, 105]. However, the use of these techniques is limited to research protocols and is not implemented in routine care so far.

1.4.4 Limitations of genotypic testing

One main limitation of genotypic testing is the complexity of interpretation, especially in the presence of multiple mutations where complex interactions between mutations cannot be excluded. Therefore, the impact of mutations on the replication capacity and on the impact of treatment outcome is constantly evaluated by panels of experts as e.g. the French ANRS AC11 Resistance group or the International AIDS society USA panel [16, 33]. Current interpretation algorithms are regularly updated as are guidelines for antiretroviral resistance testing and management of HIV drug resistance [13, 106].

1.4.5 Drug resistance mutations and mechanisms

As already mentioned earlier, mutations in the reverse transcriptase gene may result in resistance against NRTIs or NNRTIs. Accordingly, mutations in the protease gene, the integrase gene, and the envelope protein gp41 may result in resistance against PIs, INSTIs, and the fusion inhibitor enfuvirtide, respectively (see Figure 10).

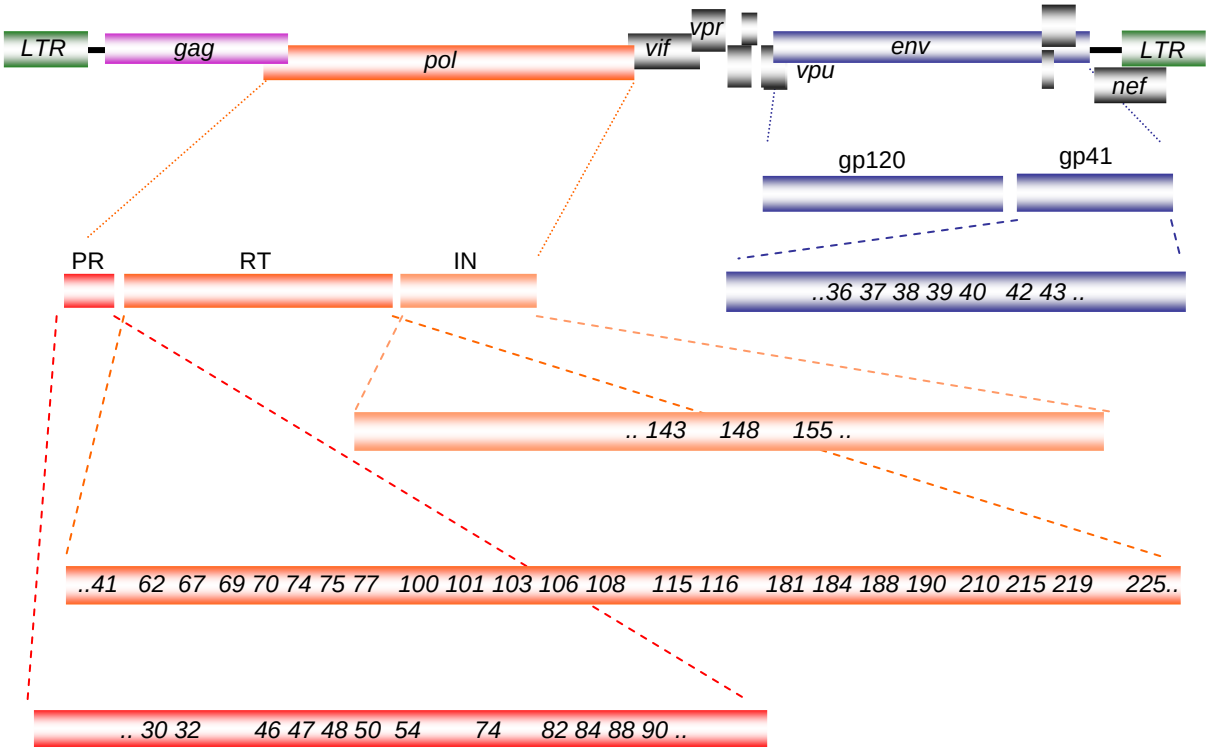

Figure 10: Drug resistance mutations in the HIV genome (adapted from [65])**.** The current drug targets of the HIV genome are the protease (PR), the reverse transcriptase (RT) and the integrase (IN) encoded by the *pol* gene as well as the envelope protein gp41 encoded by *env* gene. The positions of four targets on which drug resistance mutations occur are depicted (IAS-USA, December 2009, only major mutations are depicted). *gag*, group specific antigen, LTR, long terminal repeats. *vif, vpr, vpu and vef* encode viral accessory proteins; *tat* and *rev* encode viral regulatory proteins. Numbers in the figure correspond to amino acid positions in the encoded protein.

The envelope protein gp120 may be sequenced to determine the tropism of the virus (i.e. CCR5, CXCR4, or a mixture of both) and to predict the susceptibility to CCR5-inhibitors [107]. Only resistance mutations in the reverse transcriptase and protease genes are described in more details in the following sections because most regimens are still based on NRTIs, NNRTIs and PIs. Further, for studies presented in chapter 5 and 6 we analysed genotypic data of reverse transcriptase and protease sequences.

The protease gene encodes the 99 amino acids forming the viral enzyme protease and nearly all positions of the protease should be sequenced for clinical purposes [14, 15]. The reverse transcriptase gene encodes approximately 560 amino acids of which standard sequencing should cover amino acids in positions 41 to 236 [14, 15]. Theoretically, each wild type amino acid can be changed into 19 different amino acids. For example, the Methionine in position 46 of the protease (see example Figure 9) can be replaced not only by Leucine but also by Isoleucine, a different amino acid.

1.4.5.1 Protease gene mutations and protease inhibitor resistance

Protease gene mutations are divided into primary (major) and secondary (minor) mutations [16]. Primary/major mutations are selected under drug selective pressure and are highly specific because they are often located in the substrate cleft (active centre) of the enzyme [14, 15]. Primary mutations affect binding affinity of the inhibitor drug to the active site without totally inhibiting the physiologic protease substrate-interaction. Secondary/minor/accessory mutations are located outside the active site and usually occur later than the primary mutations. Secondary mutations may play a compensatory role for the initial decrease of viral fitness and are thought to cause an active site re-shaping through structural changes of the protease [14, 15]. Protease inhibitor resistance is characterised by the accumulation of multiple mutations, and in order to develop clinically relevant resistance typically more than one mutation is necessary [16, 108]. Currently major mutations are defined for 14 codons of the protease and minor mutations for 20 codons of the protease by the International AIDS society USA mutation list (an internationally used reference list) [16].

1.4.5.2 Reverse transcriptase inhibitor mutations and resistance to NRTIs and NNRTIs

1.4.5.2.1 NRTIs

NRTI resistance mutations consist in thymidine analog mutations (TAMs), non thymidine analog regimen mutations, multi-NRTI resistance mutations, M184V and other accessory

mutations. NRTI resistance mutations act either by blocking incorporation of the NRTI into the DNA chain or by removing the NRTI from the DNA chain [109-112]. TAMs and multi-NRTI resistance mutations affect all NRTIs currently approved by the FDA and EMEA and are found on 6 codons of the reverse transcriptase, respectively [15, 16].

1.4.5.2.2 NNRTIs

NNRTIs have a low genetic barrier to develop resistance, i.e. only one or two mutations are required for resistance. NNRTI resistance mutations reduce susceptibility to multiple NNRTIs implying a high risk for cross-resistance. NNRTI resistance mutations occur close to the active site of the reverse transcriptase at the NNRTI binding pocket [113]. Shafer *et al.* classified NNRTI resistance mutations in four classes: primary, secondary, minor non-polymorphic and polymorphic accessory mutations [15].

1.4.6 Interpretation of genotypic tests and genotypic mutations

Interpretation algorithms are designed to assist the physician in choosing an optimal drug combination for a given patient using information from drug-resistance testing. In this context, `interpretation' refers to the task of predicting a parameter of treatment response (i.e. drug activity or virological response) from genotypic data and additional characteristics.

A variety of interpretation algorithms that predict either the *in vivo* activity of a specific drug or the *in vitro* activity, i.e. the replicative drug activity measured by a phenotypic test (see Table 2), are available. Further, more recent interpretation algorithms allow for predicting the *in vivo* activity of drug combinations, i.e. THEO or EuResist [39, 114]. All lists and algorithms are mainly developed on knowledge based on HIV-1 infection and more specific for infection with a subtype B virus.

Genotyping is widely used in clinical practice and therefore algorithms predicting *in vivo* drug activity from genotypic data are of great interest to support the clinician in antiretroviral drugs selection. The main limitations in relating the viral genotype to drug activity are a lack of studies with monotherapy data and the high diversity of mutations as well as the complexity of mutational patterns.

1.4.6.1 Genotypic sensitivity score

Interpretations systems currently used in clinical practice evaluate each drug separately (beside THEO and EuResist) and therefore a genotypic sensitivity score (GSS) for a given combination of drugs is usually calculated. The GSS reflects the number of active drugs in the regimen of a patient and is calculated based on the result of a genotypic interpretation

algorithm. Usually, it is calculated by assigning a score of 1 for susceptible/potential low level resistance, 0.5 for low-level/intermediate resistance and 0 for high-level resistance. Therefore, a standard of care combination antiretroviral therapy consisting of three drugs would attain a total GSS of 3 if no resistance for a prescribed drug is present.

Table 2: Selection of freely available interpretation algorithms (for a complete overview please see [106, 115, 116])

System and Reference	Description	Output
In vivo activity of single drugs		
ANRS AC11 [33]	A rule-based algorithm established by a panel of experts. Table of rules combining mutations conferring drug resistance (i.e. from clinical studies, observational studies) and studies correlating clinical outcome and genotypic mutations	3 categories: Resistant, intermediate resistant, susceptible
HIVdb, Stanford HIV drug resistance database [34, 117]	A rule-based algorithm combining information from (i) Published studies and data linking mutations to ARV therapy; (ii) Published studies and data linking mutations to decreased ARV susceptibility; (iii) Published studies linking pre-therapy mutations with the virological response to a new ARV treatment regimen	5 categories: 1) susceptibel 2) potential low-level resistance 3) low-level resistance 4) intermediate resistance 5) high level resistance
Rega algorithm [118, 119]	A rule-based algorithm established by a panel of experts.	3 categories: Resistant, intermediate resistant, susceptible
In vitro phenotypic resistance of single drugs		
geno2pheno [38, 120]	Database derived algorithm based on information from 1100 genotype-phenotype training pairs. The algorithm is based on support vector machines.	Fold-changes in IC50% result
Virological response to combination therapy		
THEO (THErapy Optimizer) [114, 121]	Database derived algorithm based on genotypic resistance data linked to treatment outcome using logistic model trees for prediction. Links the data also to phenotypic resistance data and calculates the genetic barrier based on mutagenetic trees to add information for prediction.	Amongst others: Probability of virologic success over 24 or more weeks for selected combination therapies
EuResist [39, 122, 123]	Database derived algorithm based on information from more than 39,000 patients combining three statistical learning engines. Allows for inclusion of baseline characteristics such as viral load, age, sex and CD4 cell count for prediction.	

1.4.7 Definition of drug resistance for the evaluation of prevalence

Drug resistance can be defined as either having at least one mutation of an established list of mutations or being classified as resistant to at least one drug using a current interpretation algorithm.

Updated lists of drug resistance mutations are available, for example the IAS-USA list, which is updated on a regular basis [16]. The IAS-USA list summarises mutations described for each currently FDA approved drug but is not an interpretation algorithm. Thus, the list does not allow for determining whether a patient harbouring a virus with a given set of mutations is resistant or not. The list contains further a lot of polymorphisms that may contribute to resistance in the presence of other resistance mutations but can also be found in untreated patients because mutation frequency is high at these positions. Polymorphisms are not necessarily specific mutations that developed under drug pressure. Assessing the prevalence of transmitted drug resistance using this list could lead to an overestimation of drug resistance due to these polymorphisms. Therefore, a lot of authors use the definition of at least one *major* resistant mutation of the IAS list.

The World Health Organisation (WHO) list for surveillance of transmitted drug resistance mutations is a more restricted list of mutations that was conceived for drug resistance surveillance. The list includes only non-polymorphic mutations. Further, it aims at giving a standard list for accurate estimation of transmitted drug resistance from different regions and times [124]. To date, the WHO list is the standard list for surveillance of transmitted drug resistance mutations. In general, transmitted drug resistance is defined as having at least one mutation of this list.

Genotypic testing can detect different amino acids at one position due to the viral quasispecies (i.e. more than one major quasispecies can exist). For example, one major quasispecies can harbour a Leucine at position M46 and a second major quasispecies can harbour an Isoleucine at the same position. This would be reported in a mixture of mutations at position M46, e.g. reported as M46L/I. Mixtures between aan amino acid known to cause drug resistance and the wild type amino acid are possible as well, e.g. reported as M46M/L.

Differences in reported prevalence can thus not only be due to the use of different lists or algorithms but also to the way a mixture of mutations or a mixture between mutation and wild type on a given position were considered [125].

1.5 Epidemiology of antiretroviral drug resistance

1.5.1 Transmitted drug resistance in adults

Transmitted drug resistance is observed in most countries where antiretroviral drugs are available. The first cases of transmitted drug resistance have been reported in the 1990s [126]. Transmitted drug resistance varies according to region, study population and other factors

[127] and can also vary due to the use of different interpretation systems or surveillance lists for transmitted drug resistance [128]. The following sections focus on transmitted drug resistance for the commonly used drug classes NRTIs, NNRTIs and PIs. Knowledge about the transmission of resistance mutation to newer treatment classes (e.g. INSTIs and Fusion inhibitors) is scarce but a first case of transmission of integrase mutations has already been reported [127] as has transmitted drug resistance for enfurvitide [129].

1.5.1.1 Prevalence in Europe and North America

Prevalence of transmitted drug resistance in Europe ranges between 10% and 15% [19, 21, 23, 24, 28, 59-61]. In a large European surveillance study the prevalence was found to be stabilising around 8% in recent years (data up to 2005 included) in Europe [60]. A peak prevalence of transmitted drug resistance of around 15% in the years 2000 to 2002 was found in the UK with a decline to a prevalence of around 9% in 2004 [130]. A similar trend was found in the European SPREAD study [60]. Higher prevalence especially in earlier time periods can be due to various reasons but a selection bias cannot be excluded, given that resistance testing in treatment naïve patients was not part of standard care and might have preferentially been prescribed in patients at high risk for carrying transmitted mutations. The stabilisation of a prevalence of transmitted drug resistance around 10% especially in the latest published studies may also be a result of the use of standardised mutation lists for surveillance of transmitted drug resistance (e.g. [124, 131]).

Prevalence of transmitted drug resistance was found to be up to 25% in North America [26, 27]. The most recent study published by Wheeler *et al.* found a prevalence of 14.6% using data from 10 states and defining transmitted drug resistance with the current surveillance list of the WHO [132].

1.5.1.2 Minority variants

The prevalence of transmitted drug resistance may be underestimated due to the fact that resistance mutations are detected only in the major virus population. Some transmitted drug resistance surveillance studies using ultrasensitive resistance test methods suggest that prevalence of transmitted drug resistance could at least be two fold higher compared to population-based sequencing [105, 133]. In a recent study of Lataillade *et al.* prevalence of transmitted drug resistance was found to be 30.5% using ultra deep sequencing in treatment naïve patients included in the CASTLE study. Prevalence of transmitted drug resistance was 14.9% using population based sequencing in the CASTLE study [134].

1.5.1.3 Prevalence in resource limited settings

Individual genotypic testing is not recommended in resource limited settings by the WHO. However, a need for population-based surveillance of both transmitted and acquired drug resistance was acknowledged by the WHO [135].

Reports of surveys with small sample sizes of sub-Saharan Africa indicate an overall prevalence of transmitted drug resistance of 0% to 14.8% (for an overview see [23]). Prevalence reaching such levels in developing countries can be partly explained by genotypic testing in a highly selected population but also by the use of unadapted mutation lists. In contrast, a WHO HIV drug resistance survey including data from Ethopia, Malawi, South Africa, Swaziland, Tanzania, Thailand and Vietnam found a prevalence of <5% using a transmitted drug resistance surveillance mutation list [136]. In India, transmitted drug resistance was found to be 10% among treatment-naïve patients [137].

Access to antiretroviral treatment is steadily growing in the developing world and with a higher exposure to antiretroviral treatment the level of acquired resistance is expected to rise. Consequently, prevalence of transmitted drug resistance will probably rise at least to levels comparable to that in the developed world. A mathematic model published by Blower and colleagues predicts that levels of transmitted drug resistance will rise to levels above 5% 10 years after scaling up antiretroviral treatment or if >30% of all HIV infected patients are treated with antiretroviral therapy [138].

1.5.1.4 Prevalence of transmitted drug resistance in children

Prevalence of transmitted drug resistance in children was found to be up to 87% if the mother received single-dose nevirapine for prevention of mother to child transmission [139]. In a meta-analysis Arrivé *et al.* found a nevirapine resistance prevalence of 52.6% in 4-6 week old children if only single-dose nevirapine was administered to the mothers [140]. The nevirapine prevalence was 16.5% when other antiretrovirals had been given besides single-dose nevirapine to the mothers or to the children [140].

1.5.1.5 Impact of transmitted drug resistance on treatment outcome

The potential impact of transmitted drug resistance on treatment response remains controversial and has not been fully described (see Table 3). Some studies report no significant association between the presence of transmitted drug resistance and time to viral load suppression, proportions with viral load suppression [19, 29, 30] or with immune response [19, 26, 29, 30].

Table 3: Overview of studies investigating impact of transmitted drug resistance on treatment outcome by descending sample size.

Reference	N*	Country	Cohort name/Study population	Year	TDR	Main Results
Bansi *et al.* [141]	935	UK	UK-CHIC patients with resistance test prior to the start of cART as part of routine care	1999-2006	Stanford algorithm was used to calculate a GSS	Patients with a GSS<3 had higher risk to not suppress VL below 50 copies/mL within the first year
Chaix *et al.* [142]	350	France	ANRS CO 06 PRIMO, Genotype was not used for treatment selection	1996-2005	Resistant to ≥1 drug - ANRS	% of patients with a VL <400 cp/mL was lower in pts with resistance (at week 12 and 24)
Bannister *et al.* [19]	277	Europe	EuroSIDA, Genotype was not used for treatment selection	1996-2004	At least intermediate resistance to ≥1 drug - Stanford	No difference for % of VL <500 (at week 12 and week 24)
Oette *et al.* [29]	269	Germany	RESINA, Genotype was used to guide treatment selection	2001-2003	geno2pheno	No difference for % of VL <50 (at week 24 and week 48)
Pillay *et al.* [28]	201	Europe	Seroconverters CASCADE, Genotype was not used for treatment selection	1996-2003	At least intermediate resistance to ≥1 drug - Stanford	No impact on time to VL suppression <500 copies/mL
Grant *et al.* [31]	141	USA, San Francisco	~80% MSM, Genotype was not used for treatment selection	1996-2001	≥1 major mutation of the IAS-USA list	Time to VL suppression <500 copies/mL was longer in pts with genotypic resistance
Shet *et al.* [26]	73	USA, New York	Newly diagnosed, Genotype was used for treatment selection	2003-2004	≥1 IAS-USA mutation	No impact on time to VL suppression <50 copies/mL
Poggensee *et al.* [30]	69	Germany	German HIV-1 Seroconverter Study, Genotype was not used for treatment selection		At least intermediate resistance to ≥1 drug - Stanford	% VL <500 cp/mL was lower in patients with resistant strains but not statistically significant

*Number of patients available to assess the impact of transmitted drug resistance on virological response. TDR: transmitted drug resistance, cART: combination antiretroviral therapy, GSS: genotypic sensitivity score, VL: viral load, MSM: men having sex with men

Other studies report poorer virological response in patients with transmitted drug resistance and a significantly shorter time to viral load suppression among patients with susceptible strains [26, 30, 141, 142].

However, all these studies are hampered by a lack of statistical power due to the relatively small proportion of patients with transmitted drug resistance included. In particular, the impact of transmitted drug resistance on virological response in patients treated with a fully active regimen has not yet been explored in the context of systematic genotypic testing prior to treatment initiation in larger datasets. We have therefore investigated the impact of

transmitted drug resistance on virological and immunological outcome after the start of first line combination antiretroviral therapy in a large European study (see chapter 5).

1.5.2 Prevalence of antiretroviral drug resistance in treatment experienced patients

In a French nationwide study, prevalence of drug resistance to at least one antiretroviral drug using the ANRS interpretation algorithm was found to be 88% in treated patients with a viral load of >1000 copies/mL [17]. In patients failing a first line regimen included in the Swiss HIV cohort, resistance mutations (major mutations of the IAS-USA list) were found in 84% of patients who started a regimen containing an unboosted PI, 66% of patients who started a regimen containing an NNRTI and 30% of patients who started a ritonavir boosted PI [143].

Nevertheless, cumulative resistance to all three most commonly used drugs classes (i.e. NRTIs, NNRTIs and PIs) is rare. Costagliola *et al.* observed resistance to three classes in 4% of their study population in France and Lima *et al.* in 2% of their patients in British Columbia (Canada) [17, 144]. Development of multiclass resistance was more frequently observed in patients starting an NNRTI based regimen than with other regimens [143, 144].

Prevalence of acquired drug resistance in resource limited settings was found to be over 80% [145, 146]. In a systematic review Gupta *et al.* found that prevalence of drug resistance was 88% for patients with infrequent monitoring and 61% for those with frequent monitoring [147].

1.5.3 Persistence of drug resistance

1.5.3.1 Persistence of transmitted drug resistance

In contrast to acquired drug resistance, patients with transmitted drug resistance virus do not have a reservoir of drug susceptible (wild type) virus. This implicates that transmitted drug resistant virus can only revert to wild type by back mutation. This can occur very rapidly for specific mutations such as reverse transcriptase mutation M184V which is linked to a fitness cost [148, 149]. For other mutations this back-reversion is less common or uncommon such as reverse transcriptase mutations Y181C or M41L, respectively.

HIV resistant strains acquired at the time of primary infection massively fuel the cellular reservoir and can persist over long time periods [150-153]. Delaugerre *et al.* still found multidrug resistant HIV-1 two years after sexual transmission in a patient who was not treated during this time period [151]. A recent study even found transmitted drug resistance after 10

years in a patient without treatment [154]. Further, transmitted drug resistance mutations were found to persist even when the viral load was suppressed to undetectable levels [155].

1.5.3.2 Persistance of acquired drug resistance

After treatment discontinuation there is a rapid decline of HIV drug resistance mutations and the wild-type virus archived in the viral reservoir overgrows the resistant virus in weeks after the arrest of drug selective pressure [156-160].

However, it is important to know that mutant viruses remain incorporated in the viral quasispecies and in the viral reservoir [7-12]. These mutant viruses may “reappear” under selective pressure, e.g. if the same drug is re-introduced.

Thus, the whole treatment story and former genotypic test results, if available, should be considered for treatment optimization.

2 Statistical analysis of the impact of drug resistance on treatment response

2.1 Definition of endpoints

The definition of the endpoint of a study is crucial as the main conclusions are based on it and the sample size depends directly on it. The endpoint of a clinical study is usually a quantitative measurement(s) in relation to the objectives of the study. In other words the endpoint should address the primary question of the study. There are various desirable features for an endpoint, amongst others it should be relevant to disease process and easy to interpret. Further, ideally it should be free from measurement or assessment error and measurable within a reasonable period of time.

Various definitions for endpoints exist and some basic definitions were proposed by the Biomarkers definition Working group of the National Health Institute, USA to describe biological measurements in therapeutic development and assessment [161]:

Clinical endpoint:	A characteristic or variable that reflects how a patient feels or functions, or how long a patient survives.
Biological marker (Biomarker):	A characteristic that is objectively measured and evaluated as an indicator of normal biologic process, pathogenic process, or pharmacologic responses to a therapeutic intervention.
Surrogate endpoint:	A biomarker intended to substitute for a clinical endpoint. A clinical investigator uses epidemiologic, therapeutic, pathophysiologic, or other scientific evidence to select a surrogate endpoint that is expected to predict clinical benefit, harm, or lack of benefit or harm.

There exist more strict statistical criteria for the definition of surrogate endpoints, e.g. Prentice's criteria [162]. However, the discussion of evaluation and validation of surrogate endpoints is complex and out of scope of this thesis.

2.1.1 Multiple endpoints

Studies rarely use a single endpoint. Most often endpoints cover clinical events, symptoms, physiologic measures, side effects, quality of life, etc., some being primary and other

secondary endpoints. In general, clinical studies are powered for the primary endpoint only and analysis of multiple endpoints is linked to methodological issues such as inflation in type I error [163]. Further, secondary endpoints and primary endpoints are often related. For example, the observation of the secondary endpoint can depend directly on the primary one if the primary endpoint is survival and the secondary disease progression. Then there is a competing risk between the primary and secondary endpoint. In this case the analysis of the secondary outcome requires adapted statistical methods that condition on the primary outcome [164].

Another possibility is to define co-primary outcomes which also implie the use of adequate methods as described by DiRienzo and De Gruttola [165] and discussed in chapter 4.1.

The construction of a composite endpoint is now widely used in clinical studies. A composite endpoint combines multiple measurements into a single endpoint using a pre-specified algorithm.

2.1.2 Which endpoints are used in HIV studies and in which context?

In the HIV field, "classical" clinical endpoints are progression to AIDS or death, and frequently used biomarkers are the HIV viral load and the CD4 cell count. The field has evolved a lot in recent years so that the definition of endpoints has become more and more complex (see Figure 11). Survival of people living with HIV is approaching that of the general population, especially in patients with a CD4 cell count above 500 copies/mL [83]. However, the mortality of people living with HIV is still higher than that of the general population but not only due to AIDS events but also to non-AIDS defining events, cancer and other co-morbidities [82, 166-168]. HIV can not be eradicated yet so that, once started, antiretroviral treatment should be continued life long [1, 3]. Furthermore, adherence, pill burden, toxicity and development of drug resistance have to be considered. Another question is thus how long a specific treatment is efficient. Treatment goals are therefore more sophisticated than only to suppress the viral load and hamper disease progression. Today, progression to non-AIDS defining events, adherence, toxicity, development of resistance and the preservation of future treatment options may be equally important.

The study population plays a role for the definition of an endpoint. For example, one could imagine that the primary treatment goal differs between treatment naïve patients and experienced patients (especially patients needing salvage therapy). Further, it will depend on the general context of the study; the evaluation of a single drug for approval might claim a different definition from a study evaluating a treatment strategy.

All these considerations have led to wide use of composite endpoints in HIV clinical trials because i) using a 'simple' clinical endpoint such as death or disease progression is now difficult because of the rarity of these endpoints, ii) a suppression in viral load alone might be an imperfect surrogate for clinically relevant outcomes and might only reflect treatment efficacy at the shorter term and iii) as treatment needs to be taken life-long the duration of an initial treatment and the preservation of future treatment options have become equally important.

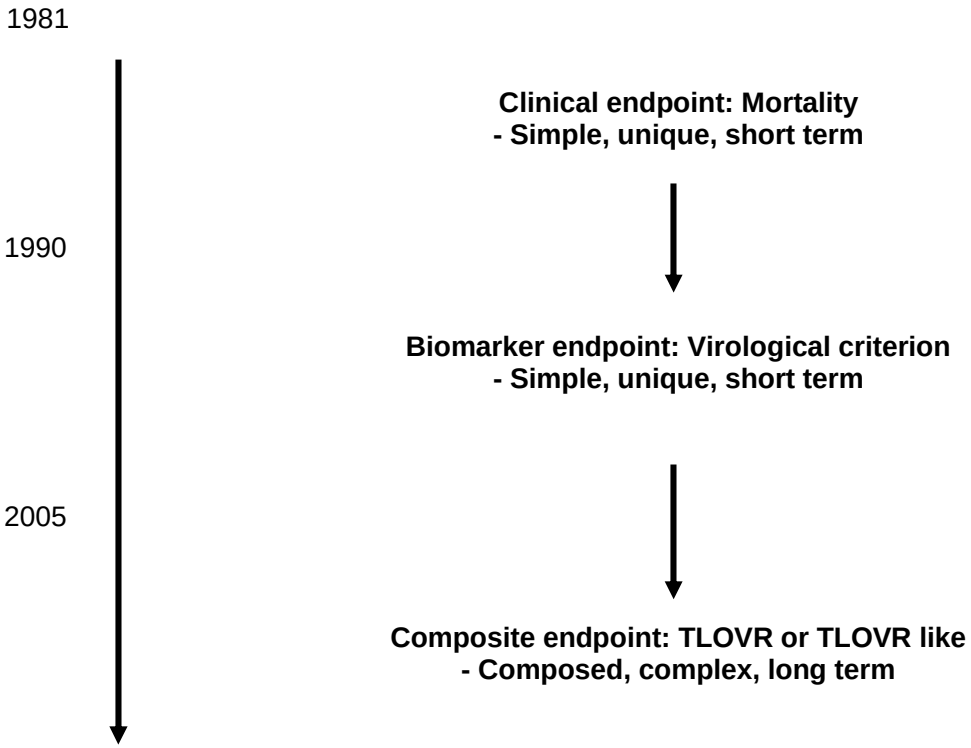

Figure 11: Simplified schema of change of endpoints in HIV clinical trials over time.
TLOVR: Time to loss of virological response.

2.1.3 Composite endpoints

The FDA currently recommends the use of a composite endpoint called Time to loss of virological response (TLOVR) that combines components relating to virological failure, loss-to-follow-up, initiation of a new treatment due to intolerance/toxicity or any other reason and death [169].

Other definitions for composite endpoints in the HIV field are available and they are now the most commonly used endpoints for HIV clinical trials. Methodological issues in the use of composite endpoints are discussed in chapter 4.1.

2.1.4 Virological endpoints

'Pure' virological endpoints are necessary in specific contexts and to answer specific questions. Definition of a pure virological endpoint is supposed to reflect the impact of

antiretroviral therapy on the replication of the virus. Thus, in the context of the evaluation of genotypic resistance mutations a 'pure' virological endpoint is the preferred one. Other situations would be the evaluation of an early antiretroviral effect to have an early potential surrogate criterion for clinical studies, to have the possibility of an early evaluation for the patient (daily practice) that may lead to treatment modification, or to have a criterion for clinical trials with an adaptive design (e.g. with possibilities of treatment optimisation).

The quantification of HIV viral load is hampered by a detection limit. The assays currently used to measure HIV viral load for standard clinical care mostly have a lower limit of detection of 50 copies/mL (ranging from 500 to 20 copies/mL).

2.1.4.1 Binary response

A very simple way to define a virological endpoint would be to define a binary response that reflects the percentage of patients below/above the detection limit at a defined time point after treatment start, e.g. 6 months.

However, such a definition will consider a patient starting with a very high viral load who stays above the detection limit up to 6 months to be in virological failure even when he had a steep decay and probably will reach detection limits at a later point. In some studies virological failure was defined as the proportion of patients with a viral load reduction of at least 1 $\log_{10}$ copies/mL [170-172]. A patient with a steep initial decay would be considered virological success with this definition.

To avoid misclassification problems due to a high baseline viral load some researchers used combined virological criteria and defined virological failure as a viral load above the detection limit and a decrease ≤ 1 $\log_{10}$ copies/mL [173, 174].

Of note, another issue is that the detection limit depends on technological progress and evolves with new generation of assays, but does not necessarily reflect a clinically relevant cut-off.

2.1.4.2 Quantitative endpoints

2.1.4.2.1 Difference of baseline viral load and follow up viral load

Some studies use the difference in viral load between baseline (treatment start) and a follow up viral load to compare the difference in viral load decay observed between groups. A simple method to deal with the problem of the detection limit would be to impute the value of the lower detection limit. Simple imputation of the detection limit leads to an underestimation of the difference and studies using such a definition are thus prone to miss-classification bias.

Therefore, Marschner *et al.* proposed to use survival analysis methods to compare the reduction in viral load accounting for the censoring of the viral load measurements [175]. Another possibility is to use a linear mixed model approach taking left censoring (viral load below the detection limit) into account [52, 53].

2.1.4.2.2 The use of viral load dynamics and mathematical models to evaluate treatment efficacy

Usually viral load is repeatedly measured after treatment start to monitor treatment efficacy. The efficacy of a treatment is supposed to have a direct influence on the viral load decline, i.e. the faster the decline the more efficient the treatment [91] but may also depend on the inhibition mechanism of the drug [176].

The use of piecewise linear mixed models extended for considering left-censored data are one possibility to analyse the viral load evolution [52, 53]. Other models based on bi-exponential models (which could be a solution of a system of differential equations) or differential equations can also be used to estimate decay rates [90, 91, 177-179]. Evaluation of early viral load decay rates in controlled clinical trials was discussed as a possibility to avoid undesired prolongation of study duration [133, 180] but results on this topic are controversy [181-184].

Another possibility to assess treatment efficacy is to use dynamical models (i.e. mechanistic models based on differential equations) to estimate the percentage of virus production blocked by the therapy [185]. This method is used in the evaluation of treatment efficacy of hepatitis C virus (HCV) treatments in HCV or HIV-HCV infected patients [186, 187] and was adapted for left-censored data [188].

In conclusion, neither the definition of a clinical meaningful endpoint nor the definition of a 'pure' virological endpoint is straightforward. We reviewed definitions of endpoints used in recent HIV clinical trials and discussed their methodological issues (chapter 4.1). Further, we summarised and discussed definitions used to analyse resistance data (chapter 4.2).

2.2 Prediction of treatment outcome using genotypic data in treatment naïve patients

In general, available interpretation algorithms (i.e. ANRS algorithm or the Stanford algorithm) are used to interprete genotypic resistance data in treatment naïve patients.

For exemple, Bansi *et al.* used the Stanford algorithm to calculate a GSS and analysed whether a GSS <3 due to transmitted drug resistance mutations was associated with treatment outcome [141]. Others used interpretation algorithms to class patients in groups, e.g. being resistant to at least one drug in their prescribed regimen [19, 28, 142].

For the analysis presented in chapter 5 we opted for a slightly different definition in order to distinguish three patient groups: 0) those with no transmitted drug resistance mutations, 1) those with at least one transmitted drug resistance mutation but predicted to receive a fully active treatment (i.e. no resistance for any of their prescribed drugs) and 2) those with transmitted drug resistance ant predicted to be resistant to at least one of their prescribed drugs (see Figure 12).

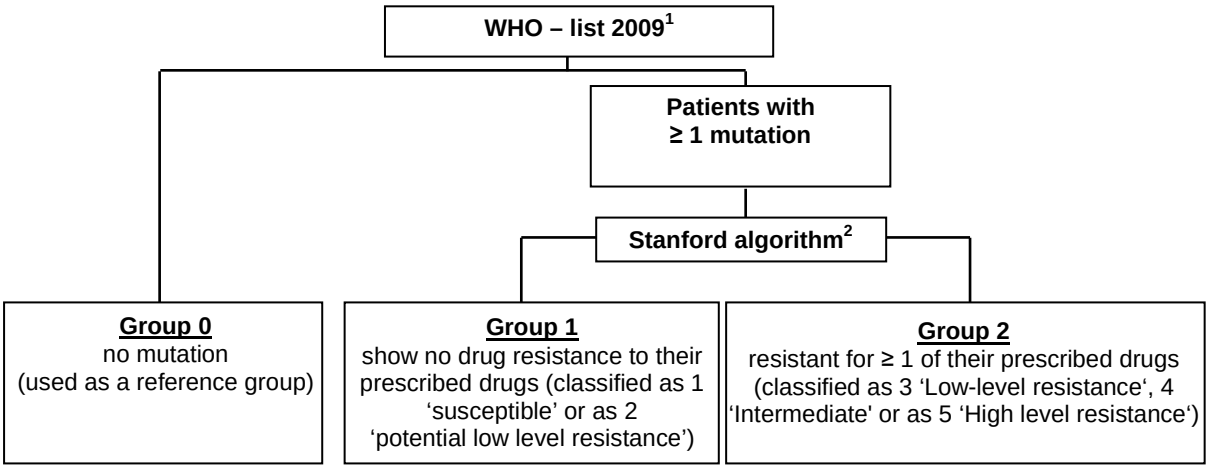

Figure 12: Classification scheme used to analyse the impact of transmitted drug resistance on treatment outcome in EuroCoord-CHAIN. [1] The World Health Organisation 2009 List of Mutations for Surveillance of Transmitted Drug Resistant HIV Strains [124], [2] Standford interpretation algorithm version 6.0.5 [34, 117].

This variable was created in two steps: First, the WHO-list 2009 [124] was used to distinguish between patients having at least one mutation of this list and having no mutation. Second, the Stanford algorithm version 6.0.5 [34, 117] was used to classify patients having at least one WHO mutation in two groups (see Figure 12).

2.3 Using genotypic resistance mutations to predict virological outcome in treatment experienced patients

2.3.1 High number of predictors/mutations relative to the number of patients included in these studies

Genotypic sequencing is now widely used in clinical practice (at least in the resource rich settings) and has led to the discovery of a still increasing number of drug resistance mutations which further can occur in diverse combinations.

The protease has 99 positions where mutations can occur and the reverse transcriptase approximately 560 positions. Given the fact that multiple mutations can occur at any position, i.e. theoretically the wild type amino acid can be exchanged to 19 other amino acids, a hugh number of predictors is the result. Of note, this scenario does not count for possible silent mutations, i.e. mutations in the nucleotide sequence that do not lead to an amino acid change but could also be a risk factor for virological failure.

Datasets for the study of genotypic resistance mutations and virological response to therapy have often a small sample size, especially when new drugs are evaluated. Thus, the number of predictors can easily be higher than that of the observations included in the study. Another issue is that resistance mutations occur not necessarily independently from each other but occur in mutation clusters.

In summary, studies that are linking baseline genotypic mutations to treatment outcome are hampered by i) the high number of mutations, ii) collinearity of the mutations and iii) the low number of patients included in such studies.

The high number of variables/mutations in relation to the low number of patients can lead to overfitting. An overfitted model will typically fail on unseen data and will have a poor prediction performance. Thus, overfitting leads to lack of external validity. To avoid overfitting one solution is to use cross-validation or to train the model on a training data set and test its performance on a completely different new dataset.

Collinearity can lead to estimation problems especially if ordinary least squares estimator are used. In the context of collinearity the estimator produces large variances, which in turn might inappropriately lead to exclusion of otherwise significant variables/mutations from the model.

2.3.2 Methods to deal with high number of predictors

Several methods have already been employed to predict either the phenotypic drug resistance or the virological outcome using genotypic resistance data as the main predictor. Some methods are so called statistical learning or machine learning methods. Statistical/machine

learning methods are algorithms that allow for deriving computational models that are able to predict for example treatment outcome from an available amount of predictors/mutations. Usually these models are derived from so called training data which comprise the predictors together with their associated response.

The following two sections give an overview of methods using genotypic data either to predict phenotypic or virological outcome in treatment-experienced patients. The distinction between phenotypic and virological outcome is factual but makes no sense for the methodology. Methods summarized can be used to predict both outcomes.

2.3.2.1 Overview of methods in the litterature using genotypic data to predict phenotypic resistance

An overview of methods applied and compared to predict phenotypic drug resistance using genotypic mutations is given in Table 4.

Support vector machines (SVM) were found to be highly predictive for phenotypic drug resistance [38]. SVMs are a group of learning methods that can be applied for classification or regression problems and can be used for linear and nonlinear data structure. In the context of genotype-phenotype data, sequences with known phenotype are mapped into a high-dimensional vector space. In this space, a hyperplane is computed that optimally approximates the genotype–phenotype relation. SVM generally provides accurate prediction models but the generated models are typically regarded as incomprehensible black-box models.

Other approaches tested to predict the phenotype are artificial neural networks [48] and decision trees [49].

Usual linear regression models with and without interaction were used by Vermeiren *et al.* and Wang *et al.* to predict phenotype and in both studies a high accuracy in predicting the phenotype was found [189, 190]. The virtual phenotype by Virco is also based on linear regression models [191].

Table 4: Overview of methods applied and compared to predict phenotypic drug resistance using genotypic mutations

Reference	Methods	Main results
Sevin *et al.* 2000 [192]	Cluster analysis, recursive partitioning, linear discriminant analysis	Recursive partitioning was found to be useful because it can identify interactions among mutations at different codons
Beerenwinkel *et al.* 2002 [49]	decision trees	Sensitivities ranged from 77.7 - 92.5% and specificities ranged from 68.7 - 97.2% depending on the drug classes
Beerenwinkel *et al.* 2003 [38]	support vector machines	Models accounted for 30 to 79% of the phenotypic variance
Wang *et al.* 2004 [190]	stepwise linear regression, decision trees, support vector machines, ANRS, Rega, Stanford	Stepwise linear regression outperformed the other methods and interpretation algorithms for predictive accuracy
Rabinowitz *et al.* 2006 [46]	ridge regression, neural networks, PCA, decision trees, stepwise selection, support vector machines, Lasso	Lasso and SVMs outperformed the other techniques
Rhee *et al.* 2006 [196]	decision trees, neural networks, least square regression, support vector regression, LARS	LARS was superior to the compared methods if the complete set of mutations present ≥2 sequences was used.
Vermeiren *et al.* 2007 [189]	linear regression	Good correlation between measured and predicted Fold change
Saigo *et al.* 2007 [197]	Itemset boosting (a non linear regression method), LARS, support vector regression, ridge regression	Itemset boosting outperformed LARS and support vector regression but only for NRTIs
Sinisi *et al.* 2007 [51]	Superlearner: LARS, CART, D/S/A, logic regression, ridge regression and linear regression	The selection of an optimal learner from candidate learners asymptotically outperformed any of the candidate estimators. D/S/A (Deletion/Substitution/Addition) and linear regression were selected as optimal learner to predict phenotypic resistance using genotypic data
Schumi and DeGruttola 2008 [194]	resampling based methods	Methods presented may allow the investigation of how mutations act in the presence of others and may be informative in suggesting candiate regression models
Kjaer *et al.* 2008 [198]	Artificial neural networks	Artificial neural networks predict phenotypic susceptibility to antiretroviral drugs to an extent that is comparable to routine phenotypic susceptibility testing

PCA: principal component analysis, Lasso: least absolute shrinkage and selection operator, LARS: least angle regression. CART: classification and regression trees, ANRS: Agence nationale de recherche sur le SIDA et les hépatites.

Further, linear discriminant analysis, cluster analysis, recursive partitioning (an iterative technique to construct decision trees) [192], non-parametric methods [193] and resampling based methods that allow the investigation of combinations of mutations [194, 195] were also applied to predict the phenotypic drug resistance.

Rabinowitz *et al.* compared most of the above listed methods and found the least absolute shrinkage and selection operator (Lasso, see also) and SVMs to be the best predictors for phenotypic drug resistance [46].

Lasso is a penalized regression technique and gives easy interpretable results compared to SVMs. The regression parameters found for each predictor/mutation can be interpreted as a different weight for each mutation for the prediction of phenotypic drug resistance [46]. Further, Rhee *et al.* found that LARS (an algorithm including a solution for Lasso) was superior to the compared methods (amongst others SVMs and decision trees) if the complete set of mutations (no pre-selection of known resistance mutations) present ≥ 2 sequences (i.e. sequences of the reverse transcriptase or protease of patients included in the study) was used for predicting phenotypic drug resistance [196].

2.3.2.2 Overview of methods in the litterature using genotypic data to predict virological outcome

Even if phenotypic drug resistance can also give helpful information, most clinicians are interested in the direct prediction of treatment outcome.

The construction of a genotypic score is a simple method to summarize the information from genotypic resistance mutation to predict the virological response [36, 37].

The genotypic score reflects the association of a set of mutations with virological response and is related to a given drug (see also section 2.3.3.2). The genotypic score is usually combined with expert knowledge and results from *in vitro* studies to create an interpretation algorithm for a given drug [33].

The HIV resistance response database initiative investigated SVMs, artificial neural networks and random forests (which are formed by many decision trees) [50] and has launched an experimental interpretation system (HIV TRePS v1.0 [203]).

Table 5: Overview of alternative methods applied and compared to predict virological outcome using genotypic mutations

Reference	Methods	Main results
De Luca *et al.* 2004 [199]	fuzzy rule based algorithm	algorithm showed independent prediction of virological outcome after adjusting for GSS made by rule based expert systems
Brun-Vezinet *et al.* and Flandre *et al.* [36, 37, 200]	1) Preselection of mutations by univariable tests 2) Selection of the set of mutations the most correlated with virological response by a test for trend	The Jonckheere test for trend was recommended for building a genotypic score when compared with the Kruskal-Wallis test but the choice may depend on the objective of the score.
Larder *et al.* 2007 [48]	artificial neural networks	The best performing models explained 69% of the variance in virological response
Yang *et al.* 2008 [195]	resampling based methods with covariate adjustment	Method was found to be useful to help determine patterns of mutation significantly associated with drug resistance in settings where there are interactions in the effects of mutations. Might be helpful when used in conjunction with regression methods for prediction of virological response.
Wang *et al.* 2009 [50]	artificial neural networks, support vector machines, random forests	Random forests and SVMs were comparable to a committee of artificial neural network models.
Bembom *et al.* 2009 [201]	targeted maximum-likelihood estimation	Targeted maximum-likelihood was considered a promising approach to select mutations associated with virological outcome.
Larder *et al.* 2010 [202]	random forests	Random forests model were accurate predictors of virological outcome and outperformed rule-based expert systems (HIVdb, ANRS, Rega)

GSS: genotypic sensitivity score, SVM : support vector machine, HIVdb: HIV drug resistance database, ANRS: Agence nationale de recherche sur le SIDA et les hépatites.

In the following we describe shortly the data in general available to analyse the impact of genotypic mutations on virological outcome, the construction of a genotypic score, the use of PCA, the use of PLS, and the use of Lasso for the analysis of genotypic resistance mutation to predict virological response.

2.3.3 Alternative methods and the construction of a genotypic score

2.3.3.1 Brief overview of the data structure available for the analysis of the impact of genotypic resistance mutations on virological outcome

In general, studies of patients adding a new drug to an existing regimen (e.g. fosamprenavir/rtv) or patients starting a new combination containing one drug administered for all patients (e.g.darunavir/rtv) are available for the analysis of acquired genotypic resistance mutations on virological response in treatment-experienced patients (see Figure 13).

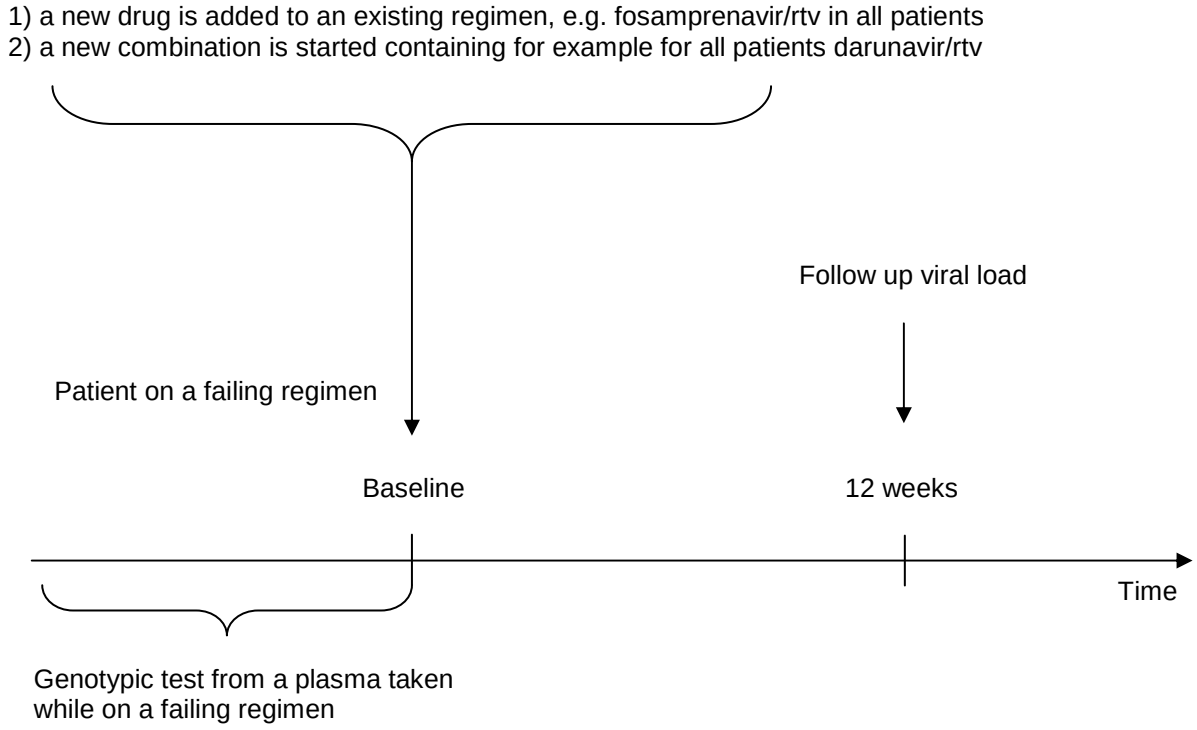

Figure 13: Simplified schema for data available for the analysis of the impact of genotypic resistance mutations on virological response.

2.3.3.1.1 Response variable

The response variable is derived from the virological response and is either defined as a binary response or a quantitative response, i.e. change in viral load.

For the observation (patient) i, we can define for example a binary response at week 12:

$$y_i \begin{cases} = 1 \text{ when viral load} > 400 \text{ copies/mL} \\ = 0 \text{ when viral load} \leq 400 \text{ copies/mL} \end{cases} \qquad i = 1 \ldots n$$

This variable will give the response vector for the whole population of n subjects:

$$y_{n\times 1} = \begin{bmatrix} y_1 \\ . \\ . \\ . \\ y_n \end{bmatrix}$$

2.3.3.1.2 Main predictor variables

The main explanatory variables will be the genotypic mutations. If patients start as a common drug a protease inhibitor main explanatory mutations will be protease mutations and if patients start a reverse transcriptase inhibitor the main predictor variable will be the mutations of the reverse transcriptase.

For a patient *i*, we can define a binary variable representing a mutation j:

$$x_{ij} = \begin{cases} = 1 \text{ if a given amino acid is present instead of the wild type amino acid} \\ = 0 \text{ otherwise} \end{cases}$$

The matrix of the k predictor variables *(j = 1…k)* for n patients *(i = 1…n)* can be written as:

$$X_{n\times k} = \begin{bmatrix} x_{11} & . & . & . & x_{1k} \\ . & . & . & . & . \\ . & . & . & . & . . \\ . & . & . & . & . \\ x_{n1} & . & . & . & x_{nk} \end{bmatrix}$$

Thus, if at one position more than one mutation is reported a binary variable for each of them will be created.

Another possibility would be to treat all possible amino acid substitutions in the same way. For example, whether at position 47 the wild-type amino acid Isoleucine is displaced by a Valine or by Alanine could be considered to be the same.

2.3.3.2 Construction of a genotypic score

The large number of possible mutations and possible collinearities lead to the application of strategies for reducing the number of predictors as e.g. backward and forward selection strategies in simple regression models. Backward selection is only applicable after pre-selection of mutations. Inclusion of all mutations would lead to too many predictors which would require extremely high numbers of patients to be included in the studies. However, even after a pre-selection of mutation, applying backward selection could be problematic if mutations are highly correlated. Forward selection in contrast could be applicable but could

eliminate useful predictors that happen to be correlated with mutations already included in the model. To circumvent these problems and to simplify the interpretation, genotypic mutations are generally related to a single drug, summarised in a genotypic score. This score is the sum of resistance mutations observed in a given patient. The combination of mutations that are represented in the sets for constructing the score can be selected by different strategies. One of the classical strategies selects mutations in two steps.

Step 1

In the first step all mutations or the mutations from the IAS panel are investigated (i.e. k mutations known to be associated with poor response *in vitro* and *in vivo*).

The first step determines a set of $p < k$ mutations, which are associated with virological failure. For each mutation, frequency and prevalence are determined and only mutations having prevalence ≥10 % and ≤90 % are considered for further analyses [36]. With these mutations univariable analyses are realised in order to determine the association of each mutation with virological failure.

Mutations providing a p-value < 0.25 are kept for further analysis [37]. These mutations form the first set of $p \geq m$ mutations that is used to calculate the first genotypic score.

The score for a patient i calculated with the first set of mutations is defined as:

$$s_i = \sum_{j=1}^{p} x_j$$

Example:

With the mutations selected in the first step the first genotypic score is calculated for each patient. For instance a first set contains the five protease mutations V32, I47, I50, V77, I84 and L90. The score is defined as S= V32 + I47 + I50 + V77 + I84, in which each mutation is defined to get the value 1 if the mutation is present and 0 if not present (S varying from 0 to 5). A patient with mutations I50, V77, I84 and L90 would thus get a score of 4.

Step 2

The next step reduces the number of mutations to m mutations for the final set. The final set of m mutations can be obtained by different selection strategies [37].

The number of included mutations can be reduced using step by step procedures. With forward or backward selection, scores are calculated for each considered subset of mutations. For each considered subset of mutations and its derived score, associations between the score values on an ordinal scale and virologic failure are assessed by a non parametric test for trend.

The set of mutations determined for the final score represents the subset providing the score with the strongest association with virological failure [37, 116].

In the following paragraph we describe a removing procedure to select the final set of mutations:

Backward selection procedure

Starting with $p \geq m$ mutations that have been kept in the first step, every mutation is removed one by one. All sets of p-1 mutations are investigated. For each possible set, the test compares groups of patients having none to p-1 mutations.

For a given set of mutations the number of patients with virological failure is defined as $y_h = \sum_{i=1}^{N_h} y_i$ for every group h with N_h individuals. The group h is defined for patients presenting the same number of mutations $S_h = 0 \ldots p$ ($S_h = 0 \ldots p-1$, respectively), i.e. the same score value.

We applied the Cochrane Armitage trend test to verify the hypothesis of an equal repartition of the probability of virological failure regarding mutation numbers as we defined a binary response variable for the study presented in chapter 6.1. The choice to use a trend test is based on a paper by Flandre *et al.* that demonstrated that the Jonckheere's trend test was superior for the selection of a subset of mutations compared to Wilcoxon-Mann-Whitney when a quantitative outcome was used [37, 200].

The proportion of virological failure for the group h is defined as $\pi_h = \frac{y_h}{N_h}$ and the proportion of virological failure is defined as $\pi = \frac{\sum_{i=1}^{n} y_i}{n}$

H_0: $\pi_0 = \pi_1 = \ldots = \pi_h =: \pi$

H_1: The proportions π_h are different to π and these proportions depend on the score value $S_h = 0 \ldots p$. The proportions tent to be higher relative to the number of mutations.

The combination providing the lowest p-value is kept. The procedure is repeated and mutations are removed one by one to compare the different combinations of p-2 mutations to the set of p-1 mutation kept. The combination providing the lowest p-value is again kept, and so on. The procedure stops when removal of a mutation does not result in a lower p-value (see see Figure 14).

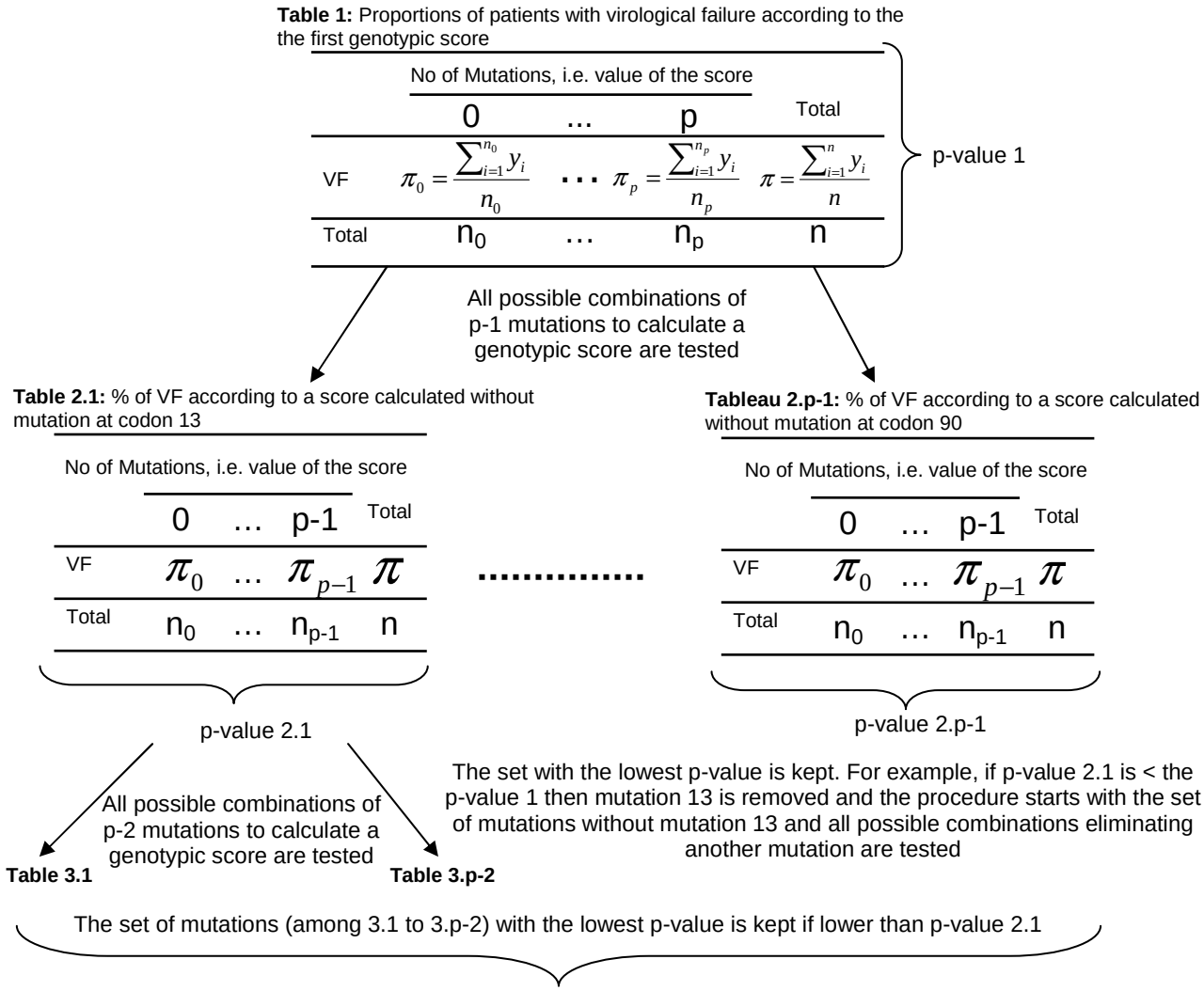

Figure 14: Scheme of the backward selection procedure realised in step 2 for the determination of a set of mutation associated with virological failure (VF).

One restriction of this strategy is that every mutation has the same weighting. Hence, no difference between the impacts of major or minor mutations on the virological response can be determined. Further, some mutations are known to lead to hypersusceptibility. Flandre *et al.* 2005 and Capdepont *et al.* 2006 defined a value of -1 for mutations associated with a better virological response in the context of constructing a score for didanosine [37, 40]. Protease inhibitor mutation V77 was described to provoke hypersusceptibility [41]. The score S in the above depicted example would then range from -1 (only V77 present) to 4 (V77 absent but all others are present). The score for the patient with mutations I50, V77, I84 and L90 would be S=1-1+1+1=2.

Another restriction of the genotypic score is that interactions between mutations are not taken into account. The effect of one mutation may be higher or weaker when another mutation is present.

Once the genotypic score is defined, a cut-off with reasonable sensibilities and specificities for the prediction of virological failure is determined by comparison with clinical parameters.

In summary, the construction of a genotypic score has several limitations. First, a pre-selection of mutations linked to virological response is needed in order to reduce the set of mutations used for constructing the genotypic score. This pre-selection can potentially lead to exclusion of mutations linked to virological response. Second, each mutations used for the calculation of the genotypic score is given the same weight, irrespectively whether it is a major or minor mutation. Third, given the number of mutations a high number of tests must be realised. Thus, the false discovery rate due to the raise in type I error may be important.

2.3.3.3 Alternative strategies

Alternative strategies such as principal component analysis (PCA) and partial least square (PLS) have been suggested to reduce the size of correlated predictors [42-44]. Moreover, these strategies may help in describing associations between mutations. Lasso is another technique which is suited for data with a high number of potentially correlated predictors and which is easy to interpret [46, 204, 205].

Advantages of these methods are that they do not need pre-selection of variables/mutations. PCA and PLS consider all potential mutations with a different weight for the prediction of treatment outcome. Lasso shrinks parameters of some mutations with no impact on treatment outcome to zero.

2.3.3.3.1 Principal Component Analysis

The objective of PCA is to find a set of "latent variables" in form of a linear transformation of the original predictors. The properties of these latent variables are that they are uncorrelated and that they account for as much of the variance of the predictor variables as possible [44]. The reduced numbers of uncorrelated latent variables are also called Principal Components (PC). PCA has been used to determine groups of mutations [45] and was used to predict phenotypic drug resistance [46].

PCA analyses the structure of the correlation matrix of the predictor variables. The objective is to determine components which are representing the variability of the predictor variables matrix, e.g. the mutations. Variables are centred and scaled before PCA analysis to prevent scaling inequalities.

The centred and scaled matrix can be written as:

$$\tilde{\tilde{X}}_{n\times k} = \begin{bmatrix} \frac{x_{11}-\bar{x}_1}{s_{\bar{x}_1}} & \cdots & \frac{x_{1k}-\bar{x}_k}{s_{\bar{x}_k}} \\ \vdots & \cdots & \vdots \\ \frac{x_{n1}-\bar{x}_1}{s_{\bar{x}_1}} & \cdots & \frac{x_{nk}-\bar{x}_k}{s_{\bar{x}_k}} \end{bmatrix} \quad \text{with} \quad \begin{cases} \bar{x}_j = \dfrac{\sum_{i=1}^{n} x_{ij}}{n} \\ s_{\bar{x}_j} = Var(\bar{x}_j) \end{cases} \quad \text{and } j = 1\ldots k$$

The correlation matrix can be denoted as $S = (s_{jk})_{k\times k}$. As the variables are centred and scaled we can write the samples correlation matrix as:

$$S_{k\times k} = \tilde{\tilde{X}}^T \tilde{\tilde{X}}$$

The eigenvalues λ and corresponding eigenvectors V_k of the correlation matrix are obtained by singular value decomposition such as:

$$S = V\Lambda V^T = \begin{bmatrix} v_{11} & \cdots & v_{1k} \\ \vdots & \cdots & \vdots \\ v_{k1} & \cdots & v_{kk} \end{bmatrix} \begin{bmatrix} \lambda_1 & 0 & 0 & 0 & 0 \\ 0 & \ddots & 0 & 0 & 0 \\ 0 & 0 & \ddots & 0 & 0 \\ 0 & 0 & 0 & \ddots & 0 \\ 0 & 0 & 0 & 0 & \lambda_k \end{bmatrix} \begin{bmatrix} v_{11} & \cdots & v_{k1} \\ \vdots & \cdots & \vdots \\ v_{1k} & \cdots & v_{kk} \end{bmatrix}$$

The principal components can be denoted as:

$$C_{n\times k} = \tilde{\tilde{X}}_{n\times k} V_{k\times k}$$

The coefficient vectors that define the principal components are the eigenvectors of the correlation matrix *S*. In order to assure that the first principal components explain as much of the variance of the columns of the matrix $\tilde{\tilde{X}}$ as possible, the corresponding eigenvalues are ordered so that $\lambda_1 \geq \lambda_2 \geq \ldots \geq \lambda_k$. Therefore the principal components can be seen as orthogonal linear spans in which the variance is the corresponding eigenvalue.

Because there are as many principal components as variables usually only some of the principal components are used *(h = 1 … k)*.

$$C_{n\times k} = \begin{bmatrix} \sum_{h=1}^{k} \tilde{\tilde{x}}_{1h} v_{h1} & \cdots & \sum_{h=1}^{k} \tilde{\tilde{x}}_{1h} v_{hk} \\ \vdots & \cdots & \vdots \\ \sum_{h=1}^{k} \tilde{\tilde{x}}_{nh} v_{h1} & \cdots & \sum_{h=1}^{k} \tilde{\tilde{x}}_{nh} v_{hk} \end{bmatrix} \quad \text{as} \quad C_{n\times k} = \left[\begin{array}{ccc|ccc} c_{11} & \cdots & c_{1s} & c_{1s+1} & \cdots & c_{1k} \\ \vdots & \cdots & \vdots & \vdots & \cdots & \vdots \\ c_{n1} & \cdots & c_{ns} & c_{ns+1} & \cdots & c_{nk} \end{array}\right]$$

Generally, *s* principal components that account for a high percentage of the total given variability are used. The *s* PCs used should explain at least 75 % of that variability so that:

$$\left[\frac{\sum_{j=1}^{s}\lambda_j}{\sum_{j=1}^{k}\lambda_j}\times 100\right] \geq 75 \qquad s \leq k$$

Another possibility is to use principal components, which are related to the response variables. The PCs chosen with this strategy are not imperatively those who account for a high percentage of the total variability.

The first *s* principal components can then for example be used in a logistic model:

$$\text{Logit}(P(y_i = 1)) = \gamma_0 + \sum_{h=1}^{s} c_{ih}\gamma_h$$

2.3.3.3.2 Partial Least Square

PLS reduces equally a set of predictor variables to a set of uncorrelated "latent variables", the so-called PLS components. The main difference between the PCA and PLS is that PLS also considers the variability of the response in order to determine the components.

Variables (predictors X and response Y) are centred and scaled before PLS analysis to prevent scaling inequalities and values equal to zero which might perturb the estimations.

$$\tilde{\tilde{X}}_{n\times k} = \begin{bmatrix} \frac{x_{11}-\bar{x}_1}{s_{\bar{x}_1}} & \cdots & \frac{x_{1k}-\bar{x}_k}{s_{\bar{x}_k}} \\ \vdots & \cdots & \vdots \\ \vdots & \cdots & \vdots \\ \vdots & \cdots & \vdots \\ \frac{x_{n1}-\bar{x}_1}{s_{\bar{x}_1}} & \cdots & \frac{x_{nk}-\bar{x}_k}{s_{\bar{x}_k}} \end{bmatrix} \quad \text{with} \quad \begin{cases} \bar{x}_j = \dfrac{\sum_{i=1}^{n} x_{ij}}{n} \\ s_{\bar{x}_j} = Var(\bar{x}_j) \end{cases} \quad \text{and } j = 1\ldots k$$

and

$$\tilde{\tilde{Y}}_{n\times 1} = \begin{bmatrix} \frac{y_{11}-\bar{y}}{s_{\bar{y}}} \\ \vdots \\ \vdots \\ \vdots \\ \frac{y_{n1}-\bar{y}}{s_{\bar{y}}} \end{bmatrix} \quad \text{with} \quad \begin{cases} \bar{y} = \dfrac{\sum_{i=1}^{n} y_i}{n} \\ s_{\bar{y}} = Var(\bar{y}) \end{cases}$$

The first component that is determined can be defined as

$$t_1 = \tilde{X} w_1 = \sum_{j=1}^{k} corr(\tilde{y}, \tilde{x}_j) \cdot x_j \qquad w_1 = \begin{bmatrix} Corr(\tilde{y}, \tilde{x}_1) \\ . \\ . \\ . \\ Corr(\tilde{y}, \tilde{x}_k) \end{bmatrix}$$

The vector w_1 represents the weight of every predictor variable (mutation) on the first PLS component. This component is used to make a regression on $\tilde{X}$ and $\tilde{y}$, respectively.

$$\tilde{y} = \hat{b}_1 t_1 + y_1$$

$$\tilde{X} = \hat{p}_1 t_1 + X_1$$

The estimated regression coefficients $\hat{b}_1$ and $\hat{p}_1$ are used to calculate the residuals.

$$\hat{y}_1 = \tilde{y} - \hat{b}_1 t_1$$

$$\hat{X}_1 = \tilde{X} - \hat{p}_1 t_1$$

The second component is determined with the residuals $\hat{y}_1$ and $\hat{X}_1$ and it can be defined as

$$t_2 = \hat{X}_1 w_2 = \sum_{j=1}^{k} \mathrm{cov}(y_1, \hat{x}_{1j}) \cdot \hat{x}_{1j} \qquad w_2 = \begin{bmatrix} Cov(y_1, \hat{x}_{11}) \\ . \\ . \\ . \\ Cov(y_1, \hat{x}_{1k}) \end{bmatrix}$$

The vector w_2 represents the weight of every predictor variable (mutation) on the second PLS component. The residuals are calculated with the estimated regression coefficients for the second component.

$$\hat{y}_2 = \hat{y}_1 - \hat{b}_2 t_2$$

$$\hat{X}_2 = \hat{X}_1 - \hat{p}_2 t_2$$

Further components are obtained in the same manner.

To be able to calculate the PLS components (h different PLS components exists) directly with the centred and scaled predictor variables one has to transform the weights w because they are related to the residuals (with exception of the weights obtained for the first PLS component).

$$t_h = X_{h-1} w_h \qquad t_h = \tilde{X} w_h^* \qquad (w_1 = w_1^*)$$

For instance, PLS components for a new patient must be calculated with w^*.

PLS components can then be used in logistic regression or other models to assess their association with virological outcome.

A logistic model can be written as

$$\text{Logit}(P(y_i = 1)) = \gamma_0 + \sum_{h=1}^{s} t_{ih}\gamma_h$$

$$t_{i1} = \tilde{x}_{i1}w_{11}^* + \tilde{x}_{i2}w_{12}^* + \ldots + \tilde{x}_{ik}w_{1k}^*$$

$$\text{Logit}(\text{P}(y_\text{i} = 1)) = \gamma_0 + \gamma_1 w_{11}^* \tilde{x}_{i1} + \gamma_1 w_{12}^* \tilde{x}_{i2} + \ldots + \gamma_1 w_{1k}^* \tilde{x}_{ik}$$

The number of PLS components to consider is usually determined by cross-validation.

Additionally, the prediction quality of the logistic model (or other models) is determined by cross-validation because PLS components are already determined using the virological response.

2.3.3.3.3 Least absolute shrinkage and selection operator (Lasso)

Lasso was first described by Tibshirani [205] and uses the l1 norm to shrink the linear regression parameters. Lasso allows for the selection of a subset of variables that together are the most effective predictors.

The parameter (b) are estimated by:

$$\hat{b} = \arg\min_b \|y - Xb\| + \lambda \sum_{j=1}^{k} |b_j|$$

λ is found by cross-validation.

The Lasso technique sets several parameters to 0 and those parameters kept in the model can be interpreted as parameters of a linear regression model.

Lasso was also expanded for the estimation of general linear models [206] and for variable selection using Cox models [207].

3 Plan and objectives

Given the background and methodological issues outlined above, this thesis addresses three objectives with regards to virological outcomes and analysis of resistance data:

The first objective was to describe definitions of endpoints used in the HIV field and to discuss their methodological limitations. Chapter 4.1 describes methodological issues in the use of composite endpoints in clinical trials and was published in the journal *Clinical Trials* in 2010. This work is the result of discussion with workpackage 4 (Trial design, Statistics) of the European AIDS treatment network (NEAT) and was helpful for study design and endpoint definition of the first NEAT randomised clinical trial (NEAT 001/ANRS143 trial, NCT01066962 [55]) evaluating two antiretroviral first-line treatment strategies with a composite virological and clinical endpoint. Endpoints used to analyse the impact of transmitted drug resistance on virological outcome as well as endpoints used in studies constructing a genotypic score are presented and discussed in chapter 4.2.

The second objective was to analyse the impact of transmitted drug resistance mutations in the first year after combination antiretroviral therapy was started. In this work we were particularly interested in the effect of transmitted drug resistance in patients receiving a treatment predicted to be fully susceptible to the genotype of the patient virus. A manuscript (chapter 5) of this work is in completion and will be submitted to the Lancet. This work was realised as a collaborative pilot project between EuroCoord (meta-collaboration of European cohorts of HIV infected patients) and CHAIN (a network of experts in HIV drug resistance).

The third objective was to investigate alternative methods for the analysis of genotypic resistance mutations in treatment experienced patients. We were especially interested in methods that allow for summarizing the information of genotypic mutations and that can be used as a predictor for virological response. Chapter 6.1 summarizes the application of PCA and PLS in comparison to the construction of a genotypic score and was published in BMC Medical Research Methodology in 2008. The adaptation of Lasso for left censored data as a perspective for the analysis of genotypic resistance mutations to predict virological outcome is presented in Chapter 6.2. This project is realised in the context of a collaboration with the Forum for collaborative HIV research (international Forum aiming at facilitating HIV research).

4 Endpoints in the HIV field

As already mentioned the definition of endpoints is very important and may vary according to the purpose of the study. In the following two chapters we show present definitions of endpoints in two different contexts. First, in the context of recent clinical trials in the HIV field realised in connection with the European AIDS treatment network (NEAT). Second, in the context of the analysis of drug resistance mutations and their impact on treatment outcome in treatment naïve and treatment experienced patients.

Although the virological response is a common denominator in both contexts the definition of endpoints are very different.

4.1 Methodological issues in the use of composite endpoints in clinical trials: examples from the HIV field

Many HIV clinical trials raise practical methodological challenges and require innovative approaches in study design in order to address issues such as limited sample sizes or complex endpoints. One objective of the methodological workpackage 4 (WP4; Trial design, Statistics) of NEAT is to support trial design. Therefore, areas of statistical methodological work which are of key practical relevance to the analysis of trials in HIV/AIDS were one priority of WP4. Methodological issues include: handling missing data (e.g. missing = failure principle and multiple imputations) for missing laboratory measurements, left censoring of viral load measurements etc.

NEAT001/ANRS143 is a phase III, randomised, open-label, multinational, multicenter trial (countries participating in NEAT) that aims at comparing the efficacy and tolerability of darunavir/ritonavir plus tenofovir/emtricitabine versus darunavir/ritonavir plus raltegravir in treatment naïve patients. For the primary endpoint of NEAT001/ANRS143 the definition of an endpoint reflecting virological efficacy, clinical progression and serious toxicity issues was of particular interest.

In collaboration with WP4 we were thus interested in an overview of endpoints used in recent clinical trials and in methodological issues linked to their definitions.

The following chapter gives an overview of methodological issues in the use of composite endpoints in clinical trials and uses examples from the HIV field.

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Methodological issues in the use of composite endpoints in clinical trials: examples from the HIV field

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Background In many fields, the choice of a primary endpoint for a trial is not always the ultimate clinical endpoint of interest, but rather some surrogate endpoint believed to be relevant for predicting the effect of the intervention on the clinical endpoint. The classic example of such a field is clinical HIV treatment research, where a variety of primary endpoints are used to evaluate the efficacy of new antiretroviral drugs or new combinations of existing drugs. The choice of endpoint reflects either the goal of therapy as recommended by treatment guidelines (e.g. rapid virological suppression) or the licensing requirements of official drug approval organizations (e.g. time to loss of virological response [TLOVR]).

Purpose To review the diversity of endpoints used in recent clinical trials in HIV infection and highlight the methodological issues.

Methods We identified articles relating to antiretroviral therapy by searching PubMed and through hand searches of relevant conference abstracts. We restricted the search to randomized controlled trials conducted in HIV-infected adults published/presented from January 2005 until March 2008.

Results We identified 28 trials in antiretroviral-naive patients (i.e. patients who were starting antiretroviral therapy for the first time at the time of randomization) and 23 trials in antiretroviral-experienced patients. Most trials were performed for purposes of drug licensing, but others were focused on strategies of using approved drugs. Most trials (40 of 51) used a composite primary endpoint (TLOVR in 13). Of note, 22 of these 40 studies reported that they had used a purely virological efficacy endpoint, but the primary endpoint was actually a composite one due to the way in which missing data and treatment switches were considered as failures.

Limitations Examples are restricted to HIV clinical trials.

Conclusions Whilst most current HIV clinical trials use composite primary endpoints, there are substantial differences in the components that make up these endpoints. In HIV and other fields where precise definitions are variable, guidelines for standardization of definition and reporting would greatly improve the ability to compare trial results. *Clinical Trials* 2010; **7**: 19–35. http://ctj.sagepub.com

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Introduction

The primary endpoint used in a randomized trial is fundamental to its design and interpretation. The main conclusion of the trial will be based on this outcome and the number of subjects recruited to the trial will depend on it. The choice of a primary endpoint is not always the ultimate clinical endpoint of interest, but rather some surrogate believed to be relevant for predicting the effect of the intervention on the clinical outcome of interest. In the HIV field, the pathogenesis is sufficiently understood so that the effect of a drug regimen on the HIV RNA level (viral load) is accepted as being relevant for understanding the contribution of the drug regimen to ultimately preventing the clinical endpoint of interest, AIDS and death from AIDS. Such endpoints are often chosen for practical purposes because the clinical endpoint of interest may be rare and take many years to occur. In addition, if the standard of practice is to change or add therapies upon the occurrence of a surrogate endpoint, this can complicate the comparison of the original treatments. Faced with this situation in the HIV infection field it has become necessary to employ the markers used in clinical practice (e.g. viral load and CD4 count [1,2]) to assess a patient's health status and define switches in therapy as endpoints. This situation will become more common in fields outside of HIV, as biomarkers for predicting disease outcome become more widely used.

In the HIV field, the introduction of more potent drugs over the past 10 years has improved the efficacy of highly active antiretroviral therapy (HAART) used to treat HIV-infected patients. HIV-infected patients receiving HAART with more than 500 T lymphocytes CD4 positive cells/mm^3 have almost the same death rates as the general population [3]. Clinical outcomes such as AIDS or death are relatively rare and clinical questions are now more focused on toxicity or drug resistance. The mortality of HIV-infected patients is linked to late presentation with HIV (i.e. presentation when the CD4 count is already low) and the occurrence of serious non-AIDS defining events such as malignancies, cardiovascular diseases, liver failure etc [4].

The choice of an appropriate primary outcome for the evaluation of antiretroviral efficacy has become complex and involves issues that are relevant to other fields. The outcome definition depends not only on the population to be tested (e.g. naive or treatment experienced) but also on whether a trial is for licensing purposes and whether a novel treatment strategy is being evaluated. This report provides a review of the primary endpoints that have been used in recent HIV clinical trials and discusses methodological issues raised by the use of these endpoints.

Search and selection strategy

We identified articles relating to antiretroviral therapy by searching PubMed. We restricted the search to randomized controlled trials conducted among HIV-infected adults and published from January 2005 until March 2008. The following request was performed in PubMed: (antiretroviral [All Fields] AND ('therapy'[Subheading] OR ('therapeutics'[TIAB] NOT Medline[SB]) OR 'therapeutics'[MeSH Terms] OR therapy[Text Word])) AND (('2005/01/01'[PDAT]: '2008/03/01' [PDAT]) AND 'humans'[MeSH Terms] AND Randomized Controlled Trial[ptyp])

We selected articles for consideration by screening the title and excluded inappropriate studies based on the abstracts and full text. We excluded articles reporting results of trials evaluating drugs in children, structured interruption therapy or effects on metabolic outcomes such as lipids. Additionally, we included manually selected abstracts from the Conference on Retroviruses and Opportunistic Infections, Interscience Conference on Antimicrobial Agents and Chemotherapy, the International AIDS Conference and the European AIDS Conference during the same time period.

The search identified a total of 51 studies for inclusion (28 in antiretroviral-naive patients (Tables 1 and 2) and 23 in antiretroviral-experienced patients (Table 3)).

Primary endpoint in trials of antiretroviral-naive patients

The recommended goal of treatment in antiretroviral-naive patients starting HAART is the achievement of a viral load < 50 copies/mL (virological suppression) within 16 (or 24) weeks and maintenance of this thereafter [1,2]. The United States Food and Drug Administration (FDA) currently recommends assessment of the proportion of trial subjects who achieve virological suppression through the 'time to loss of virological response' (TLOVR) algorithm at 24 weeks for accelerated approval and at 48 weeks for traditional approval [5]. TLOVR is a composite outcome that incorporates components relating to virological failure (confirmed with two consecutive viral load measurements), loss-to-follow-up, initiation of a new treatment due to intolerance/toxicity or any other

Table 1 Overview of primary endpoints used in HIV trials of antiretroviral-naive patients (based on information provided in published articles)

Reference	Trial	Definition of the primary endpoint	Time of evaluation week	Treatment of patients		Endpoint after analysis	Design	Blinding/ open-label
				Missing	Discontinuation			
Delfraissy *et al.* [47]	MONARK	% <400 cp/mL % <50 cp/mL	24 48	Failure	Failure	Composite	Pilot study	Open-label
Mallolas *et al.* [48]	TRIZEFAL	Failure: Virologic(failure to suppress viral load <50 cp/mL by w24 or w36 and 2 consecutive viral load >200 cp/mL after having dropped below 50 cp/mL), LTFU, Switch, disease progression, Death	72	Failure	Failure	Composite	Superiority	Open-label
Tashima *et al.* [49]		% <400 cp/mL TLOVR algorithm	168	Failure	Failure	Composite	Noninferiority	Open-label
Eron *et al.* [6]	KLEAN	% <400 cp/mL TLOVR algorithm	168	Failure	Failure	Composite	Noninferiority	Open-label
Markowitz *et al.* [50]	Merck 004	% <400 cp/mL	24	Failure	Failure	Composite	Pilot study	Open-label
Bartlett *et al.* [51]	CLASS	% <400 cp/mL Composite Time to treatment failure	96	Failure Failure	Not specified Failure	Composite	Strategy trial	Open-label
Gallant *et al.* [52]	Study 934	% <400 cp/mL TLOVR algorithm	48	Failure	Failure	Composite	Noninferiority	Open-label
Johnson *et al.* [53]		% <50 cp/mL	48	Failure	Failure	Composite	Noninferiority	Open-label
Montaner *et al.* [54]		% <50 cp/mL	48	Not specified	Not specified	Purely virological?	Noninferiority	Open-label
Gulick *et al.* [15]	A5095	Time to virological failure (defined as time to first of 2 successive HIV-RNA ≥200 cp/mL at or after w 16)	144	Censored	Analyzed regardless of treatment status	Purely virological	Superiority	Double blind
Yeni *et al.* [55]	INITIO	% <50 cp/mL	156	excluded	Not specified	Purely virological?	Strategy trial	Open-label
Markowitz *et al.* [56]	ESS40013	% <50 cp/mL Time to treatment failure after w48	96	Censored Failure	Censored Failure	Composite	Noninferiority	Open-label
Gallant *et al.* [57]	Study 903	% <400 cp/mL	48	Failure	Failure except EFV to NVP	Composite	Noninferiority	Blinded
Saag *et al.* [36]	FTC-301A Study	% <400 cp/mL % <50 cp/mL TLOVR algorithm	24 48 60	Failure	Failure	Composite	Superiority	Double blind

(*continued*)

Table 1 Continued

Reference	Trial	Definition of the primary endpoint	Time of evaluation week	Treatment of patients		Endpoint after analysis	Design	Blinding/ open-label
				Missing	Discontinuation			
Van Leth *et al.* [58]	2NN	Failure: decline <1log at W12,- HIV RNA <50 cp/ml (2 meas) after W24, HIV RNA <50 cp/ml (1 meas), disease progression, switch, except D4T/3TC backbone	48	Failure	Failure	Composite	Noninferiority	Open-label
DeJesus *et al.* [59]	EPV20001 Study	% <400 cp/mL	48	Failure	Failure	Composite	Noninferiority	Double blind
Gathe *et al.* [60]	SOLO	% <400 cp/mL	48	Failure	Failure	Composite	Noninferiority	Open-label
Riddler *et al.* [18]	ACTG 5142	(1) Time to virological failure: HIV RNA $>$ 200 cp/ml after w32 (2 measurements) or lack of suppression of plasma HIV-1 RNA by 1$\log_{10}$ or rebound before w32 (2) Time to regimen completion due to toxicity/intolerance	96	Censored	Failure (if toxicity related)	Purely virological and Composite (for the time to regimen failure endpoint) Considered as a bivariate outcome	Superiority	Open-label
Moyle *et al.* [61]	CNA30021	% <50 cp/mL TLOVR algorithm	48	Failure	Failure	Composite	Noninferiority	Double-blind

ITT, Intent to treat; cp/mL, copies/mL; TLOVR, Time to loss of virological response.

Table 2 Overview of primary endpoints used in HIV trials of antiretroviral-naive patients (based on information provided in abstracts and posters)

Reference	Trial	Definition of the primary endpoint	Time of evaluation week	Treatment of patients		Endpoint after analysis	Design	Blinding/open-label
				Missing	Discontinuation			
Lowe *et al.* [62]	ARES	% < 50 cp/mL	48	Not specified	Not specified	Purely virological?	Not reported	Not reported
Mallolas *et al.* [63]		Time to failure after switch HIV-RNA > 200 (2 meas), death, disease progression, discontinuation of second treatment	96	Not specified	Failure	Composite	Strategy trial	Open-label
Berenguer *et al.* [64]	GESIDA 3903	% < 50 cp/mL	48	Failure	Failure	Composite	Noninferiority	Open-label
Molina *et al.* [65]	CASTLE	% < 50 cp/ml	48	Failure	Failure	Composite	Noninferiority	Open label
Gathe *et al.* [66]	M05-730	% < 50 cp/ml	48	Failure	Failure	Composite	Noninferiority	Open-label
Smith *et al.* [67]	HEAT	% < 50 cp/ml	48	Failure	Included	Composite	Noninferiority	Not reported
De Jesus *et al.* [68]	ARTEMIS	% < 50 cp/ml TLOVR algorithm	48	Failure	Failure	Composite	Noninferiority	Not reported
Wamsley *et al.* [69]	GEMINI	% < 50 cp/ml	48	Failure	Not specified	Composite	Noninferiority	Open label
Malan *et al.* [70]	AI424-089	% < 400 cp/ml TLOVR algorithm	48	Failure	Failure	Composite	Not specified	Open label

ITT, Intent to treat; cp/mL, copies/mL; TLOVR, Time to loss of virological response.

reason (exceptions can be defined in the protocol for changes in the background regimen) and death [5]. By analyzing treatment modification as failure, it is possible to focus on the effects of the drugs under evaluation, rather than drugs that are taken subsequently. For example, the proportion of patients who achieved a viral load <400 copies/mL at 48 weeks according to the TLOVR algorithm was determined in the KLEAN study [6]. Responders were patients with confirmed viral load <400 copies/mL on two consecutive occasions who had not yet met any nonresponder criterion. Nonresponders were patients who never achieved a confirmed viral load <400 copies/mL on two consecutive occasions, prematurely discontinued study protease inhibitor for any reason, had confirmed viral load rebound to ≥ 400 copies/mL, or had an unconfirmed viral load ≥ 400 copies/mL on their final study visit [6] (see table 5 for an suggested approach of displaying the outcome). In our literature research, TLOVR was used as primary outcome in 13 of the 51 trials.

Composite endpoints

TLOVR is an example of composite endpoint. A total of 40 of the 51 studies reviewed used a composite endpoint. A composite outcome may include two kinds of criteria: biological/clinical criteria describing the efficacy or tolerance of the studied drugs (including treatment switch or termination) and criteria describing loss-to-follow-up and missing data. Among the biological/clinical criteria, the most commonly used in HIV clinical trials are the occurrence of toxicities (that might lead to treatment changes), clinical disease progression (including death) and other laboratory markers such as the CD4 cell count. Treatment modification can be defined in a variety of ways, including any treatment modification or modification due to specific toxicities/virological failure or of specific drugs only. Thus, the composite outcomes used are usually a combination of surrogate markers (e.g. viral load) and true clinical endpoints (e.g. death or progression to AIDS).

Composite endpoints are frequently used in many disease areas. For example, in trials of treatments for cardiovascular disease, composite endpoints may combine several events from death, nonfatal myocardial infarction, hospital admission for acute coronary syndrome, quality of life, cardiac arrest with resuscitation, hospitalization for heart failure and/or administration of intravenous inotropic or vasodilator drugs [7].

Table 3 Overview of primary endpoints used in HIV trials of antiretroviral-experienced patients (based on information provided in published articles)

Reference	Trial	Definition of the primary endpoint	Time of evaluation week	Treatment of patients		Endpoint after analysis	Design	Blinding/ open-label
				Missing	Discontinuation			
Pulido *et al.* [71]	OK04 study	Failure: VF (2 meas > 500), LTFU, Discontinuation, Change of randomized treatment other than reinduction	48	Failure	Failure	Composite	Noninferiority	Open-label
Hicks *et al.* [72]	RESIST-1	% > 1 log reduction	48	Failure	Failure	Composite	Noninferiority (% > $\log_{10}$ reduction) Superiority (time to treatment failure)	Open-label
	RESIST-2	Composite: Time to treatment failure	48					
Lazzarin *et al.* [73]	DUET-2	% < 50 cp/mL TLOVR algorithm	24	Failure	Failure	Composite	Superiority	Double-blind
Clotet *et al.* [74]	POWER-1 POWER-2	% ≥ 1 $\log_{10}$ cp/mL reduction TLOVR algorithm	48	Failure	Failure	Composite	Not clear	Open-label
Molina *et al.* [75]	POWER-3	% ≥ 1 $\log_{10}$ cp/mL reduction TLOVR algorithm	24	Failure	Failure	Composite	Strategy trial	Open-label
Johnson *et al.* [76]	BMS Study 045	Time averaged difference of plasma HIV-RNA reduction from baseline	48	Not specified	Included?	Purely virological	Noninferiority	Open-label
Markowitz *et al.* [77]	BI 1182.2	VL change from baseline	24, 48, 80	LOCF	EFV to NVP allowed	Purely virological?	Exploratory study	Open-label
		% < 400 cp/mL	24, 48, 80	Failure				
		% < 50 cp/Ml	24, 48, 80	Failure				
Nelson *et al.* [78]	TORO-1 TORO-2	VL change from baseline	48	Failure/LOCF	Failure/LOCF	Composite	Pooled analysis of TORO-1 and TORO-2 (Salvage Studies)	Open-label
Madruga *et al.* [79]	TITAN	% < 400 cp/mL TLOVR algorithm	48	Failure	Failure	Composite	Noninferiority (per protocol) If yes than Superiority (ITT)	Open-label
Madruga *et al.* [80]	DUET-1	% < 50 TLOVR algorithm	24	Failure	Failure	Composite	Not clear	Double-blind
Loutfy *et al.* [81]		% < 50 cp/mL	48	Failure	Not specified	Composite	Not specified	Open-label

Gatell *et al.* [82]	SWAN	% ≥50 copies/mL	48	Not specified	Not specified	Purely virological?	Noninferiority	Open-label
Grinsztejn *et al.* [83]	Protocol 005	VL change from baseline	24	Failure	Failure (+Failure only when discontinuation was due to treatment related toxicities +Failure only if discontinuation was related with virological failure)	Composite	Superiority	Double blind
Gulick *et al.* [84]		Time to treatment failure (time to virological failure (2 measurements > 200 cp/mL or time to treatment discontinuation, whatever came first)	48	Censored	Failure (beside protocol permitted switches)	Composite	Not specified	Open-label
Nadler *et al.* [85]	TMC125-C223	VL change from baseline	24	Failure	Failure	Composite	Pilot-study	Open-label
Fox *et al.* [86]	COLATE study	AAUCMB	48	Not specified	Not specified	Purely virological?	Strategy trial	Open-label
Gripshover *et al.* [87]	A5118	AAUCMB	24	Not specified	Not specified	Purely virological?	Not specified	Unblinding after interim review of the study design
Lamarca *et al.* [88]	CAL30001 study	AAUCMB TLOVR algorithm was used	48	Failure	Included? Failure	Composite	Noninferiority	Open-label
Arribas *et al.* [89]	OK study	% < 500 cp/mL	48	Failure	Failure	Composite	Pilot-study	Open-label
Arranz Caso *et al.* [90]		Failure: VL rebound, disease progression, Switch	48	Not specified	Failure	Composite	Not specified	Open-label
Bonjoch *et al.* [91]	SimplifiHAART study	% < 200 cp/mL	48	Censored (per protocol) Failure (ITT)	Censored (per protocol) Failure (ITT)	Purely virological composite	Noninferiority	Open-label
Swindells *et al.* [92]	NZTA4008 study	% < 400 cp/mL	24 48	Failure	Failure	Composite	Proof of concept	Open-label
Molina *et al.* [93]		VL change from baseline	24	Not specified	Included	Purely virological ITT	Superiority	Double Blind

ITT, Intent to treat; cp/mL, copies/mL; TLOVR, Time to loss of virological response; LOCF, Last observation carried forward.

Advantages of a composite endpoint

In HIV clinical trials, the motivation for a composite endpoint is to enable investigators to evaluate and compare interventions on features other than pure virological control. However, other more general advantages of composite endpoints include: (i) a reduction in sample size; (ii) an estimation of the net clinical benefit of a therapy; (iii) a better understanding of the effect of the interventions after excluding any competing risks, being able to study the effect of a given regimen, without contamination of the effect of subsequent regimens taken by the patient; (iv) the removal of the need to choose a single primary endpoint when many endpoints may be of equal importance; and (v) the removal of the need to adjust for multiple comparisons (for an overview see [8,9]).

The relevance of a composite endpoint is determined by the hypothesis being tested in the trial. As more clinical events occur with longer follow-up, these events may make a greater contribution to the composite endpoint in trials of longer duration (and so these endpoints may often be used in the context of a pragmatic trial). In the HIV field in particular, composite endpoints are often preferred, as neither the viral load or CD4 cell count alone are perfect surrogate markers [10,11] and discordant CD4/viral load responses may occur [12].

Disadvantages of a composite endpoint

In standard analyses, a composite outcome gives equal weight to each component of the outcome. For instance, in the TLOVR algorithm, virological failure has the same weight as mild treatment-limiting side effects (which can lead to treatment modification), serious irreversible toxicities, loss-to-follow-up and death. This can lead to interpretation and validity issues. These different components may not have the same clinical importance, may not occur with similar frequency and may not be affected to the same extent by the treatment under consideration [8,9,13]. Attempts to weight each component differently have already been made in cardiovascular disease field [14].

The validity of composite outcomes that include treatment termination as an endpoint may be questioned in open-label studies. Indeed, the knowledge of the received treatment and its associated side effects may influence the rate of earlier switching of the regimen in anticipation of these side effects, which might bias the comparison between treatment arms where switches are unbalanced. One approach to minimize this potential bias is to ensure that treatment switches are counted as failures only if the patient meets a series of objective criteria for switching. For example, a switch may be attributed to treatment failure when the switch is due to virological failure, treatment-related toxicities or serious adverse events, or disease progression. But how to deal with switches that occur before these criteria are met remains an issue. Additionally, all failure-defining endpoints should be evaluated by an independent committee that is unaware of the randomized treatment allocation. Of note, none of the reviewed trials stated that treatment modification was evaluated by an independent committee.

Virological endpoints

Virologic endpoints are often used as surrogate endpoints. Outcomes can be defined as achieving an undetectable viral load (e.g. <50 copies/mL at 24 weeks), as the time taken to achieve a viral load below a given threshold, or the time to the emergence of detectable viraemia after an initial virological response. An example of the use of a purely virological endpoint is the recently published A5095 study where time to virological failure was used as the primary efficacy endpoint [15]. Virological failure was defined as (i) a lack of initial response (failure to suppress viral load <200 copies/mL by week 16) or (ii) an early rebound or later relapse defined as two consecutive viral loads ≥ 200 copies/mL after week 16, ignoring treatment switches. The rationale for the use of a purely virological endpoint is the belief that the effect of the investigated therapies on plasma viral load will capture the essential information needed to define the role of the therapies in clinical practice for the target population. It gives an estimation of the antiretroviral effect (the efficacy) of the treatment, but only if a relatively small number of patients have switched to a nonrandomized treatment or are lost to follow up. Compared to a regimen termination endpoint, a purely virological endpoint is more valid (if treatment switches are ignored) because it is less dependent on physician/patient choice and less subject to bias in open-label studies.

In the reviewed trials, only 5 of the 28 clinical trials conducted in antiretroviral-naive patients, and 7 of the 23 clinical trials conducted in antiretroviral-experienced patients claimed to use a purely virological endpoint (Tables 1–3). However, for 3 of the 5 antiretroviral-naive trials and 6 of the 7 antiretroviral-experienced trials, it was unclear whether a purely virological endpoint was used as no details were provided for how missing data and treatment switches/discontinuations were handled.

Endpoints based on viral load alone can be misleading when patients who experience side effects on the randomized treatment are switched to a more potent regimen [16]. More generally, interpretation difficulties can arise if a large number of patients change their treatment regimen before reaching the virological endpoint. Gilbert *et al.* [16] used the ACTG 347 trial to demonstrate that the use of a purely virological endpoint can be misleading. The intent-to-treat (ITT) analysis of the binary endpoint (suppression of viral load < 500 copies/mL) at week 24 in this trial showed that monotherapy was superior to a triple combination arm [17]. This was due to the fact that a greater proportion of patients in the monotherapy arm switched to a more potent therapy.

Co-primary endpoints

The ACTG 5142 study used two primary endpoints, one of which was a purely virological endpoint defined as time to virological failure, and the second was a regimen termination endpoint (time to toxicity-related discontinuation) [18]. However, the adoption of a co-primary endpoint has implications for the sample size and requires appropriate statistical methods. Several possible methods of analysis were described in DiRienzo and DeGruttola [19] who compared (i) an omnibus test for testing the joint null hypotheses for the two primary endpoints, (ii) an average test, that was a combination of the two log-rank statistics, (iii) a Bonferroni correction for the type I error when testing the null hypotheses of each endpoint separately and (iv) a sequential testing procedure proposed by Marcus *et al.* [20–22]. Both the omnibus and the average tests had the disadvantage that when these tests reject the null hypotheses (i.e. suggest evidence of a difference between the two treatment arms) they do not provide information for which of the two endpoints the treatment difference was significant. In contrast, the Bonferroni correction and the sequential testing procedure both provide a decision on each null hypothesis separately. A simulation study led the authors to conclude that the best method for the analysis of the two primary endpoints of ACTG 5142 was the sequential testing procedure [19].

Primary endpoint in HIV trials of antiretroviral-experienced patients

Response to a new antiretroviral treatment is expected to be more heterogeneous in antiretroviral-experienced patients. Patients who have only experienced failure of their first or second treatment may still have several remaining treatment options. In this group, the goal of therapy remains complete suppression of viral load. Patients who have experienced failure of multiple treatments will have fewer future treatment options (such patients are said to require 'salvage' treatment) and may be less likely to achieve virological suppression on a new regimen. In trials of salvage treatments, a purely virological endpoint based on a sustained 1 $\log_{10}$ decline in viral load or mean changes in viral load may be considered [5]. This type of endpoint may lead to interpretation issues [23] as the more realistic goal of therapy may be to preserve immune function and prevent clinical progression [24] – as measured by CD4 change – rather than to achieve viral suppression. Of note, the group of patients who require salvage therapy is small as few patients fail all six currently available drug classes.

Impact of trial design and statistical analysis

Composite endpoints induced by analysis

If analyses use the intention-to-treat approach, failures can be defined as switching therapy (designated as ITT/S = F), noncompletion (ITT/NC = F) or missing outcome data (ITT/missing = F). Alternatively, with on-treatment analyses several different approaches may be taken: for example, patient follow-up can be censored at the time of treatment switch regardless of the reason for the switch, or can be censored only if viral load is undetectable and considered as failure otherwise. ITT/S = F analyses consider patients who switch either the main study drug or one of the other drugs in the regimen as failure (depending on the protocol), regardless of the reason for switching the drug.

Of the reviewed trials, treatment switch/discontinuation was considered as failure in 21 of the 28 trials conducted in antiretroviral-naive patients and in 14 of the 23 trials conducted in antiretroviral-experienced patients (22 trials as ITT/S = F and 13 trials due to TLOVR algorithm). Hill *et al.* reported that among trials conducted in antiretroviral-naive patients, of patients who were considered as failures, 27% were as a result of virological failure, 32% were as a result of treatment discontinuation for adverse events and 41% were as a result of discontinuation for other reasons [25]. As the proportion of patients discontinuing treatment was high, an endpoint which defines switches as failures is more likely to reflect the toxicity and

convenience of starting a given regimen, rather than its virological efficacy. Therefore, ITT/S = F has been criticized because it treats virological failure as equivalent to switching due to toxicity or other reasons [26]. While a patient who switches due to virological failure has a potentially higher risk of having developed drug resistance than a patient who switches due to an adverse effect with a suppressed viral load, the two will be treated equally in any comparison of outcomes. In some cases this may be reasonable even if no resistance is present (for example, if the drug has caused a severe hypersensitivity reaction which means that it cannot be used in the future). Additionally, any method that handles treatment switch/discontinuation or missing values as failures actually results in a composite endpoint. For instance, the analysis of a pure virological primary outcome at a given time (e.g. $\leq$50 copies/mL at 24 weeks) with an intent-to-treat analysis where switch is considered as failure (ITT/S = F) is the same as a composite endpoint where the first of either virological failure or treatment switch leads to the definition of failure. If switches occur in a substantial number of cases, then the study design may be affected. For instance, the sample size calculation in such a trial would have to be based not only on the expected rate of virological success but also on the possible proportion of patients who will make treatment switches.

Interpretation issues for composite endpoints

Drug toxicity is reflected in a wide range of adverse events occurring early after starting treatment or over the long-term and can be graded. The grade of toxicity may not correlate with treatment discontinuation. Furthermore, the decision to discontinue an antiretroviral drug will depend on both the clinician's perception of the importance of that toxicity and the patient's ability to tolerate it. This raises concerns about the application of switch = failure analysis (especially in open label studies), because an improved safety profile might compensate for inferior virological potency, leading to apparently similar outcomes [25,26]. As illustrated by Hill *et al.* fewer virological failures but a greater number of treatment discontinuations will lead to equivalent results in an ITT/S = F analysis [25]. In the CNAAB30005 trial [27], the ITT/S = F analysis gave virological success rates of 51% in each treatment arm while the on-treatment analysis gave rates of 94% in the zidovudine/lamivudine/indinavir arm and 86% in the zidovudine/lamivudine/abacavir arm [27]. A treatment arm may also appear to be more effective because of better tolerability even if the degree of virological failure is the same in the two treatment arms. For instance, in the BEST trial [28] indinavir was compared to ritonavir-boosted indinavir in virologically suppressed patients, each in combination with two nucleoside reverse transcriptase inhibitors. In the boosted indinavir arm 74% were classified as virological successes using an ITT/S = F analysis, compared to only 58% in the nonboosted indinavir arm; in contrast, 'on treatment' analyses revealed 93% versus 92% virological successes, respectively [28].

The most relevant approach to analyze purely virological outcomes in a superiority trial in the absence of significant amounts of missing information is the ITT analysis. The justification this approach is to preserve randomization, thus avoiding selection bias [29]. However, increasingly in the HIV field, clinical trials use a noninferiority design (22/51 of the reviewed trials). This design is mainly used when the added value of a new drug/regimen is due to ancillary benefits, such as a more convenient formulation, lower toxicity or lower cost.

In such trials (as well as equivalence trials), ITT analyses should be complemented with on-treatment analyses due to the increased possibility of a false claim of noninferiority when ITT analyses are used [30,31]. Indeed, for greater confidence, it is usually required that the results from the on-treatment and ITT analyses be consistent [31,32]; if this is not the case, the validity of the results may be questioned.

The relevance of noninferiority trials may often be debated because of issues related to the choice of the noninferiority margin [33]. Use of composite endpoints in either noninferiority or superiority trials can make their interpretation difficult, especially when the virological and nonvirological endpoints are imbalanced across treatment arms, and when components do not have the same clinical impact. For example, consider a hypothetical study in which 30% and 10% of patients on the experimental and reference arms, respectively, experience virological failure, with 5% and 25%, respectively, experiencing failure due to treatment discontinuation. As the overall failure rate (35%) is the same in both study arms, the experimental arm would be considered to be noninferior (assuming that the trial has been powered adequately so that the lower confidence limit does not overlap the noninferiority margin) despite very different virological efficacy. In practice, as the reason for discontinuation is often unknown [25], it may be difficult to assess the potential impact of this. Hill and Sabin propose that analyses are conducted using a 'nonvirological failures censored' approach that includes as failures all virological failures that occur while patients are receiving randomized treatment; any events that occur after treatment

discontinuation (i.e. where discontinuation was for nonvirological failure) are censored. Such an analysis may help to identify treatments that are virologically inferior but are associated with better tolerability [32]. However, this approach may still lead to biased treatment comparisons if censoring due to 'nonvirological failure' is not random or independent of the virological outcome.

Interpretation issues because of unbalanced outcomes included in composite outcomes have already been widely discussed; for example, for the use of composite endpoints in cardiovascular diseases [13,34,35].

Fixed time versus time to event

Although TLOVR was initially defined as an outcome for use in time-to-event analyses, it is most often analyzed at a fixed point in time (e.g. at the end of the trial). Among the 13 trials that report using the TLOVR algorithm, only one study [36] reported using a time-to-event approach (the Kaplan-Meier method) to estimate treatment differences. However, fixed endpoints may lead to a loss of information. Of note, the FDA recommends 'When assessing superiority for time to loss of virological response, the log rank test for differences in the Kaplan Meier curves, using all available follow-up data, should be performed'.

Gilbert *et al.* compared different definitions for time-to-event analysis using the Agouron 511 trial [37,38]. They found that both the choice of definition of the early failure time and the assigned failure time in this time-to-event endpoint definition influenced the results. The authors concluded that a time-from-randomization endpoint should be used that assigns subjects with early virological failure within T weeks a failure time of T weeks (where T is chosen such that >95% of responders are expected to respond by time T) [37].

Missing data

Data that are missing because of patient withdrawal from a study remains an issue for any type of outcome. Complete-case analysis, last-observation-carried-forward, censoring at the time of withdrawal or any other method of imputation all require strong assumptions to be made that should be stated explicitly and justified [39].

Composite outcomes which include 'loss-to-follow-up equals failure' give equal weight to patients with missing data and patients who really experience virological failure. In contrast, the censoring of patients with missing data requires that drop-out is independent of the risk of virological failure. As both the choice of approach and reasons for loss-to-follow-up are likely to be driven by the study population, either assumption may lead to biased results. Sensitivity analysis should be performed to study the impact of each definition on the results of the trial.

Components of composite endpoints should also appear as secondary endpoints [13,40]. However, when follow-up is discontinued after one of the components of the endpoint has been reached this may lead to substantial missing data for the analysis of the secondary endpoints. Thus, complete follow-up should be encouraged, even after virological failure or treatment switch because it will permit several complementary analyses [26,41]. Furthermore, it is important to ascertain all secondary endpoints including those not included as components in the composite endpoint, e.g. CD4 cell counts or incidence of non-AIDS defining events [26].

Time of measurement/assessment

The FDA recommend that primary efficacy endpoints should be assessed at either week 24 or week 48 [5]. The early failure time has to be chosen to give all patients the opportunity of reaching the primary virological endpoint over that time. For instance, patients with high baseline levels may not reach viral suppression $\leq$50 copies/mL by 24 weeks, which would lead to misclassification of a patient who has a steep viral load decline but who does not reach the defined threshold by week 24. Patients with slow but consistent rates of viral load decline are likely to be misclassified even at later time points. Hill *et al.* reported that the peak response for the 1 $\log_{10}$ copies/mL reduction criterion was reached by week 4 with the proportion of new patients reaching this endpoint decreasing thereafter [42]. Peak response for a viral load $\leq$400 copies/mL was reached by 16 weeks in the TORO, RESIST and POWER trials. In contrast the peak response for a viral load $\leq$50 copies/mL was reached by weeks 32, 40 and 48 in the RESIST, POWER and TORO trials, respectively [42]. In the M98-863 study, all patients who achieved a viral load $\leq$400 copies/mL did so within the first 24 weeks, 20% of those who achieved a viral load $\leq$50 copies/mL only achieved this for the first time after week 24 [43]. Thus, for the evaluation of a primary efficacy endpoint that includes a viral load $\leq$50 copies/mL, a later time point may be more appropriate.

Of note, recent studies of viral load kinetics after treatment initiation suggest that viral decay rates in the first two weeks may be good predictors of responses over the longer-term [44,45].

Table 4 Summary of endpoints and designs used in HIV trials of antiretroviral-naive and antiretroviral-experienced patients

Endpoint/Design feature	Treatment naive ($N = 28$)	Treatment experienced ($N = 23$)
Virological endpoint	5[a,b]	7[b]
Composite endpoint	24[a]	16
TLOVR[c]	7	6
Induced by analysis	14	8
Noninferiority	17	7
Superiority	4	3
Strategy or pilot study	5	7
Not specified	2	6
Open-Label	20	18

[a]One trial used a coprimary endpoint and has been counted as virological and composite endpoint. [b]For three of these five treatment-naive trials and for six of these seven treatment-experienced trials, it was unclear whether a purely virological endpoint was used as no details were provided for how missing data and treatment switches/discontinuations were handled. [c]Time to loss of virological response.

In conclusion, the definition of the endpoint may influence the time of evaluation.

However, the value of results from trials may also be limited by the relatively short duration of follow-up (48 weeks, or even 24 weeks for accelerated approval) required for licensing trials. Extended follow-up could provide useful results especially regarding the incidence of clinical events and the durability of a given treatment.

Concluding remarks

The choice of primary endpoint in a randomized trial is mainly driven by the clinical question to be answered. However, with increasing use of biomarkers in clinical practice to monitor people and an increasing number of drugs to choose there are decreasing mortality rates. Thus, in trials comparing specific drugs it will become increasingly impractical and often perceived as unethical to employ clinical endpoints. This has already occurred in the HIV field and is likely to be seen

Table 5 Suggested approach for displaying outcomes at 48-weeks outcome in trials that use a composite endpoint to define the primary endpoint (modified according to [6] and [5])

Outcome at 48 weeks	Abacavir/Lamivudine +	
	Fosamprenavir/ ritonavir	Lopinavir/ ritonavir
	$N = 434$	$N = 444$
Treatment failure overall, *n* (%)	**119 (27)**	**127 (29)**
Components of treatment failure		
Virologic failure (HIV-1 RNA ≥400 copies/mL), *n* (%)	26 (6)	30 (7)
Above assay limit, confirmed[a], *n* (%)	10 (2)	6 (1)
Confirmed rebound after achieving <400 copies/mL, *n* (%)	10 (2)	20 (5)
Never suppressed through week 24, *n* (%)	6 (1)	4 (<1)
Death[b], *n* (%)	3 (<1)	1 (<1)
Discontinuation due to other reasons, *n* (%)	90 (21)	96 (22)
Consent withdrawn, *n* (%)	0 (0)	0 (0)
Loss to follow up, *n* (%)	20 (5)	31 (7)
No data at week 48 or beyond, *n* (%)	6 (1)	12 (3)
Adverse event, *n* (%)	23 (5)	24 (5)
Patient's decision, *n* (%)	16 (4)	8 (2)
Noncompliance, *n* (%)	13 (3)	8 (2)
Pregnancy, *n* (%)	4 (<1)	2 (<1)
Protocol violation, *n* (%)	2 (<1)	2 (<1)
Insufficient viral load response, *n* (%)	0 (0)	1 (<1)
Other, *n* (%)	6 (1)	8 (2)
Other possible endpoint components could be (but were not in KLEAN [6]):		
AIDS defining events (new or recurrent), *n* (%)	7 (2)	10 (2)
Serious non AIDS defining events, *n* (%)	Not available	Not available

[a]Patients who had unconfirmed HIV-1 RNA ≥400 copies/mL on final study visit were deemed virological failure. [b]There was one additional death in the fosamprenavir/ritonavir group but this patient met the virological endpoint before being discontinued from the study due to death and was counted as a virological failure in this table.

in other fields. The choice of what endpoint to use is not straightforward. A composite endpoint can be of great interest in pragmatic trials. In the HIV setting pragmatic trials are of particular interest because the question may not only be whether a drug or drug combination demonstrates virological efficacy, but whether it also prevents clinical progression over the long-term, is safe, well-tolerated and does not lead to resistance or toxicities that may lead to the loss of future drug options. This is similar to clinical trials in oncology where drugs are required to demonstrate anti-tumor activity, tolerability, cost-effectiveness and an impact on quality-of-life [46].

Current clinical questions in the HIV field are based on the use of composite endpoints in trials of both antiretroviral- naive and antiretroviral-experienced patients (see Table 4 for an overview). The endpoint chosen should at least include components relating to virological response, death from any cause, any new or recurrent AIDS-defining event and any new serious non-AIDS-defining event. The definition of the composite endpoint should be clearly stated to avoid any confusion between a defined composite endpoint and a purely virological endpoint that has, in practice, become a composite endpoint through the approach used to deal with patients who switch treatments or are noncompleters. Furthermore, the clinical meaning of an endpoint that incorporates loss-to-follow-up may be difficult to establish. As treatment changes usually reflect some negative aspect of the regimen (e.g. tolerability or adherence issues), they may usefully be incorporated into a composite endpoint. However, whether any change of any component of the randomized regimen is of interest, or whether only changes to specific drugs should be counted as failure may depend on the underlying clinical question and the trial design. Any trial report should explicitly provide details of all outcomes, e.g. numbers of patients experiencing virological failure, treatment switch, loss-to-follow-up, missing outcomes, death, discontinuation due to an AIDS-defining event [5,8,40] (see, for e.g.,Table 5).

The other issue is the primary purpose of the trial; if it is to license a drug then there are stronger reasons for treating switch as failure, so that the effect of the drug can be isolated. For postlicensing trials there is a stronger argument for ignoring treatment switches in the primary analysis and focusing on the viral load, which is more relevant for clinical practice. Of course, such trials should also describe treatment changes to assess how they

Box 1 Synopsis

Definition of primary endpoints in HIV clinical trials
- depends on the underlying clinical questions
- regarding current clinical questions the most reasonable primary endpoints in both naive and treatment experienced patients may be a composite endpoint.

Components that may be considered
- virological failure (e.g. viral load >50 copies/mL)
- death due to any cause
- any new or recurrent AIDS defining event
- any serious non AIDS defining event
- Treatment change
  - whether the change of any component of the initial randomised regimen or only the change of the main drug should be counted as failure may depend on the underlying clinical question and the trial design.

Statistical analysis
- a time to event analysis should be preferred
  - time to failure as defined by the composite endpoint (that means that the time to the earliest event will be analysed)
  - time to virological response
  - time to treatment discontinuation
- Lost-to-follow-up
  - censoring: patients are considered to be successfully treated until the date of last contact
  - failure: patients are considered to have failed therapy at the date of last contact
- ITT or per protocol
  - depends on superiority or non-inferiority design
  - both types of analysis should always be performed when a non-inferiority design has been used
- Nonvirological failure censored analysis
  - to help identify treatments that virologically are inferior but better tolerated than the control arm

Reporting results
- detailed description of each component of the endpoint should be given, e.g. exact numbers of patients should be detailed for any reason that defines treatment failures
- detailed description of lost-to-follow-up should be provided
- reasons for censoring should be specified

could have influenced the outcome. Lastly, trials with clinical endpoints remain possible to conduct, and indeed necessary, in the HIV field when the comparison is between strategies rather than particular drugs.

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4.2 Definition of a virological endpoint for the analysis of the impact of drug resistance mutations

Genotypic resistance mutations directly or indirectly influence the replicative capacity of the virus under drug pressure, and render the antiretroviral effect of the treatment defective. Thus, to analyse the impact of genotypic resistance mutations on treatment response the definition of a 'pure' virological endpoint is required. In other words, we are interested in a definition that is highly specific for virological response as it gives an estimation of the antiretroviral effect of the treatment.

4.2.1 Endpoint definitions in treatment naïve patients

In recent studies, for the analysis of the impact of transmitted genotypic drug resistance on virological outcome either a binary response (% of patients below the detection limit) was used or the time to virological suppression below the detection limit was analysed (see Table 7).

Table 7: Virological endpoints used in recent studies (by descending year of publication) that investigated the impact of transmitted genotypic drug resistance on virological outcome in treatment naïve patients (by descending year of publication).

				Treatment of patients with	
Reference	**Cohort name/ Study population**	**Endpoint**	**Time**	**Missing data of VL**	**treatment change**
Bansi *et al.* [141]	CHIC	Time to VL<50 cp/mL		excluded	adjusted with a time updated co-variable
Bannister *et al.* [19]	EuroSIDA	% VL<500cp/mL	24 weeks 48 weeks	= failure; for sensitivity analysis excluded	not specified
Chaix *et al.* [142]	ANRS CO 06 PRIMO	% VL <400 cp/mL	12 weeks 24 weeks	excluded	not specified
Poggensee *et al.* [30]	German HIV-1 Seroconverter Study	% VL <500 cp/mL	24 weeks	excluded	not specified
Oette *et al.* [29]	RESINA	% VL<50cp/mL or <400 cp/mL	24 weeks 48 weeks	= failure; for sensitivity analysis excluded	not specified
Shet *et al.* [26]	Newly diagnosed, New York City	Time to VL <50 cp/mL		excluded	not specified
Pillay *et al.* [28]	CASCADE	Time to VL <500 cp/mL		excluded	not specified
Grant *et al.* [31]	~80% MSM, San Francisco	Time to VL <500 cp/mL		excluded	censored*

*patients with complete treatment stop were censored at the date of treatment stop. VL: viral load, MSM: men having sex with men, cp: copies.

4.2.1.1 Binary response

The analysis of binary virological response can probably be hampered by the amount of information still available after the follow-up. This implicates that one needs to define how to handle observations with missing data on the follow-up viral load. Bannister *et al.* defined observations with missing data as endpoint failures but as already mentioned in chapter 4.1 this definition is in reality the same than defining a composite outcome including missing = failure. Thus, the endpoint is not a 'pure' virological one anymore. Further, whether to consider patients that change any treatment may be dependent on the underlying question but patients stopping treatment should be excluded as done by Grant *et al.* as stopping treatment naturally results in a raise in viral load [31]. For all but one study summarised above it was not specified how patients changing or stopping treatment were taken into account.

4.2.1.2 Time to event

4.2.1.2.1 Time to suppression

Time to event analysis are all hampered by the issue how to handle observations of people who died, and the fact that patients who are lost-to-follow-up before reaching the above defined endpoint should be censored. Patients who completely stop treatment should be excluded as a treatment stop will lead to a raise in viral load. Treatment changes could be ignored if treatment change is not supposed to be due to virological failure. Another probability is to censor patients at the date of a treatment change with the caveat to miss some of the virological failures. Sensitivity analysis accounting for this problem should be performed.

The analysis of the time to virological suppression to below the detection limit is supposed to reflect the initial efficacy of a treatment. Further, the interest to look at the initial response is because it is supposed to be directly correlated with the response at a longer term i.e. 12 months or 24 months.

4.2.1.2.2 Time to failure

If the question is to study the risk of virological failure after a given time of therapy, the time to virological failure could be analysed. Failure could for example be defined as the time to a viral load above the detection limit after the patient was given a supposedly sufficient long time to suppress its viral load first, e.g. after 6 months of therapy. There are also some methodological issues with such a definition. First, patients never suppressing their viral load below the limit of detection will have as earliest failure time 6 months. Second, patients

starting with very high viral load who take longer time to suppress may not be "real" failures but would be considered as such. Third, an imbalance in the number and timing of measurements (e.g. viral load measurements) between compared groups may lead to an over or underestimation of the effect when time-to-event analyse are used in the context of observational databases [208, 209]. Fourth, if a lot of treatment changes occur before the defined virological failure endpoint is reached it may be difficult to assign failure to the initial treatment. Therefore sensitivity analyses should be done. A simple way would be to censor all treatment changes but another possibility is discussed below.

To account for patients that stopped or changed treatment due to virological failure a sort of composite endpoint could be created. For example, this endpoint could combine virological failure as defined by a confirmed viral load above the detection limit after 6 months of therapy or having a viral load above the detection limit before switching or stopping antiretroviral treatment. Of note, if the treatment switch occurs very early after treatment start a viral load above the detection limit may not necessarily indicate virological failure. The analysis of this endpoint as a time to event endpoint would implicate that some patients are truncated, i.e. they cannot fail earlier than 6 months. In contrast, patients who stopped or changed treatment before 6 months could also have a failure date before 6 months. Thus, estimations of hazards ratios are not reliable using this definition. However, a binary response could be created that assigns patients that reached either endpoint up to a given time, i.e. 12 months or 24 months as failures. Patients who died or who are lost to follow up before 12/24 months are not at risk for virological failure at 12/24 months anymore and thus should be excluded from this analysis (including those with a viral load above the detection limit before dieng or before lost-to-follow-up may be discussed). Further, patients who completely stopped antiretroviral treatment should be excluded as well as a treatment stop ultimately leads to a rise in viral load. Such an analysis would be a modified 'nonvirological failures censored/excluded' approach as proposed by Hill and Sabin [210].

4.2.2 Endpoint definitions in treatment experienced patients

For the analysis of the impact of genotypic resistance mutations on virological response in treatment experienced patients and for the construction of a genotypic score, an early virological endpoint is of interest. An early virological endpoint avoids the response to be affected by other factors such as adherence, toxicity, plasma drug levels, the evolution of the baseline genotype or the emergence of undetectable minority resistant strains [36].

None of the studies listed in Table 8 used a time of evaluation later than 12 weeks after treatment start. Three studies used a simple binary outcome, two studies a composite binary outcome taking into account suppression of viral load below detection or a reduction of more than 1 log10 copies/mL, and three studies used a quantitative outcome, i.e. the difference between baseline and a follow-up viral load.

Table 8: Virological endpoints used in selected studies evaluating the impact of genotypic resistance mutations on treatment outcome in treatment experienced patients and used to construct a genotypic score.

Reference	Drug for which a genotypic score was constructed	Endpoint	Time of evaluation
Descamps *et al.* [211]	darunavir	% <200 cp/mL	12 weeks
Marcelin *et al.* [173]	tipranavir	% <LOQ or decay of $\geq$1 $\log_{10}$	12 weeks
Masquelier *et al.* [41]	fosamprenavir	VL difference (VL at baseline minus VL at 12 weeks)	12 weeks
Pellegrin *et al.* [174]	fosamprenavir	% <400 cp/mL or decay of $\geq$ 1 $\log_{10}$	12 weeks
Capdepont *et al.* [40]	didanosine	% <50 cp/mL	12 weeks
Pellegrin *et al.* [212]	atazanavir	% <50 cp/mL	12 weeks
Marcelin *et al.* [213]	didanosine	VL difference (VL at baseline minus VL at 4 weeks)	4 weeks
Marcelin *et al.* [214]	saquinavir	VL difference (VL at baseline minus VL at 12 weeks)	12 weeks

LOQ: lower limit of detection, VL: viral load, cp: copies.

A simple binary outcome might lead to misclassification especially when evaluated very early after treatment start because not all patients may have reached the detection limit. A viral load difference endpoint without considering left-censoring may lead to biased estimation of the initial decay [52-54].

5 Impact of transmitted drug resistance on virological and immunological outcome to initial combination antiretroviral therapy

EuroCoord is a European platform for the integration of four ongoing cohorts or cohort collaborations related to clinical, virological and epidemiological HIV research. Partners are three EU-funded Coordination Actions, i.e. CASCADE, EuroSIDA, PENTA-EPPICC, and COHERE.

The joint project on transmitted drug resistance (TDR) and their impact on treatment response between EuroCoord and the EU funded FP7 CHAIN (Collaborative HIV and Anti HIV resistance network) was identified as a pilot project because a large number of patients was required to address the scientific question raised. The objective was to compare virological, immunological and clinical outcome up to 12-16 months following initiation of cART, according to markers of virus variability (specific mutations, subtypes), and relevant to the drugs in the regimen.

This joint project is a proof of concept of the collaboration between the four founding partners involved in EuroCoord (mainly epidemiologists, statisticians and clinicians) and CHAIN (mainly virologists).

For the following analysis we assessed the impact of TDR on virological and immunological response in the first year of cART in adults and children. In particular we focussed our analysis on response in patients with TDR receiving a fully active treatment in terms of regimens containing 2 NRTIs with either a ritonavir boosted PI or an NNRTI because these regimens are recommended as first-line treatments in high income countries.

Impact of transmitted drug resistance on virological and immunological response to initial combination antiretroviral therapy

The EuroCoord-CHAIN writing committee

Abstract

Background:

The impact of transmitted drug resistance (TDR) on first line treatment of HIV-1 infected patients requires further study, particular in the context of optimal drug regimens to use in such cases. We investigated the impact of TDR on treatment outcome in the first year of combination antiretroviral therapy (cART) in patients within a large European cohort collaboration.

Methods:

HIV-1 infected patients regardless of age, who started cART after 1.1.1998 and had ≥1 sample for genotypic testing taken whilst antiretroviral naïve, were included. We used the World Health Organisation (WHO) drug resistance list (2009) and the Stanford algorithm (v6.0.5) to classify patients in three resistance categories: no TDR, ≥1 mutation but receiving a fully active cART, ≥1 mutation and resistant to ≥1 prescribed drug. Virological failure (VF) was defined as time to the first of two consecutive viral load measurements >500 cp/mL after 6 months of therapy.

Findings:

Of 10,056 patients from 25 cohorts, 9102 (90.5%) patients harboured HIV-1 without TDR, 475 (4.7%) had ≥1 mutation but received a fully active cART and 479 (4.8%) had ≥1 mutation and were classified as resistant to ≥1 drug. Patients infected with TDR and resistant to ≥1 drug had a 3.3 fold higher risk of VF (95% confidence interval [CI]: 2.5; 4.4, $P<10^{-4}$) compared to patients without TDR. Overall, there was no significant difference between patients with TDR receiving a fully active cART and patients without TDR (HR: 1.4, 95% CI: 0.9; 2.3, P=0.17). In stratified analysis, those receiving 2NRTIs+1NNRTI with TDR but still predicted to receive a fully active cART tended to have a higher risk for VF (HR: 2.0, 95% CI: 0.9; 4.7, P=0.09).

Interpretation:

TDR caused a poor virological response when patients received cART containing ≥1 drug classified with at least low-level resistance. Even when an active regimen was used in the presence of TDR we found a potential higher risk of VF if 2NRTIs+1NNRTI were used, though not if a boosted PI+2 NRTIs were prescribed.

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Introduction

In Europe, widespread combination antiretroviral therapy (cART) use has been associated with a dramatic improvement in survival. However, this trend is paralleled by increased transmission of antiretroviral drug resistance: Between 10% and 15% of antiretroviral-naive patients are estimated to carry viruses with ≥ 1 drug-resistance mutation(s) [1-7]. In North America recent observational studies reported prevalence up to 25% [8, 9].

Mutations in the genome of HIV conferring drug resistance are a major reason for virological or immunological failure of antiretroviral therapy (as measured either by HIV RNA levels or CD4 cell counts, respectively). Recent treatment guidelines recommend genotypic testing in naïve patients to detect the presence of transmitted drug resistance (TDR) and to adapt their first line treatment [10-12]. However, the potential impact of TDR on virological and immunologic response remains controversial and has not been fully described. Some studies report no significant association between presence of TDR and either time to HIV RNA load suppression or proportions with HIV RNA suppression [2, 13, 14] or with immunological response [2, 8, 13, 14]. Other studies report poorer virological response in patients with TDR and a significantly shorter time to HIV RNA suppression among patients with susceptible strains [8, 14-18]. However, all of these studies are hampered by a lack of statistical power due to the relatively small proportion of patients with transmitted drug resistance included. In particular, the impact of TDR on virological response in patients treated with a fully active regimen has not yet been explored in the context of systematic genotypic testing prior to treatment initiation in larger datasets. Furthermore, there are also concerns about the possible impact of minority resistance strains (not detectable by standard genotypic testing) on treatment outcome especially for patients with minority non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance mutations receiving an NNRTI based regimen predicted to be fully active based on population genotypic test results (detectable TDR mutations) [19-24].

We assessed the impact of TDR on virological and immunological response in the first year of combination antiretroviral therapy (cART) in adults and children within a very large European collaboration of HIV observational cohorts (EuroCoord) and the European collaborative HIV and Anti HIV resistance network (CHAIN). In particular we focussed our analysis on response in patients with TDR receiving a fully active treatment as well as regimens containing two nucleoside reverse transcriptase inhibitors (NRTIs) with either a ritonavir boosted protease inhibitor (PI) or an NNRTI because these regimens are recommended as first-line treatments in high income countries [10, 25, 26].

Methods

Study population

The collaborative HIV cohorts CASCADE, COHERE, EuroSIDA and PENTA-EPPICC are the four founding networks of EuroCoord (The European Coordinating Committee for the Integration of Ongoing Coordination Actions Related to Clinical and Epidemiological HIV Research). CHAIN (Collaborative HIV and Anti HIV resistance network) and EuroCoord joined their collaborative efforts for this project.

The 25 cohorts participating through the four funding networks (and listed in the Appendix) submitted a defined dataset to their network-specific Coordination Centre, using the HIV Cohort Data Exchange Protocol (HICDEP) [27]. The dataset included patient demographics, use of cART, CD4 counts and HIV RNA measurements up to 16 months post-cART start, clinical (AIDS and death) events and genotypic resistance tests. Genotypic tests results were submitted as nucleotide sequences for protease and reverse-transcriptase or as lists of mutations for protease and reverse-transcriptase. Duplicates were removed prior to merging

the 4 network datasets into one overall EuroCoord-CHAIN database (final merger October 2009).

Each Coordinating Centre ensured that their participating cohorts had documented evidence of the ethics approval for such a project, and that the holding of data complied with local and national data protection requirements. All data proved by cohorts was anonymised, i.e. it was not possible to identify any infected individual using data submitted.

HIV infected patients were included regardless of age if they were antiretroviral naïve prior to starting cART for the first time after the 1 January 1998 and if they had ≥1 sample for a genotypic test taken before the initiation of cART. Time between genotypic testing and cART was not restricted, i.e. test results were not necessarily used to optimize first line treatment. For the main analysis a viral load measurement after 6 months was required if patients did not die or were lost to follow up before 6 months.

If more than one genotypic test result was available results were cumulated. Subtype of the virus was used as reported by the cohorts or determined by the Rega subtyping tool version 2 [28] for patients with missing data on subtype but an available nucleotide sequence.

Statistical analyses

Definition of TDR

TDR was defined in two steps. First, the World Health Organisation (WHO) drug resistance surveillance list [29] was used to distinguish between patients harbouring a virus with ≥1 TDR mutation of this list and patients harbouring a virus with no TDR mutation of this list referred to as 'no TDR'. Second, for patients harbouring a virus with ≥1 TDR mutation the Stanford algorithm version 6.0.5 [30] was used to classify patients into those receiving a fully active cART (Stanford levels 1: susceptible, 2: potential low level resistance for all prescribed drugs) referred to as 'TDR and fully active cART' or patients harbouring a resistant HIV strain (Stanford levels 3: low level resistance, 4: intermediate, 5: high level resistance) affecting ≥1 of their prescribed drugs refferd to as 'TDR and resistant'. For robustness analyses we further distinguished between patients with high level resistance (level 5) to ≥1 of their prescribed drugs, patients with low level/intermediate resistance (level 3,4) to ≥1 of their drugs and patients receiving a fully active cART (level 1,2). The prevalence of patients having ≥1 mutation of the WHO list was described; specifying also prevalence according to treatment classes: ≥1 NRTI mutation, ≥1 NNRTI mutation and ≥1 PI mutation. Furthermore, patients having ≥1 mutation to two different treatment classes (NRTI and NNRTI or NRTI and PI or NNRTI and PI) or having at ≥1 to three different treatment classes (NRTIs, NNRTIs and PIs) were calculated.

Virological response

Virological failure was defined as two consecutive viral loads >500 cp/mL after 6 months of therapy (date of first viral load >500 cp/mL was considered as failure date) (virological endpoint 1). Patients were censored if they died, were lost to follow up as defined by each cohort or stopped cART. In the absence of above defined events patients were censored at their last available viral load date in a six to 16 months window (patients with only one viral load after 6 months were censored at the date of viral load measurement either having a viral load ≤500 or >500 cp/mL). The time to virological failure is described by Kaplan Meier curves and analysed by Cox proportional Hazards model stratified by cohort. Baseline is defined as date of cART initiation. Proportional hazard assumption was graphically checked by plotting the log negative log survival time against the log time.

For sensitivity analysis, virological failure was defined as two consecutive viral loads > 500 cp/mL after 6 months of therapy, one viral load >500 cp/mL after six months where only one viral load available or one viral load >500 cp/mL in a two months period prior to a treatment

change/treatment stop (virological endpoint 2). A binary variable that assigned virological failure for those experiencing virological failure up to 12 months was created. Patients who died and patients who were lost to follow up before 12 months were excluded for this analysis. Further, patients stopping cART before 12 months without a viral load measurement >500 cp/mL before stopping were also excluded. Factors associated with virological failure at 12 months were assessed using logistic regression. The method of generalised estimating equations was used to estimate odds ratios and a compound symmetry covariance matrix was used to take intra-cohort correlation into account.

Immunological outcome

We modelled the difference between follow up CD4 counts and pre-treatment CD4 count of patients included in the main virological survival analysis. All CD4 cell counts measured after start of cART and before 12 months were used, and CD4 counts taken after treatment stops or changes were excluded. Children aged less than five years were excluded from this analysis because the marked differences in the variation of absolute CD4 count in this group has been previously described [31]. We used a piecewise linear mixed model with two slopes. The first slope was defined up to one month and the second slope after one month up to 12 months based on graphically observed slope change at one month. We took inter-patient correlation into account using random effects on the first and second slope. Correlation between the first and second slope was taken into account via an unstructured covariance matrix. Residuals distribution was graphically checked.

All multivariable models were adjusted for gender, age, pre-treatment viral load ($\log_{10}$ transformed) and CD4 count, subtype (B, non B, unknown), origin (African, European, other/unknown), year of treatment start (1998-1999, 2000-2005, ≥2006), previous AIDS diagnosis (yes, no, unknown) and HIV transmission risk group (homosexual/bisexual, heterosexual, injection drug use, perinatal, other/unknown). Analyses were performed using SAS 9.1 (SAS Institute, Inc., Cary, NC). P-values are double-sided.

Role of the funding source

The sponsors had no role in data collection, design, data analysis, data interpretation and writing of the report.

Results

Patient characteristics

Of 12,016 patients starting cART after the first January 1998 and had a resistance test performed on a plasma sample taken whilst ART-naïve, 10,056 patients had sufficient follow up data and were included in the main analysis (table 1). Of 10,056 patients, 6126 (60·9%) had ≥1 nucleotide sequence available. A plasma sample for a genotypic test was taken before ART in all patients but the date the sample was tested was after start of cART in some cases: for 3722 (37·0%) the genotypic test was assessed before initiation of cART (median 2 months, interquartile range [IQR]: 0.6-9.2); 2536 (25·2%) patients had a test assessed retrospectively after initiation of cART(median 34 months, IQR: 2-76); and for 3798 (37·8%) of the patients the genotypic test date was unknown. Median time between diagnosis of HIV and treatment start was 11 months (IQR: 2; 42) and median time between diagnosis and time of plasma sample for genotypic testing was 1.6 months (IQR: 0·4; 24). Of 10,056 patients included, 4845 received 2 NRTIs + 1 NNRTI (48·2%), 3117 2 NRTIs + 1 ritonavir boosted PI (31·1%), 1220 2 NRTIs + 1 unboosted PI (12·1%), 282 patients received NRTIs only (3 or 4 NRTIs, 2·8%) and 592 received other combinations (5·9%) (table 1 and table 2 of supplements). Complete cART stop was observed for 9·8% (95%CI: 9·2; 10·4) and 13·6%

(95% CI: 12·9; 14·3) of the patients at 6 and 12 months, respectively. Reasons for complete treatment stop were treatment failure in 3·6% of patients stopping cART up to 16 months (N=1479), toxicity and tolerance issues in 23·8%, other reasons (i.e. non-compliance, patient's decision etc.) in 42·4% and 30·2% stopped for unknown reasons. Treatment change of ≥1 drug was observed for 25·4% (95%CI: 24·5; 26·2) and 37.7% (38·7; 36·7) of the patients at 6 and 12 months, respectively. Reasons were treatment failure in 4·3%, tolerance/toxicity in 30·9%, other reasons in 35·5% and for 29·3% the reason was unknown.

Genotypic characteristics

At least one TDR mutation was found in 954 (9·5%, 95% confidence interval (CI): 8·9; 10·0) of patients. Of those 954 patients with TDR, 475 (49·8%) received a fully active cART and 479 (50·2%) were resistant to ≥1 prescribed drug. Of 479 patients with resistance, 334 (70·0%) patients had resistance to their prescribed NRTIs only, 56 (11·7%) patients to their prescribed NNRTI only, 14 (2·9%) patients to their prescribed PI only, 31 (6·5%) patients were resistant for their prescribed NRTIs and NNRTIs, 41 (8·6%) patients were resistant for NRTIs and PIs in their regimen and three patients (1·7%) had resistance against NRTIs, NNRTIs and PIs in their regimen. Further, of 479 patients with resistance for ≥1 prescribed drug, 157 (32·8%) patients had Stanford level 5 for ≥1 prescribed drug, 136 (28·4%) Stanford level 4 for ≥1 prescribed drug and 186 (38·8%) Stanford level 3 for ≥1 prescribed drug.
The prevalence for ≥1 NRTI mutation, ≥1 NNRTI mutation and ≥1 PI mutation were 6·2% (95% CI: 5·8; 6.7), 3·0% (95% CI: 2·7; 3·4) and 2.4% (95% CI: 2·1; 3·7), respectively. In total, 180 (1·8%) patients harboured a virus with ≥1 TDR mutation to two different treatment classes and 38 (0·4%) had ≥1 TDR mutation to three treatment classes. The most common mutations were for NRTIs M41L (n=215 (2·1 %)) followed by T215D (120 (1·1%)), for NNRTIs K103N (n=183 (1·8%)) followed by Y181C (n=67 (0·7%) and for PIs L90M (n=79 (0·8%)) followed by M46I (n=49 (0·5%)) (table 3 supplements). Most of the patients were infected with a subtype B (n=6906, 68·7%) although other subtypes were subtype C (n=725, 7·1%), circulating recombinant form CRF01_AE (n=594, 5·9%), CRF02_AG (n=458, 4·6%), subtype A (n=451, 4·5%) and subtype G (n=145, 1·4%). For 124 patients other CRFs (1·2%), for 179 other subtypes (1·8%) were found and for 474 patients (4·7%) the subtype was unknown. The prevalence of transmitted drug resistance varied between 4·8% and 5·8% for subtypes C and A, respectively and 9·8% for subtype B infected patients.

Virological outcome

All patients

Cumulative Kaplan Meier estimates for virological failure at 12 months were 4·2% (95% CI: 3·8; 4·7), 4.7% (2·9; 7·5) and 15·1% (11·9; 19·0) for patient groups no TDR, TDR and fully active cART and TDR and resistant, respectively (figure 1 A, Log-rank P<0·0001). In the adjusted Cox proportional hazards model the virological response differed significantly according to the TDR groups (*Global* P<0·0001). Patients of the TDR and resistant group had a 3·3 fold higher risk of virological failure (95% CI: 2·5; 4·4 P<0·0001) compared to patients of the no TDR group (table 2). In contrast, the risk of virological failure was not significantly different between patients of the TDR and fully active cART group and patients of the no TDR group (adjusted HR: 1·4, 95% CI: 0·9; 2·3, P=0·1724) (table 2). Then, in patients predicted with resistance for ≥1 prescribed drug we distinguished between patients classified with at least low-level/intermediate or fully resistance for ≥1 prescribed drug (figure 1 B Log-rank P<0·0001). Relative to patients of the no TDR group, a significantly higher risk of virological failure was observed as soon as patients received ≥1 drug classified with low-level/intermediate resistance (adjusted HR: 2·2, 95%CI: 1·5; 3·3, P=0·0001) and patients receiving ≥1 drug classified with high level resistance had a 6.3 fold higher risk for

virological failure (95%CI: 4·2; 9·4, P<0·0001). Other factors associated with a significant lower risk for virological failure included: a treatment start in recent years (≥2006), HIV transmission risk group: heterosexual/bisexual and European origin (table 2).

Treatment strata

Patients receiving 2 NRTIs + PI/rtv were more likely to be women, more likely to have initiated cART ≥2006, less likely to be homosexual, more likely to be of European origin and had higher pre-treatment viral loads and lower pre-treatment CD4 counts compared to patients receiving 2 NRTIs + 1 NNRTI (table 1). The cumulative Kaplan Meier estimates for virological failure for patients receiving 2 NRTIs plus 1 NNRTI at 12 months were 2·8%, 4·3% and 10·6% for the groups no TDR, TDR and fully active cART and the TDR and resistant group, respectively. The risk for virological failure of patients receiving 2 NRTIs + PI/rtv were 2·7%, 2·7% and 10·9% for patients in the groups no TDR, TDR and fully active cART and TDR and resistant group, respectively.

Patients with TDR receiving a fully active cART in the strata 2 NRTIs + 1 NNRTI tended to have a higher risk (HR: 2·0, 95% CI: 0·9; 4·7,*P*=0·0928) compared to patients of the no TDR group. In contrast, the risk for virological failure for patients of the TDR and fully active treatment group receiving a PI/rtv containing regimen was similar to the group no TDR (HR: 0·9, 95% CI: 0·4; 2·0, *P*=0·7302) (figure 2). The interaction between TDR and the two treatment strata 2NRTIs+ either NNRTI or PI/rtv was not significant (Global P=0·3439). For patients receiving other treatments with a low genetic barrier, i.e. 2 NRTIs + an unboosted PI and 3 or 4 NRTIs, a tendency for a higher risk for virological failure was seen for patients with TDR but predicted to receive a fully active treatment (table 6 supplements). The interaction between TDR and these four treatment strata was significant in unadjusted analysis (*P*=0·0105).

Sensitivity analyses

All patients

In all patients, exclusion of children younger than 13 and patients harbouring a virus with the mutation M184V (as a potential sign for non reported treatment exposure) revealed the same results than the main analysis. Frequencies of virological failure at 12 months in sensitivity analysis (virological endpoint 2) considering patients having one viral load over 500 cp/mL before stopping cART or before changing ≥1 drug as a virological failure were 12·2%, 12·4% and 30·4% for patients of the groups no TDR, TDR and fully active cART and TDR and resistant group, respectively. In adjusted analysis, the risk for virological failure was not significantly higher compared to the no TDR group in patients of the TDR and fully active cART group (OR: 1·3, 95% CI: 0·95; 1·8, P=0·1009) but it was for the TDR and resistant group (OR 3·1, 95% CI: 2·4; 4·0, P<0·0001). All adjusted models were consistent with the main results (table 4 supplements).

Treatment strata

Frequencies of virological failure at 12 months (virological endpoint 2) were 8·6%, 10·2% and 27·8% for patients receiving 2 NRTIs plus 1 NNRTI and 11·7%, 10·1% and 24·3% for patients receiving 2 NRTIs plus PI/rtv in patients of the groups no TDR, TDR and fully active cART and TDR and resistant group, respectively. In adjusted analysis, the risk of virological failure (ORs) for patients starting 2NRTIs + 1NNRTI and for patients starting 2NRTIs + 1PI/rtv were 1·4 (95% CI: 0·8; 2·4,*P*=0·2349) and 0·99 (95% CI: 0·6; 1·6,*P*=0·9593) in the group TDR and fully active treatment compared to the group no TDR. ORs for virological failure in the group TDR and resistant were 3·8 (95% CI: 2·6; 5·7, P<0·0001) and 2·5 (95%

CI: 1·6; 4·2, P=0·0002) compared to the no TDR group for patients in the NNRTI and PI/rtv treatment strata, respectively (table 5 supplements).

Impact on immune response

All patients

Median CD4 cell increase between start of cART and 12 months was 183 cells/µL (IQR: 105; 282). Unadjusted evolution according to the three classes of TDR is depicted in figure 3. Using a piecewise linear mixed model adjusted for potential cofounders there was neither a significant difference of CD4 cell increase in the first month after first line treatment start (*Global P* = 0·4013) nor a significant difference of CD4 cell increase after M1 neither (*Global P* = 0.0689), regardless of presence TDR and predicted susceptibility of cART received. Relative to patients of the no TDR group, the estimated difference in increase of CD4 count cells/µL per 12 months after one month of therapy were + 8 cells/µL/12months (95% CI: -11; 27, *P*=0·4308) for patients of the TDR and fully active cART group and -25 cells/µL/12months (95% CI: -48; -2, *P*=0·0326) for patients of the TDR and resistant group.

Treatment strata

Compared to the no TDR group, patients receiving 2 NRTIs + 1 PI/rtv had an estimated difference of +16 cells/µL/12months (95% CI: -10; 42, *P*=0.22) in the TDR and fully active cART group and -18 cells/µL/12months (95% CI: -60; 23, *P*=0.39) in the TDR and resistant group. For patients receiving 2NRTIs + 1NNRTI the estimated difference was -7 cells/µL/12months (95% CI: -37; 22, *P*=0·6312) in the TDR and fully active cART group compared to the no TDR group and -34 cells/µL/12months (95% CI: -68; 0, *P*=0·0514) in the TDR and resistant group versus the no TDR group.

Discussion

This is the largest evaluation of the clinical impact of TDR to date. We found that TDR was strongly associated with virological failure in particularly in those who received ≥1 drug to which the virus had lost susceptibility. Our findings confirm a poorer virological response in patients with TDR [8, 14-16, 18]. This is the first study that provides strong evidence that the selection of an initial regimen should be based on resistance testing in treatment naïve patients as recommended in recent treatment guidelines [10-12]. Furthermore, we showed that the prescription of a drug classified even with low-level resistance is associated with a significantly higher risk for virological failure which underscores the need of ≥3 fully active antiretroviral drugs in order to optimize the virological response to first-line regimen. A stratified analysis showed a tendency for a higher risk of virological failure in patients starting a 2 NRTIs + 1 NNRTI containing regimen if the patient harboured a virus with TDR even when the prescribed treatment was predicted to be fully active. In contrast, patients starting a regimen containing 2 NRTIs + 1 PI/rtv receiving a fully active treatment in presence of TDR had the same risk for virological failure as patients harbouring a virus with no TDR mutations.

Our study has several limitations. Genotypic testing was realised after treatment start for some patients. Thus, therapy was not necessarily guided by resistance testing and this could be one explanation why some suboptimal cART regimens have been used. Clinicians could have prescribed drugs that they were not aware to be incompletely active. We used a recent interpretation algorithm to predict the susceptibility of the prescribed treatment for all patients irrespective of the date of treatment start and time of genotypic testing. Interpretation algorithms evolve over time and even patient predicted to be susceptible to a specific drug at their treatment start could now have been classified as resistant if the algorithm for this drug

changed. This could be another explanation for the relatively high proportion of patients predicted to have at least low level resistance to ≥1 of their prescribed drugs.
Furthermore, patients classified to harbour a virus with no mutations of the WHO list could in fact harbour other resistance mutations not listed by the WHO but used by the Stanford algorithm. Indeed, there were 20 patients harbouring a virus with no mutation of the WHO list but predicted to have some resistance by the Stanford algorithm to their prescribed drugs (Stanford level 3, n=16; Stanford level 4, n=2 and Stanford level 5, n=2). Exclusion of these patients had no impact on the results (data not shown).
Patients predicted to have at least low level resistance to ≥1 of their prescribed drugs could have received ≥3 active drugs. Indeed there were 53 patients receiving ≥3 drugs predicted to have ≥3 active drugs but classified in the category at least low level resistance to ≥1 drug. Excluding those patients had no impact on the results (data not shown).

The findings for patients receiving 2 NRTIs + 1NNRTI could be partly explained by the presence of minority NNRTI resistant strains. These results would support previous findings that the presence of minority NNRTI resistance mutations can be related to virological failure if patients start a NNRTI based regimen [19-22, 24]. All sensitivity analyses consistently found a tendency for a higher risk of virological failure for patients receiving 2 NRTIs + 1NNRTI in the presence of TDR even when the regimen was predicted to be fully active. However, we can not exclude that other factors such as a poor adherence could explain our findings. For instance, if patients receiving 2 NRTIs + 1 NNRTI with TDR were less adherent than patients receiving 2 NRTIs + 1 boosted PI with TDR this could explain the higher failure rate in patients receiving 2 NRTIs + 1 NNRTI. Furthermore, the higher rate of virological failure in patients starting 2NRTIs + 1 NNRTI must not be necessarily linked to minority NNRTI mutations but could also be due to minority NRTI mutations impacting on the efficiency of the NRTI backbone in the regimen[32]. For other treatment combinations with a low genetic barrier, i.e. 2NRTIs plus an unboosted PI and NRTI monoclass therapy, patients with TDR predicted to receive a fully active cART tended also to have a higher risk for virological failure compared to those harbouring a virus with no TDR. This finding supports the hypothesis that detection of TDR at population sequencing level may be a sign of hidden resistant minority species.

The other finding is that patients receiving a boosted PI + 2NRTIs had the same risk for virological failure in presence of TDR if the treatment was adapted to the baseline reflects the higher genetic barrier of boosted PIs when compared to NNRTIs [33]. This is in agreement with previous studies that even when minority PI resistant variants were detected by ultra-deep sequencing or when NRTI mutations were present detected by allele specific PCR during primary infection the virological response was not affected if the patient received a boosted PI [32, 34]. Of note, from a clinical point of view if drug resistance mutations are detected before treatment initiation, a ritonavir boosted PI should be included in the first treatment regimen whose higher genetic barrier could better protect from the risk of virological failure due to potential invisible resistant minority species.

Patients having TDR and resistant to at least one prescribed drug had the same increase in CD4 cell count up to one month and tended to have a lower CD4 cell increase after one month compared to patients with no TDR. This finding may reflect the higher virological failure rate in these patients as we only adjusted for pre-treatment viral loads and is thus probably not a direct effect of TDR on CD4 cell count. For patients having with TDR receiving a fully active cART there was no significant difference in CD4 cell count increase neither up to one month

nor after one month compared to patients with no TDR This finding is in accordance with previous studies that found no impact of TDR on immunological outcome [2, 8, 13, 14].

We found an improved virological response for patients starting ≥2006-2008 that is probably due to the use of more potent regimen in recent years and are in accordance with recent findings of an increased proportion of patients with virological success over time [35]. The reasons for a better response in the homosexual transmission risk group and for patients with European origin are not straight forward but could be due to a better socioeconomic status, different health-seeking behaviour or adherence.

In conclusion, transmitted drug resistance was associated with a poorer virological response if the treatment was not adapted to the viral genotype. In the presence of TDR and when an active regimen was used we found a potential higher risk of virological failure if a combination of 2 NRTIs + 1NNRTI was used, though not if a boosted PI + 2 NRTIs were prescribed. Our results underscore that genotypic testing in treatment naive patients in regions with medium to high prevalence TDR is important to select a fully active regimen for treatment initiation. In regions where genotypic testing is not routinely available but high prevalence of TDR is suspected, first line regimens containing boosted PI should be considered.

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Table 1: Patient characteristics at the time of starting cART (N=10,056) in all patients and in those starting 2NRTIs + 1NNRTI (N=4845) or 2NRTIs + PI/rtv (N=3117).

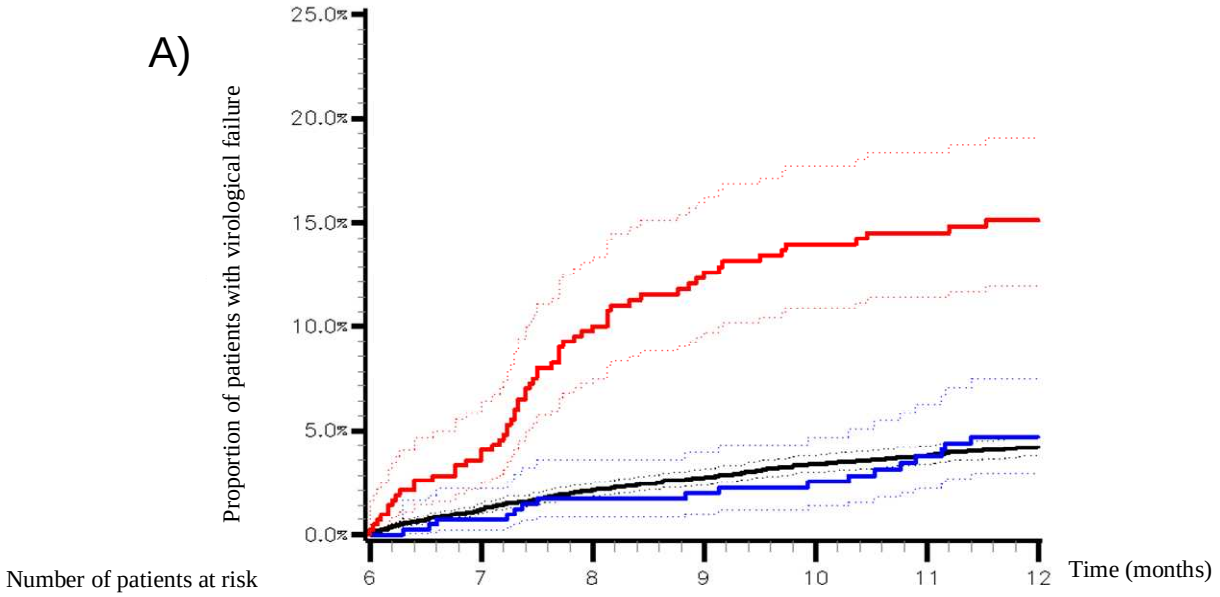
		Statistics[†]		
		All	2NRTIs+1NNRTI	2NRTIs+1PI/r
Female gender[a]		2404 (23·9)	976 (20·1)	782 (25·1)
Age, years	**median (IQR)**	38 (32; 44)	38 (32; 45)	38 (32; 44)
	<2	63 (0·6)	8 (0·2)	9 (0·3)
	3-5	60 (0·6)	11 (0·2)	3 (0·1)
	6-12	90 (0·9)	39 (0·8)	5 (0·2)
	13-17	46 (0·5)	22 (0·5)	12 (0·4)
	18-29	1702 (16·9)	767 (15·8)	552 (17·7)
	30-39	4099 (40·8)	1922 (39·7)	1263 (40·5)
	40-49	2710 (26·9)	1408 (29·1)	873 (28·0)
	50-59	952 (9·5)	496 (10·2)	298 (9·6)
	≥60	334 (3·3)	172 (3·6)	102 (3·3)
Year of treatment start	**1998-1999**	1041 (10·4)	179 (3·7)	96 (3·1)
	2000-2002	1578 (15·7)	633 (13·1)	282 (9·0)
	2003-2004	2087 (20·8)	1136 (23·4)	663 (21·3)
	2005-2006	3349 (33·3)	1781 (36·8)	1322 (42·4)
	2007-2008	2001 (19·9)	1116 (23·0)	754 (24·2)
Transmission risk group	**Homosexual/Bisexual men**	5025 (50·0)	2693 (55·6)	1525 (48·9)
	Injecting drug user	754 (7·5)	254 (5·2)	242 (7·8)
	heterosexual contact	3259 (32·4)	1481 (30·6)	1060 (34·0)
	perinatal	214 (2·1)	62 (1·3)	19 (0·6)
	Other/unknown	804 (8·0)	355 (7·3)	271 (8·7)
Origin	**Africa**	1002 (10·0)	448 (9·2)	283 (9·1)
	Europe	5653 (56·2)	2375 (49·0)	1829 (58·7)
	Other/unknown	3401 (33·8)	2022 (41·7)	1005 (32·2)
Previous aids diagnosis	**Yes**	1451 (14·4)	596 (12·3)	520 (16·7)
	No	7679 (76·4)	3877 (80·0)	2248 (72·1)
	unknown	926 (9·2)	372 (7·7)	349 (11·2)
Subtype	**Non B**	2676 (26·6)	1293 (26·7)	843 (27·0)
	B	6906 (68·7)	3345 (69·0)	2141 (68·7)
	unknown	474 (4·7)	207 (4·3)	133 (4·3)
Pre-treatment VL log10 cp/mL[b]	**median (IQR)**	5 (4·4; 5·4)	4.9 (4·4; 5·3)	5 (4·5; 5·5)
	<4	1292 (13·5)	595 (12·9)	398 (13·3)
	≥4 and <4.5	1342 (14·0)	690 (15·0)	388 (13·0)
	≥4.5 and <5	2421 (25·2)	1249 (27·1)	670 (22·5)
	≥5 and <5.5	2507 (26·1)	1271 (27·6)	751 (25·2)
	≥5.5 and <6	1533 (16·0)	652 (14·1)	548 (18·4)
	>6	506 (5.3)	152 (3·3)	228 (7·6)
Pre-treatment CD4 count cells/µL[c]	**median (IQR)**	218 (124; 310)	216 (137; 289)	207 (101; 313)
	<50	1062 (11·3)	388 (8·5)	436 (14·8)
	≥50 and <200	3103 (32·9)	1597 (35·0)	954 (32·4)
	≥200 and <350	3449 (36·6)	1970 (43·1)	955 (32·5)
	≥350 and <500	1043 (11·1)	396 (8·7)	355 (12·1)
	≥500	768 (8·1)	215 (4·7)	241 (8·2)
Time of genotypic testing[d]	**Before treatment start**	3722 (37·0)	2114 (46·6)	1275 (40·9)
	After treatment start	2536 (25·2)	893 (18·4)	777 (24·9)
	unknown	3798 (37·8)	1838 (40·0)	1065 (34·2)

[a] ALL: N=10,053, 2NRTIs+1NNRTI: N=4845, 2NRTIs+1PI/rtv: N=3114, [b] ALL: N=9601, 2NRTIs+1NNRTI: N= 4609, 2NRTIs+1PI/rtv: N=2983, [c] ALL: N=9425, 2NRTIs+1NNRTI: N=4566, 2NRTIs+1PI/rtv: N= 2941, [d]all samples for genotypic testing were taken before treatment start, [†]Frequencies (percentages) are reported unless stated otherwise. PI: Protease inhibitors, PI/rtv: ritonavir boosted Protease inhibitor, NRTI: Nucleotide reverse transcriptase inhibitors, NNRTI: Non nucleotide reverse transcriptase inhibitors, AIDS: Acquired immunodeficiency syndrome. IQR: Interquartile range.

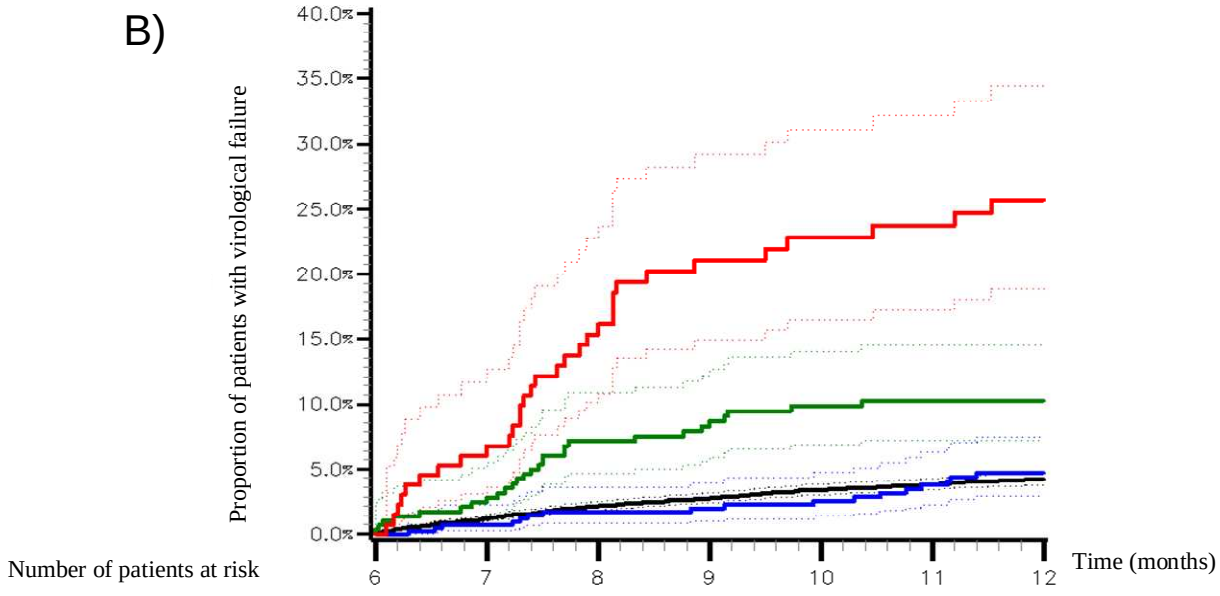
Table 2: Univariable and multivariable analysis of risk factors for time to virological failure using a Cox proportional Hazards model stratified by cohort.

		Univariable			Multivariable		
		HR	**(95% CI)**	***P***	**HR**	**(95% CI)**	***P***
TDR	No TDR	1·00		<0·0001	1·00		<0·0001
	TDR and fully active cART	1·15	(0·72; 1·83)	0·5574	1·40	(0·86; 2·26)	0·1724
	TDR and resistant	3·30	(2·52; 4·32)	<0·0001	3·30	(2·46; 4·43)	<0·0001
Gender	Female	1·00			1·00		
	Male	0·83	(0·67; 1·03)	0·0902	1·05	(0·81; 1·37)	0·7057
age	per additional year	0·98	(0·97; 0·99)	<0·0001	0·99	(0·98; 1·00)	0·1038
Pre-treatment viral load	per additional log10 cp/mL	1·02	(0·91; 1·14)	0·7428	1·04	(0·92; 1·17)	0·5444
Pre-treatmen CD4 cell count	per additional 100 cells/µL	0·99	(0·95; 1·03)	0·5460	0·97	(0·93; 1·01)	0·1052
Year of treatment start	≥2006	1·00		<0·0001	1·00		<0·0001
	1998-1999	3·60	(2·60; 4·99)	<0·0001	3·64	(2·56; 5·18)	<0·0001
	2000-2005	1·78	(1·37; 2·30)	<0·0001	1·58	(1·19; 2·08)	0·0013
Subtype	B	1·00		0·1904	1·00		0·0653
	Non B	1·10	(0·87; 1·38)	0·4170	0·85	(0·63; 1·14)	0·2776
	Unknown	0·71	(0·46; 1·10)	0·1263	0·54	(0·32; 0·92)	0·0228
Previous AIDS diagnosis	No	1·00		0·0102	1·00		0·0932
	Yes	1·41	(1·10; 1·81)	0·0067	1·32	(1·00; 1·73)	0·0483
	Unknown	0·73	(0·31; 1·76)	0·4876	0·79	(0·30; 2·13)	0·6467
Transmission risk group	heterosexual	1·00		<0·0001	1·00		<0·0001
	IDU	1·16	(0·82; 1·63)	0·4123	1·07	(0·73; 1·56)	0·7353
	Homosexual men	0·65	(0·52; 0·83)	0·0004	0·64	(0·47; 0·88)	0·0052
	Perinatal	8·46	(4·07; 17·62)	<0·0001	7·44	(3·21; 17·24)	<0·0001
	Other/unknown	0·93	(0·63; 1·37)	0·7076	0·92	(0·60; 1·40)	0·6923
Origin	Europe	1·00		0·0062	1·00		0·0653
	African	1·63	(1·20; 2·22)	0·0019	1·56	(1·07; 2·28)	0·0219
	Other/Unknown	1·28	(0·91; 1·78)	0·1529	1·19	(0·83; 1·70)	0·3495

*Due to exclusion of patients with missing values for pre-treatment viral load, pre-treatment CD4 cell count or gender N=9236. TDR: Transmitted drug resistance, no TDR: no mutation of the World Health Organization 2009 list of mutations for surveillance of transmitted drug resistant HIV strains (WHO list), TDR and fully active cART: ≥1 mutation of the WHO list and Stanford levels 1, 2 to all prescribed drugs, TDR and resistant: ≥1 mutation of the WHO list and resistant to at least one drug in the prescribed regimen (Stanford levels 3, 4, 5), HR: Hazards ratio, CI: confidence interval, AIDS: Acquired immunodeficiency syndrome, IDU: Injection drug user. cART: combination antiretroviral therapy.



Number of patients at risk							
No TDR (black line)	8086	7701	7367	7066	6717	6346	5786
TDR and fully active cART (blue line)	427	403	383	361	337	312	284
TDR and resistant (red line)	420	394	356	331	310	298	269



Number of patients at risk							
No TDR (black line)	8086	7701	7367	7066	6717	6346	5786
TDR and fully active cART (blue line)	427	403	383	361	337	312	284
TDR and resistant to drug (Stanford 3, 4; green line)	287	273	250	239	225	217	199
TDR and resistant to ≥1 prescribed drug (Stanford 5; red line)	133	121	106	92	85	81	70

Figure 1: Proportion of patients with virological failure according to transmitted drug resistance (TDR) (crude Kaplan Meier estimates, dotted lines 95% confidence intervals). A) Risk of virological failure according to three group of patients: no TDR (no mutation of the World Health Organization 2009 list of mutations for surveillance of transmitted drug resistant HIV strains (WHO list), TDR and fully active cART: ≥1 mutation of the WHO list and Stanford 1,2 to all prescribed drugs and TDR and resistant: ≥1 mutation of the WHO list and resistant to ≥1 prescribed drug (Stanford level 3, 4, 5). Log rank P<0·0001. B) Risk of virological distinguishing patients with intermediate and high level resistance. Log rank P<0·0001. cART: combination antiretroviral therapy.

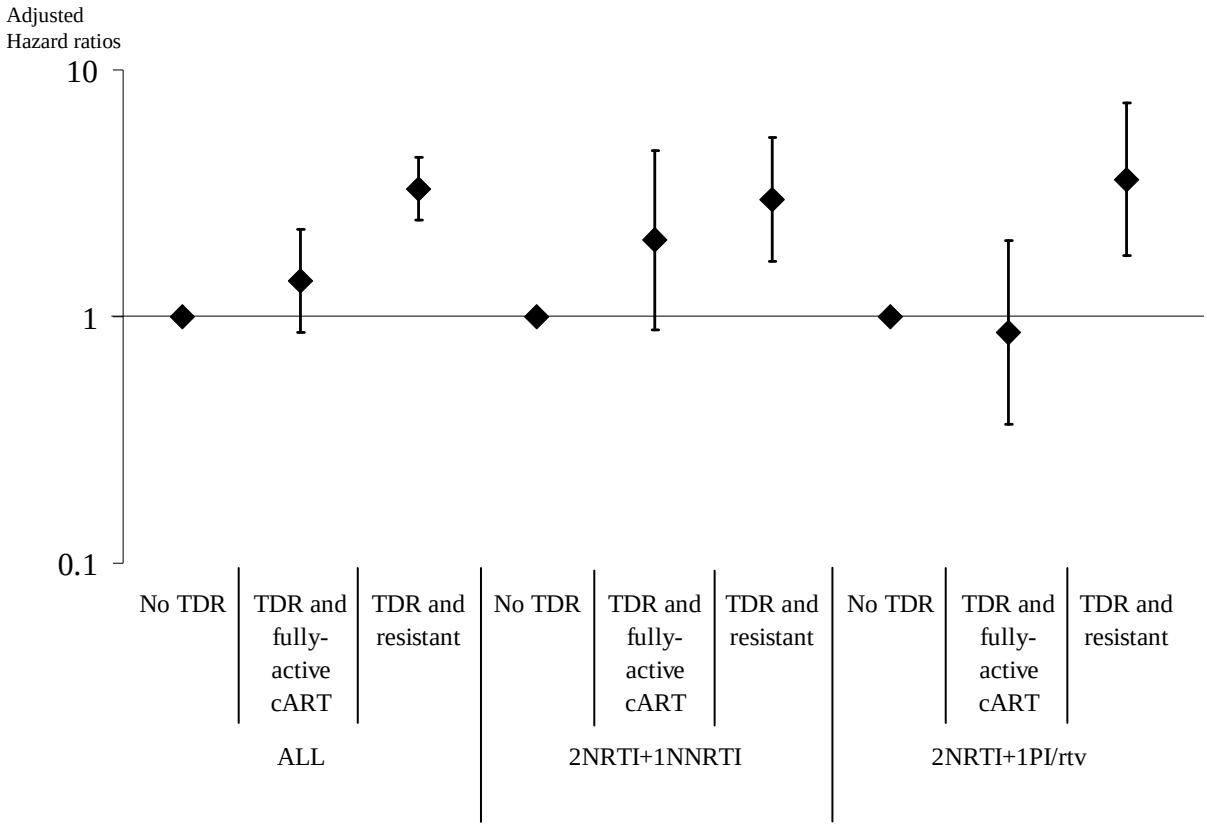


Figure 2: Adjusted Hazard ratios (log scale) in all patients and patients starting a regimen containing 2 NRTIs plus either 1 NNRTI or 1 PI/rtv

Squares: Adjusted Hazards ratios, horizontal bars: 95% confidence intervals, NRTI: nucleotide reverse transcriptase inhibitors, NNRTI: non nucleotide reverse transcriptase inhibitors, PI/rtv: ritonavir boosted protease inhibitor, No TDR: no mutation of the World Health Organization 2009 list of mutations for surveillance of transmitted drug resistant HIV strains (WHO list).TDR and fully active cART: ≥ 1 mutation of the WHO list and Stanford level 1, 2to all prescribed drugs. TDR and resistant: ≥1 mutation of the WHO list and resistant (Stanford level 3, 4, 5) to ≥1 prescribed drugs. All models are stratified by cohort and multivariable models ajusted for: Gender, age, pre-treatment viral load and CD4 count, year of treatment start, previous AIDS diagnosis, subtype, HIV transmission risk group and origin. TDR: Transmitted drug resistance. cART: combination antiretroviral therapy

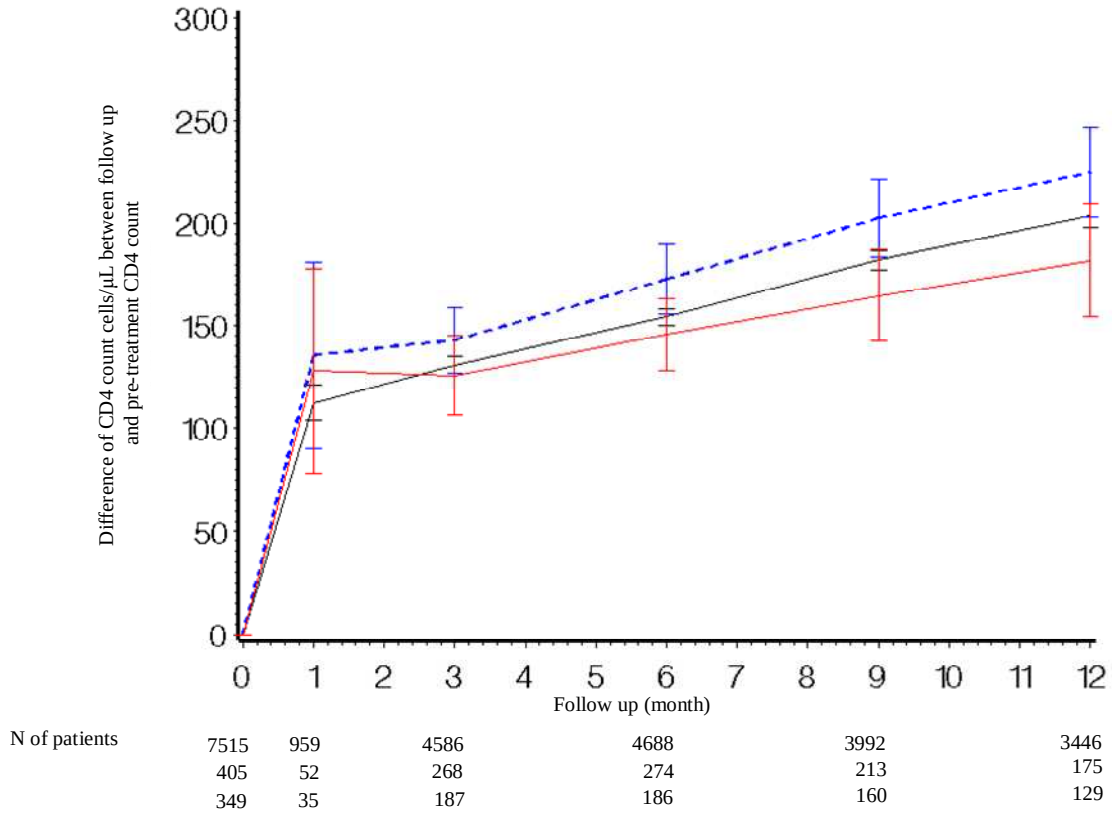


Figure 3: Observed difference of CD4 cell count cells/µL between follow up and pre-treatment CD4 count. black line: no mutation of the World Health Organization 2009 list of mutations for surveillance of transmitted drug resistant HIV strains (WHO list). Blue line: ≥1 mutation of the WHO list and Stanford level 1, 2 to all prescribed drugs. red line: ≥1 mutation of the WHO list and resistant (Stanford level 3, 4, 5) to ≥1 prescribed drugs). Horizontal bars: 95% confidence intervals

Supplements

Table 1: Treatment combinations prescribed at the time of starting cART in patients included in the main analysis (N=10,056)

Treatment combination prescribed		N	(%)
1 class treatments	3 or 4 NRTIs	282	(2.8)
2 class treatments	2 NRTIs + 1 NNRTI	4845	(48.2)
	2 NRTIs + 1 RTV boosted PI	3117	(31.0)
	2 NRTIs + 1 PI	1220	(12.1)
	≥ 3 NRTIs + 1 NNRTI	134	(1.3)
	≥ 3 NRTIs + 1 RTV boosted PI	107	(1.1)
	≥ 3 NRTIs + 1 PI	31	(0.3)
	0ther	34	(0.3)
3 class treatments	≥ 1 NRTI + 1NNRTI + 1 RTV boosted PI	201	(2.0)
	≥ 1 NRTI + 1NNRTI + 1 PI	54	(0.5)
	Other	24	(0.2)
4 class treatments	2 NRTI + 1NNRTI + 1 RTV boosted PI + 1FI	5	(0.0)
	1 NRTI + 1NNRTI + 1 RTV boosted PI + 1INSTI	1	(0.0)
	1 NRTI + 1 RTV boosted PI + 1FI + 1INSTI	1	(0.0)

NRTI: Nucleoside reverse transcriptase inhibitors, NNRTI: Non nucleoside reverse transcriptase inhibitor, PI: protease inhibitor, RTV: ritonavir, FI: Fusion inhibitor, INSTI: Integrase strand transfer inhibitor. cART: combination antiretroviral therapy.

Table 2: Antiretroviral drugs prescribed at the time of starting cART in patients included in the main analysis (N=10,056) and in those starting 2NRTIs + 1NNRTI (N=4845), 2NRTIs + PI/rtv (N=3117), 2NRTIs + 1PI (N=1220) and 3 or 4 NRTIs (N=282).

		All		2 NRTIs + 1 NNRTI		2 NRTIs + 1 PI/rtv		2 NRTIs + 1 PI		3 or 4 NRTIs	
		N	(%)	N	(%)	N	(%)	N	(%)	N	(%)
NRTIs	**Lamivudine**	6776	(67.4)	3011	(62.1)	2048	(65.7)	991	(81.2)	262	(92.9)
	Zidovudine	4099	(40.8)	1559	(32.2)	1297	(41.6)	718	(58.9)	215	(76.2)
	Tenofovir	3821	(38.0)	2247	(46.4)	1271	(40.8)	44	(3.6)	87	(30.9)
	Emtricitabine	2906	(28.9)	1674	(34.6)	979	(31.4)	29	(2.4)	56	(19.9)
	Abacavir	1717	(17.1)	733	(15.1)	419	(13.4)	87	(7.1)	237	(84.0)
	Stavudine	729	(7.2)	173	(3.6)	118	(3.8)	385	(31.6)	13	(4.6)
	Didanosine	653	(6.5)	292	(6.0)	100	(3.2)	169	(13.9)	16	(5.7)
	Zalcitabine	21	(0.2)	1		2	(0.1)	17	(1.4)	-	-
PIs	**Lopinavir**	2320	(23.1)	-	-	2042	(65.5)	-	-	-	-
	Nelfinavir	926	(9.2)	-	-	-	-	864	(70.8)	-	-
	Atazanavir	588	(5.8)	-	-	515	(16.5)	48	(3.9)	-	-
	Indinavir	426	(4.2)	-	-	143	(4.6)	250	(20.5)	-	-
	Saquinavir	290	(2.9)	-	-	246	(7.9)	14	(1.1)	-	-
	Fosamprenavir	129	(1.3)	-	-	123	(3.9)	1	(0.1)	-	-
	Amprenavir	47	(0.5)	-	-	30	(1.0)	1	(0.1)	-	-
	Tipranavir	8	(0.1)	-	-	5	(0.2)	-	-	-	-
	Darunavir	15	(0.1)	-	-	13	(0.4)	-	-	-	-
	Ritonavir alone	46	(0.5)	-	-	-	-	42	(3.4)	-	-
	Ritonavir boost	3464	(34.4)	-	-	3117	(100.0)	-	-	-	-
NNRTIs	**Efavirenz**	4226	(42.0)	3927	(81.1)	-	-	-	-	-	-
	Nevirapine	1031	(10.3)	918	(18.9)	-	-	-	-	-	-
	Delavirdine	2		-	-	-	-	-	-	-	-
	Etravirine	1		-	-	-	-	-	-	-	-
Other	**Raltegravir**	4		-	-	-	-	-	-	-	-
	Enfurvirtide	21	(0.2)	-	-	-	-	-	-	-	-
	Maraviroc	16	(0.2)	-	-	-	-	-	-	-	-

PI: protease inhibitors, PI/rtv: ritonavir boosted protease inhibitor, NRTI: nucleotide reverse transcriptase inhibitors, NNRTI: non nucleotide reverse transcriptase inhibitors, cART: combination antiretroviral therapy.

Table 3: Prevalence of mutations listed by the World Health Organization list of mutations for surveillance of transmitted drug resistant HIV strains.

NRTI mutations			NNRTI mutations			PI mutations		
Mutation	**N**	**(%)**	**Mutation**	**N**	**(%)**	**Mutation**	**N**	**(%)**
M41L	215	(2.14)	**L100I**	4	(0.04)	**L23I**	3	(0.03)
K65R	11	(0.11)	**K101E**	33	(0.33)	**L24I**	8	(0.08)
D67N	87	(0.87)	**K101P**	4	(0.04)	**D30N**	13	(0.13)
D67G	16	(0.16)	**K103N**	183	(1.82)	**V32I**	4	(0.04)
D67E	8	(0.08)	**K103S**	10	(0.10)	**M46I**	49	(0.49)
T69D	35	(0.35)	**V106M**	7	(0.07)	**M46L**	42	(0.42)
T69INS	0		**V106A**	6	(0.06)	**I47V**	7	(0.07)
K70R	45	(0.45)	**V179F**	1	(0.01)	**I47A**	2	(0.02)
K70E	4	(0.04)	**Y181C**	67	(0.67)	**G48V**	3	(0.03)
L74V	17	(0.17)	**Y181I**	1	(0.01)	**G48M**	1	(0.01)
L74I	7	(0.07)	**Y181V**	1	(0.01)	**I50V**	2	(0.02)
V75M	4	(0.04)	**Y188L**	13	(0.13)	**I50L**	0	
V75T	3	(0.03)	**Y188C**	2	(0.02)	**F53L**	11	(0.11)
A75A	3	(0.03)	**Y188H**	3	(0.03)	**F53Y**	4	(0.04)
V75S	0		**G190S**	6	(0.06)	**I54L**	2	(0.02)
F77L	15	(0.15)	**G190A**	38	(0.38)	**I54V**	31	(0.31)
Y115F	2	(0.02)	**G190E**	2	(0.02)	**I54M**	1	(0.01)
F116Y	7	(0.07)	**P225H**	4	(0.04)	**I54T**	2	(0.02)
Q151M	5	(0.05)	**M230L**	4	(0.04)	**I54A**	3	(0.03)
M184V	103	(1.02)				**I54S**	1	(0.01)
M184I	15	(0.15)				**G73S**	12	(0.12)
L210W	97	(0.96)				**G73T**	2	(0.02)
T215Y	55	(0.55)				**G73C**	0	
T215F	16	(0.16)				**G73A**	0	
T215I	17	(0.17)				**L76V**	4	(0.04)
T215S	102	(1.01)				**V82A**	37	(0.37)
T215C	32	(0.32)				**V82T**	6	(0.06)
T215D	120	(1.19)				**V82F**	6	(0.06)
T215V	10	(0.10)				**V82S**	2	(0.02)
T215E	30	(0.30)				**V82C**	0	
K219Q	61	(0.61)				**V82M**	0	
K219E	30	(0.30)				**V82L**	11	(0.11)
K219N	18	(0.18)				**N83D**	3	(0.03)
K219R	10	(0.10)				**I84V**	13	(0.13)
						I84A	0	
						I84C	0	
						I85V	14	(0.14)
						N88D	11	(0.11)
						N88S	4	(0.04)
						L90M	79	(0.79)

NRTI: nucleotide reverse transcriptase inhibitors, NNRTI: non nucleotide reverse transcriptase inhibitors, PI: protease inhibitors. Mutations: The wild type amino acid is given followed by the position of the reverse transcriptase gene for NRTI and NNRTI mutations and the protease gene for PIs. After the position number the amino acid substitution conferring resistance is given. Amino acid abbreviations: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine, G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

Table 4: Overview of sensitivity analysis in all patients

Definition of virological endpoint		N*	% VF M12**	HR	(95% CI)	*P*
	No TDR	9102	4.2	1		
	TDR and fully active cART	475	4.7	1.40	(0.86; 2.26)	0.1724
Virological endpoint 1	TDR and resistant	479	15.1	3.30	(2.46; 4.43)	<0.0001
	No TDR	9102	4.2	1		
	TDR and fully active cART	475	4.7	1.41	(0.87; 2.28)	0.1644
	TDR and resistant (stanford level 3, 4)	322	10.2	2.19	(1.47; 3.27)	0.0001
Virological endpoint 1	TDR and resistant (stanford level 5)	157	25.7	6.30	(4.22; 9.4)	<0.0001
	No TDR	8907	3.7	1		
	TDR and fully active cART	472	4.7	1.43	(0.88; 2.31)	0.1498
Virological endpoint 1 (children <13 excluded)	TDR and resistant	464	14.3	3.67	(2.72; 4.97)	<0.0001
	No TDR	9102	4.2	1		
	TDR and fully active cART	462	4.3	1.29	(0.77; 2.14)	0.3290
Virological endpoint 1 (Patients harbouring M184V excluded)	TDR and resistant	389	12.5	2.90	(2.06; 4.08)	<0.0001
		N*	**% VF M12*** **	**OR**	**(95% CI)**	***P***
	No TDR	7724	4	1		
	TDR and fully active cART	411	4.1	1.41	(0.86; 2.31)	0.1743
Virological endpoint 1b	TDR and resistant	396	14.7	3.55	(2.5; 5.04)	<0.0001
	No TDR	7918	12.2	1		
	TDR and fully active cART	421	12.4	1.29	(0.95; 1.76)	0.1009
Virological endpoint 2	TDR and resistant	420	30.4	3.12	(2.44; 3.98)	<0.0001

Virological endpoint 1: Virological failure was defined as two consecutive viral loads >500 cp/mL after 6 months of therapy (date of first viral load >500 cp/mL was considered as failure date). Patients were censored if they died, were lost to follow up as defined by each cohort or stopped cART. In the absence of above defined events patients were censored at their last available viral load date in a six to 16 months window (patients with only one viral load after 6 months were censored at the date of viral load measurement either having a viral load ≤500 or >500 cp/mL). Virological endpoint 1b: Virological failure was defined as under virological endpoint 1 but a binary variable that assigned virological failure for those experiencing virological failure up to 12 months was created. Patients who died, patients who were lost to follow up and patients who stopped treatment were excluded. Virological endpoint 2: For sensitivity analysis, virological failure was defined as two consecutive viral loads > 500 cp/mL after 6 months of therapy, one viral load >500 cp/mL after six months where only one viral load available or one viral load >500 cp/mL in a two months period prior to a treatment change/treatment stop. A binary variable that assigned virological failure for those experiencing virological failure up to 12 months was created. Patients who died and patients who were lost to follow up before 12 months were excluded for this analysis. Further, patients stopping cART before 12 months without a viral load measurement >500 cp/mL before stopping were also excluded. VF: Virological failure, HR: adjusted Hazards ratio, OR: adjusted Odds ratio, CI: confidence interval, M12: 12 months. No TDR: no mutation of the World Health Organization 2009 list of mutations for surveillance of transmitted drug resistant HIV strains (WHO list). TDR and fully active cART: ≥ 1 mutation of the WHO list and susceptible (stanford level 1, 2) to all prescribed drugs. TDR and resistant: ≥1 mutation of the WHO list and resistant (stanford level 3, 4, 5) to ≥1 prescribed drugs. cART: combination antiretroviral therapy. * Numbers included in univariable analysis, **Univariable Kaplan Meier estimates, ***Univariable frequencies of virological failure.

Table 5: Overview of sensitivity analysis in patients receiving 2 NRTIs + either 1 NNRTI or a ritonavir boosted PI

		2 NRTIs + 1 NNRTI					2 NRTIs + 1 PI/rtv				
Definition of virological endpoint		**N***	**% VF M12****	**HR**	**(95% CI)**	***P***	**N***	**% VF M12****	**HR**	**(95% CI)**	***P***
	No TDR	4509	2.8	1			2748	2.7	1		
	TDR and fully active cART	160	4.3	2.05	(0.89; 4.72)	0.0928	237	2.7	0.86	(0.36; 2.03)	0.7302
Virological endpoint 1	TDR and resistant	176	10.6	2.99	(1.67; 5.34)	0.0002	132	10.9	3.60	(1.76; 7.34)	0.0004
		N*	**% VF M12*****	**OR**	**(95% CI)**	***P***	**N***	**% VF M12*****	**OR**	**(95% CI)**	***P***
	No TDR	4003	2.5	1			2257	2.6	1		
	TDR and fully active cART	144	3.5	1.75	(0.75; 4.06)	0.1943	204	2.5	0.72	(0.23; 2.25)	0.5681
Virological endpoint 1b	TDR and resistant	152	10.5	3.42	(1.84; 6.36)	0.0001	108	11.1	4.32	(1.89; 9.89)	0.0005
	No TDR	4063	8.6	1			2318	11.7	1		
	TDR and fully active cART	147	10.2	1.39	(0.81; 2.38)	0.2349	207	10.1	0.99	(0.61; 1.60)	0.9593
Virological endpoint 2	TDR and resistant	162	27.8	3.82	(2.56; 5.68)	<0.0001	115	24.3	2.54	(1.56; 4.16)	0.0002

Virological endpoint 1: Virological failure was defined as two consecutive viral loads >500 cp/mL after 6 months of therapy (date of first viral load >500 cp/mL was considered as failure date). Patients were censored if they died, were lost to follow up as defined by each cohort or stopped cART. In the absence of above defined events patients were censored at their last available viral load date in a six to 16 months window (patients with only one viral load after 6 months were censored at the date of viral load measurement either having a viral load ≤500 or >500 cp/mL). Virological endpoint 1b: Virological failure was defined as under virological endpoint 1 but a binary variable that assigned virological failure for those experiencing virological failure up to 12 months was created. Patients who died, patients who were lost to follow up and patients who stopped treatment were excluded. Virological endpoint 2: For sensitivity analysis, virological failure was defined as two consecutive viral loads > 500 cp/mL after 6 months of therapy, one viral load >500 cp/mL after six months where only one viral load available or one viral load >500 cp/mL in a two months period prior to a treatment change/treatment stop. A binary variable that assigned virological failure for those experiencing virological failure up to 12 months was created. Patients who died and patients who were lost to follow up before 12 months were excluded for this analysis. Further, patients stopping cART before 12 months without a viral load measurement >500 cp/mL before stopping were also excluded. VF: Virological failure, HR: adjusted Hazards ratio, OR: adjusted Odds ratio, CI: confidence interval, NRTI: Nucleoside reverse transcriptase inhibitor, NNRTI: Non nucleoside reverse transcriptase inhibitor, PI/rtv: ritonavir boosted Protease inhibitor. M12: 12 months. No TDR: no mutation of the World Health Organization 2009 list of mutations for surveillance of transmitted drug resistant HIV strains (WHO list). TDR and fully active cART: ≥ 1 mutation of the WHO list and susceptible (stanford level 1, 2) to all prescribed drugs. TDR and resistant: ≥1 mutation of the WHO list and resistant (stanford level 3, 4, 5) to ≥1 prescribed drugs. cART: combination antiretroviral therapy. * Numbers included in univariable analysis, **Univariable Kaplan Meier estimates, ***Univariable frequencies of virological failure.

Table 6: Overview of sensitivity analysis in patients receiving 2 NRTIs plus an unboosted PI and patients receiving 3 or 4 NRTIs

		2 NRTIs + 1 PI					3 or 4 NRTIs[§]				
Definition of virological endpoint		**N***	**% VF M12****	**HR**	**(95% CI)**	***P***	**N***	**% VF M12****	**HR**	**(95% CI)**	***P***
Virological endpoint 1	No TDR	112	12.8	1			242	9.1	1		
	TDR and fully active cART	35	15.5	2.52	(0.89; 7.12)	0.082	19	20.5	4.62	(1.09; 19.65)	0.038
	TDR and resistant	73	24.7	1.71	(0.92; 3.17)	0.090	21	32.5	4.91	(1.76; 13.70)	0.002
		N*	**% VF M12*****	**OR**	**(95% CI)**	***P***	**N***	**% VF M12*****	**OR**	**(95% CI)**	***P***
Virological endpoint 1b	No TDR	869	12.5	1			188	9.0	1		
	TDR and fully active cART	28	14.3	1.54	(0.48; 4.99)	0.470	15	20.0	2.54	(0.66; 9.83)	0.177
	TDR and resistant	56	23.2	1.48	(0.69; 3.15)	0.314	19	31.6	4.65	(1.57; 13.79)	0.006
Virological endpoint 2	No TDR	906	23.2	1			198	17.2	1		
	TDR and fully active cART	29	27.7	1.60	(0.64; 3.95)	0.313	15	26.7	1.87	(0.57; 6.11)	0.302
	TDR and resistant	61	39.3	1.73	(0.97; 3.11)	0.065	19	31.6	2.33	(0.83; 6.49)	0.107

Virological endpoint 1: Virological failure was defined as two consecutive viral loads >500 cp/mL after 6 months of therapy (date of first viral load >500 cp/mL was considered as failure date). Patients were censored if they died, were lost to follow up as defined by each cohort or stopped cART. In the absence of above defined events patients were censored at their last available viral load date in a six to 16 months window (patients with only one viral load after 6 months were censored at the date of viral load measurement either having a viral load ≤500 or >500 cp/mL). Virological endpoint 1b: Virological failure was defined as under virological endpoint 1 but a binary variable that assigned virological failure for those experiencing virological failure up to 12 months was created. Patients who died, patients who were lost to follow up and patients who stopped treatment were excluded. Virological endpoint 2: For sensitivity analysis, virological failure was defined as two consecutive viral loads > 500 cp/mL after 6 months of therapy, one viral load >500 cp/mL after six months where only one viral load available or one viral load >500 cp/mL in a two months period prior to a treatment change/treatment stop. A binary variable that assigned virological failure for those experiencing virological failure up to 12 months was created. Patients who died and patients who were lost to follow up before 12 months were excluded for this analysis. Further, patients stopping cART before 12 months without a viral load measurement >500 cp/mL before stopping were also excluded. VF: Virological failure, HR: adjusted hazards ratio, OR: adjusted odds ratio, CI: confidence interval, NRTI: Nucleoside reverse transcriptase inhibitor, NNRTI: Non nucleoside reverse transcriptase inhibitor, PI: unboosted protease inhibitor. M12: 12 months. No TDR: no mutation of the World Health Organization 2009 list of mutations for surveillance of transmitted drug resistant HIV strains (WHO list). TDR and fully active cART: ≥ 1 mutation of the WHO list and susceptible (stanford level 1, 2) to all prescribed drugs. TDR and resistant: ≥1 mutation of the WHO list and resistant (stanford level 3, 4, 5) to ≥1 prescribed drugs. cART: combination antiretroviral therapy. * Numbers included in univariable analysis, **Univariable Kaplan Meier estimates, ***Univariable frequencies of virological failure, [§]Only Univariable Hazard rations and odds rations are presented.

6 Analysis of the impact of genotypic mutations on virological response in treatment experienced patients

We were interested in methods that are suitable for a high number of potentially correlated predictors that give mutations different weights for the prediction of virological outcome. Further, we were interested in easily interpretable methods.

First, we investigated the use of principal component analysis and partial least square (PLS) to predict virological outcome. Both methods determine linear combinations (principal components and PLS components) of the genotypic mutations that summarize best the variance structure of the mutations. The difference is that PLS takes the response variable into account to determine its components whereas principal components are based on the information given by the mutations only. Both, principal components and PLS components can then be used in regression models to assess their correlation with the virological outcome and their predictive abilities [42]. Second, we were also interested in Lasso since this method has showed a good performance for the prediction of phenotypic resistance and has also the advantage to be easy interpretable [46].

Our hypothesis was that the use of methods that attribute different weights to different mutations would result in a better prediction of the virological outcome compared to the construction of a genotypic score.

6.1 Alternative methods to analyse the impact of HIV genotypic mutations on virological response to antiretroviral therapy in treatment experienced patients

We hypothesized that a better consideration of mutations (e.g. different weights) related to virological failure may lead to a higher quality of the predictions of treatment outcome.

The objective was to compare strategies that analyse the impact of protease gene mutations on virological responses related to fosamprenavir/ritonavir treatment.

We used data from the Zephir study, a substudy of the ANRS CO3 Aquitaine Cohort [174]. The Zephir study was designed to study the impact of protease mutations on virological outcome in treatment experienced patients starting a fosamprenavir/ritonavir based cART.

For the following work we focussed on the use of principal component analysis, partial least square and compared their predictive ability to the construction of a genotypic score. This work was published in BMC Medical Research Methodology.

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Alternative methods to analyse the impact of HIV mutations on virological response to antiviral therapy

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Abstract

Background: Principal component analysis (PCA) and partial least square (PLS) regression may be useful to summarize the HIV genotypic information. Without pre-selection each mutation presented in at least one patient is considered with a different weight. We compared these two strategies with the construction of a usual genotypic score.

Methods: We used data from the ANRS-CO3 Aquitaine Cohort Zephir sub-study. We used a subset of 87 patients with a complete baseline genotype and plasma HIV-1 RNA available at baseline and at week 12. PCA and PLS components were determined with all mutations that had prevalences >0. For the genotypic score, mutations were selected in two steps: 1) p-value < 0.01 in univariable analysis and prevalences between 10% and 90% and 2) backwards selection procedure based on the Cochran-Armitage Test. The predictive performances were compared by means of the cross-validated area under the receiver operating curve (AUC).

Results: Virological failure was observed in 46 (53%) patients at week 12. Principal components and PLS components showed a good performance for the prediction of virological response in HIV infected patients. The cross-validated AUCs for the PCA, PLS and genotypic score were 0.880, 0.868 and 0.863, respectively. The strength of the effect of each mutation could be considered through PCA and PLS components. In contrast, each selected mutation contributes with the same weight for the calculation of the genotypic score. Furthermore, PCA and PLS regression helped to describe mutation clusters (e.g. 10, 46, 90).

Conclusion: In this dataset, PCA and PLS showed a good performance but their predictive ability was not clinically superior to that of the genotypic score.

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Background

The development of HIV resistance mutations is one of the major problems for optimizing treatment of HIV-infected patients. Therefore, resistance testing before starting highly active antiretroviral therapy (HAART) or before switching to a new antiretroviral component is widely recommended [1-4] and now routinely implemented in industrialised countries. Resistance is due to mutations in the viral genome, e.g. mutations in the reverse transcriptase (RT), protease or integrase genes that cause resistance to nucleoside RT inhibitors (NRTIs) and non-nucleoside RT Inhibitors (NNRTIs), protease inhibitors (PIs), or integrase inhibitors, respectively. Genotypic and phenotypic resistance testing are the two commonly used tests. The impact of genotypic mutations on virological response in patients treated with a particular drug regimen are based on *in vitro* informations or on the virological response reported in patients who switched to that particular regimen. Before the initiation of an optimized treatment, a genotype of the main (major) patients' virus populations (only virus species present at >20–30% are detected and therefore analysed) is assessed. Statistical analyses aim at finding the baseline genotypic mutations associated with virological response in order to predict whether a patient who will switch to a similar regimen is resistant or not. Noteworthy, data are mostly analysed for the main drug of a given regimen only, i.e. NNRTI and/or PI.

However, traditional statistical analyses of the association between genotypic mutations and virological response are hampered by i) the high number of potential mutations, ii) the correlations between mutations and iii) the low number of patients usually available for this type of study. Specifically, the analysis of the effect of high number of mutations measured in a limited number of patients may lead to over-fitting issues. Hence, inflated variances result in non-significant associations. In order to circumvent these problems and to simplify the interpretation, genotypic mutations are summarised in a so-called genotypic score. This score is the sum of observed resistance mutations at baseline for the given drug in a given patient. The mutations composing the score are selected by different strategies [5,6]. The drawbacks of this analysis are that a preselection of mutations is required and that every mutation has the same weighting. Alternative strategies such as principal component analysis (PCA) and partial least square (PLS) regression have been suggested for the sake of size reduction of correlated predictors [5,7-9] and may present advantages to improve the description of associations between mutations. The two techniques do not lead to a selection of mutations but to a different weighting of each mutation presented in the dataset. We aimed at comparing these two strategies with the usual construction of a genotypic score using data from an existing study evaluating the impact of protease mutations on the virological response in patients switching to a fosamprenavir/ritonavir-based HAART [10].

Methods

Data

The Zephir study was designed to investigate the impact of baseline protease genotypic mutations in HIV-1 infected PI-experienced patients on virological response. All patients had baseline HIV-1 RNA levels >1.7 $\log_{10}$ copies/mL and switched to a ritonavir-boosted fosamprenavir-based HAART [10]. Patients included were followed at the Bordeaux University hospital and at four other public hospitals in Aquitaine, south western France, all participating to the ANRS CO3 Aquitaine Cohort. We used a subset of 87 patients with a complete baseline genotype and plasma HIV-1 RNA available at baseline and at week 12. Virological failure was defined as a HIV-1 RNA ≥400 copies/mL and <1 $\log_{10}$ copies/mL decrease of HIV-1 RNA between baseline and week 12 (virological success: HIV-1 RNA <400 copies/mL or ≥1 $\log_{10}$ copies/mL reduction). A mutation was defined as a difference between the amino acid sequence of the studied virus and the wild type (HXB2) virus. In total, we created 69 dummy variables (69 mutations among the 99 possible protease mutations were encountered at least once).

Statistical analysis

Construction of a genotypic score

The genotypic score was created in two steps. The first step considered mutations with prevalences ≥10% and ≤90% [5] to assess their association with virological failure. Mutations associated with a p-value ≥ 0.01 (univariable logistic regression) were selected. Second, the backwards procedure selected the combination with the strongest association with virological response [6]. These m selected mutations were used to calculate the first genotypic score for each patient. For instance, a first set contains the six mutations V32, I47, I50, V77, I84 and L90. The score is defined as $S = I_{V32} + I_{I47} + I_{I50} + I_{V77} + I_{I84} + I_{L90}$ (S varying from 0 to 6). During the backwards selection procedure every mutation was removed one by one and all combinations of (m-1) mutations were investigated. The Cochran-Armitage test for linear trends in proportions was used to compare the probability of virological failure in patients having none to (m-1) mutations [11]. The combination providing the lowest p-value was kept and the procedure was repeated with all combinations of (m-2) mutations. The procedure stopped when removal of a mutation did not result in a lower p-value.

We performed 200 bootstrap samples from the original data set to analyze the variability in mutations' selection. We assumed that variability in the selection of mutations due to the restricted sample size might essentially play a

role in the first selection step. Therefore, a bootstrap analysis was performed only to the first selection criteria. In each sample the prevalence of each mutation was calculated. A univariable logistic regression was performed to determine the association of each mutation with virologic failure in each sample. Then we calculated the frequencies of selection of each mutation in the 200 bootstrap samples under the conditions mentioned above (prevalence between 10% and 90% and a p-value < 0.01 in univariable analysis).

Principal component analysis (PCA)

Each principal component is a linear combination of the original variables, with coefficients equal to the eigenvectors of the correlation or covariance matrix [7,9]. Principal components analysis determines components which are representing the variability of the mutations. The association between the principal components and the response variable was tested with the Wald test statistics of the estimated regression coefficient related to the principal components. We only tested principal components with an eigenvalue >2 reflecting that ≥3% of the variability of the mutations was explained. Any principal component was kept when it was related to the virological response using a logistic regression according to the Wald test.

Partial least square (PLS) regression

PLS regression is a technique widely used for dealing with numerous correlated explanatory variables [8,12]. PLS regression aims also at identifying components explaining as much as possible the variance of the predictor variables. These components are simultaneously correlated with the response variable. Over-fitting issues were controlled with a leave-one-out cross-validation during the construction process. The number of factors chosen is usually the one that minimizes the predicted residual sum of squares (PRESS) [13].

Comparison

The probability of virological failure at week 12 was studied using a logistic regression model adjusted for either the genotypic score or the principal components or the PLS components as explanatory variables. The performance of each strategy was compared using the cross-validated AUC [7,8]. We used 5-fold cross-validation. We split the dataset in five equal parts. That way we selected five times a dataset with 1/5 of the patients as 'validation set' and the remaining 4/5 of the patients served as 'test set'. In the test set, we determined i) the genotypic score ii) the principal components and iii) the PLS components. The selected mutations were then used to calculate the genotypic score for the patients included in the validation set. The weights for each mutation derived by PCA and PLS were applied to calculate the score of the principal component and the PLS component respectively for the patients of the validation set. For each validation set the AUC under the ROC curve was calculated by means of a logistic regression for the three different methods. Thus, we obtained for each method 5 AUCs and the cross-validated AUC was calculated as the mean of these 5 AUCs. This approach allows to avoid over-fitting because the performance of the methods is tested in a subset of patients that were not used to determine the genotypic score and the weights of mutations in the PCA and PLS components.

Statistical analyses were performed using SAS® version 9.1 software (SAS Institute, Inc., Cary, NC). We used the procedures PROC PRINCOMP for principal component analysis and PROC PLS for partial least square regression. Principal components and PLS components were determined considering all mutations being present in at least one patient.

Results

Study population characteristics have been reported before [10]. We used a subset of 87 patients with a complete baseline genotype and plasma HIV-1 RNA available at baseline and at week 12. Virological failure was observed in 46 (53%) patients at week 12. Mutations at codon 63 had the highest prevalence in this population 80% followed by mutations at codons 10 (58%), 71 (51%), 46 (47%), 54 (47%), 37 (47%), 35 (41%), 82 (40%) and 90 (40%). Mutations at codons 11, 12, 13, 14, 15, 19, 20, 32, 33, 34, 36, 41, 43, 47, 55, 57, 60, 61, 62, 64, 69, 72, 73, 77, 84, 89 and 93 had prevalences between 10% and 40%. Mutations at codons 10, 46, 54, 82 and 90 showed the highest association with virological failure in univariable analysis ($p < 10^{-5}$). All patients with virological failure presented a mutation at codon 84.

Genotypic score

Among mutations occurring in more than 10% and less than 90% of the patients, 27, 18 and 11 mutations were selected according to p-value thresholds of < 0.25, < 0.05 and < 0.01, respectively. The backward selection procedure using the Cochrane Armitage trend test was started with the 11 mutations (10, 33, 36, 46, 54, 62, 71, 73, 82, 84, 90) selected with the most restrictive criteria ($p < 0.01$) to avoid computational issues. The stability of this selection step was checked on 200 bootstrap samples. Seven (10:100%, 46: 100%, 54: 100%, 71: 95.5%, 82: 97%, 84: 100%, 90: 96%) of the 11 mutations were selected in over 90% of the samples. The other four mutations were selected between 50% and 90% (33: 88%, 36: 68%, 62: 50%, 73: 68.5%). Mutations not included in the IAS list [14] were in general not selected in the bootstrap samples (exceptions: 19: 36.5%, 37: 19% and 41: 19%). This additional bootstrap analysis confirmed that mutations known to be associated with virological failure were cho-

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sen for further steps. Mutations (also known as polymorphisms) that also occur occasionally in untreated patients, thus generally without any relation to antiretroviral treatment, were chosen in less than 3% of the bootstrap samples.

During the backward selection procedure the following six mutations 10, 36, 46, 62, 84, and 90 were selected for the calculation of a genotypic score. The genotypic score calculated with these six mutations was significantly associated with virological failure (OR = 4.1 for a difference of one mutation, $CI_{95\%}$ [2.4; 7.0]; $p < 10^{-4}$; cross-validated OR = 4.9).

Principal component analysis

The first and second principal components explained 11% and 6% of mutations variability. Principal components accounted for a small variability overall. Therefore, their interpretation was difficult. The correlation of the mutations amongst them and to the principal components allowed identifying some clusters as for example mutations 10, 46 and 90 or mutations 32 and 47 already known to be associated together (figure 1). Figure 2 represents the relative weight of each mutation in the dataset to calculate the first principal component. The relative weight of each mutation to calculate the PCA 'score' ranged between 0% (e.g. mutation at codon 22) and 4.3% (e.g. mutations at codons 10 and 54). The sum of the relative weights of mutations represented in the IAS list was 70%, meaning that mutations of the IAS list contributed the most to calculate the first principal component. The mutations at the following six positions 10, 33, 46, 54, 82 and 90 contributed mostly to the first component (figure 2). Among others, mutations at positions 77, 88 and 30 contributed with a negative scoring coefficient to the first component, meaning that the presence of such mutation would decrease the value of the score. Medians of the first and the second principal component were -0.10 (IQR: -0.5–0.84) and 0 (IQR: -0.53–0.40), respectively. The first principal component was significantly associated with virological failure with an OR of 11.9 ($CI_{95\%}$ [4.8; 29.7], $p < 10^{-4}$) for a difference of one unit whereas the second was not OR = 1.1 ($CI_{95\%}$ 0.7; 1.7, p = 0.62).

Partial least Square

One PLS component was chosen according to the PRESS criterion. This component explained 11% of the variability of the mutations and 60% of the variability of the response variable. The median of the first PLS component was -0.17 (IQR: -2.69–2.64). This PLS component was significantly associated with virological failure OR = 2.6 ($CI_{95\%}$1.8; 3.9 $p < 10^{-4}$). Figure 3 represents the relative weight of each mutation in the dataset to calculate the first PLS component. Mutations at positions 10, 46, 54, 82, 84, and 90 had the highest contribution to the calculation of the first component (figure 3). Negative weight for the calculation of the first PLS component was amongst others given by mutations 77, 30 and 48. Mutation at codon 69 contributed with the smallest relative weight (0.03%) and mutation at codon 10 with the highest (4.7%). The contribution of mutations included into the IAS list was 69% (i.e. the sum of relative weights). Thus, mutations already known to be associated with virological failure were given more weight than polymorphisms (mutations that also occur occasionally generally without association to antiretroviral treatment).

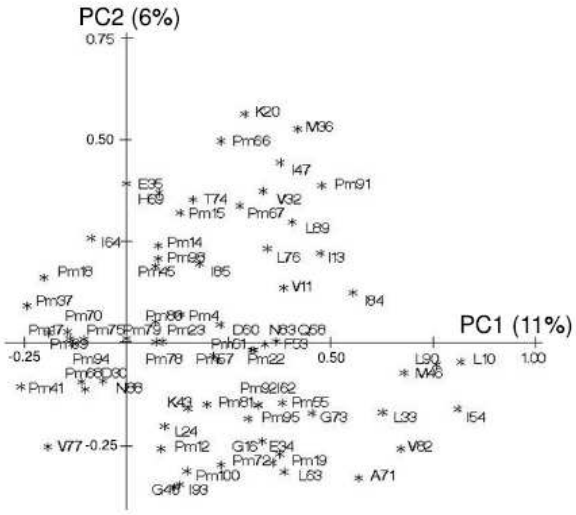

Figure 1

Mutations on the first and second principal components. All mutations having prevalences different from 0 are depicted. The wild type amino acid is cited before the codon of the mutation. Interpretation: PC1: First principal component (representing 11% of the variability), PC2: Second principal component (representing 6% of the variability). Mutations are represented by the component when they are close to the corresponding axis. When two mutations are far from the center, then, if they are: i) Close to each other, they are significantly positively correlated; ii) If they are in a rectangular position, they are not correlated; iii) If they are on the opposite side of the center, then they are negatively correlated. When the mutations are close to the center, it means that some information is carried on other axes.

Comparison

We compared the results of the PCA and PLS with the results obtained using the classical strategy to build a genotypic score. Mutations 10, 46 and 90 were found among the six mutations contributing with the highest weight for the calculation of the first PC, the first PLS component and were selected for the genotypic score. Major mutations 54 and 82, which were found among the mutations

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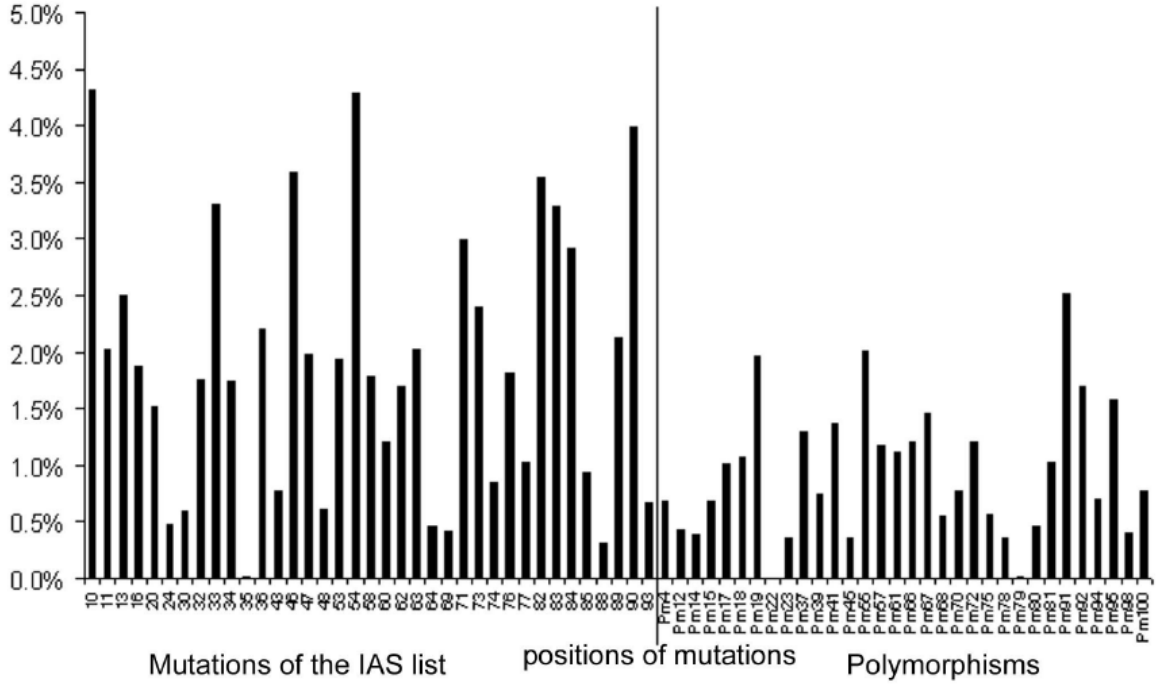

Figure 2
Relative weights of each mutation to calculate the 'score' of the first principal component. Black line: separation of mutations represented in the IAS list [14] and polymorphisms.

with the highest association to virological failure in univariable analysis, were also found among the six mutations contributing with the highest weight for the calculation of the first PC and the first PLS component. In contrast, these two mutations were eliminated from the score during the backward selection procedure (figure 4). Therefore, one first advantage of methods based on PCA and PLS is that they helped in reducing the number of predictors without neglecting mutations that could play a significant role.

We compared the performance of these three methods with the area under the ROC curve. The cross-validated AUCs for the PCA, PLS and genotypic score were 0.880, 0.868 and 0.863, respectively. The model with the first principal component slightly outperformed the model with one PLS component. The predictive quality of the genotypic score was slightly lower than the two AUCs obtained for PCA and PLS but still showed a very good performance.

To compare the methods in an illustrative way we used a patient presenting the following 21 protease gene mutations at baseline: mutations at positions 33, 54, 82, 90 defined as major, mutations at positions 10, 13, 20, 35, 36 43, 53, 60, 63, 64, 74 defined as minor and mutations at positions 14, 15, 19, 37, 67, 98 defined as polymorphisms. Virological failure was observed for this patient. The genotypic score was $S = I_{10}+I_{36}+I_{90} = 3$ and the probability of virological failure was 77% using this score. The main difference between the genotypic score and the principal component value or the PLS component value is that with the latter methods we can take in consideration the fact that the patient has 21 protease gene mutations and give them different weights. For instance, the relative weights for mutations 10, 36, 90 were 4.4%, 2.2%, 4.1% and 4.7%, 2.4%, 4.4% for the PCA and PLS 'score', respectively (figure 2 and 3). The predicted probability of virological failure was 94% and 96% using the PC "score" and the PLS "score", respectively.

Discussion

We investigated PCA and PLS regression to analyse associations between baseline protease mutations and virological failure. PCA and PLS are easily applicable because they are implemented in standard statistical analyses programs such as SAS (SAS Institute, Inc., Cary, NC).

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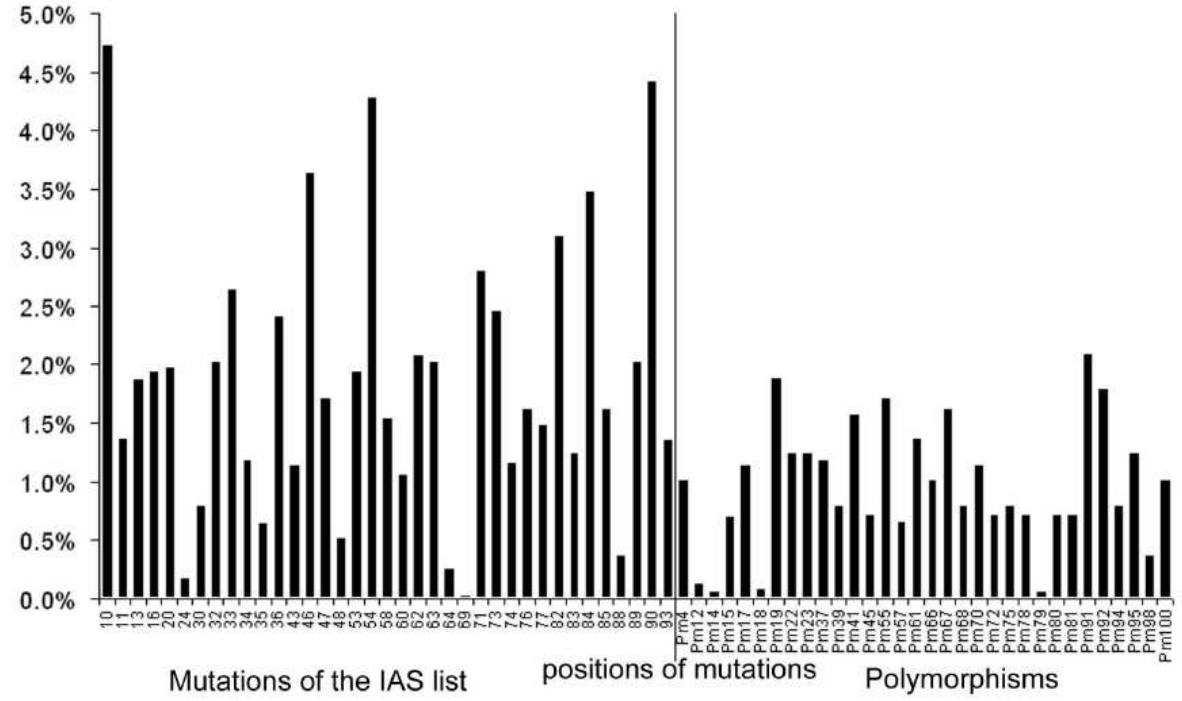

Figure 3
Relative weights of each mutation to calculate the 'score' of the first PLS component. Black line: separation of mutations represented in the IAS list [14] and polymorphisms.

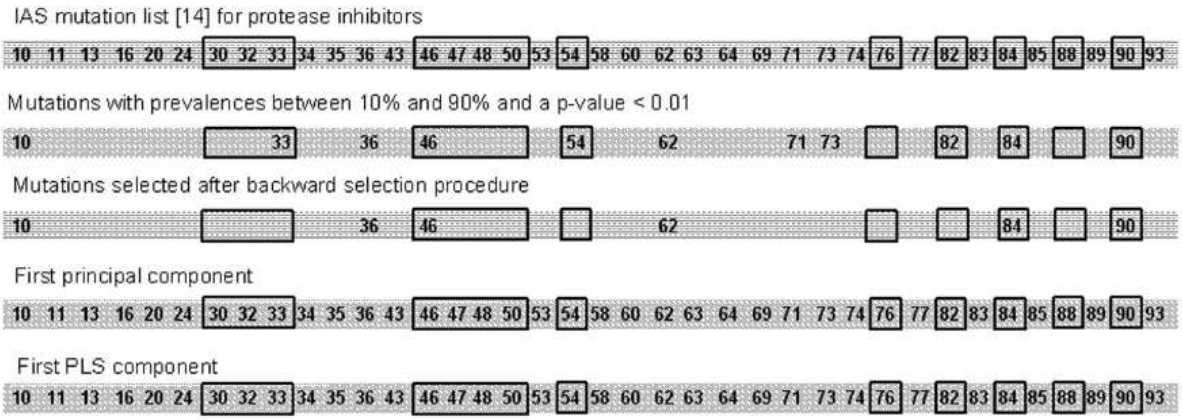

Figure 4
Codons of mutations taken into consideration by the presented methods to predict virological failure(Codons at which polymorphisms occur are not depicted). The IAS mutation list shows all codons which have been described to be related with resistance to any of the protease inhibitors. Black boxes: Codons where major mutations occur.

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We compared these two techniques with the construction of a genotypic score because they allow considering each mutation with a different weight. The objective of PCA is to find a set of new "latent variables" in form of a linear transformation of the original predictors. The properties of these latent variables are that they are uncorrelated and that they account for as much of the variance of the predictor variables as possible. PCA has been recently used to determine clusters of mutations in patients that were treated with at least one PI [15] and to predict the phenotypic fold change from genotypic information [16]. PLS regression reduces also a set of predictor variables to a set of uncorrelated "latent variables", the so-called PLS components. The main difference between the two techniques is that PLS also considers the strength of each mutation effect on the virological response to construct the components. Hence, these two methods can help solving the issues of the high number of predictors and their different effects. They may also help in describing the relationship between mutations by detecting potential groups of mutations. PLS was mentioned to be a useful analysing strategy for genotypic mutation data [5] but neither applications nor comparisons had been published yet.

In this study population, these two methods were able to identify some mutations that were expected to contribute with higher weights to virologic failure (e.g. mutations at codons 10, 82 and 90 which contribute to resistance to at least 7 of the 8 currently used PIs [5]). Furthermore, known clusters of mutations could be described. Recent papers including co-variation analysis [15,17-19] found some correlated pairs and clusters which are associated with a specific treatment. Two of them used PCA to visualise correlations of mutations. We identified some clusters of mutations, e.g. mutations at codons 10, 46, and 90 and at codons 33, 46, 54 and 82, which were also found to be correlated with each other. Mutations 32 and 47 had the highest correlation coefficient (r = 0.78) in this population and are known to be key mutations for amprenavir [20] and lopinavir [14]. The cluster of mutations at positions 10, 46, 90 [19] and a high correlation between 32 and 47 were also determined by Wu et al and Kagan et al [19,21]. The mutations 10, 33, 46, 54, 71, 82, 84 and 90 are separated from all other mutations by the PCA and are contributing with the highest weight to calculate this component. The cluster 10, 46, 54, 71, 90 was recently described [17] to appear under lopinavir treatment and these mutations are also related to amprenavir-resistance [22]. We found that PCA had indeed detected this latter cluster in our patient's population previously treated by lopinavir or amprenavir (25% and 32% of the patients, respectively). Furthermore, the fact that the principal component was related to virological response highlights that PCA can detect mutation clusters on the way to lopinavir and fosamprenavir resistance although principal component analysis did not consider the virologic response for the construction of the component. As mentioned above, PLS searches latent variables but takes into account the response variable. Consequently one might expect differences for the distribution of the weights given by the mutations. Actually, the mutations found to contribute the highest weight on the PLS component are almost the same. Among the six mutations contributing with the highest weight, mutations at codons 10, 46, 54 82 and 90 were found for the principal component and the PLS component. Mutation at codon 33 was found on the principal component, while mutation 84 was found on the PLS component. In addition, the mutations which contributed with a higher weight for the calculation of the first principal and first PLS components are those which showed the highest association with virological response in univariable analysis. In conclusion, the weightings of the mutations found were consistent across these alternative strategies. A possible explanation is that the patients were mainly pre-treated with two PIs known to induce similar mutation patterns than fosamprenavir. In other cases, PLS might outperform PCA when a drug induces completely different mutations since the virological response is considered during the construction of the component.

The above presented example (patient presenting 21 protease gene mutations) highlights the advantage of taking into account all mutations and giving them different weights by either PCA or PLS. This results in a better prediction of virological failure. After cross-validation the first principal component and the first PLS component only slightly outperformed the genotypic score in the prediction ability. However, it has to be stated that the cross-validated AUCs showed no clinical relevant difference. In this study population this might partly be explained by the fact that there was an explicit subset of mutations strongly associated with virological failure. This was also substantiated by the bootstrap analyses in which four of the six mutations remaining in the final genotypic score had been selected in over 95% of the bootstrap samples. This clear separation between mutations associated with virological failure from those which are not, could have facilitated the detection of a predictive subset using the classical strategy to construct a genotypic score.

One of the reasons to apply PCA and PLS analyses to these kind of data was that these approaches do not need a preselection of variables (i.e. mutations) as they are summarized in predictors. Hence, all mutations can be considered even when they are present in a small proportion of patients. Among others, the attempt to study these approaches was to study whether considering all mutations has an advantage and if mutations known to be associated with virologic failure are given higher weights.

However, the slightly better performance of the alternative approaches may be simply linked with the use of a larger amount of information. This was the minimum expected gain of these approaches compared to the usual one.

Therefore, it would be very helpful to study the performance of PCA and PLS in other, potentially bigger, trials considering other antiretroviral regimen/patients.

Conclusion

PCA and PLS regression were helpful in describing the association between mutations and to detect mutation clusters. PCA and PLS showed a good performance but their predictive ability was not clinically superior to that of the genotypic score.

Appendix

Aquitaine Cohort composition

Scientific Committee: J. Beylot, M. Dupon, M. Longy-Boursier, J.L. Pellegrin, J.M. Ragnaud and R. Salamon (Chair).

Scientific Coordination: M. Bruyand, G. Chêne, F. Dabis (Coordinator), S. Lawson-Ayayi, C. Lewden, R. Thiébaut.

Medical Coordination: N. Bernard, M. Dupon, D. Lacoste, D. Malvy JF. Moreau, P. Mercié, P. Morlat, D. Neau, JL. Pellegrin, and JM. Ragnaud.

Data Management and Statistical Analysis: E. Balestre, L. Dequae-Merchadou, V. Lavignolle-Aurillac.

Technical Team: MJ. Blaizeau, M. Decoin, S. Delveaux, D. Dutoit, C. Hanappier, L. Houinou, S. Labarrère, G. Palmer, D. Touchard, and B. Uwamaliya.

Participating Hospital Departments (participating physicians): Bordeaux University Hospitals: J. Beylot (N. Bernard, M. Bonarek, F. Bonnet, D. Lacoste, P. Morlat, and R. Vatan), P. Couzigou, H. Fleury (ME. Lafon, B. Masquelier, and I. Pellegrin), M. Dupon (H. Dutronc, F. Bocquentin, and S. Lafarie), J. L. Pellegrin (O. Caubet, E. Lazaro C. Nouts, and J. F. Viallard), M. Longy-Boursier (D. Malvy, P. Mercié, T. Pistonne and C. Receveur), J. F. Moreau (P. Blanco), J. M. Ragnaud (C. Cazorla, D. Chambon, C. De La Taille, D. Neau, and A. Ochoa); Dax Hospital: P. Loste (L. Caunègre); Bayonne Hospital: F. Bonnal (S. Farbos, and M. C. Gemain); Libourne Hospital: J. Ceccaldi (S. Tchamgoué); Mont-de-Marsan Hospital: S. de Witte.

Abbreviations

ANRS: Agence Nationale de Recherche sur le SIDA; AUC: Area under the receiver operating characteristics curve; CI: Confidence interval; HAART: Highly active antiretroviral therapy; HIV: Human immunodeficiency virus; IAS: International AIDS society; IQR: Interquartiles range; NNRTI: Non-nucleoside reverse transcriptase inhibitor; NRTI: Nucleoside reverse transcriptase inhibitor; OR: Odds ratio; PC: Principal component; PCA: Principal component analysis; PLS: Partial least square; PRESS: Predicted residual sum of squares; RT: Reverse transcriptase.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LW carried out the statistical analysis and drafted the manuscript. RT and DC participated in the statistical analysis and helped to draft the manuscript. IP, DB, DN, DL, JLP, GC and FD performed the clinical trial and helped to draft the manuscript. All authors read and approved the final manuscript.

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6.2 Perspectives

6.2.1 Collaboration with the Forum for Collaborative HIV Research

Founded in 1997, The Forum for Collaborative HIV Research is a public/private partnership at the University of California, Berkeley Washington Campus. The Forum's mission is to enhance and facilitate HIV research and this is accomplished by bringing together all relevant stakeholders (i.e. government, industry, patient advocates, health care providers, academia and foundations) to address emerging issues in HIV/AIDS [215].

We started a collaboration with the Forum for Collaborative HIV Research and in particular with the drug resistance working group (Standardization and Clinical Relevance of HIV Drug Resistance Testing Project). Beside the comparison of existing genotypic interpretation algorithms and external validation of existing rules [216, 217] the drug resistance working group is also interested in comparison of quantitative methods for the analysis of genotypic resistance data [194, 195, 218]. We obtained a dataset containing observations of treatment experienced patients who started abacavir plus additional antiretroviral drugs having a genotype at baseline [217].

6.2.2 Application of Lasso, PCA and PLS to data from the Forum for Collaborative HIV Research

The objective of this ongoing work is to compare principal component analysis (PCA), partial least square (PLS) and Lasso with an existing genotypic interpretation system (ANRS) to analyse the impact of HIV reverse transcriptase (RT) mutations on virological response.

6.2.2.1 Methods

In preliminary analyses we used a subset of 574 patients initiating an abacavir-based regimen (baseline) not including protease inhibitors. We calculated the genotypic score for abacavir using the ANRS algorithm (version 17, July 2008). Virological success was defined as 1) a viral load ≤400 cps/mL at week 8 or 2) a VL ≤400 cps/mL at week 8 or at least 1 $\log_{10}$ reduction between baseline and week 8. We selected known RT mutations (IAS USA list December 2008 and those listed by Shafer *et al.* [15]) to determine the first principal component and the first PLS component. A logistic regression model was used to determine the performance of the first PC and PLS component. For Lasso we used the same known RT mutations using the Akaike Information Criterion (AIC) to select the model. We used 5-fold

cross-validation to assess the area under the receiver operating curve (AUC of ROC) for PCA, PLS and Lasso. Results presented are unadjusted for covariates.

6.2.2.2 Results

Median number (IQR) of drugs in regimen (including abacavir) were 2 (1; 3). Median CD4 cell count and viral load at baseline were 271 (152; 428)/mm^3 and 4.3 (3.7; 5.0) $\log_{10}$ cp/mL, respectively. Of 574 patients, 272 (47%) had a viral load ≤400 cps/mL at week 8 and 369 (64%) had a viral load ≤400 cps/mL at week 8 or at least 1 $\log_{10}$ reduction between baseline and week 8.

AUCs were overall fairly low but AUCs calculated using PCA, PLS and Lasso suggested a better predictive performance compared to the ANRS abacavir score (see Table 9). Further investigation of these methods are ongoing.

Table 9: Cross-validated area under the receiver operator curve (AUC (standard deviation))

Definition of virological response	Method applied			
	ANRS	**PCA**	**PLS**	**Lasso**
viral load ≤400 cps/mL at week 8	0.56 (0.03)	0.65 (0.04)	0.67 (0.05)	0.64 (0.04)
viral load ≤400 cps/mL at week 8 or at least 1 $\log_{10}$ reduction between baseline and week 8	0.60 (0.05)	0.67 (0.02)	0.67 (0.02)	0.65 (0.03)

6.2.3 Ongoing - Lasso and left censuring

Continuous viral load change from baseline give the greatest amount of information of the effect of drug resistance mutations on treatment efficacy and methods taking left censoring into account should be considered for their evaluation [36].

We are currently working on the adaptation of Lasso for left-censored data.

6.2.3.1 Definition of response

We can define the virological response as the difference between a follow-up viral load (VL) at time t (e.g. at 8 weeks) and the viral load at baseline (t_0).

$Y = VL(t) - VL(t_0)$

The viral load at time t is either observed or censored due to the detection limit. All baseline viral loads are assumed to be detectable.

6.2.3.2 The Model

We consider a usual regression situation. We have data (x_i, y_i), $i = 1 \ldots n$, where $x_i = (x_{i1} \ldots x_{ik})$ and y_i are the k predictors and response for the i^{th} observation. The model parameter for the i^{th}

observation represent a vector $b = (b_1 \ldots b_k)^T$. The model for the i^{th} observation can be written as follows:

$$y_i = x_i b + e_i \quad (1)$$

We assume that e_i is independently normally distributed $e_i \sim N(0, \sigma^2)$.

6.2.3.3 The Likelihood

Let y_i^o be the difference between an observed viral load at t and a baseline viral load and y_i^c be the difference between a censored viral load at t and a baseline viral load.

According to the model (1) y_i has a Gaussian probability density function. Then, the contribution o the likelihood for a patient with observed viral loads takes the form:

$$L_i = \frac{1}{\sqrt{2\pi\sigma^2}} e^{-\frac{1}{2}\frac{\left(y_i^o - x_i b\right)^2}{\sigma^2}} \qquad i = 1 \ldots n_i^o \quad (2)$$

The contribution to the likelihood for a censored observation y_i^c can be written as:

$$L_i = P(y_i^c < c) = \int_{-\infty}^{c} \frac{1}{\sqrt{2\pi\sigma^2}} e^{-\frac{1}{2}\left(\frac{y_i^c - x_i b}{\sigma}\right)^2} dy_i^c \qquad i = 1 \ldots n_i^c \quad (3)$$

Thus, the log-likelihood has the following form:

$$LL = \sum_{i=1}^{n_i^o} \left[-\frac{1}{2}\ln(2\pi) - \frac{1}{2}\ln\sigma^2 - \frac{1}{2\sigma^2}(y_i^o - x_i b)^2 \right] + \sum_{i=1}^{n_i^c} \ln\left[\int_{-\infty}^{c} \frac{1}{\sqrt{2\pi\sigma^2}} e^{-\frac{1}{2}\left(\frac{y_i^c - x_i b}{\sigma}\right)^2} dy^c \right] \quad (4)$$

6.2.3.4 Estimation and Computation

The estimation of the parameters is realised by the maximization of the log-lihelihood penalized by the norm 1 (L_1) of b and by a tuning parameter determined by cross-validation.

$$\arg\max_b \left(LL - \lambda \|b\|_1 \right) \quad (5)$$

There exist different algorithms to find a solution for (5) [207, 219]. The R package "penalized" is a package for fitting possibly high dimensional penalized regression models developed by Jelle J. Goeman [220]. The penalty structure can be any combination of a L_1 penalty (lasso), a L2 penalty (ridge) and a positivity constraint on the regression coefficients. The package supports linear regression models, logistic and poisson regression and the Cox Proportional Hazards model. The algorithm used in the penalized package is based on a novel algorithm that efficiently computes L_1 penalized (lasso) estimates of parameters in high-dimensional models [221]. In collaboration with Marta Avalos, Pierre Seiter and Daniel Commenges in our team we modified this package in order to take left censoring into account. Currently, we work on the evaluation of this approach.

7 Conclusion

Knowing the impact of genotypic resistance mutations on treatment outcome is important both in treatment naïve and treatment experienced patients. However, the statistical analysis of the impact of genotypic mutations on treatment outcome is hampered by i) the high number of possible mutations, ii) the potential collinearity between mutations and iii) the low number of patients. Further, the definition of an endpoint is not straightforward and may depend on the study population (naïve or treatment experienced), the purpose of the study (e.g. licencing a drug) and the scientific question to be answered. We were thus interested in epidemiological and methodological questions regarding the analysis of genotypic resistance mutations.

The analysis of the impact of transmitted drug resistance on first line antiretroviral treatment is an important epidemiological question as the choice of the initial treatment is crucial and may further limit future treatment options. Several existing studies are controversial and may suffer from limited power [19, 26, 28-31, 141, 142]. We investigated the impact of transmitted drug resistance on virological and immunological outcome in patients starting at least three antiretroviral drugs in a large-scale European collaboration. To our knowledge, this is the most ample evaluation of the clinical impact of TDR to date. We particularily focused on i) patients starting 2 NRTIs + 1 NNRTI or 2 NRTIs + 1 PI/ritonavir and ii) patients receiving treatment predicted to be fully active. We found strong evidence that the selection of an initial regimen should be based on resistance testing in treatment naïve patients as recommended in recent treatment guidelines [2, 3, 13]. This is the first study showing that the prescription of a drug classified even as encountering low-level resistance is associated with a significantly higher risk for virological failure. This finding underlines the need of ≥3 fully active antiretroviral drugs in order to optimize the virological response to first-line regimen. In the presence of transmitted drug resistance and when an active regimen was used, we found a potential higher risk of virological failure compared to patients with no transmitted drug resistance mutations if a combination of 2 NRTIs + 1 NNRTI was used, though not if a boosted PI + 2 NRTIs were prescribed. The findings for patients receiving 2 NRTIs + 1 NNRTI could be partly explained by the presence of minority NNRTI resistant strains. These results support previous findings that the presence of minority NNRTI resistance mutations can be related to virological failure if patients start a NNRTI based regimen [222-226].

In conclusion, genotypic testing in treatment naive patients in regions with medium to high prevalence transmitted drug resistance is important to select a fully active regimen for

treatment initiation. In regions where genotypic testing is not routinely available but high prevalence of transmitted drug resistance is suspected, first line regimens containing a ritonavir boosted PI should be considered.

The definition of an endpoint for the study presented above was not straightforward. We opted for a time-to-event analysis but as discussed these implicates methodological issues. Therefore, we realised several sensitivity analyses in order to account among other aspects for patients switching treatment. The evaluation of viral load evolution using measurements taken before any treatment stop/switch only would be a powerful alternative. Preliminary results using piecewise linear random effect models in order to analyse the viral load decay gave comparable results (data not show) and might be a helpful tool to evaluate virological efficacy of a treatment.

As the definition of an endpoint is variable in different contexts and may implicate various methodological issues we summarized endpoints 1) used in recent clinical trials and 2) used in studies to evaluate the impact of genotypic resistance mutations to predict virological outcome.

We reviewed recent clinical trials in antiretroviral naive and treatment experienced patients to give an overview of the endpoints and to discuss the resulting methodological problems. Composite endpoints were the most used endpoints in recent HIV clinical trials. The change of endpoint definition over time in the HIV field is amongst others due to the amelioration of antiretroviral treatment efficacy. The use of clinical endpoints, e.g. disease progression or death may now be unfeasible as these endpoints became rare and very large long-term trials to observe these endpoints would be required. More easily observed laboratory measurements, such as HIV viral load can be used as biomarkers for treatment efficacy. However, good virological response may not be a sufficient definition in the context of pragmatic trials where toxicity, quality of life or preservation of future drug options are also of interest. We observed considerable differences in what made up these endpoints, and reporting would benefit from standardization. All components of the endpoint should be reported to allow a better understanding of eventual differences between compared groups [169, 227].

'Pure' virological endpoints were rarely used in clinical trials but are of interest for the evaluation of genotypic drug resistance and their impact of treatment outcome [36]. Further, a quantitative measurement may better reflect the direct impact of drug resistance mutation on the replicative capacity of the virus. However, the definition of a quantitative outcome is hampered by the detection limit of current viral load assays. Methods allowing the

consideration of left-censoring for the evaluation of viral load evolution or the first viral load decay have been proposed [52-54] and are less biased then using simple imputation based on the detection limit.

We compared principal component analysis and partial least square with the construction of a genotypic score as described by Flandre *et al.* and Brun-Vézinet *et al.* [36, 37] in order to predict the virological outcome using genotypic resistance data in treatment experienced patients. We compared these two techniques with the construction of a genotypic score because they allow considering each mutation with a different weight. Principal component analysis and partial least square showed a good performance but had only a slightly better predictive capacity than the genotypic score. However, both methods provided a helpful tool in describing the association between mutations and to detect mutation clusters. The latter finding is in accordance with other studies, which used similar techniques to detect co-variation and cluster of protease and reverse transcriptase mutations [45, 228]. However, many other techniques to analyse genotypic mutations have been described and were applied either to predict phenotypic drug resistance or virological outcome. Some of these methods have been integrated in data-driven prediction engines [38, 39, 121].

Lasso was identified as one of the best performing methods to predict phenotypic drug resistance [46] and has not yet been used to predict virological outcome. Further, preliminary investigations using different endpoint definitions to construct a genotypic score but also using different statistical methods showed that prediction accuracy but also the selection/weighting of mutations varied between outcome definitions (data not shown). This led to the idea of adapting Lasso for left-censored data in order to evaluate this method using a quantitative virological outcome.
The comparison of different methods using different virological criteria could be another perspective of this work. Further, the use of PCA, PLS and Lasso are also of interest for other studies with high-dimensional data, for example in the context of “omics” data.

In total, our work is relevant for clinical care of HIV-1 infected patients starting their first antiretroviral therapy and may also be relevant to improve adequate design and analysis of future drug resistance studies.

Abbreviations

AIDS	Acquired immune deficiency syndrome
ART	Antiretroviral therapy
AUC	Area under the receiver operator curve
cART	combination antiretroviral therapy
CART	classification and regression trees
CD4	T lymphocytes CD4 receptor positive
CI	confidence interval
DNA	desoxyribonucleic acid
env	envelope
e.g.	exempli gratia
FDA	Food and drug administration
gag	group antigen
gp	glycoprotein
GSS	genotypic sensitivity score
HAART	highly active antiretroviral therapy
HIV	Human immunedeficiency virus
HCV	Hepatitis C virus
HR	Hazards ratio
IAS	International AIDS society
i.e.	id est
INSTI	Integrase Strand transfer inhibitor
IN	Integrase
LARS	Least angle regression
Lasso	Least absolute shrinkage and selection operator
LTR	long terminal repeat
NRTI	Nucleoside and nucleotide reverse transcriptase inhibitor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
OR	Odds ratio
PCA	Principal component analysis
PC	principal component
PI	Protease inhibitor
PI/rtv	ritonavir boosted protease inhibitor
PLS	Partial least square
Pm	Polymorphism
pol	polymerase
PR	protease
RT	reverse transcriptase
RNA	ribonucleic acid
SVM	support vector machines
TAM	Thymidin analog mutations
TDR	transmitted drug resistance
VL	viral load
WHO	World Health Organization

Glossary

Amino acid Amino acids are the structural units that make up proteins. One letter code amino acid abbreviations of the 20 amino acid directly encoded by the genetic code: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine, G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

Base pair Two nucleotides on opposite complementary DNA or RNA strands are a so-called base pair.

CASCADE **C**oncerted **A**ction on **S**ero**C**onversion to **A**IDS and **D**eath in **E**urope.
It is currently a network of epidemiologists, statisticians, virologists and clinicians from lead HIV institutions in 15 European countries, Australia and Canada. Seroconverters are enrolled into the individual cohorts locally and nationally and are typically followed up life-long. CASCADE's main aim is to monitor newly infected individuals and those already enrolled in studies, covering the entire duration of HIV infection.

CHAIN Collaborative HIV and Anti-HIV Drug Resistance Network.
CHAIN is a large scale integrating project aimet to effectively and durably combat new and existing anti-HIV drug resistance in clinical settings, with a special emphasis on Eastern Europe and in heavily affected resource-poor regions in Africa.

CHAIN received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement n° 223131.

Codon Three nucleotides that encode one amino acid.

COHERE **C**ollaboration of **O**bservational **H**IV **E**pidemiological **R**esearch **E**urope.
COHERE is a structure that was organised in 2005 and is a collaboration of 33 cohorts from 30 European countries. The mission of COHERE is: To conduct epidemiological research on the prognosis and outcome of HIV-infected people from across Europe including pregnant mothers, children, and adults. The research will focus on scientific questions requiring a large sample size of patients which the contributing cohorts cannot answer individually and which do not overlap with existing collaborations between participating COHERE cohorts.

Cross-validation A method to assess prediction accuracy. The dataset is devided into equal-sized parts, for example five. A model is assessed using four parts of the data and is then tested on the 5^{th} part (e.g. the prediction accuracy is tested). The procedure is repeated 5 times so that each part served as test-set.

DNA Deoxyribonucleic acid is a chain of nucleotides, i.e. adenine: A, cytosine: C, guanine: G and thymine: T. DNA contains the genetic intstructions and one of the main roles is the long term storage of information.

EuroCOORD The collaborative HIV cohorts CASCADE, COHERE, EuroSIDA and PENTA-EPPICC are the four founding networks of EuroCoord (The European Coordinating Committee for the Integration of Ongoing Coordination Actions Related to Clinical and Epidemiological HIV Research).

EuroSIDA The EuroSIDA study is a prospective observational cohort study of more than 16.505 patients followed in 103 hospitals in 32 European countries plus Israel and Argentina. The main objective of the study is to assess the impact of antiretroviral drugs on the outcome of the general population of HIV-infected patients living in Europe.

Forum of Collaborative HIV Research Founded in 1997, The Forum for Collaborative HIV Research is a public/private partnership at the University of California, Berkeley Washington Campus. The Forum's mission is to enhance and facilitate HIV research and this is accomplished by bringing together all relevant stakeholders (i.e. government, industry, patient advocates, health care providers, academia and foundations) to address emerging issues in HIV/AIDS.

Genetic barrier The number of mutations required to overcome drug-selective pressure.

Genetic code The genetic code defines the mapping between codons and amino acids. The genetic code has redundancy. For example, codons AAA and AAG both specify lysine (redundancy) but neither of them specifies any other amino acid (no ambiguity).

HXB2 The viral strain HXB2 is a commonly used laboratory HIV-1 wild-type virus (GenBank Accession Number K03455). The HXB2 sequence is used as the reference sequence to determine changes in the genome of the HIV-1 virus.

Logit

The logit transformation is defined

$$\text{logit}\,(P(y=1)) = \ln\left(\frac{P(y=1)}{1-P(y=1)}\right)$$

Mutation

Change in the genetic code. In this report mutations refers to a change in an amino acid. Silent mutations, i.e. mutations in the nucleotide sequence without leading to change in the amino acid are not considered.

NEAT

Eurpean AIDS Treatment Network. NEAT's mission is to strengthen European HIV clinical research capacity by building a clinical and laboratory network in HIV therapeutics. NEAT will catalyse a critical mass of resources and expertise, with the direct involvement of 41 core partners from 16 countries and over 350 affiliated centres of established international reputation. NEAT is committed to designing the roadmap for a durable reshaping of the way clinical research is carried out in Europe so as to achieve a progressive and lasting integration. In doing so, NEAT will pave the way toward the creation of the European Research Area. The Network will also spread expertise and resources provide training and mobility of scientists at all levels and will foster lasting collaborations across Europe.

PENTA

Paediatric **E**uropean **N**etwork for **T**reatment of **A**IDS. Penta was established in 1991 as a collaboration between paediatric HIV centres in Europe.

PCR

Polymerase Chain reaction.
PCR is a technique used in molecular biology to amplify a piece of DNA.

Polymorphism

Polymorphisms in the HIV genome occurs at several codons even without drug selective pressure and occur commonly in untreated patients as naturally occurring variants.

Protein

Amino acid sequence. Structure and function of proteins are various. For example, the protease of HIV is a protein which catalyzes cleavage of other viral proteins, i.e. the protease is an enzyme. The proteins forming the envelope or the nucleopasid of HIV are structural proteins.

Replicative capacity

The ability of the virus to reproduce itself. Viral fitness is a synonym.

RNA Ribonucleic acid is a chain of nucleotides that differ slightly from that used to form a DNA molecule, i.e. adenine: A, cytosine: C, guanine: G and uracil: U. Uracil is used at the place of thymidine. Many different RNA molecules exist. One example is the messenger RNA (mRNA) that carries information about a protein sequence. Further, RNA can carry genetic information (as DNA) and RNA viruses have genomes composed of RNA such as HIV.

Transcription In biology, transcription refers to the creation of an equivalent RNA copy from a DNA.

Translation In biology, transcription refers to the step of protein synthesis. The nucleotide sequence of a messenger RNA is translated into an amino acid seuquence forming a protein.

Silent mutation A change in the nucleotide seuquence that does not change the amino acid seuquence. Silent mutations can occur due to the redundancy of the genetic code. For example, a change of the codon AAA to AAG does not change the amino acid sequence, i.e. bot codons encode lysine.

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Annexe

List of publications and communications not linked to the thesis

Articles

Wittkop L, Grün-Bernhard S, Schwarz A, Delaleau M, Rabe B, Cazenave C, Gerlich W, Glebe G, Kann M. Proteinkinase C Activity in capsids of Hepadnaviruses decreases capsid stability and enhances virus secretion. *Cell Microbiol.* 2010 Jul;12(7):962-75.

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Pellegrin I, Wittkop L, Joubert LM, Neau D, Bollens D, Bonarek M, Girard PM, Fleury H, Winters B, Saux MC, Pellegrin JL, Thiébaut R, Breilh D. Virological response to darunavir/ritonavir-based regimens in antiretroviral-experienced patients (PREDIZISTA study). *Antivir Ther* 2008,13:271-279**. (co-first author)**

Communications

Oral presentations

V Svicher, V Cento, R Salpini, F Mercurio, M Fraune, B Beggel, Y Han, C Gori, L Wittkop, A Bertoli, V Micheli, G Gubertini, R Longo, S Romano, M Visca, V Gallinaro, N Marino, F Mazzotta, GM De Sanctis, P Trimoulet, M Angelico, XX Zhang, J Verheyen, F Ceccherini-Silberstein, CF Perno. The HBV genetic barrier and the overlapping structure of HBV genome synergistically modulate drug resistance emergence and immune escape potential of HBV genotypes. Abstracts of the XVIII International HIV & Hepatitis Drug Resistance Workshop & curative strategies, June 08-12, 2009, Dubrovnik, Croatia. *Antivir Ther* 2010, 14 Suppl 2: A23

Posters

L Wittkop, A Cassany, M Delaleau, P Trimoulet, J Foucher, R Thiébaut, H Fleury, M Kann Impact of Genotypic Mutations of the Hepatitis B Virus Capsid on Viral Load and Liver Enzymes in Hepatitis B Virus-Infected Patients. Abstracts of the XVII International HIV Drug Resistance Workshop: Basic Principles and Clinical Implications, June 09-13, 2009, Fort Meyers, Florida. *Antivir Ther* 2009, 14 Suppl 1: A70.

S Reigadas, ML Andreola, G Anies, P Recordon-Pinson, L Wittkop, R Thiébaut, B Masquelier and H Fleury. Evolution of 2-LTR episomal HIV-1 DNA in Raltegravir-treated patients and in *in vitro* infected cells. XVII International HIV Drug Resistance Workshop: Basic Principles and Clinical Implications, June 09-13, 2009, Fort Meyers, Florida. *Antivir Ther* 2009, 14 Suppl 1: A97.

D Da Silva, I Pellegrin, G Anies, D Breilh, L Wittkop, P Morlat, M Dupon, D Neau, JL Pellegrin, H Fleury, and B Masquelier Mutational patterns in the HIV-1 integrase related to virological failures on raltegravir-containing regimens. Abstracts of the XVII International HIV Drug Resistance Workshop: Basic Principles and Clinical Implications, June 10-14, 2008, Sitges, Spain. *Antivir Ther* 2008,13 Suppl 3: A14.

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G Anies, D Da Silva, P Recordon-Pinson, S Reigadas, L Wittkop, D Neau, P Morlat, H Fleury, and Bernard Masquelier. Clonal Analysis of Raltegravir-resistant Patterns Including Mutations at Positions 143 and 155 in the HIV-1 Integrase. 16th Conference on Retroviruses and Opportunistic Infections, February 8-11, 2009, Montreal, Canada (Poster 619).

L Wittkop, G Anies, D da Silva, D Breilh, P Duffau, P Mercié, I Raymond, H Fleury, MCSaux, F Dabis, C Fagard, R Thiébaut, B Masquelier, I Pellegrin. Viro-immunological response in patients starting a raltegravir-based antiretroviral therapy, ANRS CO3 Aquitaine cohort. International workshop on HIV observational databases, March 26-28, 2009, Lisbon, Portugal

G Anies, P Recordon-Pinson, S Reigadas, L Van Wesenbeeck, K Van Baelen, L Stuyver, E Rondelez, L Wittkop, H Fleury, B Masquelier. Genotypic and phenotypic resistance to raltegravir and elvitegravir in a cohort of ARV experienced patients receiving raltegravir-containing regimens. 7th European HIV drug resistance workshop, March 25-27 2009, Stockholm, Sweden

L Wittkop, R Thiebaut, G Chêne, G Raguin, L Morand-Joubert, D Commenges. Early virological parameters and their prediction performance for indetectability of HIV-1 RNA by week 26. ANRS 2e Forum de recherches fondamentales et cliniques sur le VIH "Stratégies de contrôle du VIH : nouvelles perspectives, nouvelles questions", 14.-15. April 2008, Paris, France.